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Brenneria tiliae sp. nov., isolated from symptomatic *Tilia* × *moltkei* and *Tilia* × *europaea* trees in the UK

Helene Kile[1], Dawn Arnold[2], Joel Allainguilaume[1], Sandra Denman[3] and Carrie Brady[1,*]

Abstract

Several strains of a previously undescribed bacterial species were isolated from mature *Tilia* hybrid trees suffering from bleeding cankers at various geographic locations in the UK. The strains were Gram-negative, facultatively anaerobic, and partial sequencing of the *gyrB* gene revealed that the strains belong to the genus *Brenneria* with the closest phylogenetic neighbours being *Brenneria corticis* and *Brenneria nigrifluens*. Further investigation using a polyphasic approach was undertaken to determine the taxonomic position of the novel species. Phylogenies based on the 16S rRNA gene and multilocus sequence analysis of partial housekeeping gene sequences of *gyrB*, *rpoB*, *infB* and *atpD* revealed that the strains formed an independent cluster within the genus *Brenneria*. The phenotypic and chemotaxonomic assays demonstrated that the strains could be differentiated from the closest relatives. Genome analysis of representative strains revealed *in silico* DNA–DNA hybridization values below the threshold for species delimitation, although the average nucleotide identity values obtained when compared to *B. corticis* (95.9–96%) were slightly higher than the suggested cut-off value of 95%. However, as all other data suggests that the strains belong to a novel taxon that can be differentiated from the closest relatives, we propose that the strains represent a novel species in the genus *Brenneria*, *Brenneria tiliae* sp. nov. (type strain WC1b.1^T=LMG 32575^T=NCPPB 4697^T).

INTRODUCTION

In the late 1990s, several bacterial phytopathogens belonging to the genus *Erwinia* were transferred to the novel genus *Brenneria* by Hauben *et al.* [1]. In the following years, several novel *Brenneria* species were described and the genus currently consists of eight validly published bacterial species that are renowned for their ability to cause disease on woody hosts such as willow, alder, poplar, oak and walnut [2]. Common disease symptoms caused by *Brenneria* species include bleeding cankers with dark exudate and tissue necrosis, wilt and watermark [3–6]. In the UK, *Brenneria goodwinii* and *Brenneria roseae* subsp. *roseae* have been isolated from oaks affected by acute oak decline while *Brenneria salicis* is the causative agent of watermark disease of willow [3, 7, 8].

The genus *Tilia*, commonly known as lime, consists of around 23 species of broad-leaved temperate forest trees with a nearly circumboreal distribution [9]. Only three species are native to the UK, including the large-leaved lime (*Tilia platyphyllos*), the small-leaved lime (*Tilia cordata*) and their hybrid the common lime (*Tilia* × *europaea*). In the UK, the species occur naturally in old woodlands and are among the most frequently planted tree species in urban areas, overall providing a range of ecosystem services [10–15].

There have been increasing reports in recent years of cankers observed in *Tilia* hybrids in the UK; however, the cause is currently unknown although *Phytophthora* species are suspected to play a role in some cases (S. Denman, personal communication). During a study to compare the cultivable bacterial microbiome of healthy and symptomatic *Tilia* hybrids [16], several strains

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Keywords: *Brenneria tiliae*; *Brenneria*; *Enterobacterales*; lime; taxonomy; *Tilia*.
Abbreviations: ANI, average nucleotide identity; FAME, fatty acid methyl ester; GBDP, genome BLAST distance phylogeny; *is*DDH, *in silico* DNA–DNA hybridization; LB, Luria–Bertani; MC1, *Tilia x europaea* located at Holy Trinity Church, Minchinhampton; MC11, *Tilia x europaea* located at Holy Trinity Church, Minchinhampton; MLSA, multilocus sequence analysis; TSA, tryptic soy agar; TW2, *Tilia x europaea* located at Tidworth Garrison; WB1, *Tilia x moltkei* located at Westonbirt Arboretum.
The GenBank/EMBL/DDBJ accession numbers for sequences generated in this study are: OM505019–OM505020 (16S rRNA), OM523104–OM523118 (*atpD*), OM523119–OM532133 (*gyrB*), OM523134–OM523148 (*infB*), OM523149–OM523163 (*rpoB*) and JAKPCA000000000, JAKPCB000000000 and JAKPBZ000000000 (whole genome).
Four supplementary figures and two supplementary tables are available with the online version of this article.

