A Canker Disease of *Populus × euramericana* in China Caused by *Lonsdalea quercina* subsp. *populi*

Yong Li, The Key Laboratory for Silviculture and Conservation of Ministry of Education, Beijing Forestry University, Beijing 100083, and The Key Laboratory of State Forestry Administration on Forest Protection, Research Institute of Forest Ecology Environment and Protection, Chinese Academy of Forestry, Beijing 100091; **Wei He** and **Feijuan Ren**, The Key Laboratory for Silviculture and Conservation of Ministry of Education, Beijing Forestry University, Beijing 100083; **Limin Guo** and **Jupu Chang**, Forestry Research Institute of Puyang, Henan Puyang, China; **Ilse Cleenwerck**, BCCM/LMG Bacteria Collection, Ghent University, Laboratory of Microbiology, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium; **Yuchao Ma**, Beijing Forestry University, Beijing 100083; and **Haiming Wang**, Forest Protection Station, Heze, Shandong province, China

Abstract

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In 2006, a new canker was observed on trees of *Populus × euramericana* '74/76' and *P. × euramericana* 'Zhonglin 46' in the Henan and Shandong provinces of China. The disease, which is characterized by canker with white exudates dripping from the bark, occurred mainly in the summer. A particular gram-negative, rod-shaped bacterium was repeatedly isolated from the infected samples and proven to infect trees

of *P. × euramericana* by Koch's postulates. Through a polyphasic taxonomic approach using sequence, DNA-DNA hybridization, chemotaxonomic, and phenotypic data, the poplar isolates were identified as *Lonsdalea quercina* subsp. *populi*, a subspecies very recently described based on isolates from oozing bark canker of poplar (*P. × euramericana*) trees in Hungary.

Populus × euramericana, an interspecific hybrid poplar between *Populus nigra* and *P. deltoids*, was introduced into China in the 1980s and has become one of the most widely grown trees in China, mainly due to its fast-growing nature and good wood property as paper pulp. In 2006, poplar trees more than 5 years old showing bark canker with abundant white, sour exudates were observed for the first time in China's Henan and Shandong provinces. Diseased plants had stem or branch bark that cracked and exuded frothy fluid and, when the disease progressed, many cankers (50 to 150 by 3 to 8 cm, width by length) rapidly appeared (Fig. 1). In serious cases, diseased trees even died.

Four cultivars of *P. × euramericana* were found with this bark canker disease in China. Of four susceptible cultivars, *P. × euramericana* 'Zhonglin 46' is the most susceptible, with disease incidence up to 70% in some stands. Cultivars '74/76' and *P. deltoides* 'Zhonghe 1' are intermediate, with up to 50% disease incidence in the most serious cases. *P. × euramericana* 'Robusta' seems relatively resistant, with disease incidence up to 5% in a pure stand of 9-year-old trees. The resistance of other poplar cultivars in China to this disease has not been investigated.

In a preliminary report (13), the fungus *Fusarium solani* was identified as the causal agent of this canker disease. This conclusion was based on inoculation of water-cultured excised stem of *P. × euramericana* 74/76 in a greenhouse (28°C incubator), in which a *F. solani* strain, isolated from canker tissue, proved to be pathogenic. However, when *F. solani* was subsequently tested in field experiments during the course of this study, symptoms at inoculation points of *P. × euramericana* 74/76 and *P. × euramericana* Zhonglin 46 trees were not typical of naturally infected trees. This result indicated that *F. solani* alone was not the causal agent.

In the 1990s, poplar trees (*P. × euramericana*) showing bark cankers with copious brownish exudations, somewhat similar to the disease in China, were observed in several plantations in Spain (3,4). The causal agent of these cankers was identified as "*Brenneria populi*" using API 20E, API 20NE, API 50CH, API ZYM, and fatty acid analyses (4). The genus *Brenneria*, which was established in 1998 (11,12), was originally composed of six recognized species (*B. alni*, *B. nigrifluens*, *B. paradisiaca*, *B. quercina*, *B. rubrifaciens*, and *B. salicis*) that cause blight canker, wilt, and necrosis of various trees, including oak, willow, and walnut (2,10,15,22). Recently, *B. quercina* was transferred into a novel genus, *Lonsdalea*, as *Lonsdalea quercina* comb. nov., and three subspecies were described based on isolates that were proven or supposed pathogens of oak trees (*L. quercina* subsp. *quercina*, *L. quercina* subsp. *iberica*, and *L. quercina* subsp. *britannica*; 5). Very recently, a fourth subspecies was described (*L. quercina* subsp. *populi*) for isolates from oozing bark canker of *P. × euramericana* trees in Hungary (25).

In order to determine the real causal agent of the poplar canker disease observed in China, more samples were investigated using different methods of isolation, and inoculation tests were performed in both the greenhouse and the field. In addition, "*B. populi*" National Collection of Plant-Pathogenic Bacteria (NCPBPB, Food and Environment Research Agency, Sand Hutton, York, UK) 4299 of *P. × euramericana* causing bark canker in Spain, *L. quercina* subsp. *quercina* American Type Culture Collection (ATCC) 29281^T of *Quercus* sp. causing drippy nut disease in the United States, *L. quercina* subsp. *iberica* LMG 26264^T (Belgian Coordinated Collections of Microorganisms [BCCM]/LMG Bacteria Collection, Ghent University, Belgium) from *Quercus ilex* showing bark canker in Spain, and *L. quercina* subsp. *britannica* LMG 26267^T of *Q. ruber* causing acute oak decline in Britain were also selected for field inoculation. The microorganisms that were pathogenic in the field experiment were subjected to a polyphasic taxonomic analysis.

Materials and Methods

Isolation of the pathogen. Diseased poplar tissues of *P. × euramericana* 74/76 and Zhonglin 46 were collected between 2009 and 2010 from three locations in Henan and Shandong provinces.

Corresponding author: W. He, E-mail: hewei@bjfu.edu.cn

The first and second authors contributed equally to this work.

