Lonsdalea quercina subsp. populi subsp. nov., isolated from bark canker of poplar trees

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Seven Gram-negative bacterial strains were isolated from oozing bark canker of poplar (Populus × euramericana) trees in Hungary. They showed high (>98.3 %) 16S rRNA gene sequence similarity to Lonsdalea quercina; however, they differed from this species in several phenotypic characteristics. Multilocus sequence analysis based on three housekeeping genes (gyrB, atpD and infB) revealed, and DNA–DNA hybridization analysis confirmed, that this group of bacterial strains forms a distinct lineage within the species Lonsdalea quercina. A detailed study of phenotypic and physiological characteristics confirmed the separation of isolates from poplars from other subspecies of L. quercina; therefore, a novel subspecies, Lonsdalea quercina subsp. populi, type strain NY060T (= DSM 25466T = NCAIM B 02483T), is proposed.

The bacterial genus Lonsdalea was proposed by Brady et al. (2012) with the transfer of Brenneria quercina (Hildebrand & Schroth 1967; Hauben et al., 1998) into a novel genus. At the time of writing, the genus contains only one species, Lonsdalea quercina, with three subspecies (Lonsdalea quercina subsp. quercina, Lonsdalea quercina subsp. iberica and Lonsdalea quercina subsp. britannica). All the known isolates belonging to the genus Lonsdalea are proven or supposed pathogens of oak trees: L. quercina subsp. iberica has been found in Quercus ilex and Quercus pyrenaica (Biosca et al., 2003), L. quercina subsp. britannica has been found in Quercus robur and Quercus petrea (Brady et al., 2012), and L. quercina subsp. quercina has been found in Quercus agrifolia and Quercus wislizenii (Hildebrand & Schroth 1967). The members of the genus Brenneria are pathogens of various deciduous tree species: Brenneria salicis infects Salix alba and Salix caprea, Brenneria nigrifluous and Brenneria rubrifaciens infect Juglans regia (Hauben et al., 1998), Brenneria alni infects Alnus cordata and Alnus glutinosa (Surico et al., 1996), and Brenneria goodwinii infects Q. robur and Q. petrea (Denman et al., 2012).

Since 2009, a specific symptom has been detected in poplar (Populus × euramericana) stands in the central part of Hungary. The bark of symptomatic trees is vertically cracked, and a sticky, brown-coloured fluid oozes from the canker. Typically, huge numbers of the free-living, bacteriophagous nematode Panagrellus redivivus feed on the oozing fluid and on the creamy mass under the cracked bark, suggesting that high numbers of bacteria are present in the bark canker. During 2011, many bacterial strains were obtained from oozing bark cracks and creamy slime under the bark of poplar trees at three different sites in Hungary. The isolates were grouped by morphological and phenotypic characteristics (API 20E and Biolog GN2 analyses). Only the members of one morphologically homogeneous group were present in all samples. This group was tentatively identified as L. quercina (formerly Brenneria quercina) based on partial 16S rRNA sequencing; however, it differed from L. quercina in several phenotypic traits. In the present study the taxonomic position of isolates belonging to this group was investigated using multilocus sequence analysis (MLSA) of three housekeeping genes (gyrB, infB and atpD), DNA–DNA hybridization analysis and phenotypic assays.

In total, seven strains (including NY060T) were characterized by physiological features and carbon source utilization. Cell morphology was observed under a Zeiss AxioStar light microscope at × 1000 magnification, with cells grown for 3–4 days on tryptic soy agar plates (TSA) at 28 °C. Physiological properties and carbon source utilization profiles were analysed with API 20E system (bioMérieux) and Biolog GN2 analysis according to the manufacturer’s instructions.

All seven strains (including NY060T) obtained from poplar trees grew well on TSA plates. Cells are Gram-negative.

Abbreviation: MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are: J0291573–J0291575 (16S rRNA), J0291576–J0291578 (atpD), J0291579–J0291581 (gyrB) and J0291582–J0291584 (infB); for each gene the accession numbers are for Lonsdalea quercina subsp. populi NY011, NY041 and NY060T in that order.