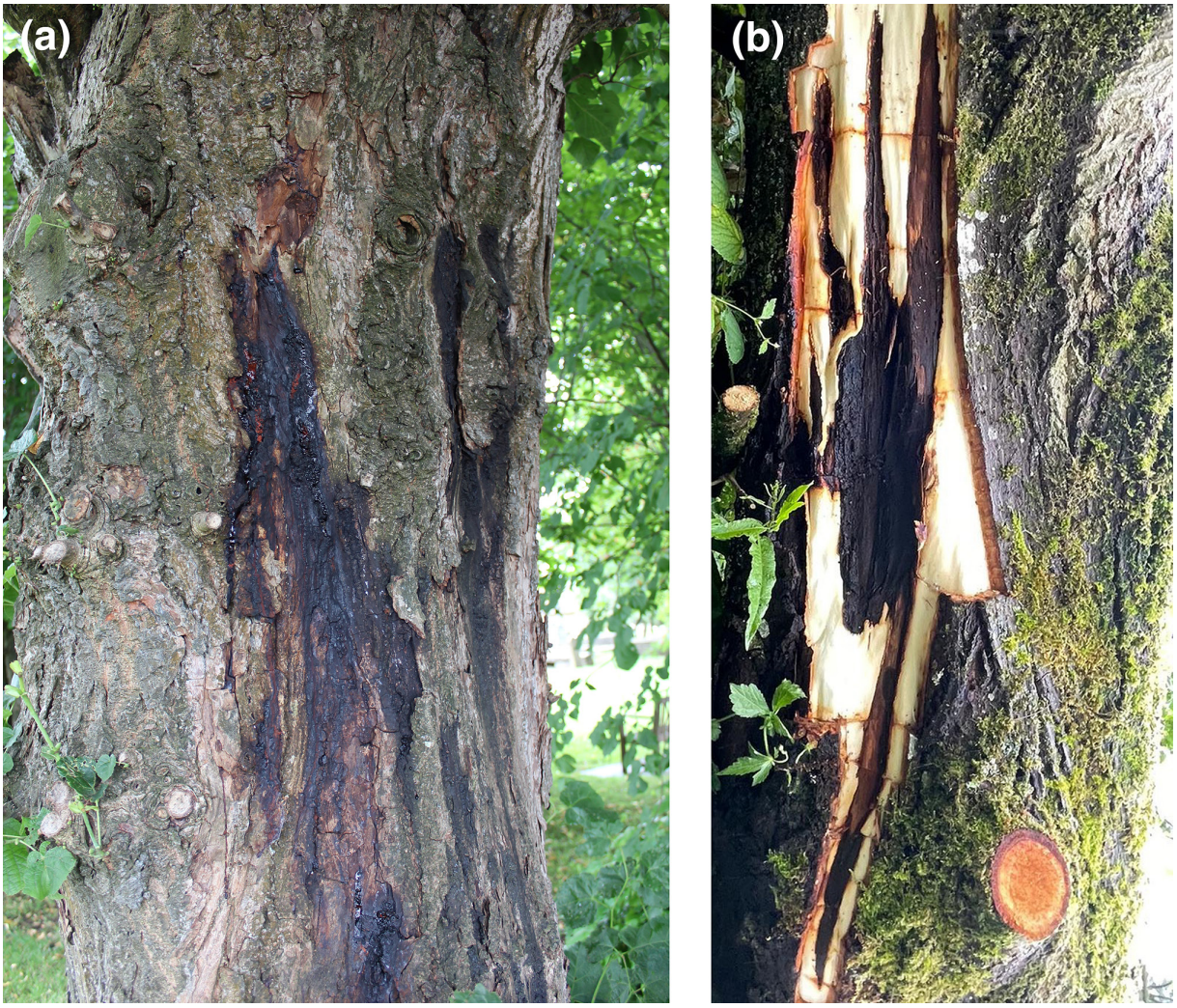

Fig. 1. Cankers of symptomatic *Tilia* hybrids. (a) External symptoms of the bleeding cankers of *Tilia* × *europaea* located at Minchinhampton, Gloucestershire (MC1). (b) Fully exposed internal lesion of the *Tilia* × *moltkei* located at Westonbirt Arboretum, Gloucestershire (WB1).

of a potential novel *Brenneria* species closely related to *Brenneria nigrifluens* and *Brenneria corticis* were isolated. Following a polyphasic approach in the present study, we confirm that the strains belong to a novel taxon within the genus *Brenneria* for which the name *Brenneria tiliae* sp. nov. is proposed.

ISOLATION SOURCE AND SAMPLE PROCESSING

The bacterial strains were isolated from one symptomatic *Tilia* × *moltkei* (WB1) located at Westonbirt Arboretum, Gloucestershire, one symptomatic *Tilia* × *europaea* (TW2) at Tidworth, Wiltshire, and two symptomatic *Tilia* × *europaea* (MC1 and MC11) at Minchinhampton, Gloucestershire (Fig. S1, available in the online version of this article). Sampling was undertaken in September 2020 and July 2021.

Strains were isolated from symptomatic tissue only, including swabs of exudate and inner bark panels (7–30×5–15×2–3 cm) taken from diseased tissue. The cankers were identified by dark brown, nearly black discolouration of the outer bark presumably due to tissue necrosis. Some cankers appeared dry while others had wet and slimy exudate of dark brown-rust colour weeping from the cankers. Removal of the bark from WB1 revealed a black lesion of necrotic tissue extending longitudinally for at least 110 cm in length and tapering into thinner lesions (Fig. 1). The lesion was shallow (<5 mm deep), and the underlying tissue appeared healthy. After sampling, the swabs were suspended in 3 ml ¼ Ringers followed by spreading onto Luria–Bertani (LB) agar plates. From the bark panels, thin wood shavings targeting the dead-live tissue junction of the inner-bark where fresh bacterial activity was expected, were taken using a sterile scalpel and placed on LB agar. Cycloheximide (50 µg ml^{-1}) was added to the agar to prevent

fungal contamination. The plates were incubated anaerobically at 35 °C for 48 h and colonies that developed were re-streaked several times to obtain pure isolates. See Table S1 for a list of strains used in this study.

GENOTYPIC CHARACTERIZATION

Genomic DNA was extracted using an alkali lysis method and was used in subsequent PCR amplification of the 16S rRNA gene and four housekeeping genes [17]. 16S rRNA gene amplification and sequencing was performed on two representative strains (WC1b.1^T and EX1a) to obtain almost-complete 16S rRNA gene sequences following the previously described procedure by Coenye *et al.* [18]. To clarify the taxonomic position of the strains, multilocus sequence analysis (MLSA) was performed on the four housekeeping genes *gyrB*, *rpoB*, *atpD* and *infB* of 15 strains using the procedure and conditions set by Brady *et al.* [19]. For strains that failed to amplify at 55 °C, an annealing temperature of 46 or 50 °C was used. For sequence analysis, all consensus sequences were assembled using Unipro UGENE version 38.1 [20] followed by sequence alignment and trimming of overhangs using MEGA X version 10.2.5 [21]. Resulting sequencing lengths in bp were: 16S rRNA, 1346; *gyrB*, 745; *rpoB*, 637; *infB*, 615; and *atpD*, 642. Sequences of corresponding genes from the closest phylogenetic relatives were obtained from GenBank and used for references in the phylogenetic analyses. Maximum-likelihood phylogenetic analysis with 1000 bootstrap replicates was performed in PhyML 3.0 [22], following smart model selection [23]. Bootstrap values <50% were excluded from the phylogenetic trees. *Plesiomonas shigelloides* NCIMB 9242^T was used as an outgroup for the 16S rRNA phylogenetic tree while *Cronobacter sakazakii* ATCC BAA-894 was used for the concatenated MLSA tree.

