**Brenneria populi** sp. nov., isolated from symptomatic bark of *Populus euramericana* canker

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Five Gram-stain-negative, facultatively anaerobic, motile, bacterial strains were isolated from symptomatic bark tissue of *Populus euramericana* canker. Strains grew at 4–41 °C, pH 4–10 and 0–6 % (w/v) salinity. They were positive with respect to catalase activity and negative for oxidase activity, nitrate reduction and the Voges–Prokauer reaction. Analysis of 16S rRNA gene sequences indicated that these five poplar isolates belong to the genus *Brenneria*, having highest sequence similarity of 95.98 % with *Brenneria goodwinii* LMG 26270ᵀ. These five isolates formed a single cluster based on multilocus sequence analysis, indicating that they all belong to a single taxon within the genus *Brenneria*, which was confirmed by DNA–DNA hybridization. The DNA G+C content was 54.9–55.7 mol%, and the main fatty acids were C₁₆:₀, C₁₈:₁ω7c, C₁₇:₀ cyclo and C₁₆:₁ω7c/C₁₅:₀ 2-OH. Based on these results, we describe a novel species of the genus *Brenneria* with the proposed name *Brenneria populi* sp. nov. The type strain is D9-5ᵀ (=CFCC 11963ᵀ =KCTC 42088ᵀ).

The genus *Brenneria* was first established by Hauben et al. (1998a). At the time of writing, the genus *Brenneria* comprises five species with validly published names. Almost all species of the genus are plant pathogens. *Brenneria salicis* (the type species of the genus) causes willow (*Salix* spp.) watermark disease, and occurs mainly in the xylem vessels of the host plants (Hauben et al., 1998b). There are also three species which can cause plant bark canker disease. *Brenneria nigrifluens* and *Brenneria rubrifaciens* are the causal agents of bark canker and deep bark canker of walnut, respectively (Loreti et al., 2008; McClean et al., 2008). *Brenneria alni* is the causal agent of bark canker of alder (Surico et al., 1996). Moreover, another two species, namely *Brenneria goodwinii* and *Brenneria roseae* (including two subspecies), which were isolated from symptomatic oak tissues, are both associated with acute oak decline (Denman et al., 2012; Brady et al., 2014). Here, we describe the phenotypic and genotypic properties of five novel strains representing the genus *Brenneria* isolated from symptomatic bark of *Populus euramericana* canker in Henan Province of China. The *Populus euramericana* canker with abundant white, sour exudates on poplar trees more than five years old were observed for the first time in China’s Henan and Shandong provinces in 2006. Diseased plants had stem or branch bark that cracked and exuded frothy fluid. When the disease progressed, many cankers (50–150 × 3–8 cm, width by length) appeared rapidly (Li et al., 2014). *Lonsdalea quercina* subsp. *populi* proved to be the causal agent of the bark canker (Li et al., 2014).

During our research on pathogen isolation from *Populus euramericana* canker, besides the pathogen *L. quercina* subsp. *populi*, five other strains (D9-5ᵀ =CFCC 11963ᵀ, G2-2-2 =CFCC 11445, CFCC 11042, CFCC 12062, CFCC 11991) were also isolated from symptomatic poplar...
bark in Puyang City, Henan Province, China. To isolate these strains, the symptomatic bark tissue was surface-sterilized with 70% ethanol for 30 s followed by exposure to 4% (v/v) sodium hypochlorite for 2 min. After rinsing three times in sterile water, the bark tissue was ground using a sterile mortar and pestle. The resulting solution was three times in sterile water, the bark tissue was ground to 4% (v/v) sodium hypochlorite for 2 min. After rinsing sterilized with 70% ethanol for 30 s followed by exposure these strains, the symptomatic bark tissue was surface-bark in Puyang City, Henan Province, China. To isolate and the ability to produce arginine dihydrolase (except Brenneria