A supplementary figure and four supplementary tables are available with the online version of this paper.
short rods (0.5 x 1.5 μm). Colonies are light cream coloured, round and slightly convex. Strains can grow at temperatures between 8 and 44 °C; however, the optimal growing temperature is 30–34 °C. They have a distinctive biochemical and physiological characteristic pattern compared with all species and subspecies of the genera Lonsdalea and Brenneria (Table 1). Isolates from poplars utilize citrate and do not produce acid from L-arabinose, which are distinctive features of genus Lonsdalea, contrasting with the genus Brenneria. Isolates from poplars produce acid from amygdalin, which differentiates them from the three subspecies of L. quercina. Furthermore, the carbon source utilization pattern is suitable for distinguishing the poplar strains from all subspecies of L. quercina (Table S1, available in IJSEM online). The ability to utilize cellobiose, gentiobiose, pyruvic acid methyl ester and succinic acid monomethyl-ester and the inability to utilize turanose differentiate the poplar isolates from all recognized strains of species of the genus Lonsdalea, while several other features listed in Table S1 are suitable for distinguishing the poplar strains from one or two subspecies of L. quercina.

Three strains (NY060T, NY011 and NY041) originating from different poplar stands were selected for sequence analysis. Genomic DNA for sequencing was extracted using the Gentra Puregene (Qiagen) DNA extraction kit. The 16S rRNA and gyrB genes were analysed as described by Tailliez et al. (2006) and Akhurst et al. (2004), respectively. Amplification and sequencing of atpD and infB genes were carried out as previously described by Brady et al. (2008). The oligonucleotide primers used for gene amplification are listed in Table S2. In the case of the 16S rRNA gene, sequence data for strains NY060T, NY011 and NY041, type strains of the three subspecies of L. quercina, five species of the genus Brenneria and several plant pathogenic species of the family Enterobacteriaceae were used in the phylogenetic analysis. Sequence data for three poplar strains and three strains (including type strains) of each species or subspecies of the genera Lonsdalea and Brenneria were used in the MLSA phylogenetic analysis. Details of the sequences are listed in Table S3.

Partial (1331 bp) 16S rRNA gene sequences were aligned using CLUSTAL_X 2.0 (Larkin et al., 2007). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura et al., 2011). The best-fit evolutionary model for the dataset was determined using the MEGA5 software, and a maximum-likelihood tree was constructed. Bootstrap analysis with 1000 replicates was performed on the tree to assess the reliability of the clusters generated.

The three isolates from poplars showed ≥99.7% 16S rRNA gene sequence pairwise similarity to each other and 98.3–99.0% to the three subspecies of L. quercina, while they showed 94.7–96.4% similarity to the type strains of species of the genus Brenneria. Generally, the similarity values are high, the poplar isolates show ≥95.0% similarity to several species of the genus Brenneria, to species of the genus Dickeya, to Erwinia aphidicola, Erwinia toletana, Erwinia persicium and Pectobacterium atrosepticum. The poplar isolates form a single cluster on the same branch in the 16S rRNA gene phylogenetic tree as other members of the genus Lonsdalea, relatively near to B. goodwinii, B. rubrifaciens and B. salcis and far from the other two Brenneria species, B. alni and B. nigrifluens (Fig. S1). However, the tree topology is uncertain, because of low bootstrap values.

MLSA based on partial sequencing of four housekeeping genes (atpD, gyrB, infB and rpoB) was used to study the taxonomic positions of the genus Brenneria within the family Enterobacteriaceae and delimitate the genera

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**Table 1. Phenotypic characters differentiating L. quercina subsp. populi subsp. nov. from described taxa of the genera Lonsdalea and Brenneria**

Taka: 1, Brenneria salicis (n=3); 2, B. alni (n=5); 3, B. nigrifluens (n=5); 4, B. rubrifaciens (n=5); 5, B. goodwinii (n=9); 6, L. quercina subsp. quercina ATCC29281; 7, L. quercina subsp. iberica LMG 26264; 8, L. quercina subsp. britannica LMG 26267; 9, L. quercina subsp. populi subsp. nov. (n=7). All data were generated under the same conditions using API tests (bioMérieux). Data are from Brady et al. (2012) (taxa 1–4); Denman et al. (2012) (taxon 5) and this study (taxa 6–9). +, Positive reaction; −, negative reaction; V, variable reaction. In the case of taxon 9, at least six out of seven strains showed similar reactions for all the indicated features.