Comparison of the 16S rRNA gene sequences against the EzBioCloud database [24] showed that strains of the potential novel species showed the highest pairwise sequence similarity to *B. nigrifluens* (99.7–99.8%) and *B. corticis* (98.7%). In the 16S rRNA gene phylogenetic tree (Fig. S2), the strains of the novel species form a separate cluster not containing any reference strains with bootstrap support of 80%. *B. nigrifluens* is situated within the same clade with a bootstrap support of 98%, while *B. corticis* is closely located on the border of this clade with 51% support.

The concatenated maximum likelihood tree, based on the partial housekeeping gene sequences of *gyrB*, *rpoB*, *atpD* and *infB*, grouped the 15 strains isolated from symptomatic *Tilia* hybrids into one strongly supported cluster (Fig. 2), suggesting that these strains belong to a single taxon. Slight genetic variation is observed between strains within the cluster, although this is congruent with the three different geographical locations of the trees the strains were isolated from. The cluster does not contain any reference strains and is supported by a bootstrap value of 82%. The relatively short distances to the closest relatives, *B. corticis* and *B. nigrifluens*, are supported by bootstrap values of 56 and 100%, respectively.

To investigate genetic diversity between strains, the DNA fingerprinting technique ERIC PCR was used to amplify enterobacterial repetitive intergenic elements using the primers ERIC2 and ERIC1R following the previously described protocol [25]. Eight selected strains of the novel species were included along with the type strains of *B. salicis* DSM 30166^T, *B. alni* DSM 11811^T, *B. goodwinii* FRB 141^T, *B. nigrifluens* DSM 30175^T, *B. roseae* subsp. *roseae* FRB 222^T and *B. rubifaciens* DSM 4483^T. The fingerprinting results (Fig. S3) show an indication of diversity between the strains as different band patterns were observed for strains isolated from WB1 (WC1b^T, Ex1a, S1a.1, B3P2.1 and B2U2.2), TW2 (TWS2b.2ii), MC1 (MC1SB4.1) and MC11 (MCS11.3i).

GENOME FEATURES

Two strains (WC1b.1^T and EX1a) isolated from WB1 and one strain (MC1SB4.1) isolated from MC1 were selected for whole-genome sequencing. The service was provided by MicrobesNG (Birmingham, UK) using the Illumina HiSeq method following DNA extraction by cell lysis and DNA purification with solid-phase reversible-immobilization beads. Reads were adapter-trimmed using Trimmomatic 0.30 with a sliding window quality cut-off of Q15 [26]. *De novo* assembly was performed using SPAdes version 3.11.1 [27] and the resulting contigs were annotated in Prokka 1.11 [28]. The genome sequences of strains WC1b.1^T, EX1a and MC1SB4.1 were submitted to GenBank under the BioProject number PRJNA804419.

Average nucleotide identity (ANI) was calculated using FastANI [29] and *in silico* DNA–DNA hybridization (*is*DHH) values were determined using the Genome-to-Genome Distance Calculator 3.0 [30, 31]. A phylogenomic tree was reconstructed using the Type (Strain) Genome Server [32], where pairwise comparisons between the genomes were conducted using genome BLAST distance phylogeny (GBDP) and accurate intergenomic distances inferred under the algorithm 'trimming' and distance formula d_5 [30] with 100 distance replicates each. The resulting intergenomic distances were used to construct a balanced minimum evolution tree including subtree pruning and regrafting post-processing in FastME 2.1.6.1 [33]. Branch support was inferred from 100 pseudo-bootstrap replicates and the tree was rooted at the midpoint [34].

Sequence assembly of strains WC1b.1^T, EX1a and MC1SB4.1 yielded genomes with a total length of 5.16–5.23 Mbp. The DNA G+C content ranged from 56.3 to 56.5 mol% for the three genomes. Genome features and assembly accession numbers are listed in Table S2.