>goodwinii

B. alni and Brenneria. Furthermore, several characteristics of substrate utilization were also useful for

| Table 1. Phenotypic characteristics that distinguish the five poplar strains from recognized species of the genera <em>Brenneria</em> and <em>Lonsdalea</em> |
|---|---|---|---|---|---|---|---|---|
| **Species** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| **B. goodwinii** | + | - | + | + | + | + | - | - |
| **B. salicis** | + | + | + | - | - | - | - | - |
| **B. nigrofluen** | - | - | + | - | + | - | - | - |
| **B. rubrifaciens** | - | - | + | - | + | - | - | - |
| **L. quercina** | - | - | + | - | + | - | - | - |
| **B. roseae** | - | - | + | - | + | - | - | - |
| **B. nigrifluens** | - | - | + | - | + | - | - | - |
| **B. rubrifaciens** | - | - | + | - | + | - | - | - |
| **B. nigrifluens** | - | - | + | - | + | - | - | - |
| **B. roseae** | - | - | + | - | + | - | - | - |
| **B. alni** | - | - | + | - | + | - | - | - |
| **B. goodwinii** | - | - | + | - | + | - | - | - |
| **B. roseae** | - | - | + | - | + | - | - | - |
| **B. salicis** | - | - | + | - | + | - | - | - |
| **B. nigrifluens** | - | - | + | - | + | - | - | - |
| **B. rubrifaciens** | - | - | + | - | + | - | - | - |
| **B. nigrifluens** | - | - | + | - | + | - | - | - |
| **B. roseae** | - | - | + | - | + | - | - | - |
| **B. nigrifluens** | - | - | + | - | + | - | - | - |
| **B. rubrifaciens** | - | - | + | - | + | - | - | - |
| **B. alni** | - | - | + | - | + | - | - | - |
| **B. goodwinii** | - | - | + | - | + | - | - | - |
| **B. roseae** | - | - | + | - | + | - | - | - |
| **B. nigrifluens** | - | - | + | - | + | - | - | - |
| **B. rubrifaciens** | - | - | + | - | + | - | - | - |
| **B. alni** | - | - | + | - | + | - | - | - |
| **B. goodwinii** | - | - | + | - | + | - | - | - |
| **B. roseae** | - | - | + | - | + | - | - | - |
| **B. nigrifluens** | - | - | + | - | + | - | - | - |
| **B. rubrifaciens** | - | - | + | - | + | - | - | - |
| **B. alni** | - | - | + | - | + | - | - | - |
| **B. goodwinii** | - | - | + | - | + | - | - | - |
| **B. roseae** | - | - | + | - | + | - | - | - |
| **B. nigrifluens** | - | - | + | - | + | - | - | - |
| **B. rubrifaciens** | - | - | + | - | + | - | - | - |
| **B. alni** | - | - | + | - | + | - | - | - |
| **B. goodwinii** | - | - | + | - | + | - | - | - |
| **B. roseae** | - | - | + | - | + | - | - | - |
| **B. nigrifluens** | - | - | + | - | + | - | - | - |
| **B. rubrifaciens** | - | - | + | - | + | - | - | - |
| **B. alni** | - | - | + | - | + | - | - | - |
| **B. goodwinii** | - | - | + | - | + | - | - | - |
| **B. roseae** | - | - | + | - | + | - | - | - |
| **B. nigrifluens** | - | - | + | - | + | - | - | - |
| **B. rubrifaciens** | - | - | + | - | + | - | - | - |
| **B. alni** | - | - | + | - | + | - | - | - |
| **B. goodwinii** | - | - | + | - | + | - | - | - |
| **B. roseae** | - | - | + | - | + | - | - | - |
| **B. nigrifluens** | - | - | + | - | + | - | - | - |
| **B. rubrifaciens** | - | - | + | - | + | - | - | - |
| **B. alni** | - | - | + | - | + | - | - | - |
| **B. goodwinii** | - | - | + | - | + | - | - | - |
| **B. roseae** | - | - | + | - | + | - | - | - |
| **B. nigrifluens** | - | - | + | - | + | - | - | - |
| **B. rubrifaciens** | - | - | + | - | + | - | - | - |
| **B. alni** | - | - | + | - | + | - | - | - |
| **B. goodwinii** | - | - | + | - | + | - | - | - |
| **B. roseae** | - | - | + | - | + | - | - | - |
| **B. nigrifluens** | - | - | + | - | + | - | - | - |
| **B. rubrifaciens** | - | - | + | - | + | - | - | - |