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<td>Acid production from:</td>
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<td>Glycerol</td>
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*Lonsdalea* and *Brenneria* (Brady et al., 2012). Analysing the dataset containing the relevant sequences of three strains from each of the five revealed species of the genus *Brenneria* and each of the three subspecies of *L. quercina*, the partial sequence of the rpoB gene was proved to be the least effective in the separation of *Brenneria* and *Lonsdalea*: the percentage of variable nucleotide sites is only 23.2% and that of variable amino acid sites is 4.7%, while for the other three genes these values are 25.5–30.6% and 6.3–12.1%, respectively. Therefore, in this present study, concatenated sequences of three other genes (*atpD*, *gyrB* and *infB*) were used in the tree construction.

MLSA based on partial sequencing of *atpD*, *gyrB* and *infB* was performed as described by Brady et al. (2008). Following sequence alignment using CLUSTAL_X 2.0 (Larkin et al., 2007) and trimming of the overhangs, the lengths of the three housekeeping genes were: *atpD*= 566 bp, *gyrB*= 622 bp, *infB*= 525 bp. After finding the best-fit evolutionary model, the maximum-likelihood tree was constructed from the concatenated dataset by applying the Tamura–Nei model (Tamura & Nei, 1993) including the proportion of invariable sites and Gamma distribution.

The separation of the genera *Brenneria* and *Lonsdalea* from each other is clear based on MLSA, and the poplar strains form a distinct cluster in the *Lonsdalea* clade supported by a 100% bootstrap value (Fig. 1). The poplar isolates show 92.8–94.2% mean concatenated sequence pairwise similarity to the three revealed subspecies of *L. quercina* and 80.3–82.7% to *Erwinia amylovora*.

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**Fig. 1.** Molecular phylogenetic analysis of species of the genera *Lonsdalea* and *Brenneria* based on concatenated *gyrB*, *atpD* and *infB* sequences. The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura–Nei model (Tamura & Nei, 1993). The percentage of trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Erwinia amylovora* LMG 2024T served as an outgroup. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).
species of the genus *Brenneria*. The mean sequence pairwise similarities between species of the genus *Brenneria* are 81.8–86.8%, which are significantly lower than the similarity values between *L. quercina* subspecies and poplar isolates. This confirms that different taxa within *L. quercina* are separated at the subspecies level. The phylogenetic tree based on MLSA similarities between species of the genus Universiteit Gent (BCCM/LMG; Gent University, Belgium). DNA–DNA hybridization analysis was carried out by the Identification Service of the Belgian Coordinated Collections of Microorganisms/Laboratorium Voor Microbiologie – Universiteit Gent (BCCM/LMG; Gent University, Belgium). Total genomic DNA was isolated from poplar strain NY060\(^T\) and the type strains of the three subspecies of *L. quercina* with validly published names according to a modification of the procedure of Wilson (1987). Hybridizations were performed in the presence of 50% formamide at 43 °C according to a modification (Goris *et al.*, 1998; Cleenwerck *et al.*, 2002) of the method described by Ezaki *et al.* (1989). Reciprocal reactions (A × B and B × A) were performed. The DNA–DNA relatedness percentage reported is the mean of a minimum of six replications. The poplar strain NY060\(^T\) showed around 65% DNA–DNA relatedness with the type strains of the three subspecies of *L. quercina* (Table 2). This value is somewhat below 70%, generally accepted as the limit for species delimitation (Wayne *et al.*, 1987); however, it is similar to the relatedness values measured between the type strains of *L. quercina* subspecies. This result indicates that isolates from poplars are delimited from the three *L. quercina* subspecies to the same extent as they are separated from each other, which confirms that these isolates belong to a novel subspecies of *L. quercina*.

Fatty acid analysis were carried out by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) using an Agilent 6890N GC and version 6.1 of the MIDI Sherlock MIS software and TSBA40 (version 4.10) or TSBA6 (version 6.10) identification library. The method of preparation of the samples is the standard method described in MIDI Technical Note 101 (http://www.midi-inc.com/pdf/MIS_Technote_101.pdf). Bacteria were grown on trypticase soy agar at 28 °C for 24 h. The fatty acid profiles of the poplar isolates differ from those of the other members of the genera *Lonsdalea* and *Brenneria* with regard to the relatively low amounts of cyclopropane fatty acids and high amounts of summed feature 3 (C\(_{16:1\alpha9c}\) and/or iso-C\(_{15:0\,2-OH}\)). The percentages of peak areas for fatty acids are presented in Table S4.

