Monilinia Species Associated with Brown Rot of Cultivated Apple and Pear Fruit in China

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Abstract

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Monilinia isolates were collected from major apple and pear production regions in China from 2004 to 2011 and identified based on their morphological characteristics and three highly conserved loci. The 247 isolates belonged to three species: *Monilinia fructicola*, *Monilia yunnanensis*, and *Monilia polystroma*. *M. yunnanensis* was the most prevalent (77%), followed by *M. polystroma* (20%) and *Monilinia fructicola* (3%). *Monilia yunnanensis* is primarily distributed in the south, north, and west of China; *M. polystroma* is limited to the north and east; and *Monilinia fructicola* was detected only from a few samples from the north and east. Phylogenetic analysis based on internal transcribed spacer, β -tubulin, and laccase (*lcc2*) genes suggested that *Monilia yunnanensis*, *M. polystroma*, and

Monilinia fructigena are closely related, and *Monilia yunnanensis* is more distantly related. We also found that these three species do not show consistent differences in morphological characteristics, including colony morphology, colony expansion rate, conidial characteristics, and the amount of stroma produced in culture. Thus, these three species are more like phylogenetic species in the process of speciation. In addition, a set of species-specific primers based on single-nucleotide polymorphisms and deletions in the *lcc2* gene region were designed and a conventional polymerase chain reaction method successfully developed for differentiating *Monilinia fructicola*, *Monilia yunnanensis*, *M. polystroma*, and *Monilinia laxa* from the other species.

Brown rot is one of the most economically important diseases of *Malus* spp. Mill. and *Pyrus communis* L., causing severe yield losses at pre- and postharvest stages (Byrde and Willetts 1977). Brown rot, caused by *Monilinia* spp., not only affects the fruit but also causes blossom and leaf blight (Berrie and Holb 2014; Byrde and Willetts 1977). In Europe, preharvest losses from 7 to 25% were found in conventional and organic apple orchards, respectively (Berrie and Holb 2014).

To date, six closely related species of brown rot fungi have been reported on stone and pome fruit, including *Monilinia fructicola* (G. Winter) Honey, *M. laxa* (Aderh. & Ruhland) Honey, *M. fructigena* Honey, *Monilia polystroma* G. C. M. van Leeuwen, *M. yunnanensis* M. J. Hu & C. X. Luo, and *M. mumecola* Y. Harada, Y. Sasaki & T. Sano (Byrde and Willetts 1977; Harada et al. 2004; Hu et al. 2011; van Leeuwen et al. 2002). The geographic distribution of these species differs across the world. *Monilinia laxa* is commonly found in many places worldwide. *M. fructigena* is mostly restricted to European countries. *M. fructicola* used to be documented mainly in America and New Zealand but it was recently found in southern France (OEPP/EPPO 2002), Hungary (Petróczy and Palkovics 2006), Switzerland (Bosshard et al. 2006), the Czech Republic (Duchoslavová et al. 2007), Spain (De Cal et al. 2009), Italy (Pellegrino et al. 2009), Slovenia (Munda and Marn 2010), Germany (Grabke et al. 2011), Switzerland (Hilber-Bodmer et al. 2010), Serbia (Vasić et al. 2012), Poland (Poniatowska et al. 2013), several regions in China (Hu et al. 2011; Yin et al. 2015; Zhu et al. 2005, 2011), and, recently, on apple in Pennsylvania (Peter et al. 2015). *Monilia polystroma*, a species closely related to *Monilinia fructigena*, was first described in Japan (van Leeuwen et al. 2002) and then reported in Hungary

(Petróczy and Palkovics 2009; Petróczy et al. 2012), China (Zhu and Guo 2010), Croatia (Di Francesco et al. 2015), and Slovenia (Munda 2015). *Monilia yunnanensis* (Hu et al. 2011), closely related to *Monilinia fructigena* (Zhu et al. 2011), was recently found in China. *Monilia mumecola*, another newly identified species, was first found in Japan (Harada et al. 2004) and was detected recently in China (Hu et al. 2011; Yin et al. 2014, 2015).

China has the world's largest pome fruit industry. In 2013, apple production was about 40 million metric tons and pear production was about 17 million metric tons, accounting for 50 and 69% of the world's total yield, respectively (<http://faostat3.fao.org/download/Q/QC/E>). In recent studies, five species (*Monilinia fructicola*, *Monilia yunnanensis*, *M. polystroma*, *M. mumecola*, and *Monilinia laxa*) have been found on stone fruit in China (Fan et al. 2010; Hu et al. 2011; Yin et al. 2014; Zhu et al. 2011), suggesting that the population structure of brown rot fungi in this region is more complex than previously recognized (Zhu et al. 2008). However, little is known about the species composition of brown rot fungi on apple and pear in China. Furthermore, previous research has shown that brown rot pathogens have a certain degree of host specificity (Byrde and Willetts 1977). For example, *M. fructicola* mainly infects peach and nectarine, *M. fructigena* commonly attacks apple and pear, and *M. laxa* frequently occurs on apricot (EPPO/CABI 1997). Therefore, it is possible that the species composition of brown rot fungi on apple and pear in China is different from that on stone fruit.