*The type strain is positive.*
differentiation of the five poplar strains from the closely related species *B. goodwinii* (listed in Table 1). Physiological and biochemical characteristics, metabolic properties and substrate utilization results for the five strains are given in detail in Table 1 and in the species description.

For 16S rRNA gene sequence analysis, PCR amplification was carried out with primers 8F/1525R (5'-AGAGTTTGATCCTGGCTCAG-3'/5'-AAGGAGGTGATCCAGGCTG-3') as described by Lane (1991) and Baker et al. (2003). A 1400 bp sequence, corresponding to nucleotide positions 53–1453 in the *Escherichia coli* numbering system, was used for sequence similarity calculations (Li et al., 2013) using the CLUSTAL W program with LaserGene software 7 (DNASTAR). The 16S rRNA gene sequences of the reference strains were retrieved from GenBank. The five strains were retrieved from GenBank. The five strains shared 99.9–100 % 16S rRNA gene sequence similarity with each other, and the type strain shared highest sequence similarity with *B. goodwinii* LMG 26270T (95.98 %). The type strain had 95.93, 95.93, 95.71, 95.71 and 95.68 % sequence similarity with *Pectobacterium cacti cida* LMG 17936T, *B. salicis* LMG 2698T, *B. nigrifluens* LMG 2698T, *Gibbsiella dentisursi* NUM 1720T and 'B. roseae' subsp. *roseae* FRB 222, respectively. A trimmed sequence length of 1344 bp (sequence corresponding to nucleotide positions 80–1424 in the *E. coli* numbering system) was used for sequence similarity calculations for 'B. roseae' because only 1344 bp sequence was available for these strains. The five novel isolates shared less than 95.68 % sequence similarity with all species with validly published names (http://eztaxon-e.ezbiocloud.net; Kim et al., 2012). A trimmed sequence length of 1344 bp (corresponding to nucleotide positions 80–1424 in the *E. coli* numbering system) was used for phylogenetic analysis of the 16S rRNA gene, which was performed using MEGA 5.1 (Tamura et al., 2011). Neighbour-joining and maximum-likelihood trees were reconstructed using Cronobacter sakazakii as the outgroup, by applying the Jukes–Cantor model including proportion of invariable sites and gamma distribution (partial deletion of gaps/missing data treatment and a site coverage cut-off of 90 %). The resulting trees were evaluated by 1000 bootstrap replicates.

The topology of the two phylogenetic trees was similar. In both phylogenetic trees, the five poplar strains formed one cluster with 99 % and 100 % bootstrap support, respectively, and were distinct from the closely related neighbours *B. alni* and *B. nigrifluens*. In the maximum-likelihood tree (Fig. 1), the novel species, *B. alni* and *B. nigrifluens* formed a clade, whereas *B. salicis* and *B. rubrifaciens* formed another clade with *B. goodwinii*, clustering on the border of the genus *Lonsdalea*. The 'B. roseae' cluster was separate, on
the border of the genus Dickeya. In this tree, all of these clades had low bootstrap support (less than 50 %). However, in the neighbour-joining tree (Fig. S1, available in the online Supplementary Material), strains of 'B. roseae' were clustered together with species of the genus Dickeya, closely related to the Brenneria clade, whereas the Lonsdalea clade formed one distinct cluster. In both phylogenetic trees, 'B. roseae' subsp. roseae' and 'B. roseae' subsp. americana' were not clustered together with the Brenneria clade. Like many genera in the family Enterobacteriaceae, Brenneria is polyphyletic when analysis is based on 16S rRNA gene sequence. It is possible that a degree of homoplasly exists in the hypervariable regions of this gene as a result of tolerance to mutation. Alternatively, horizontal gene transfer could have taken place in these regions without affecting gene function, but disrupting the phylogenetic signal (Naum et al., 2008; Denman et al., 2012).