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Samples collected in the field were transferred to laboratories of the Beijing Forestry University in Beijing for isolation. Samples from asymptomatic poplar trees were also collected. The tissue from poplar stems was surface disinfected with 4.5% sodium hypochlorite, washed three times with sterile water, and cut into 3-by-3-mm segments with a sterile razor blade. Tissue segments were then put in a tube containing 5 ml of sterile distilled water (SDW) and shaken for about 10 min. The resulting suspension was serially diluted in SDW and spread onto nutrient agar medium. After incubation at 30°C for 2 days, single bacterial colonies were selected for subculturing to obtain pure cultures for storage and identification.

Pathogenicity tests in poplars. Pathogenicity tests were first conducted on water-cultured excised stems in a greenhouse (28°C incubator), after which the isolates that induced canker symptoms were tested in field studies.

In a greenhouse at Beijing Forestry University, stems of 2-year-old water-cultured seedlings of *P. × euramericana* 74/76 or Zhonglin 46 were cut into 30-cm-long segments and the top ends wrapped with plastic film to prevent desiccation. The segments were inoculated and placed in a tray that was subsequently covered with parafilm (Sunway Kordis Ltd.) to retain humidity. The segments were kept in darkness at 28°C, and the canker length and width were measured 14 days after inoculation.

In a field study at the Experimental Station of the Forestry Research Institute of Puyang, Henan province (where the disease occurs naturally), 3-, 4-, or 5-year-old trees of *P. × euramericana* 74/76 or Zhonglin 46 were inoculated with pure cultures of isolates

that induced canker symptoms in the greenhouse. In addition, *B. populi* NCPPB 4299, *L. quercina* subsp. *quercina* ATCC 29281^T, *L. quercina* subsp. *iberica* LMG 26264^T, and *L. quercina* subsp. *britannica* LMG 26267^T were used in the field studies. Field inoculations were executed in the bark on the stem of the trees, and the wounds were wrapped with plastic foil for 3 days and then unwrapped.

Each bacterial strain was inoculated on six stems of 2-year-old seedlings in the greenhouse, and on the stems of six trees of one cultivar in the same forest plantation. Prior to inoculation, each strain was grown in Luria-Bertani medium at 30°C for 24 h and then centrifuged for 10 min at 11,700 × *g* to pellet the cells. The cells were resuspended in SDW and adjusted to a concentration of 10⁷ CFU/ml before inoculation. Bark was surface disinfected with 75% ethanol, before cross-shaped wounds (5 mm long in the greenhouse and 2 cm long in the field) were made in the bark (in the middle of the segments in the greenhouse or at a height of 1.5 m on the stem of trees in the field) with a sterile knife. The suspension of bacteria (100 µl per site) was introduced into the wound with a sterile dropper. Control plants were treated with SDW only.

Field inoculations were conducted from June to September 2009 to 2012, when air temperature in the field was suitable for disease development. Poplar isolate N-5-1, obtained in 2009, was inoculated in the field in 2009. In 2010, isolates N-5-1, 4-4, and HZ1031, the latter two obtained in 2010, were inoculated in the field in *P. × euramericana* Zhonglin 46. In 2011, strains N-5-1 and 4-4 were inoculated on *P. × euramericana* 74/76 to test their

Fig. 1. Symptoms of canker disease of *Populus × euramericana* '74/76' caused by *Lonsdalea quercina* subsp. *populi* and observed in the field in Puyang city, Henan province. **A**, Initial symptoms with abundant white, sour exudate; **B**, medium-term symptoms 3 weeks after initial appearance.

pathogenicity to this cultivar. The reference strains *B. populi* NCPPB 4299, *L. quercina* subsp. *quercina* ATCC 29281^T, *L. quercina* subsp. *iberica* LMG 26264^T, and *L. quercina* subsp. *britannica* LMG 26267^T were tested in the field in 2012 on *P. × euramericana* Zhonglin 46. Strain N-5-1 was then used as a positive control. The results of the field pathogenicity tests were re-

corded 10, 20, and 30 days after inoculation. Diseased poplar tissue was sampled for reisolation of the inoculated bacteria.

Strains and DNA extraction. The poplar isolates and reference strains investigated in this study are listed in Table 1. Reference strains were obtained from the BCCM/LMG, NCPPB, and ATCC. All bacterial strains were cultured on tryptic soy agar (TSA; Ox-

Table 1. Poplar strains of *Lonsdalea* and reference bacterial strains investigated in this study