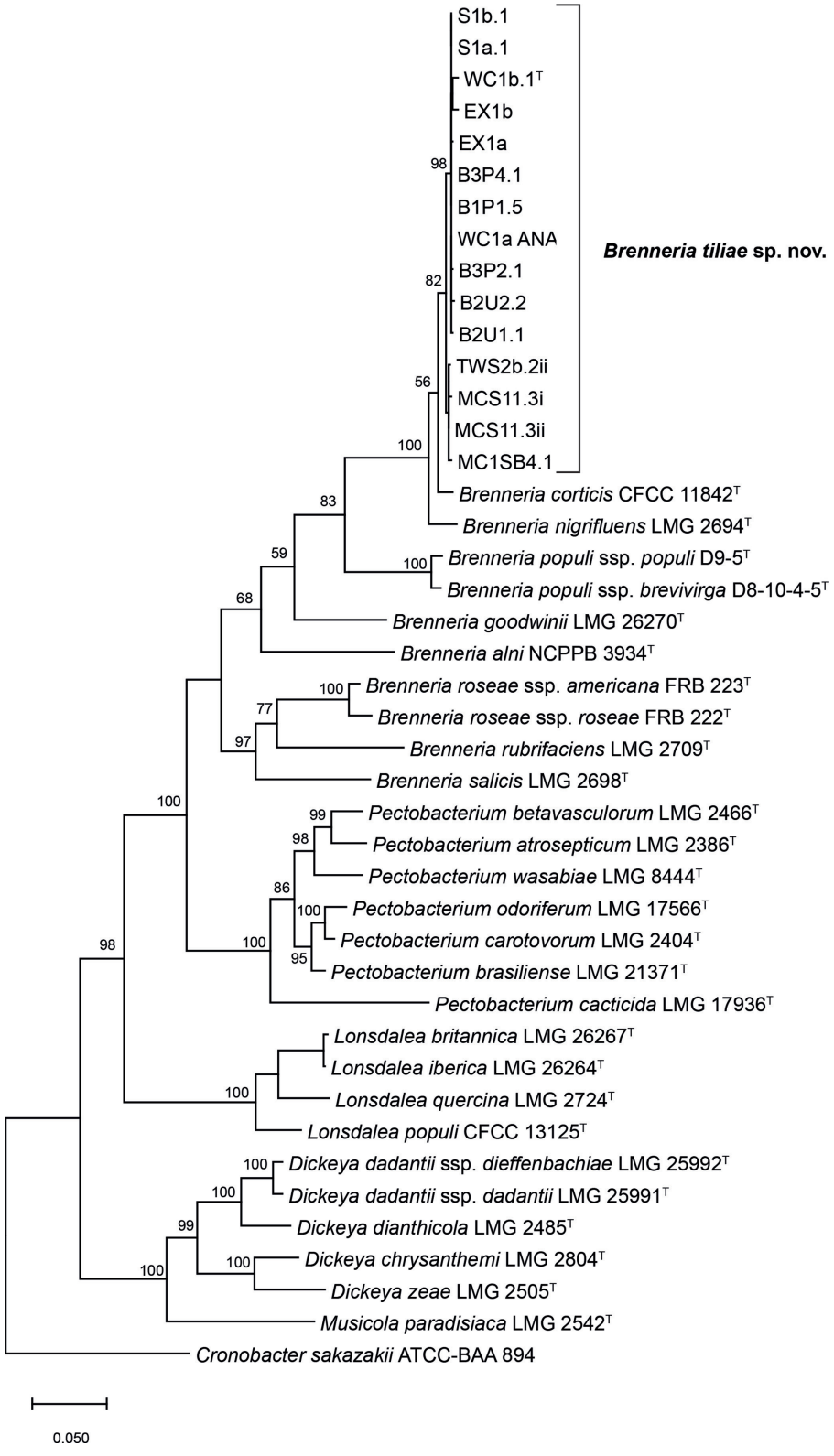

Fig. 2. Maximum-likelihood tree based on concatenated partial *gyrB*, *rpoB*, *atpD* and *infB* gene sequences of Brenneria *tiliae* sp. nov., existing *Brenneria* species and the closest phylogenetic relatives. Bootstrap values after 1000 replicates are expressed as percentages (values > 50% shown). *Cronobacter sakazakii* (ATCC-BAA 894) is included as an outgroup. The scale bar indicates the fraction of substitutions per site. T, type strain.

Table 1. DNA–DNA similarity values between Brenneria *tiliae* sp. nov. and existing species of the genus *Brenneria* based on average nucleotide identity (fastANI – lower left) and *in silico* DNA–DNA hybridization (*is*DDH – upper right)

Strains: 1, *Brenneria tiliae* WC1b.1^T (JAKPCB000000000); 2, *Brenneria tiliae* Ex1a (JAKPCA000000000); 3, *Brenneria tiliae* MC1SB4.1 (JAKPBZ000000000); 4, *Brenneria salicis* ATCC 15712^T (GCA_003315515); 5, *Brenneria alni* NCPPB 3934^T (GCA_003666245); 6, *Brenneria corticis* CFCC 11842^T (GCA_003115855); 7, *Brenneria goodwinii* FRB 141^T (GCA_002291445); 8, *Brenneria nigrifluens* DSM 30175^T (GCA_005484965); 9, *Brenneria roseae* subsp. *roseae* LMG 27714^T (GCA_003115845); 10, *Brenneria roseae* subsp. *americana* LMG 27715^T (GCA_003115815); 11, *Brenneria rubrifaciens* 6D370 (GCA_005484945). Percentages above cut-off values for species delimitation (>95% for ANI and >70% for *is*DDH) are in bold text.

Strain	1	2	3	4	5	6	7	8	9	10	11
1	**100**	**100**	**85.6**	23.7	28.5	67.6	27.4	61.6	24.9	24.9	24.4
2	**100**	**100**	**85.6**	23.7	28.5	67.6	27.4	61.7	24.9	24.9	24.4
3	**98.3**	**98.2**	**100**	23.8	28.3	67.9	27.3	61.7	24.8	24.8	24.3
4	81.7	81.6	81.6	**100**	23.8	23.7	23.7	23.8	30.3	30.2	27.6
5	84.4	84.3	84.5	81.6	**100**	28.2	27.7	28.7	24.9	24.7	24.4
6	95.9	96.0	96.0	81.6	84.4	**100**	27.5	62.8	24.8	24.6	24.1
7	83.8	83.8	83.7	81.4	84.0	83.7	**100**	27.8	26.1	25.6	24.2
8	94.9	94.9	95.0	81.7	84.6	95.0	83.8	**100**	24.6	24.5	24.3
9	82.1	82.1	82.1	85.8	82.2	82.2	82.5	81.8	**100**	72.3	31.1
10	82.0	82.0	81.9	85.6	82.1	81.9	82.5	81.9	96.6	**100**	31.0
11	81.7	81.7	81.6	84.3	82.1	81.4	81.4	81.9	85.9	85.7	**100**