Multilocus sequence analysis (MLSA), based on partial sequences of the protein-encoding genes gyrB, rpoB, infB and atpD, was performed on the five poplar isolates. Amplification and sequencing of the four genes of these five isolates were performed as described by Brady et al. (2008), and the resulting sequences were compared with those of reference strains of the genera Brenneria, Dickeya, Lonsdalea, Pantoea and Pectobacterium, downloaded from GenBank. The GenBank accession numbers for all the housekeeping gene sequences determined in this study and downloaded from GenBank are listed in Table S1. The lengths of the four genes were: gyrB, 742 bp; rpoB, 637 bp; atpD, 657 bp; and infB, 615 bp. Sequence analysis and tree reconstruction were executed on the concatenated dataset as described for the 16S rRNA gene.

The five isolates shared a high degree of sequence similarity (99.7–100 %) to each other for all four housekeeping genes. In the concatenated nucleotide phylogenetic trees (Figs 2 and S2), the poplar isolates were located in the Brenneria clade along with all species of the genus with validly published names, with a bootstrap value of 70 %. The five poplar isolates formed a single cluster with 100 % bootstrap support, and were distinct from the closely related B. goodwinnii LMG 26270T and B. nigrifluens LMG 2694T. This not only confirms that the five isolates are members of the genus Brenneria, but also shows that they represent a novel species in this genus.

For cellular fatty acid analysis, cells were harvested from stationary phase cultures grown on TSAor 24 h at 28 °C. Fatty acid methyl esters were prepared and analysed using the methods of Sasser (1990). The main fatty acids detected

**Fig. 2.** Maximum-likelihood tree based on concatenated partial gyrB, rpoB, atpD and infB gene sequences of the five poplar strains and of phylogenetically related species. Bootstrap percentage values based on 1000 resampled datasets are shown at nodes; only values above 50 % are given. Cronobacter sakazakii is included as an outgroup, and gene sequences were obtained from the genome sequencing database of GenBank (http://www.ncbi.nlm.nih.gov/nuccore/156530483); see Table S1 for accession numbers and strain details. Bar, 0.05 nucleotide changes per site.
in the five strains were C_{16:0}, C_{18:1\omega 7c}, C_{17:0} cyclo and C_{16:1\omega 7c}/iso-C_{15:0} 2-OH. The percentages of fatty acids C_{16:0} and C_{18:1\omega 7c} in the novel strains allowed the differentiation of these strains from other species of genus *Brenneria*. Moreover, C_{17:0} detected from the novel strains was also useful for distinguishing members of the novel species from other species of the genus *Brenneria*. The fatty acids contents of the five strains and other related reference strains are presented in Table 2.

To determine DNA G+C content and DNA–DNA similarity, genomic DNA of the five poplar strains was isolated and purified according to the method described by Marmur (1961). The DNA G+C contents of the five strains, measured by the thermal denaturation method of Marmur & Doty (1962), were 54.9–55.7 mol% and were consistent with classification in the genus *Brenneria* (Hauben *et al.*, 1998a). DNA–DNA hybridization was determined using the microtitre plate method (Ezaki *et al.*, 1989) with minor modifications (Cleenwerck *et al.*, 2002). The hybridization temperature was 42 °C. Reciprocal reactions (A × B and B × A) were performed for each possible DNA pair. Four poplar isolates (D9-5^T^, G2-2-2, CFCC 11991, CFCC 12062) were hybridized amongst each other, and to *B. goodwinii* LMG 26270^T^ and *B. salicis* LMG 2698^T^. Levels of DNA–DNA relatedness of the four strains (D9-5^T^, G2-2-2, CFCC 11991, CFCC 12062) were 92.4–97.8 % (see Table S2), confirming that the strains belong to a single taxon. The four strains showed 31.6–33.8 % and 32.5–34.2 % DNA–DNA relatedness with *B. goodwinii* LMG 26270^T^ and *B. salicis* LMG 2698^T^, respectively, below the novel genotypic species threshold of 70 % (Wayne *et al.*, 1987), indicating that the five strains represent a new taxon distinct from *B. goodwinii* and *B. salicis*.