Species, strain ^b	Source	Location	GenBank accession numbers ^a				
			<i>gyrB</i>	<i>rpoB</i>	<i>infB</i>	<i>atpD</i>	<i>gapA</i>
<i>Lonsdalea quercina</i> subsp. <i>populi</i>							
LMG 27349 ^T = NY060 ^T	<i>Populus</i> × <i>euramericana</i>	Mikebuda, Hungary	JQ291581	KC481243#	JQ291584	JQ291578	KC481244#
LMG 27397= NY011	<i>P. × euramericana</i>	Mikebuda, Hungary	JQ291579	KF279544#	JQ291582	JQ291576	/
LMG 27396= NY041	<i>P. × euramericana</i>	Mikebuda, Hungary	JQ291580	KF279545#	JQ291583	JQ291577	/
N-5-1	<i>P. × euramericana</i>	Henan, China	JX392905#	JX392900#	JX392895#	JX392910#	JF424603#
4-4	<i>P. × euramericana</i>	Henan, China	JX392906#	JX392901#	JX392896#	JX392911#	JX39288325#
PY7-2	<i>P. × euramericana</i>	Henan, China	JX392907#	JX392902#	JX392897#	JX392912#	JX39288425#
HZ1031	<i>P. × euramericana</i>	Shandong, China	JX392908#	JX392903#	JX392898#	JX392913#	JX499271#
107-18	<i>P. × euramericana</i>	Shandong, China	JX392909#	JX392904#	JX392899#	JX392914#	JX 499270#
<i>Brenneria quercina</i> pv. <i>lupinicola</i>							
W3L1	<i>Lupinus albus</i>	United States	/	/	/	/	/
W3L7	<i>L. albus</i>	United States	/	/	/	/	/
W3L8	<i>L. albus</i>	United States	/	/	/	/	/
W3L22	<i>L. leucophyllus</i>	United States	/	/	/	/	/
<i>Lonsdalea quercina</i> subsp. <i>quercina</i>							
ATCC 29281 ^T	<i>Quercus agrifoli</i>	United States	JF311656	JF311881	JF311769	JF311543	JX39287725#
LMG 2725	<i>Quercus</i> sp.	United States	JF311657	JF311882	JF311770	JF311544	JX39287825#
LMG 5952	<i>Quercus</i> sp.	United States	JF311660	JF311885	JF311773	JF311547	/
<i>L. quercina</i> subsp. <i>iberica</i>							
LMG 26264 ^T	<i>Q. ilex</i>	Madrid, Spain	JF311665	JF311890	JF311778	JF311552	JX39288025#
LMG 26265	<i>Q. pyrenaica</i>	Madrid, Spain	JF311662	JF311887	JF311775	JF311549	JX39287925#
LMG 26266	<i>Q. ilex</i>	Madrid, Spain	JF311663	JF311888	JF311776	JF311550	/
<i>L. quercina</i> subsp. <i>britannica</i>							
LMG 26267 ^T	<i>Q. robur</i>	Booth Wood, UK	JF311666	JF311891	JF311779	JF311553	JX39288225#
LMG 26268	<i>Q. robur</i>	Booth Wood, UK	JF311667	JF311892	JF311780	JF311554	JX39288125#
LMG 26269	<i>Q. robur</i>	Gorse Covert, UK	JF311669	JF311894	JF311782	JF311556	/
<i>B. populi</i>							
NCPPB 4296	<i>P. × euramericana</i>	Spain	/	/	/	/	JX39287025#
NCPPB 4297	<i>P. × euramericana</i>	Spain	/	/	/	/	JX39286925#
NCPPB 4299 ^T	<i>P. × euramericana</i>	Spain	/	/	/	/	JX39286825#
<i>B. alni</i>							
NCPPB 3934 ^T	<i>Alnus cordata</i>	Italy	JF311627	JF311852	JF311740	JF311514	JX39286525#
<i>B. nigrifluens</i>							
LMG 2694 ^T	<i>Juglans regia</i>	United States	JF311612	JF311837	JF311725	JF311499	JX39286525#
<i>B. rubrifaciens</i>							
LMG 2709 ^T	<i>J. regia</i>	United States	JF311617	JF311842	JF311730	JF311504	JX39286725#
<i>B. salicis</i>							
LMG 2698 ^T	<i>Salix alba</i>	United Kingdom	JF311622	JF311847	JF311735	JF311509	JF424605#
<i>Dickeya chrysanthemi</i>							
LMG 2804 ^T	<i>Dianthus caryophyllus</i>	United Kingdom	JF311636	JF311861	JF311749	JF311523	JX39287125#
<i>Dickeya dadantii</i> subsp. <i>dieffenbachiae</i>							
LMG 25992 ^T	<i>Dieffenbachia</i> sp.	United States	JF311652	JF311877	JF311765	JF311539	GQ891968
<i>Dickeya dianthicola</i>							
LMG 2485 ^T	<i>Dianthus caryophyllus</i>	United Kingdom	JF311648	JF311873	JF311761	JF311535	JX39287325#
<i>Dickeya paradisiaca</i>							
LMG 2542 ^T	<i>Musa paradisiaca</i>	Colombia	JF311640	JF311865	JF311753	JF311527	JX39287225#
<i>Erwinia amylovora</i>							
LMG 2024 ^T	<i>Pyrus communis</i>	United Kingdom	HQ393608	HQ393632	HQ393620	HQ393596	JX39287425#
<i>E. mallotivora</i>							
LMG 2708 ^T	<i>Mallotus japonicus</i>	Japan	HQ393601	HQ393625	HQ393613	HQ393589	JX39287525#
<i>E. pyrifoliae</i>							
LMG 25888 ^T	<i>Pyrus pyrifolia</i>	South Korea	HQ393609	HQ393633	HQ393621	HQ393597	NC_017390
<i>E. tracheiphila</i>							
LMG 2906 ^T	<i>Cucumis melo</i>	United States	HQ393603	HQ393627	HQ393615	HQ393591	JX39287625#
<i>Pantoea agglomerans</i>							
LMG 1286 ^T	Human	Zimbabwe	EF988798	EF988970	EF988884	EF988711	JX39288525#
<i>P. anthophila</i>							
LMG 2558 ^T	<i>Impatiens balsamina</i>	India	EF988812	EF988984	EF988898	EF988725	JX39288625#
<i>P. cypripedii</i>							
LMG 2657 ^T	<i>Cypripedium</i> sp.	United States	FJ187830	FJ187840	FJ187835	FJ187825	JX39288725#
<i>P. stewartii</i> subsp. <i>stewartii</i>							
LMG 2715 ^T	<i>Zea mays</i>	United States	EF988831	EF989003	EF988917	EF988744	JX39288825#
<i>Pectobacterium wasabiae</i>							
LMG 8444 ^T	<i>Eutrema wasabi</i>	Japan	JF311608	JF311833	JF311721	JF311495	FJ895855
<i>P. carotovorum</i> subsp. <i>carotovorum</i>							
LMG 2404 ^T	<i>Solanum tuberosum</i>	Denmark	JF311601	HM359006	JF311714	JF311488	JX392889#

^a Symbols: # = sequences determined in this study and / = sequences not available.

^b LMG = BCCM/LMG Bacteria Collection, Ghent University, Belgium; ATCC = American Type Culture Collection, Manassas, KS; and NCPPB = National Collection of Plant Pathogenic Bacteria, Food and Environment Research Agency, Sand Hutton, York, United Kingdom.

oid) at 30°C for 24 h. Genomic DNA for polymerase chain reaction (PCR) was extracted from bacterial cells using the cetyltrimethylammonium bromide protocol (1).