Historically, *Monilinia* spp. have been differentiated based on morphological and cultural methods (Byrde and Willetts 1977; van Leeuwen and van Kesteren 1998). However, morphological characteristics vary with growth conditions and often are not reliable enough to be used in routine species-level identification. As a result, several polymerase chain reaction (PCR)-based methods have been established to identify *Monilinia* spp. according to sequence differences in markers such as simple-sequence repeats (Ma et al. 2003), random amplified polymorphic DNA (Cote et al. 2004; Gell et al. 2007), and some gene regions such as internal transcribed spacer (ITS) regions 1 and 2 (Gell et al. 2007; Ioos and Frey 2000), β -tubulin (*TUB*) (Fan et al. 2014; Hu et al. 2011; Ma et al. 2003), and the *cytBI* gene (Hily et al. 2011; Miessner and Stammer 2010). However, because of the recent discovery of new species and the fact that the sequence differences of these *Monilinia* spp. are restricted to

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Table 1. Isolates used for multigene analysis and morphological characterization^x

Isolates, species	Isolate	Location	Host	Year	GenBank accession number		
					ITS	TUB	lcc2
This study							
<i>Monilinia fructicola</i>	LS19-1 ^{y,z}	Laiyang, Shandong	Apple	2006	KR778925	KR778963	KT194355
<i>M. fructicola</i>	MX4 ^y	Laiyang, Shandong	Apple	2006	KR778926	KR778964	KT194356
<i>M. fructicola</i>	LBF1 ^y	Fangshan, Beijing	Pear	2005	KR778927	KR778965	KT194357
<i>M. fructicola</i>	SPL6 ^y	Pingyi, Shandong	Pear	2007	KR778928	KR778962	KT194358
<i>M. fructicola</i>	CX27-1 ^{y,z}	Laiyang, Shandong	Pear	2006	KR778929	KR778966	KT194359
<i>M. fructicola</i>	MX5 ^y	Laiyang, Shandong	Pear	2006	KR778930	KR778967	KT194360
<i>M. fructicola</i>	SY4 ^y	Laiyang, Shandong	Pear	2006	KR778931	KR778968	KT194361
<i>Monilia polystroma</i>	SDA2 ^{y,z}	Shandong	Apple	2004	KR778903	KR778941	KT194334
<i>M. polystroma</i>	SXP1 ^y	Yiyuan, Shandong	Apple	2007	KR778904	KR778942	KT194335
<i>M. polystroma</i>	LS10-2 ^y	Laiyang, Shandong	Apple	2006	KR778905	KR778943	KT194336
<i>M. polystroma</i>	LHX3 ^{y,z}	Xinji, Hebei	Pear	2005	KR778906	KR778944	KT194337
<i>M. polystroma</i>	MX2 ^y	Laiyang, Shandong	Pear	2006	KR778907	KR778945	KT194338
<i>M. polystroma</i>	SXL-1-1 ^y	Taian, Shandong	Pear	2007	KR778908	KR778946	KT194339
<i>M. polystroma</i>	SY3 ^y	Laiyang, Shandong	Pear	2006	KR778909	KR778947	KT194340
<i>M. yunnanensis</i>	ABC1 ^y	Changpin, Beijing	Apple	2005	KR778910	KR778948	KT194341
<i>M. yunnanensis</i>	ASJ1 ^y	Jugong, Shaanxi	Apple	2005	KR778911	KR778949	KT194342
<i>M. yunnanensis</i>	AST1 ^{y,z}	Taiyukou, Shaanxi	Apple	2005	KR778912	KR778950	ND
<i>M. yunnanensis</i>	BJCC2-1 ^y	Baoji, Shaanxi	Apple	2006	KR778913	KR778951	KT194343
<i>M. yunnanensis</i>	CLA3-1 ^y	Changli, Hebei	Apple	2006	KR778914	KR778952	KT194344
<i>M. yunnanensis</i>	YN1-2-02 ^{y,z}	Zhaotong, Yunnan	Apple	2006	KR778915	KR778953	KT194345
<i>M. yunnanensis</i>	YS1-1 ^y	Yongshou, Shaanxi	Apple	2006	KR778916	KR778954	KT194346
<i>M. yunnanensis</i>	BCB-1 ^y	Changping, Beijing	Pear	2006	KR778917	KR778955	KT194347
<i>M. yunnanensis</i>	CLP26 ^y	Changli, Hebei	Pear	2006	KR778918	KR778956	KT194348
<i>M. yunnanensis</i>	GX32 ^y	Yanqing, Beijing	Pear	2005	KR778919	KR778957	KT194349
<i>M. yunnanensis</i>	JC-LH-9 ^y	Jingchuan, Gansu	Pear	2007	KR778920	ND	KT194350
<i>M. yunnanensis</i>	LBD1 ^{y,z}	Daxing, Beijing	Pear	2005	KR778921	KR778958	KT194351
<i>M. yunnanensis</i>	LBH1-1 ^y	Haidian, Beijing	Pear	2005	KR778922	KR778959	KT194352
<i>M. yunnanensis</i>	LSB1 ^y	Binxian, Shaanxi	Pear	2005	KR778923	KR778960	KT194353
<i>M. yunnanensis</i>	YXS4 ^{y,z}	Lanzhou, Gansu	Pear	2007	KR778924	KR778961	KT194354
<i>M. yunnanensis</i>	AK9-1	Akesu, Xinjiang	Apple	2011	KR778935	KR778977	KT194371
<i>M. yunnanensis</i>	AL2-1	Akesu, Xinjiang	Pear	2011	KR778936	KR778978	KT194372
<i>M. yunnanensis</i>	WS8-2	Alaer, Xinjiang	Apple	2011	KR778934	KR778976	KT194370
<i>M. polystroma</i>	MW2 ^y	Laiyang, Shandong	Apple	2006	KR778902	KR778940	KT194333
Reference							
<i>Monilinia fructicola</i>	Ft ^{y,z}	France	N/A	N/A	HQ846967	KR778979	KT194373
<i>M. fructicola</i>	NE18 ^y	New Zealand	N/A	N/A	HQ846919	KR778980	KT194374
<i>M. fructicola</i>	LVN8 ^y	USA	N/A	N/A	HQ846966	KR778981	KT194375
<i>M. fructigena</i>	EA-1 ^{y,z}	United Kingdom	N/A	N/A	ND	ND	KT194364
<i>M. fructigena</i>	CBS101500 ^{y,z}	Poland	N/A	N/A	KR778933	KR778973	KT194365
<i>M. fructigena</i>	CBS101499 ^{y,z}	Spain	N/A	N/A	KR778932	KR778974	KT194368
<i>M. fructigena</i>	EP-5 ^{y,z}	United Kingdom	N/A	N/A	ND	KR778975	KT194369
<i>M. fructigena</i>	A2 ^{y,z}	France	N/A	N/A	ND	ND	ND
<i>M. fructigena</i>	P1 ^y	France	N/A	N/A	HQ846946	KR778982	KT194376
<i>M. fructigena</i>	GENA4 ^y	Italy	N/A	N/A	HQ846945	ND	ND
<i>M. laxa</i>	P3 ^{y,z}	France	N/A	N/A	HQ846947	KR778983	KT194377
<i>M. laxa</i>	CBS298.31 ^y	Ireland	N/A	N/A	HQ846949	KR778984	KT194378
<i>M. laxa</i>	MDA12 ^y	United States	N/A	N/A	HQ846948	KR778985	KT194379
<i>Monilia mumecola</i>	ML-1a	Wuhan, Hubei	Peach	2008	HQ908786	N/A	N/A
<i>M. mumecola</i>	HXL10-1a	Xiangfan, Hubei	Nectarine	2010	HQ908787	N/A	N/A
<i>M. mumecola</i>	HWL10-13b	Wuhan, Hubei	Peach	2010	N/A	HQ908775	N/A
<i>M. mumecola</i>	HWL10-1b	Wuhan, Hubei	Peach	2010	N/A	HQ908774	N/A
<i>M. polystroma</i>	HML3 ^{y,z}	Mudanjiang, Heilongjiang	Plum	2008	GU067539	KR778970	KT194363
<i>M. polystroma</i>	CBS102686 ^{y,z}	Japan	N/A	N/A	HQ846944	KR778971	KT194366
<i>M. polystroma</i>	2319 ^{y,z}	Japan	N/A	N/A	HQ856916	KR778972	KT194367
<i>M. yunnanensis</i>	GP18 ^{y,z}	Yanqing, Beijing	Peach	2005	HQ856917	KR778969	KT194362
<i>Monilinia fructicola</i>	XP1	Chaoyang, Beijing	Peach	2005	KR778937	ND	ND
<i>Monilia yunnanensis</i>	KY-1	Kaiyuan, Yunnan	Peach	2009	HQ908788	ND	N/A
<i>M. yunnanensis</i>	YKG10-61c	Kunming, Yunnan	Peach	2010	N/A	HQ908773	N/A
<i>Sclerotinia sclerotiorum</i>	HAY30 ^y	Unknown	Rape	N/A	HQ846942	KR778938	KT194381
<i>Botrytis cinerea</i>	BCE4 ^y	Beijing	Tomato	N/A	HQ846943	KR778939	KT194380