The DNA–DNA similarity values generated from the whole genome comparison of the three strains are 98.2–100% for ANI and 85.6–100% for *is*DDH, confirming that they correspond to a single taxon. When the genome sequences of the proposed novel species were compared to the genomes of other *Brenneria* species, the ANI values ranged from 81.6 to 96.0% and the *is*DDH values from 23.7 to 67.9% (Table 1). All *is*DDH values are below the threshold of 70% for species delimitation, thus supporting the classification as a novel species [30]. The majority of ANI values are well below the 95 % threshold for species delimitation, however some values obtained when strains of the potential novel species were compared to *B. corticis* and *B. nigrifluens* (95.0–96.0%) fall within the 'fuzzy' zone of the species boundary [35]. Additionally, the ANI value of 95.0% between *B. corticis* and *B. nigrifluens* is significantly higher than the 84.3% initially reported [36]. In the case of *Brenneria*, it appears that the ANI species cut-off of 95% cannot be directly applied, and that ANI should be considered an integral part of the polyphasic approach but not the deciding factor for species delimitation [37]. The phylogenomic tree (Fig. 3) agrees with the topology of the MLSA phylogenetic tree, demonstrating that the novel species occupies a unique position within the genus *Brenneria* as a close relative of both *B. corticis* and *B. nigrifluens*.

MORPHOLOGICAL, PHENOTYPIC AND CHEMOTAXONOMIC CHARACTERIZATION

To determine morphological features of the bacterial cells, a light microscope and the microscopy imaging software CellSens version 1.11 (Olympus Life Science) were used, while the flagella arrangement was observed by transmission electron microscopy (FEI Tecnai 12 120kV BioTwin Spirit TEM) following negative staining. Copper grids (200 mesh) were floated on mid-log phase bacterial suspensions for 2 mins, washed three times in distilled water, stained with 3% uranyl acetate for 30 s and washed again three times before wicking away excess liquid and air drying. To investigate colony morphology the bacteria were incubated aerobically for 48 h at 28 °C followed by characterization. To assess growth at various temperatures, strains were plated onto tryptic soy agar (TSA) and incubated for 48 h at 4, 10, 28, 37 and 41 °C. To determine the salt tolerance of the strains, saline-free nutrient broth (3 g l^{-1} beef extract, 5 g l^{-1} peptone) was supplemented with increasing volumes (increments of 1% w/v) of NaCl to obtain salt concentrations from 1–10%. For pH growth conditions, sodium acetate/acetic acid and carbonate/bicarbonate buffers were added to tryptic soy broth to regulate the pH from 4 to 10. For both tests, the modified broth was inoculated with a selection of strains (WC1b.1^T, EX1a, B2U.2.2, TWS2b.2ii, MCS11.3i and MC1SB4.1) and incubated at 28 °C for 24 h with rotational shaking. Results for temperature, pH and NaCl growth ranges were determined visually. Catalase and oxidase activity were determined by bubble production in 3% v/v H_2O_2 and staining with Kovács reagent (1 % tetra-methyl-*p*-phenylenediamine dihydrochloride), respectively.

Microscopy of the strains revealed short rod-shaped cells with sizes of 0.7–1.1×1.5–2.8 µm, which were motile by means of peritrichous flagella (Fig. 4). On LB agar, colonies were glistening cream and translucent, circular with entire margins, convex and smooth with a size of 1–1.3 mm in diameter. Growth was observed at 10–41 °C, at pH 6–8 and in salt concentrations up