PCR amplification and DNA sequencing. The following genes were studied: the 16S ribosomal RNA (rRNA) gene; glyceraldehyde-3-phosphate dehydrogenase A (*gapA*); DNA gyrase B subunit (*gyrB*); RNA polymerase, β subunit (*rpoB*); initiation translation factor 2 (*infB*); and ATP synthase F1, β subunit (*atpD*). The 16S rRNA gene of five poplar isolates obtained during this study and three *B. populi* strains obtained from NCPPB were amplified from genomic DNA using the universal primers 27f/1492r. Amplification and sequencing were performed as previously published (14). Amplification and sequencing of the *gapA* gene was performed for the same five poplar isolates and selected strains of species belonging to the genera *Lonsdalea*, *Brenneria* (including *B. populi*), *Erwinia*, *Dickeya*, *Pantoea*, and *Pectobacterium*, as previously described (17). Amplification and sequencing of *gyrB*, *rpoB*, *infB*, and *atpD* were performed for the five poplar isolates, as previously described (5,6).

Phylogenetic analyses. Almost complete 16S rRNA gene sequences of the five poplar isolates and the three “*B. populi*” strains were compared with those of reference strains of species belonging to the genera *Lonsdalea*, *Brenneria*, *Erwinia*, *Dickeya*, *Pantoea*, and *Pectobacterium* downloaded from GenBank. The sequences were aligned using ClustalW (24) and MEGA5.1 (<http://www.megasoftware.net>) (23). Neighbor-joining, maximum parsimony, and maximum likelihood trees were constructed using MEGA 5.1. A bootstrap analysis with 1,000 replicates was performed to test the stability on randomly chosen sets of positions.

Partial *gapA* gene sequences determined in this study were compared with sequences of species belonging to the same genera as selected for the 16S rRNA gene sequence analyses downloaded from GenBank. Sequence analysis and tree construction were executed as described for the 16S rRNA gene sequences.

Multilocus sequence analysis (MLSA) was conducted using partial sequences of *gyrB*, *rpoB*, *infB*, and *atpD* determined in this study or downloaded from GenBank (for reference strains of species belonging to the genera *Lonsdalea*, *Brenneria*, *Erwinia*, *Dickeya*, and *Pectobacterium*). Analysis based on each single nucleotide gene sequence was also performed. Sequence analysis and tree construction were executed on the concatenated data set as described for the 16S rRNA gene sequences.

DNA-DNA hybridization and DNA G + C content. Two isolates from poplar canker in Puyang city (N-5-1 and 4-4); one isolate from poplar canker in Heze city (HZ1031); and the type strains

of *L. quercina* subsp. *populi* LMG 27349^T, *L. quercina* subsp. *quercina* ATCC 29281^T, *L. quercina* subsp. *iberica* LMG 26264^T, and *L. quercina* subsp. *britannica* LMG 26267^T were selected for DNA-DNA hybridization experiments. DNA for these experiments was extracted and purified using the method described by Marmur (18). The initial renaturation rate method was used for DNA-DNA hybridization as described by De Ley et al. (8). The DNA-DNA relatedness values presented are based on a minimum of three replicates. The DNA G+C content of the same three poplar canker isolates was determined using the thermal denaturation protocol, as described by De Ley et al. (8).

Fatty acid analysis. Fatty acid profiles were determined for the poplar canker isolate N-5-1 and the type strains of the subspecies of *L. quercina* (LMG 27349^T, ATCC 29281^T, LMG 26264^T, and LMG 26267^T) using identical conditions. Cultivation of the strains, extraction, and analysis of the fatty acid methyl esters were performed according to the recommendations of the Microbial Identification System (20), Sherlock version 3.10 (MIDI). Extraction of the fatty acids was done from cells grown on TSA for 24 h at 28°C. Peaks of the profiles were identified using TSBA50 identification library version 5.0.

Phenotypic tests. The poplar canker isolates obtained in this study were cultured for 24 h on TSA (Oxoid) at 30°C for colony characterization. Initial tests for identification included Gram stain, flagella stain, ability to grow as a facultative anaerobe, catalase test, H₂S and indole production test, oxidase test, cellulose-degrading enzyme test, Voges-Proskauer test, and amylase test. All tests were performed as described (9,26). Commercial biochemical tests API 20E, API 50CHB/E (bioMérieux), and BIOLOG GN2 plates (Biolog) were also performed on the five poplar canker isolates and on the type strains of the subspecies of *L. quercina* as described in the manufacturer's instructions. All tests were performed at least twice in different sessions and repeated when inconsistent results were obtained.

Results

Isolation of the pathogen. More than 10 different bacterial colony types were isolated; however, 1 circular, domed, and whitish-color colony type was dominant. Five isolates of this colony type (N-5-1 = LMG 27203, 107-18, 4-4, HZ1031, and PY7-2, with N-5-1 selected as representative strain) were obtained; among them, one isolate (107-18) was from an asymptomatic poplar. In all samples collected from the different locations in Puyang city, Henan province, bacteria like N-5-1 were consistently isolated from diseased poplar bark tissues, whereas only one such isolate (107-18)

Table 2. Pathogenicity tests of bacteria strains for *Populus × euramericana* in greenhouse and in field experiments

			Strains ^a										
			1		2		3		4	5	6	7	8
Date	Cultivar ^b	Time ^c	Lesion (cm)	N	Lesion (cm)	N	Lesion (cm)	N	N	N	N	N	N
2009-08-04	2-year-old stem, 74/76	14	3–9×1–3	6/6 (re)	...	...	...	...	...	...	...	...	0/6
2009-09-05	3-year-old tree, ZL 46	10	5–7.9×3.4–5	6/6 (re)	...	...	...	...	...	...	...	...	0/6
		20	9–18.2×4.2–7.1	6/6	...	...	...	...	...	...	...	...	
		30	13–25.2×5–7.6	6/6	...	...	...	...	...	...	...	...	
2010-06-16	2-year-old stem, 74/76	14	3.5–8×1.1–2.5	5/6	3.5–8.5×2.3–4.5	6/6 (re)	4.2–8.6×3.5–4.7	6/6 (re)	...	...	...	0/6	
2010-06-28	5-year-old tree, ZL 46	10	4–7.2×3.6–4.5	6/6	3.6–9×2.6–4.4	6/6	4.5–8×3.8–4.5	6/6	...	...	...	...	0/6
		20	8–12×4.1–5.8	6/6	5–12.4×3.1–6.3	6/6	7–12.5×4.4–5.6	6/6	...	...	...	...	
		30	14–20×4.8–7	6/6	16–20×5.2–7.1	6/6	11–17×4.8–7.7	6/6	...	...	...	...	
2011-08-05	5-year-old tree, 74/76	10	8–13.9×4.6–5.6	6/6	5.6–9×3.6–5.2	6/6	...	...	...	...	...	...	0/6
		20	12–25×5.2–7.2	6/6	11–21.4×4.1–7.3	6/6	...	...	...	...	...	...	
		30	14–32×5.2–8	6/6	13–26×5–7.8	6/6	...	...	...	...	...	...	
2012-7-28	2-year-old stem, ZL 46	14	4–10×1–2	6/6	...	...	...	...	0/6	0/6	0/6	0/6	0/6
2012-8-19	4-year-old tree, ZL 46	10	4.6–18×2.7–5.2	6/6	...	...	...	...	0/6	0/6	0/6	0/6	0/6
		20	12.3–41×4.6–7.2	6/6	...	...	...	...	...	...	...	...	...
		30	14.2–44×5.1–7.8	6/6	...	...	...	...	...	...	...	...	...