^x ITS = internal transcribed spacer, TUB = β -tubulin, lcc2 = laccase, N/A = not available, and ND = not determined.^y Isolates used for multigene analysis.^z Isolates used for morphological characterization.

a small region on these genes, none of the reported methods has been able to differentiate all six related *Monilinia* spp. mentioned above.

Therefore, the objectives of this study were to (i) identify the species of brown rot fungi associated with apple and pear based on molecular and morphological methods, (ii) determine their pathogenicity on apple, and (iii) develop a PCR-based method to differentiate *Monilinia* spp.

Materials and Methods

Fungal isolates. Diseased apple and pear fruit with typical brown rot symptoms were collected mostly from orchards located in major production areas in China from 2004 to 2011. Fungi were isolated and preserved following the method described by Zhu et al. (2011). Specifically, a sterilized wire loop was used to transfer conidia 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 conidia were aseptically transferred with the aid of a dissecting microscope 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 Mycology Lab of China Agricultural University. In addition, some previously identified isolates and reference isolates of *M. fructicola*, *M. fructigena*, *M. laxa*, *Monilia polystroma*, *M. yunnanensis*, and *M. mumeicola* were used in this study (Table 1).

DNA extraction, PCR amplification, and sequencing. Mycelia grown in pea broth at $22 \pm 2^\circ\text{C}$ for 5 to 7 days in darkness were collected by filtration and used for DNA extraction following the method described by Zhu et al. (2011). The ITS regions, including ITS1, ITS2, and the 5.8S ribosomal RNA gene of ribosomal DNA, were amplified using primers ITS1/ITS4 (White and Bruns 1990). Part of the *TUB* gene was amplified using primers Bt2a/Bt2b (Glass and Donaldson 1995). A partial laccase gene (*lcc2*) was amplified using primers LacaF/LacaR developed in this study, based on gene sequences of *lcc2* from Hirschhäuser and Fröhlich (2007) (Table 2). The PCR of ITS, *TUB*, and *lcc2* was performed in a 50- μl volume containing 200 μM each dNTP, 0.2 μM respective primers, 2.0 mM MgCl_2 , 2.0 U of *Taq* polymerase (CNS Bioservices Co. Ltd.), and 20 to 40 ng of genomic DNA. PCR cycling parameters were 94°C for 3 min; followed by 30 cycles of 94°C for 30 s, 50 to 55°C for 30 s, and 72°C for 1.5 min; and a final 72°C for 10 min in an Icyler Thermal Cycler (Bio-Rad Laboratories Inc.). PCR products were separated by electrophoresis 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. The amplified fragments were purified using

the PCR product Purification Kit (Dingguo Inc.) and both strands were sequenced using the PCR primers by Beijing Sunbiotech Co. Ltd. The nucleotide sequences were read with software Bioedit 7.0.4.1 (Hall 2011). Consensus sequences obtained in this study were deposited in GenBank (Table 1).