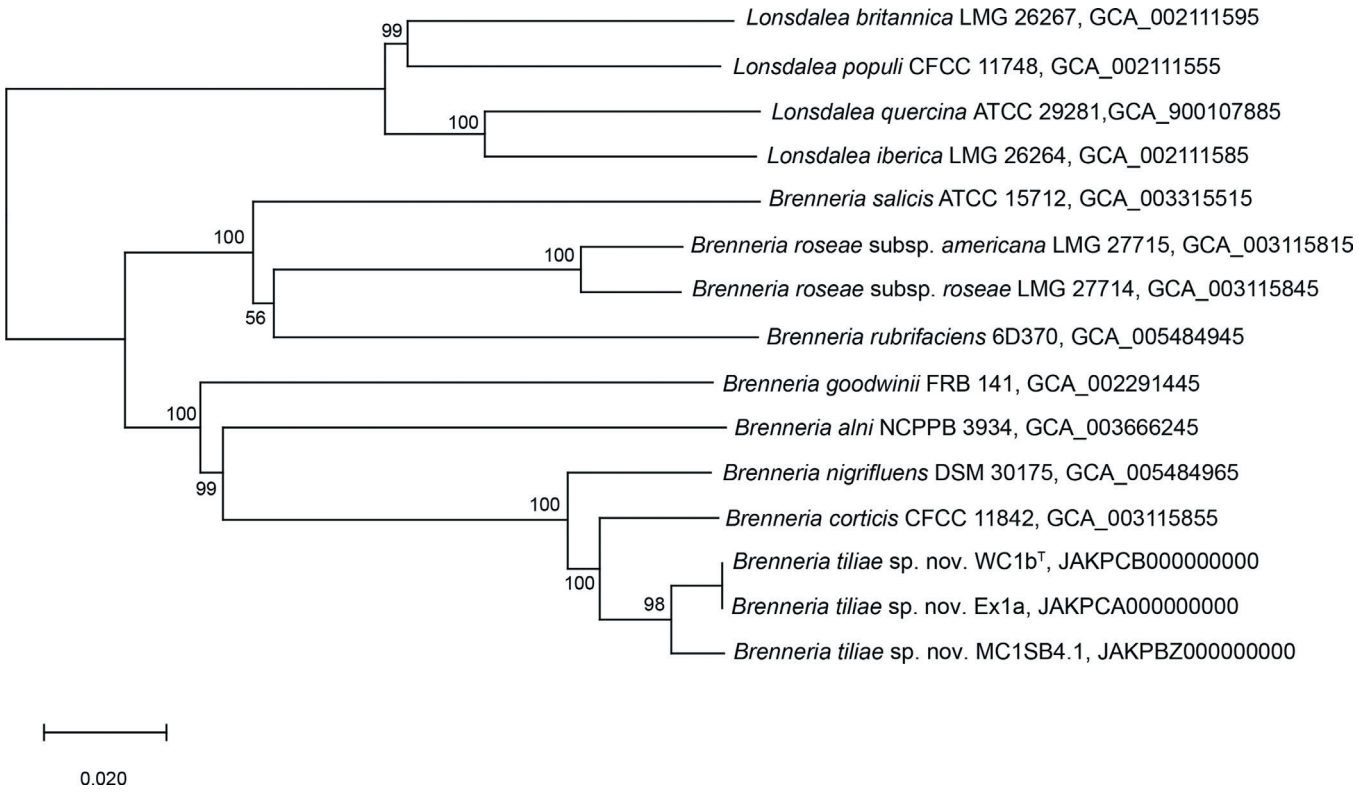

Fig. 3. Phylogenomic tree of Brenneria *tiliae* sp. nov., existing *Brenneria* species and the closest phylogenetic relatives. GBDP pseudo-bootstrap support values > 60% shown at the nodes (from 100 replicates), with an average branch support of 85.4%. The branch lengths are scaled in terms of GBDP distance formula d5 and the tree is rooted at the midpoint. T, type strain.

to 6%. All strains were oxidase-negative and catalase-positive. In contrast, the closest phylogenetic relative, *B. corticis*, has been described with one flagellum, growth was observed at 4 °C, at pH 4–11 and in salt concentrations up to 3% [36].

Phenotypic tests were performed on selected strains of the novel *Brenneria* species with *B. nigrifluens* DSM 30175^T was used as a positive control. The *B. corticis* strain is unavailable for distribution from the culture collections and was therefore not included in the comparative assays. For physiological and biochemical analysis API 20 E and API 50 CHB/E (bioMérieux) test kits were used according to the manufacturer's protocol. After inoculation with bacterial strains (WC1b.1^T, EX1a, EX1b, WC1aANA, S1a.1, S1b.1, TWS2b.2i and MC1SB4.1) the tests were incubated for 24 h (API 20E) and 48 h (API 50CHB/E) before interpretation. To investigate for differences in utilisation of carbon sources through metabolic reduction chemistry, GENIII GN/GP MicroPlates (Biolog) were inoculated with selected strains (WC1b.1^T, EX1a, EX1b and S1b.1) according to the manufacturer's instructions. The results were interpreted visually 24 h after inoculation with bacterial strains. The data was compared with that previously published for *Brenneria* species and generated under the same conditions [4, 36, 38].

The novel species shares all the common phenotypic traits that are typical for the genus *Brenneria* [2]. The phenotypic results also demonstrated that strains of the novel *Brenneria* species can be differentiated from its closest phylogenetic neighbours by reactions to acetoin, inositol, sorbitol, melibiose, glycerol, D-mannose, cellobiose, potassium 2-ketogluconate, D-serine, glucuronamide, α-hydroxy-butyric acid and α-keto-butyric acid (differentiation from *B. corticis*); meliobiose, glycerol, D-arabinose, D-galactose, inositol, amgydalin, maltose, potassium gluconate, potassium 5-ketogluconate dextrin, maltose, turanose and 3-methyl glucos (differentiation from *B. nigrifluens*). A summary of the most distinguishing characteristics suitable for differentiation of the novel *Brenneria* species from the closest relatives is presented in Table 2.

Fatty acid methyl ester (FAME) analysis was performed on strains WC1b.1^T, Ex1a.1 and MC1SB4.1 by Fera Science Ltd. (York, UK). Strains were cultivated on TSA at 28 °C for 24 h and the protocol followed was based on the Sherlock Microbial Identification System version 6.4 (MIDI). The results obtained were compared against the library RTSBA6 6.21. The FAME composition of the novel species is similar to those reported for *Brenneria* species [2], with the major fatty acids identified as $C_{16:0}$, $C_{18:1}$ *ω7c*, $C_{17:0}$ cyclo, and summed features 2 (iso-$C_{16:1}$ and/or $C_{14:0}$ 3-OH) and 3 ($C_{16:1}$ *ω7c* and /or $C_{16:1}$ *ω6c*). The complete fatty acid profile is presented in Table 3.