In the phylogenetic trees based on 16S rRNA gene sequence and MLSA data, the five isolates formed a single cluster within the genus *Brenneria*, indicating that they all belong to a single taxon within the genus *Brenneria*, which was confirmed by DNA–DNA hybridization. Several phenotypic characteristics support the distinctiveness of these five isolates from recognized species of the genus *Brenneria* (see Table 1), such as acid production from amygdalin and dulcitol, the ability to produce arginine dihydrolase, and the inability to produce β-galactosidase. Based on these analyses, it is proposed that the five isolates should be assigned to a novel species. The name proposed for this species is *Brenneria populi* sp. nov.

### Description of *Brenneria populi* sp. nov.


Cells are Gram-negative, facultatively anaerobic, catalase-positive and oxidase-negative, motile short rods. Cells are 0.6 × 1.3–1.5 μm in size. Colonies are milk-white, circular with entire margins, smooth and approximately 1–1.2 mm in diameter after incubation for 2 days at 30 °C on TSA plates. Growth occurs 4–41 °C and the optimum growth temperature is 30 °C. Able to grow at pH 4 to 10 with optimal growth at pH 6.5. Growth is present under 1–6 % (w/v) salinity. Positive for arginine dihydrolase and production of acetoin, but negative for activities of lysine decarboxylase, ornithine decarboxylase, urease, β-galactosidase, tryptophan deaminase and gelatinase, and production of indole and H₂S. Citrate is not utilized. Nitrate is not reduced to nitrite. Positive for acid production from glycerol, L-arabinose, D-ribose, D-xylene, D-galactose, D-glucose, D-fructose, D-mannose, dulcitol, D-mannitol, D-sorbitol, methyl D-D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, maltose, melibiose, sucrose and raffinose (API 50CHB/E).

Reactions for acid production from L-rhamnose, inositol, cellobiose, lactose, trehalose, gentiobiose, potassium gluconate and potassium 5-ketogluconate are variable, and the type strain is negative except for L-rhamnose and D-trehalose. Positive for assimilation of (Biolog GN2): dextrin, N-acetyl-D-glucosamine, L-arabinose, D-fructose, D-galactose, z-D-glucose, maltose, D-mannitol, melibiose, methyl β-D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, turanose, xylitol, methyl pyruvate, monomethyl succinate, acetate acid, formic acid, D-gluconic acid, z-hydroxybutyric acid, z-ketobutyric acid, D, L-lactic acid, succinic acid, bromosuccinic acid, L-asparagine, L-aspartic acid, uridine, thymidine, 2,3-butenediol, glycerol and D, L-α-glycerol. Reactions for assimilation of cellobiose,
l-fucose, D-mannose, myo-inositol, trehalose, D-galactonic acid lactone, 2-ketobutyric acid, D-saccharic acid, sebacic acid, L-glutamic acid, L-proline, glucose 1-phosphate and glucose 6-phosphate are variable, and the type strain is positive except for l-fucose, myo-inositol, sebacic acid and L-proline. The main fatty acids are C₁₆:₀, C₁₈:₁ω7c, C₁₇:₀ cyclo and C₁₆:₁ω7c/iso-C₁₅:₀ 2-OH. DNA G+C content is 54.9–55.7 mol%.

The type strain is D9-5T (=CFCC 11963T=KCTC 42088T), isolated from symptomatic bark of Populus × euramericana canker.

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References


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