^a N = number and Lesion = lesion size for strains 1, N-5-1; 2, 4-4; 3 HZ1031; 4, NCPPB 4299; 5, LMG 26264^T; 6, LMG 26267^T; 7, ATCC 29281^T; 8, control; re, reisolation.

^b Inoculated stem segments and trees of *Populus × euramericana* ‘74/76’ and ‘Zhonglin 46’ (ZL 46).

^c Time in days.

was obtained from more than 100 bark samples from asymptomatic poplar collected from 2010 to 2011. All isolates were purified by repeated dilution streaking and stored in 20% sterile glycerin at -70°C in the Lab of Forest Pathology of Beijing Forestry University.

Pathogenicity tests in poplar. Of all isolates tested in the greenhouse, three (N-5-1, 4-4, and HZ1031) induced canker symptoms in the inoculated stem segments. Symptoms on the 30-cm-long inoculated segments appeared 4 days after inoculation. Initially, the cortical layers of the inoculation site were macerated. One day later, a yellow-whitish-colored liquid flowed from the inoculation site, and the lesion expanded further in the following days. Inoculation with other isolates obtained in this study and with *B. populi* NCPPB 4299, *L. quercina* subsp. *quercina* ATCC 29281^T, *L. quercina* subsp. *iberica* LMG 26264^T, *L. quercina* subsp. *britannica* LMG 26267^T, and the control did not show formation of water-soaked lesions (Table 2). Necrosis was limited to the wound site.

The three poplar isolates that were pathogenic in the greenhouse study (N-5-1, 4-4, and HZ1031); in addition, *B. populi* NCPPB 4299, *L. quercina* subsp. *quercina* ATCC 29281^T, *L. quercina* subsp. *iberica* LMG 26264^T, and *L. quercina* subsp. *britannica* LMG 26267^T were subsequently tested in field studies. Typical field symptoms, including bark canker with abundant white sour exudate, were invariably reproduced with the three bacterial iso-

lates (Fig. 2A; Table 2). The development of symptoms paralleled those of natural infections. In addition, the same bacterium obtained through reisolation from the inoculated diseased plants reproduced typical symptoms like those of the natural infection of the disease when inoculated into 5-year-old trees. Typical symptoms were not observed in 4-year-old trees of *P. × euramericana* Zhonglin 46 inoculated with NCPPB 4299, ATCC 29281^T, LMG 26264^T, or LMG 26267^T. In addition, the wounds on these inoculated trees healed after 2 weeks (Fig. 2B, C, E, and F, respectively), and the controls remained healthy (Fig. 2D).

Phylogenetic analyses. Pairwise comparison of 16S rRNA gene sequences revealed 100% nucleotide similarity among the sequences of the five poplar isolates N-5-1, 107-18, 4-4, HZ1031, and PY7-2, and 99.7, 99.5, 98.8, and 98.4% with those of *L. quercina* subsp. *populi* NY060^T, *L. quercina* subsp. *quercina* LMG 2724^T, *L. quercina* subsp. *iberica* LMG 26264^T, and *L. quercina* subsp. *britannica* LMG 26267^T, respectively. They also showed 98.5 to 98.6% nucleotide similarity with sequences of strains of *B. quercina* pv. *lupinicola*, which causes drippy pod disease of white lupine (16). Similarity to other species of the Enterobacteriaceae family was less than 97%. In the 16S rRNA gene phylogenetic analyses, three methods yielded congruent trees. As shown in the tree generated using maximum likelihood tree (Fig. 3), the five poplar isolates fall in a large cluster composed of *Lonsdalea* strains

Fig. 2. Pathogenicity tests in the field on trees of *Populus × euramericana* 'Zhonglin 46'. **A**, *Lonsdalea* subsp. *populi* N-5-1; **B**, *L. quercina* subsp. *quercina* ATCC 29281^T; **C**, *L. quercina* subsp. *iberica* LMG 26264^T; **D**, control; **E**, *Brenneria populi* NCPPB 4299; **F**, *L. quercina* subsp. *britannica* LMG 26267^T. Symptoms are 10 days after inoculation.

and the *B. quercina* pv. *lupinicola* strains, with strong bootstrap support (99%) separating them from the other species. This cluster is further divided into four subgroups, with the five poplar isolates forming a single clade with the type strain of *L. quercina* subsp. *populi* (supported with a bootstrap value of 78%), and the *B. quercina* pv. *lupinicola* strains clustering with *L. quercina* subsp. *quercina* strains (with a bootstrap value of 74%). The strains of *L. quercina* subsp. *iberica* and *L. quercina* subsp. *britannica* form the other two subgroups.

In pairwise comparisons of the *gapA* gene sequences, the five poplar isolates obtained in China had 99 to 100% nucleotide similarity to each other and 99.5, 94.6, 96.4, and 95.9% nucleotide

similarities with *L. quercina* subsp. *populi* LMG 27349^T, *L. quercina* subsp. *quercina* LMG 2724^T, *L. quercina* subsp. *iberica* LMG 26264^T, and *L. quercina* subsp. *britannica* LMG 26267^T, respectively. With the other species tested in this study, *gapA* gene sequence similarities were 76 to 89%. Topology of the *gapA* phylogenetic tree (Fig. 4) is similar to the phylogenetic tree based on 16S rRNA gene sequences for the *L. quercina* clade, with this clade being divided into the same four subgroups, with bootstrap support values of 93 to 99%.