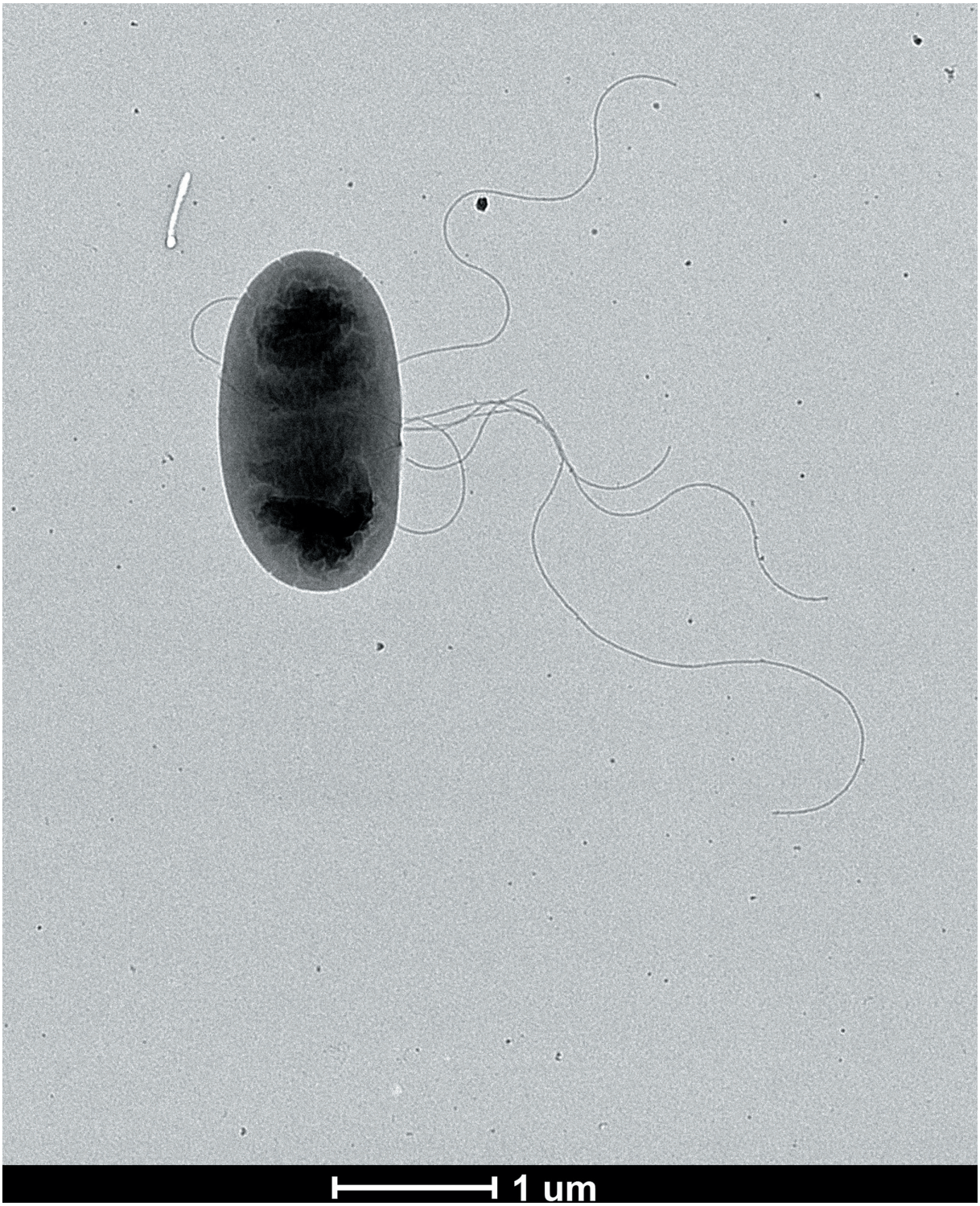

Fig. 4. Transmission electron microscopy of Brenneria *tiliae* sp. nov. displaying the peritrichous flagella arrangement. Scale bar, 1 µm.

PATHOGENICITY

Overnight cultures of two strains of the novel *Brenneria* species in LB broth were washed, re-suspended in ¼ Ringers solution and the OD adjusted to OD_{600}=1.0. Twigs (20–30 cm long) from a healthy *Tilia* × *moltkei* tree were surface sterilized using 70 % ethanol and three longitudinal incisions (3–4 cm long) per twig were made using a sterile scalpel to create bark flaps. Bacterial

Table 2. Phenotypic differences that can distinguish strains of Brenneria *tiliae* sp. nov. from those of its closest phylogenetic relatives

Strains: 1, *Brenneria tiliae* sp. nov. (*n*=6 APIs and *n*=4 for Biolog); 2, *Brenneria corticis* (*n*=1); 3, *Brenneria nigrifluens* (*n*=5 for APIs and *n*=4 for Biolog). Data for 2 and 3 taken from [36, 38]. *n*, Number of strains. +, 90–100% strains positive; –, 91–100% strains negative; v, variable; ND, not determined.

Characteristic	1	2	3
Acetoin production	–	+	–
Fermentation of (API 20E):			
Inositol	–	+	–
Sorbitol	–	+	–
Rhamnose	v	+	–
Sucrose	+	+	v
Melibiose	+	–	–
Acid production from (API 50 CBH/E):			
Glycerol	–	+	+
D-Arabinose	+	+	–
D-Galactose	+	+	–
D-Mannose	+	–	+
Inositol	–	+	+
Amygdalin	+	+	–
Cellobiose	–	+	–
Maltose	+	+	–
Potassium gluconate	+	+	–
Potassium 2-ketogluconate	–	+	–
Potassium 5-ketogluconate	+	+	–
Utilization of (Biolog):			
Dextrin	+	+	–
Maltose	+	ND	–
Cellobiose	v	ND	+
Turanose	+	ND	–
N-Acetyl-D-galactosamine	–	–	v
3-Methyl glucose	+	ND	–
1% Sodium lactate	+	ND	–
D-Serine	–	+	–
Glucuronamide	+	–	+
D-Lactic acid methyl ester	+	+	v
L-Lactic acid	+	+	v
Citric acid	+	ND	–
α-Keto-glutaric acid	–	–	v
Lithium chloride	+	ND	–
Tween 40	v	–	v
α-Hydroxy-butyric acid	–	+	–

Continued

Table 2. Continued

Characteristic	1	2	3
α-Keto-butyric acid	–	+	–
Formic acid	+	+	–
Aztreonam	–	ND	+
Sodium butyrate	v	ND	–

suspension (20 µl) was inoculated along the wound using a sterile pipette and Parafilm was used to seal the flaps. A separate twig was treated with sterile distilled water as a negative control. The tips of the twigs were sealed with wax, while the ends were placed in small volumes of water at room temperature. The inoculated twigs were covered with a plastic bag and left at room temperature (~22 °C). Twigs were assessed after 11 weeks, and bacteria from the inoculated tissue were re-isolated and the *gyrB* gene sequenced as described above. Necrotic lesions were observed on four twigs inoculated with strains EX1a and WC1a (Fig. S4), spreading outwards from the incision. A narrow area of discolouration was observed for the water control, likely due to damage from the scalpel when creating the bark flap. Back-isolation of the novel *Brenneria* species from the necrotic lesions was confirmed by partial *gyrB* gene sequencing. The extent of the role the novel *Brenneria* species plays in necrotic lesion formation is still unclear. Further pathogenicity trials on a larger scale on saplings and logs are underway.