Species-specific primers design. Sequences of *TUB* and *lcc2* genes of test isolates were aligned and compared using DNAMAN 7 (Lynnon Corporation). Primer pairs ClaF/ClaR, LlaF/LlaR,

Table 3. Rate of lesion expansion caused by *Monilinia fructicola*, *M. fructigena*, *Monilia yunnanensis*, and *M. polystroma* on apple between the second and fourth day after inoculation

Species, isolate	Host ^z	Location	Expansion rate (mm day ⁻¹) ^y	
			Exp. I	Exp. II
<i>Monilinia fructicola</i>				
CX27-1	Pear	Shandong	12.4 gh	11.2 gh
LS19-1	Apple	Shandong	10.0 h	11.4 fgh
Mean	...	...	11.2 (b)	11.3 (a)
<i>M. fructigena</i>				
A2	N/A	France	12.4 gh	9.8 h
CBS101499	N/A	The Netherlands	18.3 a	18.9 a
CBS101500	N/A	The Netherlands	16.2 abcd	15.9 abcd
EP-5	N/A	United Kingdom	18.2 a	17 ab
EA-1	N/A	United Kingdom	16.8 abc	14.1 bcdefg
Mean	...	...	16.4 (a)	15.1 (a)
<i>Monilia polystroma</i>				
CBS102686	Apple	Japan	17.7 ab	15.9 abcd
LHX3	Pear	Hebei	17.3 ab	14.6 bcdef
SDA2	Apple	Shandong	17.9 ab	16.8 abc
HML-3	Plum	Heilongjiang	17.5 ab	14.6 bcdef
2319	Apple	Japan	12.6 fg	10.8 gh
Mean	...	...	16.6 (a)	14.5 (a)
<i>M. yunnanensis</i>				
AST1	Apple	Gansu	14.8 cdef	13.5 cdefg
GP18	Peach	Beijing	14.2 defg	12.0 efgh
LBD1	Pear	Beijing	15.8 bcde	15.2 bcde
YXS4	Apple	Gansu	13.4 efg	12.2 efgh
YN1-2-02	Apple	Yunnan	14.3 defg	13.3 defg
Mean	...	...	14.5 (ab)	13.2 (a)

^y For experiments (Exp.) I and II, numbers followed by different letters indicate that they were significantly different ($P \leq 0.05$).

^z N/A = not available.

Table 2. Primers used in this study

Primer ^z	Primer sequences (5'-3')	Descriptions	References
ITS			
ITS1	TCCGTAGGTGAACCTGCGG	To amplify and sequence the ITS	White and Bruns 1990
ITS4	TCCTCCGCTTATTGATATGC	Same as ITS1	White and Bruns 1990
Tubulin			
Bt2a	GGTAACCAAATCGGTGCTGCTTTC	To amplify and sequence the β -tubulin	Glass and Donaldson 1995
Bt2b	ACCCTCAGTGTAGTGACCCCTTGGC	Same as Bt2a	Glass and Donaldson 1995
Laccase 2			
LacaF	GCATCTGCATCTGCTATTCCAGCT	To amplify and sequence laccase 2	This study
LacaR	CTTACCGCCACCAACGCAGTT	Same as LacaF	This study
Species ID			
ClaF	GGTAAGCGACATCGCATTCTCAC	Species-specific for <i>Monilinia fructicola</i>	This study
ClaR	GAGGTAAATGTGGTAAATATGCAG	Same as ClaF	This study
LlaF	CCACTCCTTCAAATACCACTT	Species-specific for <i>Monilinia laxa</i>	This study
LlaR	GCTAGACTGGTACAAAAGGATGTTT	Same as LlaF	This study
GPlaF	CCACTTCCAACATCACTC	Species-specific for <i>Monilinia fructigena</i> / <i>Monilia polystroma</i>	This study
GlaR	GTAAAGGTGGCGTAATTTGGATT	Species-specific for <i>Monilinia fructigena</i>	This study
PlaR	CCCAGATTTCAAAAGCGGATTC	Species-specific for <i>Monilia polystroma</i>	This study
GClaf	CCACTTCTTCGAATAACA	Species-specific for <i>M. yunnanensis</i>	This study
GClar	GGTTCCGTATTGAGTAACCTTA	Same as GClaf	This study

^z ITS = internal transcribed spacer and Species ID = primers for species identification.

GplAF/PlaR, GClAF/GClAR, and GplAF/GlaR were designed to distinguish *Monilinia fructicola*, *M. laxa*, *Monilia polystroma*, *M. yunnanensis*, and *Monilinia fructigena*, respectively (Table 2). Closely related species *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and other fungi, including *Colletotrichum* sp., *Alternaria* sp. (218(2)), *Valsa mali* (Accc30052), *Cladosporium fulvum*, and *Neofusicocum ribis* (CBS118822), and oomycete *Phytophthora cactorum*, were used in experiments for testing primer specificity. PCR assays were performed in a 25- μ l volume containing 200 μ M each dNTP, 0.2 μ M each forward and reverse primer, 2.0 mM MgCl₂, 1.0 U of *Taq* polymerase (CNS Bioservices Co. Ltd.), and 20 to 40 ng of genomic DNA. PCR cycling parameters were 95°C for 3 min; followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min; and a final 72°C for 10 min in an Icyler Thermal Cycler. PCR products were separated by electrophoresis in 1.5% agarose gels as described above.

Phylogenetic analysis. The ITS regions were initially sequenced and analyzed; then, representative isolates were further selected according to the ITS sequences, host, geographic location of the isolates, and difference in colony characteristics for amplifying and sequencing the *TUB* and *lcc2* genes (Table 1). Reference sequences of *M. fructicola*, *M. laxa*, *M. fructigena*, *Monilia yunnanensis*, *M. polystroma*, and *M. mumecola* were included (Table 1). Consensus sequences of the selected isolates were deposited in GenBank (Table 1). Sequence alignments were conducted in DNAMAN 7 (Lynnon Corporation) and alignments of each dataset were deposited in TreeBase (<https://treebase.org/treebase-web/home.html>). Maximum-parsimony (MP) analyses were performed for each dataset of the ITS, *TUB*, *lcc2*, and combined datasets of the three genes in PAUP 4.0b10 (Swofford 2000). Gaps were treated as missing data. Trees were inferred using the heuristic searches with Tree-bisection-reconnection branch swapping and 100 random sequence additions. Branch stability was evaluated in a bootstrap analysis with 1,000 replicates, each with 100 replicates of random stepwise addition of taxa. A second

phylogenetic analysis using the Markov Chain Monte Carlo (MCMC) algorithm was conducted to generate trees with Bayesian posterior probabilities in MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). Nucleotide substitution models were determined using Modeltest v 3.7 (Posada and Crandall 1998) for each gene region and included in the analysis. Two analyses of four MCMC chains were tested from random trees for at least 1 million generations and sampled every 100 generations until the average standard deviation of split frequencies was lower than 0.01. The first 25% of trees were discarded as the burn-in phase of each analysis and posterior probabilities determined from the remaining trees. The phylogenetic trees were viewed using Treeview 1.6.6 (Page 1996). In addition, to determine whether the combined sequences could be used to construct phylogenetic analyses, statistical congruence was examined by applying