Field pathogenicity tests are under way using three different *Populus euramericana* clones to determine whether these isolates induce bark cracking and necrosis, which can be observed in several poplar stands in Hungary. The preliminary results (not shown) indicate that strain NY060\(^T\) can cause bark necrosis; however, the frequency and intensity of the symptoms depend on the poplar clones.

The poplar isolates investigated in this study form a single novel subspecies that belongs to the species *L. quercina* based on gene sequencing. The novel subspecies shares most of the phenotypic features that are characteristic for *L. quercina*, but can also be distinguished from other *L. quercina* subspecies by several physiological and phenotypic traits. Therefore, we propose to classify these isolates as *L. quercina* subsp. *populi* subsp. nov., with NY060\(^T\) as the type strain (=NCAIM B 02483\(^T\)=DSM 25466\(^T\)).

### Description of Lonsdalea quercina subsp. populi subsp. nov.

*Lonsdalea quercina* subsp. *populi* (po’pu.li. L. gen. n. *populi* of poplar, the principal host trees of the bacterium).

The subspecies shares all the major characteristics of the species. Cells are Gram-negative short rods (0.5 × 1.5 μm), motile by peritrichous flagella. Colonies are light cream coloured, round and slightly convex. Strains can grow at temperatures between 8 and 44 °C; the optimal growth temperature is 30–34 °C. Oxidase-negative, catalase-positive. Positive for acetoin and citrate utilization, but negative for β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H\(_2\)S, urease, tryptophan deaminase, indole and gelatinase production. Nitrate is not reduced to nitrite. Acid is produced from glucose, mannose, sucrose and amygdalin (API 20E).

The following carbon sources are utilized: N-acetyl-D-glucosamine, cellobiose, D-fructose, D-galactose, gentiobiose, z-D-glucose, D-mannitol, D-mannose, β-methyl-D-glucoside, D-psicose, sucrose, trehalose, pyruvic acid methyl ester, D-gluconic acid, z-ketoglutaric acid, succinic acid, bromo-succinic acid, L-aspartic acid, L-glutamic acid, glycerol and

### Table 2. DNA–DNA relatedness values amongst the type strains of recognized subspecies of *L. quercina* and the type strain of *L. quercina* subsp. *populi* subsp. nov.

Values are expressed as percentages (values given in parentheses are the differences between the mean value of A × B and that of B × A).

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<tr>
<td>1. <em>L. quercina</em> subsp. <em>populi</em> subsp. nov. NY060(^T)</td>
<td>100</td>
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<tr>
<td>2. <em>L. quercina</em> subsp. <em>quercina</em> LMG2724(^T)</td>
<td>64 (8)</td>
<td>100</td>
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<tr>
<td>3. <em>L. quercina</em> subsp. <em>iberica</em> LMG26264(^T)</td>
<td>64 (17)</td>
<td>72 (5)</td>
<td>100</td>
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<td>4. <em>L. quercina</em> subsp. <em>britannica</em> LMG26267(^T)</td>
<td>66 (5)</td>
<td>67 (14)</td>
<td>68 (11)</td>
<td>100</td>
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D-glucose-6-phosphate. The following carbon sources are not utilized: cyclodextrin, dextrin, glycogen, Tween 40 and 80, l-arabinose, myo-inositol, l-arabinose, maltose, l-rhamnose, d-sorbitol, turanose, xyliot, acetic acid, citric acid and proline (Biolog GN2). Major fatty acids are C14:0, C16:0, C18:1ω7c and summed features 2 (iso-C16:1 and/or C14:0 3-0H) and 3 (C16:1ω7c and/or iso-C15:0 2-0H).

The type strain is NY060T (=DSM 25466T =NCAIM B 02483T), isolated from Populus × euramerica in Mikebud, Hungary (19°40′ 17.2° N, 47°09′ 43.6° E).

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References