In the MLSA phylogenetic tree (Fig. 5) based on concatenated partial sequences of *gyrB*, *rpoB*, *infB*, and *atpD* genes, the poplar isolates are contained in a single cluster with the type strain of *L.*

Fig. 3. Maximum likelihood tree based on almost complete 16S ribosomal RNA gene sequences of members of *Lonsdalea* and of phylogenetically related species. Bootstrap percentage values based on 1,000 resampled data sets are shown at the nodes; only values above 50% are given. *Cronobacter sakazakii* is included as outgroup. The scale bar indicates a 0.01 nucleotide changes per site.

quercina subsp. *populi* LMG 27349^T (with 100% bootstrap support), distinct from the other three *L. quercina* subspecies. The same topology was observed in single nucleotide-based trees (*data not shown*). Based on the *gapA*, *gyrB*, *rpoB*, *infB*, and *atpD* gene sequence data, the poplar isolates obtained in China should be classified as *L. quercina* subsp. *populi*.

DNA-DNA hybridization and DNA G + C content. The three poplar isolates N-5-1, HZ1031, and 4-4 from China displayed a high DNA-DNA relatedness to each other ($90 \pm 5\%$) and to the type strain of *L. quercina* subsp. *populi* LMG 27349^T ($92 \pm 2\%$ with N-5-1). DNA-DNA relatedness values to the type strains of other *L. quercina* subspecies were close to the recommended spe-

cies delineation value of 70% (28); specifically, $67 \pm 5\%$ with *L. quercina* subsp. *quercina* ATCC 29281^T, $66 \pm 10\%$ with *L. quercina* subsp. *britannica* LMG 26267^T, and $66 \pm 12\%$ with *L. quercina* subsp. *iberica* LMG 26264^T. The DNA-DNA hybridization data confirm that the poplar isolates obtained in China are members of *L. quercina* subsp. *populi*. The G + C content of the five poplar isolates obtained in this study was 54.5 to 54.9 mol%, which was previously observed for strains belonging to *L. quercina* (54.5 to 55.1 mol%) (5).

Fatty acid analysis. The major fatty acids of the five bacterial poplar isolates N-5-1, 107-18, 4-4, HZ1031, and PY7-2 and of *L. quercina* subsp. *populi* LMG 27349^T were C_{14:0}, C_{16:0}, C_{17:0} cyclo,

Fig. 4. Maximum likelihood tree based on *gapA* gene sequences of members of *Lonsdalea* and of phylogenetically related species. Bootstrap percentage values based on 1,000 resampled data sets are shown at the nodes; only values above 50% are given. *Cronobacter sakazakii* is included as outgroup. The scale bar is 0.01 nucleotide changes per site.

and summed features 2 (iso- $C_{16:1}$ or $C_{14:0}$ 3-OH), and 3 ($C_{16:1}$ $\omega 7c$ or iso- $C_{15:0}$ 2-OH). The peak areas percentages of the fatty acids are presented in Table 3. The percentages of the fatty acids $C_{14:0}$, $C_{16:0}$, $C_{17:0}$ cyclo, and $C_{19:0}$ cyclo were similar to those of the type strains of the other subspecies of *Lonsdalea*, and allow differentiation of *Lonsdalea* strains from *B. salicis* (LMG 2698^T), *Dickeya chrysanthemi* (LMG 2804^T), and *Pectobacterium carotovorum* subsp. *carotovorum* (LMG 2404^T).

Phenotypic tests. Phenotypic characteristics enabling differentiation of *Lonsdalea quercina* subsp. *populi* from three other subspecies of *L. quercina* are listed in Table 4. Poplar isolates N-5-1, 107-18, 4-4, HZ1031, and PY7-2 were gram-negative, non-sporulating, motile short rods (0.5 to 1.0 by 1.0 to 2.0 μm) that occurred singly, in pairs, or in groups and had four peritrichous flagella. On TSA, the isolates produced circular, domed, convex,

and smooth, white to cream-yellow colonies, with entire margins, 0.8 to 1 mm in size after 24 h of growth at 30°C. They were facultatively anaerobic and had an optimum growth at 30°C. They were negative for oxidase, amylase, phenylalanine dehydrogenase, β -galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, cellulose-degrading enzyme activity, and astaxanthin. Acetoin was not produced. They were positive for catalase, methyl-red, and the Voges-Proskauer tests, and utilized citrate. They did not produce H_2S , urease, indole, or gelatinase. Nitrates were not reduced to nitrites. Acid was produced from *N*-acetylglucosamine, arbutin, D-fructose, glycerol, D-glucose, D-mannitol, D-mannose, esculin ferric citrate, methyl- α D-glucopyranoside, D-ribose, salicin, D-sucrose, D-galactose, D-trehalose, and potassium gluconate. The following carbon sources were utilized at 28°C: *N*-acetyl-D-

Fig. 5. Maximum likelihood tree based on concatenated partial *gyrB*, *rpoB*, *atpD*, and *infB* gene sequences of members of *Lonsdalea* and of phylogenetically related species. Bootstrap percentage values based on 1,000 resampled data sets are shown at the nodes; only values above 50% are given. *Cronobacter sakazakii* is included as outgroup, and gene sequences were obtained from the genome sequencing database of GenBank. The scale bar is 0.01 nucleotide changes per site.

glucosamine, D-fructose, D-galactose, D-glucose, D-mannitol, D-mannose, β -methyl-D-glucoside, D-psicose, D-trehalose, sucrose, pyruvic acid methyl-ester, succinic acid mono-methyl-ester, succinic acid, D-gluconic acid, bromosuccinic acid, L-aspartic acid, glycerol, and D-glucose-6-phosphate. The following carbon sources were not utilized at 28°C: citric acid, α -keto glutaric acid, dextrin, glycogen, adonitol, L-arabinose, D-arabitol, D-cellobiose, erythritol, L-fucose, gentiobiose, inositol, lactose, lactulose, maltose, turanose, D-melibiose, L-rhamnose, D-sorbitol, D-raffinose, formic acid, DL-lactic acid, L-glutamic acid, L-histidine, L-leucine, xylitol, galacturonic acid, and L-serine.