Table 4. Information of the sequence dataset and maximum-parsimony trees for each locus or combined sequences

Information	Locus ^z			
	ITS	<i>TUB</i>	<i>lcc2</i>	Combined
Total alignable characters (<i>n</i>)	443	402	883	1,728
Total variable characters (<i>n</i>)	406	304	599	1,320
Parsimony-informative characters (<i>n</i>)	25	49	143	204
Most-parsimonious trees (<i>n</i>)	500	1	4	24
Tree length	46	131	370	530
Consistency index	0.8478	0.8931	0.8946	0.8943
Homoplasy index	0.1522	0.1069	0.1054	0.1057
Retention index	0.9646	0.9545	0.9673	0.9651
Rescaled consistency index	0.8179	0.8525	0.8653	0.8632

^z ITS = internal transcribed spacer, *TUB* = β -tubulin, *lcc2* = laccase, and Combined = combined gene sequences.

Fig. 1. Geographic distribution of brown rot pathogens on apple and pear in China. Species of *Monilinia fructicola*, *Monilia polystroma*, and *M. yunnanensis* are indicated as F, P, and Y, respectively.

a partition homogeneity test in PAUP 4.0b10 using 1,000 replicates and the heuristic standard search options (Swofford 2000).

Morphological characterization. The colony morphology of all 247 isolates collected in this study and that of the 11 reference isolates (Table 1) was determined on PDA. Colony expansion rate and conidial characteristics were assessed on representative isolates of *M. yunnanensis* and *M. polystroma* and reference isolates of *Monilinia fructigena*. To obtain uniform colonies for each of the isolates, a culture 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 replicated 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 (12/12-h regime) or in 24 h of darkness (24-h regime). Colony diameters were measured from two erected directions at 2 and 4 days after inoculation. After 6 days, critical characteristics such as the color of the culture and shape of the culture edge as described by Byrde and Willetts (1977), Lane (2002), and van Leeuwen et al. (2002) were determined for each isolate. After 37 days of incubation in darkness, the amount of stroma produced by each isolate was observed from the bottom side of each petri dish. After photographs were taken, the stroma areas in each dish were assessed by Image-Pro Plus (Media

Cybernetics Inc.) in comparison with the total area of each petri dish. The experiment was conducted twice.

To characterize conidia, they were harvested from inoculated apple fruit and from petri dish cultures with the mycelia scratching method described by Zhu et al. (2014). The length and width of 50 conidia/isolate were measured under a compound microscope at $\times 400$ (Olympus Model BX41TF).

Pathogenicity tests. Pathogenicity of representative isolates of *M. fructicola*, *M. fructigena*, *Monilia yunnanensis*, and *M. polystroma* was determined on apple fruit (Table 3). Mature apple ('Fuji') were purchased commercially and then washed in tap water, surface sterilized with 0.128% NaClO for 15 min, and rinsed three times with sterile distilled water. Each fruit was wounded with a nail (2 mm in diameter and 3 mm in depth) before placing a mycelial plug (5 mm in diameter), cut from the edge of a 6-day-old colony, on the wound. Fruit inoculated with plain PDA plugs were used as controls. Inoculated fruit were placed on two layers of moist paper towels on a wire rack in a plastic container at room temperature ($24.5 \pm 2^\circ\text{C}$). Two fruit were used as a replicate and three replicates were used for each tested isolate. Symptoms were observed 2 days after inoculation, lesion diameters were measured 2 and 4 days after inoculation, and lesion expansion rate (millimeters per day) was calculated

Fig. 2. Phylogram (1 of 500) of the *Monilinia* spp. and *Monilia* species complex based on maximum-parsimony analysis with internal transcribed spacer (ITS) sequences. Tree length = 46, consistency index = 0.8478, retention index = 0.9646, rescaled consistency index = 0.8179, and homoplasy index = 0.2121. *Botrytis cinerea* and *Sclerotinia sclerotiorum* were used as outgroups. Bootstrap support values (1,000 replicates) above 70% and posterior probabilities above 0.95 for Bayesian analysis were shown at the branches. Species are followed by strain numbers.

based on the two measurements. The experiment was conducted twice.

Statistical analysis. Mean growth rate, area of stroma, conidial dimensions, and lesion expansion rate were analyzed for each isolate. Data from *M. yunnanensis*, *M. polystroma*, and *Monilinia fructigena* were analyzed with SAS 8.0 (SAS Institute) using the general linear models (Proc GLM) at $P \leq 0.05$. Means were compared using Tukey's least significant difference test when effects were found to be statistically significant.

Results

Isolate collection and colony morphology. In all, 247 isolates were obtained from apple and pear fruit with typical brown rot symptoms, originating from 51 orchards in 10 provinces (or cities), including Beijing, Shandong, Hebei, Henan, Gansu, Shaanxi, Shanxi, Yunnan, Xinjiang, and Heilongjiang (Table 1; Fig. 1). Colony characteristics were separated into three groups with respective reference isolates of *M. fructicola* (Ft, CBS167.24), *M. fructigena* (CBS101499, P1)/*Monilia yunnanensis* (GP18), and *M. polystroma* (CBS102686, 2319). Isolates in group I developed light-grayish to grayish zonate colonies with entire margins and abundant sporulation on the surface, similar to *Monilinia fructicola*. Isolates in group II had white to grayish colonies with entire margins but with little or no

stroma, similar to *M. fructigena* and *Monilia yunnanensis*. Isolates in group III had white to grayish colonies with entire margins and abundant stroma, similar to *M. polystroma* (Supplementary Fig. S1).

Phylogenetic analysis. The ITS regions of 150 of the 247 isolates, which covered all the hosts, sampled regions, and colony groups, were amplified, sequenced, and analyzed with the reference isolates. Thirty-one representative isolates were further selected for sequencing *TUB* and *lcc2* genes and used in phylogenetic analysis with the reference species (Table 1). The obtained sequences were approximately 537 bp for the ITS region, 450 bp for the *TUB* gene, and 850 bp for the *lcc2* gene. The aligned sequences of each dataset of ITS (57 taxa, 421 characters) (Supplementary Fig. S2), *TUB* (58 taxa, 402 characters), and *lcc2* (54 taxa, 762 characters) gene regions were deposited in TreeBase. The combined datasets including ITS, *TUB*, and *lcc2* were also analyzed through the partition homogeneity test before catenation, and no significant conflicts ($P \geq 0.05$) were found among these datasets. The statistical data resulting from the parsimony analysis of individual trees are summarized in Table 4. For Bayesian analysis, model test analysis recommended appropriate models as follows: a TrNef + I model (Lset Base = equal, Nst = 6, Rmat = [1.0000 2.1072 1.0000 1.0000 7.6511], Rates = equal, Pinvar = 0.8027) for the ITS region, an HKY + I model (Lset Base = [0.2484 0.2550

Fig. 3. Phylograms of the *Monilinia* spp. and *Monilia* complex based on maximum-parsimony analysis with the β -tubulin gene. Tree length = 131, consistency index = 0.8931, retention index = 0.9545, rescaled consistency index = 0.8525, and homoplasy index = 0.1892. *Botrytis cinerea* and *Sclerotinia sclerotiorum* were used as outgroups. Bootstrap support values (1,000 replicates) above 70% and posterior probabilities above 0.95 for Bayesian analysis were shown at the branches. Species are followed by strain numbers.

0.2066], Nst = 2, TRatio = 2.9282, Rates = equal, Pinvar = 0.6159) for the *TUB* gene region, a TrN + I model (Lset Base = [0.2593 0.2679 0.1832], Nst = 6, Rmat = [1.0000 2.5588 1.0000 1.0000 4.5592], Rates = equal, Pinvar = 0.4261) for the *lcc2* gene region, and a TrN + I model (Lset Base = [0.2521 0.2565 0.2000], Nst = 6, Rmat = [1.0000 3.0584 1.0000 1.0000 4.7838], Rates = equal, Pinvar = 0.5820) for combined sequences of the three gene regions. The topologies of trees resulting from each gene region and the combined gene regions were basically similar in the MP and Bayesian inference.

Four phylogenetic trees were reconstructed from MP analysis (Figs. 2, 3, 4, and 5) and the tested isolates resided in three groups (Figs. 2, 3, 4, and 5) with reference sequences of *Monilinia fructicola*, *Monilia yunnanensis*, and *M. polystroma*, respectively. All the isolates in morphology group I were clustered together with reference isolates of *Monilinia fructicola*. All the isolates in morphology group II with colony characteristics of *M. fructigena* or *Monilia yunnanensis* were clustered together with *M. yunnanensis* but not *Monilinia fructigena*. However, isolates in morphology group III that showed colony characteristics similar to those of *Monilia polystroma* were divided into two clusters. One was grouped with *M. yunnanensis* and the others grouped with *M. polystroma*. Phylogenetic analysis also showed that *M. polystroma*, *M. yunnanensis*, and *Monilinia fructigena* were closely related (Figs. 2, 3, 4, and 5) and *Monilia polystroma* was more closely related to *Monilinia fructigena* than *Monilia yunnanensis*.

Morphological characteristics of *M. yunnanensis*, *M. polystroma*, and *Monilinia fructigena*. In the 12/12-h regime, mean growth rates were 12.43 mm day⁻¹ for *Monilia yunnanensis*, 12.41 mm day⁻¹ for *M. polystroma*, and 10 mm day⁻¹ for *Monilinia fructigena*. In the 24-h regime, mean growth rates were 12.14, 10.98, and 9.75 mm day⁻¹, respectively. Among these species, *Monilia yunnanensis* grew significantly faster than *Monilinia fructigena* ($P < 0.05$) in both light regimes (Table 5), *Monilia polystroma* grew significantly faster than *Monilinia fructigena* in the 12/12-h regime but not in the 24-h regime ($P < 0.05$) (Table 5), and *Monilia yunnanensis* and *M. polystroma* had similar growth rates at both light regimes ($P < 0.05$) (Table 5).

When conidia produced in vitro and in vivo were compared, the length and width of conidia produced by *Monilinia fructigena* were significantly larger than those of *Monilia yunnanensis* and *M. polystroma* ($P < 0.05$; Table 5). However, consistent differences in the ratio of length and width of conidia among these three species were not observed. The stroma was commonly observed in cultures of *M. polystroma* and *M. yunnanensis* after 7 to 10 days of incubation and the amount of stroma increased with culture age but little or no stroma was observed for isolates of *Monilinia fructigena* after 37 days of incubation. The amount of stroma formed in petri dishes ranged from 0.66 to 7.02 cm² with *Monilia yunnanensis*, 5.76 to 15.74 cm² with *M. polystroma*, and 0 to 2.71 cm² with *Monilinia fructigena* (Table 5). Although isolates of *Monilia polystroma* produced significantly more stroma than those of *Monilinia fructigena*

Fig. 4. Phylogram (one of four) of the *Monilinia* spp. and *Monilia* complex based on maximum-parsimony analysis with the laccase gene. Tree length = 370, consistency index = 0.8946, retention index = 0.9673, rescaled consistency index = 0.8653, and homoplasy index = 0.1054. *Botrytis cinerea* and *Sclerotinia sclerotiorum* were used as outgroups. Bootstrap support values (1,000 replicates) above 70% and posterior probabilities above 0.95 for Bayesian analysis were shown at the branches. Species are followed by strain numbers.

and *Monilia yunnanensis* in general ($P < 0.05$), variation in the ability to form stroma within each species was obvious and the ranges of the amount of stroma produced by *M. yunnanensis* overlapped with those of *Monilinia fructigena* and *Monilia polystroma* (Table 5). Therefore, within a species, *Monilinia fructigena*, *Monilia polystroma*, and *M. yunnanensis* do not show consistent differences in morphological characteristics, including colony morphology, colony expansion rate, conidial characteristics, and the amount of stroma produced in culture.