Discussion

In a preliminary report by He et al. (13) in 2009, strains of fungi and bacteria were isolated from diseased poplars in China. How-

ever, as some fungal stromata appeared around the lesions of the poplar cankers, fungi were thought to be the causal agent and, therefore, isolation focused on fungi. Although several fungi were isolated, only two bacterial isolates were obtained. In addition, inoculation tests with the isolates, performed only in a greenhouse, revealed that an isolate of *F. solani* induced some symptoms, whereas two bacterial isolates and several other fungi were not pathogenic to water-cultured excised stems. Subsequent inoculation with *F. solani* in field studies revealed no typical symptoms in the inoculated trees. Based on the report of a canker on *Populus* $\times$ *euramericana* in Spain caused by *B. populi*, a genus that contains causal agents of diseases on various trees, we decided to investigate more samples of diseased poplar in China and use different isolation methods. As a result, more bacterial isolates, including the pathogenic ones, were obtained.

Table 3. Fatty acid composition (percentage of peak areas) of *Lonsdalea quercina* subsp. *populi*, *L. quercina* subsp. *quercina*, *L. quercina* subsp. *iberica*, *L. quercina* subsp. *britannica*, and selected type strains of *Brenneria*, *Dickeya*, and *Pectobacterium*

Fatty acid	Species ^a							
	1	2	3	4	5	6	7	8
Saturated fatty acids								
12:0	0.84 (0.01)	0.82 (0.02)	0.75 (0.05)	0.0 (0.00)	0.82 (0.07)	4.03	0.33	9.12
14:0	11.70 (0.18)	12.43 (0.11)	10.84 (0.18)	11.49 (0.29)	10.34 (0.34)	5.35	6.84	1.41
16:0	32.75 (1.10)	31.80 (0.26)	31.57 (0.13)	34.68 (0.38)	32.04 (0.16)	29.04	26.65	24.28
18:0	0.33 (0.08)	0.19 (0.01)	1.42 (0.32)	1.89 (0.07)	5.02 (1.48)	0.00	0.00	0.00
Unsaturated fatty acids								
18:1 ω 7c	4.52 (0.14)	4.87 (0.18)	10.73 (0.27)	8.55 (0.15)	11.15 (0.34)	15.32	16.21	13.12
Cyclopropane fatty acids								
17:0	9.99 (0.02)	16.74 (0.40)	9.08 (0.71)	16.05 (1.82)	8.73 (0.21)	9.58	0.7	0.0
19:0	0.74 (0.00)	2.14 (0.09)	1.8 (0.07)	4.86 (0.04)	2.44 (0.09)	0.53	0.0	0.0
Sum in feature								
2:16:1 iso I/14:0 3OH	11.69 (1.10)	11.45 (0.78)	9.43 (0.77)	9.47 (0.23)	9.24 (1.79)	11.13	12.03	14.09
3:16:1 ω 7c/iso-C15:0 2-OH	26.62 (0.05)	18.83 (0.77)	19.47 (1.63)	10.09 (0.32)	17.47 (0.06)	23.21	34.72	30.85

^a Strains: 1, *L. quercina* subsp. *populi* (LMG 27349^T); 2, *L. quercina* subsp. *populi* (N-5-1); 3, *L. quercina* subsp. *iberica* (LMG 26264^T); 4, *L. quercina* subsp. *britannica* (LMG 26267^T); 5, *L. quercina* subsp. *quercina* (ATCC 29281^T); 6, *Brenneria salicis* (LMG 2698^T); 7, *Dickeya chrysanthemi* (LMG 2804^T); and 8, *Pectobacterium carotovorum* subsp. *carotovorum* (LMG 2404^T). Mean (%) and standard deviation of three extractions. Results of *Brenneria salicis* (LMG 2698^T), *Dickeya chrysanthemi* (LMG 2804^T), and *Pectobacterium carotovorum* subsp. *carotovorum* (LMG 2404^T) were taken from Brady et al. (5). Other results were obtained in this study.

Table 4. Phenotypic characteristics enabling differentiation of *Lonsdalea quercina* subsp. *populi* from three other subspecies of *L. quercina*

Characteristic	Subspecies ^a				
	1	2	3	4	5
Voges-Proskauer	v	+	–	+	+
Acid from ^b					
Glycerol	–	+	+	+	+
D-Galactose	+	+	–	+	+
D-Trehalose	–	–	+	+	+
D-Turanose	+	+	+	–	–
Potassium gluconate	+	–	+	+	+
D-Raffinose	w	–	–	–	–
Carbon source utilization ^c					
D-Raffinose	–	+	–	–	–
D-Trehalose	–	+	+	+	+
Turanose	+	+	+	–	–
Pyruvic acid methyl-ester	–	–	–	+	+
Succinic acid mono-methyl-ester	–	–	–	+	+
Citric acid	+	+	+	–	–
Formic acid	–	+	+	–	–
α -Keto glutaric acid	–	+	+	+	+
DL-Lactic acid	–	+	+	–	–
Succinic acid	–	+	+	+	+
L-Glutamic acid	–	–	+	+	–
L-Histidine	+	–	–	–	–
L-Leucine	+	–	–	–	–
L-Phenylalanine	+	–	–	–	...
L-Threonine	+	–	–	–	...

^a Strains: 1, *L. quercina* subsp. *quercina* (number of strains [n] = 2, ATCC 29281^T, LMG 2725); 2, *L. quercina* subsp. *iberica* (n = 2, LMG 26264^T, LMG 26265); 3, *L. quercina* subsp. *britannica* (n = 2, LMG 26267^T, LMG 26268); 4, *L. quercina* subsp. *populi* (n = 1, LMG 27349^T = NY060^T); and 5, *L. quercina* subsp. *populi* (n = 5, N-5-1, HZ1031, 107-18, 4-4, and PY7-2). Symbols: + = positive, – = negative, v = variable reaction, and w = weak.

^b API 50CHB/E.

^c Biolog GN2.

Nine different fungal colony types were isolated, with one (DQZ2-3) being dominant, representing 49.1% of all fungal isolates. Strain DQZ2-3, isolated from canker tissue of *P. × euramericana* 74/76, Puyang Henan, was identified as *F. solani* based on its morphology and molecular characteristics. Colonies similar to those of DQZ2-3 were consistently isolated from diseased poplar bark collected from different locations. This result was congruent with the preliminary report (13). However, when *F. solani* isolates (MZ01 from our previous study and DQZ2-3) were tested in a field experiment, only some water or mucus appeared at inoculation sites of *P. × euramericana* Zhonglin 46 and 74/76 at 3 to 10 days after inoculation, and a lesion with a diameter less than 1 cm was observed in the margin of the inoculated wound (*data not shown*). Subsequently, lesions did not expand and began to heal, which is in contrast to the typical symptoms of naturally infected trees (bark canker with abundant white, sour exudates). These results showed that *F. solani* was not the causal agent of *P. × euramericana* canker in China. However, because *F. solani* expressed weak pathogenicity to *P. × euramericana* and frequently occurred in canker tissues, its role in canker disease development needs to be studied further.

In this study, 3-, 4-, and 5-year-old field trees of *P. × euramericana* 74/76 or Zhonglin 46 were chosen for inoculation with pure cultures of *L. quercina* from poplar in China. The results of 4 years of field inoculation experiments revealed that trees inoculated with the bacterial isolate N-5-1 exhibited typical symptoms of naturally infected trees. Isolate 4-4 was also pathogenic to *P. × euramericana*, as shown in 2 years of field inoculation experiments. The same bacteria obtained through reisolation of the inoculated diseased trees reproduced the typical symptoms when inoculated into 5-year-old trees again, whereas the controls remained healthy. Inoculation of N-5-1 and the fungal isolate DQZ2-3 resulted in symptoms similar to those of N-5-1 only. Therefore, this bacterium is the causal agent of poplar canker in China.

The three isolates that were pathogenic in the field tests (N-5-1, HZ1031, and 4-4) and two additional isolates (PY7-2 and 107-18) were subjected to a polyphasic taxonomic study and identified as *L. quercina* subsp. *populi*. In 2011, based on 16S rRNA gene sequencing, MLSA, phenotypic testing, and DNA-DNA hybridizations, *B. quercina* was reclassified into a novel genus, *Lonsdalea* gen. nov. (5). This genus currently consists of one species, *L. quercina*, that is composed of four subspecies (*L. quercina* subsp. *quercina*, *iberica*, *britannica*, and *populi*). Strains of this species are proven or suspected pathogens that initially were believed to be restricted to *Quercus* spp., until a bacterial disease (drippy pod of white lupine) was reported to be caused by *B. quercina* pv. *lupinicola* (16). In the 16S rRNA nucleotide sequence tree (Fig. 3), four strains of *B. quercina* pv. *lupinicola* originating from the United States form a single branch with a bootstrap value of 74% with two strains of *L. quercina* subsp. *quercina*, also originating from the United States. The results show that the causal agent of drippy pod of white lupine belongs to *L. quercina* and should be named *L. quercina* pv. *lupinicola*. It is notable that white lupine is an annual herbaceous plant host of *L. quercina*. Very recently, *L. quercina* subsp. *populi* was described based on isolates of bark canker of poplar trees in Hungary (25). The data obtained in this study show that the poplar isolates obtained in China are very similar to those obtained in Hungary.

The canker on *P. × euramericana* in Spain caused by *B. populi* is somewhat similar to the disease observed in China. In this study, *B. populi* NCPPB 4299 did not cause symptoms on trees of *P. × euramericana* Zhonglin 46 under the conditions used. Three strains isolated from *P. × euramericana* cankers in Spain (NCPPB 4296, 4297, and 4299) were selected for phylogenetic analysis based on 16S rRNA and *gapA* gene sequences. The phylogeny trees revealed that these strains cluster close to the type strain of *B. salicis* and distant from the poplar canker isolates from China and Hungary. These results suggest that the pathogen of poplar canker in Spain is different from the pathogen of poplar canker in China.

Recently, analysis of multiple protein-encoding housekeeping genes, including *gapA*, *rpoB*, *gyrB*, *atpD*, and *infB*, and the MLSA

of such genes, have become widely applied tools for the investigation of bacterial taxonomic relationships (5–7,17,19,27). The high degree of sequence divergence of housekeeping genes is superior for identification purposes, because they are less conserved than rRNA genes. MLSA, like DNA-DNA hybridization, is a suitable technique for species delineation and for assessing relationships at the intraspecific level (19). In this study, the trees based on single nucleotide gene sequences of *gapA* (Fig. 4), *gyrB*, *rpoB*, *atpD*, and *infB* have groupings largely similar to those of the concatenated nucleotide-sequence-based tree (*gyrB*, *rpoB*, *atpD*, and *infB*).

P. × euramericana 74/76 is one of the most widely grown poplar cultivars in China. The bacterial canker disease observed to occur in northern Henan and western Shandong on such trees often caused a great loss. The occurrence of the bacterial canker disease on *P. × euramericana* 74/76 emerged as a barrier for expanding cultivation of the poplar trees. It is not clear how the disease spreads and where the pathogen overwinters. It was reported that the drippy pod bacterial disease can be spread by wind, splashing rain, and the feeding activities of insects (16,21). In this study, several insects were observed on the diseased plants in China. It is possible that they act as a vector for the bacterial pathogen. In our study of endophytic bacteria, only one isolate (107-18) of *L. quercina* subsp. *populi* was obtained from more than 100 bark samples of *P. × euramericana* asymptomatic trees from 2010 to 2011. Therefore, it is not likely that *L. quercina* subsp. *populi* is an endophytic bacterium in poplars.

Thus far, the canker observed in China naturally occurred only on *P. × euramericana* 74/76, *P. × euramericana* Zhonglin 46, *P. deltoides* Zhonghe 1, and *P. × euramericana* Robusta. It is unclear from the literature which *P. × euramericana* cultivar is infected in Hungary. Also, it is unclear whether other poplar species and cultivars can be infected. A detailed comparison of the isolates of China and Hungary is needed and may help to trace the source of the bacterial pathogen.

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