Discrimination of *Monilinia* spp. from other related species by conventional PCR. Sequence variation in *TUB* and *lcc2* genes was found among the six closely related species of brown rot pathogens. For the *TUB* gene region, a total of 9 bp differences were found between *M. yunnanensis* and *Monilinia fructigena*, and 9 bp differences were observed between *Monilia yunnanensis* and *M. polystroma* (Supplementary Fig. S3). However, just 4 bp differences were observed between *M. polystroma* and *Monilinia fructigena*. For the *lcc2* gene region, 31 bp differences were found between *Monilia yunnanensis* and *Monilinia fructigena*, 25 bp differences were observed between *Monilia yunnanensis* and *M. polystroma*, but just 8 bp differences were observed between *M. polystroma* and *Monilinia fructigena* (Supplementary Fig. S4). Subsequently, species-specific primers were designed based on the differences observed in the *lcc2* gene (Table 2), and the expected PCR products of 597, 731, 387, and 152 bp were produced in *Monilia yunnanensis*, *M. polystroma*, *Monilinia fructicola* and *M. laxa*, respectively (Fig. 6), whereas no

product was produced from *M. fructigena*, *Monilia mumecola*, or reference isolates of other species. The PCR method using these primers was then implemented to identify the remaining 97 isolates collected from pome fruit, and PCR products were produced from all the isolates examined. Among the 97 isolates, 68 were identified as *M. yunnanensis*, which were amplified only by the primer pair GClAF/GClAR with a product of 597 bp; the other 29 isolates were identified as *M. polystroma*, which were amplified only by the primer pair GPlAF/PlAR with a product of 731 bp.

***Monilinia* spp. on cultivated apple and pear in China.** Based on the morphological characteristics, sequence, and phylogenetic analysis of the ITS, *TUB*, and *lcc2* gene regions, and the molecular identification, 191 of the 247 isolates were *M. yunnanensis*, 49 were *M. polystroma*, and 7 were *Monilinia fructicola*. All three species were detected in Shandong province (Fig. 1). Both *Monilia yunnanensis* and *M. polystroma* were detected in Hebei province (Fig. 1). *M. yunnanensis* and *Monilinia fructicola* were found in Beijing. A single species (*Monilia polystroma*) was detected in the northeast, including Heilongjiang and Liaoning provinces (Fig. 1), whereas a different single species (*M. yunnanensis*) was found in the west, including Xinjiang, Gansu, Shaanxi, Shanxi, Yunnan, and Henan provinces (Fig. 1).

Pathogenicity tests. All apple fruit inoculated with tested isolates produced typical symptoms of brown rot, whereas no decay was developed on the controls. In experiment I, the average expansion rate of lesions caused by *M. polystroma*, *Monilinia fructigena*, and

Fig. 5. Phylogram (1 of 24) of the *Monilinia* and *Monilia* species complex based on maximum-parsimony analysis with a combination of internal transcribed spacer, β -tubulin, and laccase gene sequences. Tree length = 530, consistency index = 0.8943, retention index = 0.9651, rescaled consistency index = 0.8632, and homoplasy index = 0.1867. *Botrytis cinerea* and *Sclerotinia sclerotiorum* were used as outgroups. Bootstrap support values (1,000 replicates) above 70% and posterior probabilities above 0.95 for Bayesian analysis were shown at the branches. Species are followed by strain numbers.

Monilia yunnanensis was 16.6, 16.4, and 14.5 mm day⁻¹, respectively, which were significantly larger than that (11.2 mm day⁻¹) caused by *Monilinia fructicola* ($P < 0.05$) (Table 3). However, this difference was not significant in experiment II ($P < 0.05$) (Table 3).

Discussion

This study represents the first report of the identification to species of an extensive collection of brown rot fungi from apple and pear fruit in China. Based on a combination of morphological characterization, multigene sequences, and a conventional PCR method, we found that three species were present. Among them, *Monilia yunnanensis* was the most prevalent (77%), followed by *M. polystroma* (20%) and *Monilinia fructigena* (3%). Pathogenicity tests showed that all three species caused fruit decay on wounded apple fruit. Moreover, a set of species-specific primers was successfully developed in this study for identifying *M. fructicola*, *Monilia polystroma*, *M. yunnanensis*, and *Monilinia laxa*.

Among the three brown rot pathogens discovered in this study, *Monilia polystroma* is a species that was recently differentiated from *Monilinia fructigena* based on stroma formation, growth rate, conidia size, and ITS sequence (van Leeuwen et al. 2002), and *Monilia yunnanensis* is a new species recently reported on stone fruit and is differentiated from *Monilinia fructigena* based on spore size, colony morphology, lesion growth rate, and sporulation (Hu et al. 2011; Zhu et al. 2011). Previously, *Monilia polystroma* has been found on plum in Heilongjiang (Zhu and Guo 2010) and *M. yunnanensis* has been found on peach in Yunnan (Hu et al. 2011) but this is the first time that they have been found on pome fruit. Previously, *Monilinia fructigena* and *M. laxa* were reported on cultivated stone and pome fruit by Wang et al. (1998); however, neither were found on pome fruit in this study or in other extensive surveys conducted recently on stone fruit in China (Hu et al. 2011; Yin et al. 2015; Zhu et al. 2011). Because no preserved cultures from Wang et al. (1998) are available, reidentification is not possible. Considering that *Monilia yunnanensis* and *M. polystroma* are new species that recently differentiated from *Monilinia fructigena* and given the prevalence of *Monilia yunnanensis* and *M. polystroma* in this study, the *Monilinia*

fructigena that Wang et al. (1998) reported most likely was *Monilia yunnanensis* or *M. polystroma*, depending on the sampling location of the isolates, because these two species had unique geographic distributions, as indicated in data from this study.

The results from the current investigation showed that, although *M. yunnanensis*, *M. polystroma*, and *Monilinia fructigena* can be differentiated from each other based on the sequence differences in ITS, *TUB*, and *lcc2* gene regions, they do not show distinct differences in their morphological characteristics, including colony morphology, colony expansion rate, conidial characteristics, and the amount of stroma produced (Hu et al. 2011; van Leeuwen et al. 2002). Phylogenetic analysis based on multigenes suggested that these three species are closely related, and *Monilia yunnanensis* is more distantly related to the others. The three species also showed differences in geographic distribution. Thus, they are more like phylogenetic species in the process of speciation.

Previously, *Monilinia fructicola* was detected as the major species on stone fruit in China (Hu et al. 2011; Zhu et al. 2011) but it was detected on only a few samples of pome fruit, even in Shandong, where *M. fructicola* is widely distributed and peach and nectarine are grown in orchards adjacent to apple. This suggests that *M. fructicola* preferentially attacks stone fruit even though its virulence on wounded apple is not significantly different from that of *M. fructigena*, *Monilia yunnanensis*, and *M. polystroma*. Similarly, like *Monilinia fructigena*, *Monilia yunnanensis* and *M. polystroma* tend to attack pome fruit in situ (EPPO/CABI 1997).

In this study, a set of species-specific primers based on differences in the *lcc2* gene was designed and a conventional PCR method was established that successfully differentiated *Monilinia fructicola*, *Monilia polystroma*, *M. yunnanensis*, and *Monilinia laxa* from other *Monilinia* spp. The primer designed for *M. fructigena* failed to differentiate it from *Monilia polystroma*. Similarly, the universal PCR method developed by Ioos and Frey (2000) based on ITS sequence differences did not differentiate *Monilinia fructicola* and the other brown rot fungi (Fan et al. 2007; Hu et al. 2011; Zhu et al. 2011). Previously, a real-time PCR method could distinguish *M. fructicola* from *M. laxa*, *M. fructigena*, and *Monilia polystroma* (van Brouwershaven et al. 2010) when *M. yunnanensis* was not included in the test. Therefore,

Table 5. Biological characteristics of *Monilinia fructigena* (MF), *Monilia polystroma* (MP), and *M. yunnanensis* (MY)

Characteristics ^v	Species ^u		
	MF (6)	MP (7)	MY (6)
Growth rate (mm day ⁻¹) ^w			
12/12-h regime ^x			
Mean ± SD	10.00 ± 1.4 b	12.41 ± 2.8 a	12.43 ± 0.3 a
Range	7.89–11.39	9.11–16.50	12.0–12.86
24-h regime ^y			
Mean ± SD	9.75 ± 1.93 b	10.98 ± 0.72 ab	12.14 ± 0.93 a
Range	7.31–12.33	10.17–12.17	10.70–13.28
Conidial size (μm) ^z			
L × W (mean ± SD)	18.52 ± 2.68 × 12.75 ± 1.87 a	15.33 ± 1.56 × 11.20 ± 1.28 b	15.18 ± 0.74 × 11.38 ± 0.85 b
Range	16.90–21.62 × 11.00–14.71	13.65–17.89 × 9.6–13.18	13.94–15.91 × 10.43–12.18
L/W (mean ± SD)	1.46 ± 0.10 a	1.37 ± 0.03 ab	1.34 ± 0.05 b
Range	1.35–1.55	1.34–1.42	1.28–1.41
From mycelia scratching			
L × W (mean ± SD)	15.58 ± 2.44a × 11.41 ± 1.62a	14.61 ± 2.30ab × 10.84 ± 1.65b	13.02 ± 1.75b × 9.85 ± 1.55b
Range	10.28–22.44 × 8.36–15.27	7.51–18.97 × 7.10–13.78	8.92–15.30 × 7.84–14.94
L/W (mean ± SD)	1.37 ± 0.11 a	1.35 ± 0.15 a	1.33 ± 0.14a
Range	1.17–1.50	1.03–1.53	1.09–1.46
Stromata			
Mean ± SD (cm ²)	1.20 ± 1.09b	10.97 ± 2.85 a	3.57 ± 3.31b
Range	0–2.71	5.76–15.74	0.66–7.02

^u Numbers in parenthesis after species name abbreviations indicate the number of isolates tested. Numbers followed by different letters are significantly different (t test) at $P = 0.05$.

^v SD = standard deviation, Range = minimum to maximum, L = length, and W = width.

^w Colony growth rate.

^x Replicated dishes kept in 12 h of light and 12 h of darkness.

^y Replicated dishes kept in 24 h of darkness.

^z Grown on apple.

sequence differences on other gene regions or a more sensitive method such as real-time PCR should be investigated.

In summary, brown rots on cultivated apple and pear fruit in China are caused by three species: *Monilinia fructicola*, *Monilia polystroma*, and *M. yunnanensis*, with *M. yunnanensis* being dominant. *M. yunnanensis*, *M. polystroma*, and *Monilinia fructigena* are closely related, with distinct sequence difference in several genes, but do not

show consistent differences in morphological characteristics. Thus, they are more like phylogenetic species in the process of speciation. A set of species-specific primers and a conventional PCR method were successfully developed in this research for differentiating *M. fructicola*, *Monilia polystroma*, *M. yunnanensis*, and *Monilinia laxa* from other *Monilinia* spp. Results of this study provide useful inputs for species identification of brown rot pathogens.

Fig. 6. Electrophoresis of polymerase chain reaction (PCR) products obtained using species-specific primers that were designed in this study. **A**, ClaF/ClaR for *Monilinia fructicola*. PCR product was about 450 bp. **B**, GClaf/GClar for *Monilia yunnanensis*. PCR product was about 600 bp. **C**, GPlaF/PlaR for *M. polystroma*. PCR product was about 750 bp. **D**, LlaF/LlaR for *Monilinia laxa*. PCR product was about 150 bp.

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