In conclusion, the genotypic, genomic, phenotypic and chemotaxonomic results presented here clearly demonstrate that the bacterial strains isolated from bleeding cankers on *Tilia* hybrids constitute a novel *Brenneria* species, for which we propose the name *Brenneria tiliae* sp. nov.

DESCRIPTION OF *BRENNERIA TILIAE* SP. NOV.

Brenneria tiliae (ti'li.ae. L. gen. n. *tiliae*, of the lime or linden tree).

Gram-negative, cells are short, rod-shaped (0.7–1.1×1.5–2.8 µm) and motile by peritrichous flagella. Catalase-positive, oxidase-negative and facultatively anaerobic. Colonies on LB agar are glistening cream and translucent, circular with entire margins,

Table 3. Major fatty acid composition (percentage of peak areas) of Brenneria *tiliae* sp. nov. and closest phylogenetic relatives

Strains: 1, *Brenneria tiliae* sp. nov. (*n*=3); 2, *Brenneria corticis* gBX10-1-2^T; 3, *Brenneria nigrifluens* LMG 2695^T; 4, *Brenneria populi* subsp. *populi* D9-5^T; 5, *Brenneria populi* subsp. *brevivirga* D8-10-4-5^T. Data for strains 2–5 taken from [36]. *n*, number of strains.

Fatty acid	1	2	3	4	5
Saturated:					
$C_{12:0}$	3.3 (±0.1)	3.2	3.6	5.8	6.8
$C_{13:0}$	–	1.2	–	–	3.4
$C_{14:0}$	5.9 (±0.3)	3.3	6.3	4.1	3.6
$C_{16:0}$	34.2 (±0.7)	21.5	35.1	26.2	28.9
$C_{17:0}$	–	3.9	–	2.0	1.5
Unsaturated:					
$C_{17:1}$ ω8*c*	–	5.8	–	–	1.2
$C_{18:1}$ ω7*c*	11.4 (±0.3)	12.1	9.9	21.4	21.2
Cyclopropane:					
$C_{17:0}$	13.5 (±1.3)	11.3	15.1	11.7	4.7
$C_{19:0}$ ω8*c*	–	1.1	2.4	2.1	–
Summed features:					
2 ($C_{14:0}$ 3-OH and/or iso-$C_{16:1}$)	8.6 (±0.2)	8.4	11.1	9.7	7.6
3 ($C_{16:1}$ ω7*c* and/or $C_{16:1}$ ω6*c*)	20.4 (±1.5)	13.9	15.4	15.4	18.4

convex and smooth with a size of 1.0–1.3 mm in diameter. Optimum growth is at 30 °C although strains can growth at 10–41 °C, at pH 6–8 and at supplemented saline concentrations of up to 6 %.

Negative for β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H_2S production, urease, tryptophan deaminase, indole production, acetoin production and gelatinase. Citrate utilization is variable. Positive for fermentation of glucose, mannitol, sucrose, melibiose, amygdalin and arabinose. Nitrate is not reduced to nitrite or N_2 gas. Acid is produced from D-arabinose, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, D- mannitol, D-sorbitol, *N*-acetylglucosamine, amygdalin, arbutin, aesculin ferric citrate, salicin, maltose, melibiose, sucrose, trehalose, D-raffinose, gentiobiose, D-arabitol, potassium gluconate and potassium 5-ketogluconate (API 50CHB/E). Strains can utilize the following carbon sources: dextrin, maltose, trehalose, gentiobiose, sucrose, turanose, raffinose, melibiose, methyl β-D-glucoside, D-salicin, *N*-acetyl-D-glucosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, 3-methyl glucose, L-rhamnose, D-sorbitol, D-mannitol, D-arabitol, myo-inositol, glycerol, D-glucose-6-phosphate, D-fructose-6-phosphate, D-aspartic acid, L-aspartic acid, L-glutamic acid, L-serine, pectin, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, methyl pyruvate, D-lactic acid methyl ester, L-lactic acid, citric acid, D-malic acid, L-malic acid, bromo-succinic acid, acetic acid and formic acid, but variable for cellobiose, tween 40 and sodium butyrate (Biolog GEN III).

Major fatty acids are $C_{16:0}$, $C_{18:1}$ *ω7c*, $C_{17:0}$ cyclo, and summed features 2 (iso-$C_{16:1}$ and/or $C_{14:0}$ 3-OH) and 3 ($C_{16:1}$ *ω7c* and /or $C_{16:1}$ *ω6c*).

The type strain is WC1b.1^T (LMG 32575^T=NCPPB 4697^T) and was isolated from symptomatic tissue of a *Tilia* × *moltkei* tree suffering from cankers located at Westonbirt Arboretum, UK.

The DNA G+C content of the type strain is 56.5 mol% and the genome size is 5.17 Mbp.

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H.K. was involved in the conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization and writing of the work. C.B. was involved in the conceptualization, data curation, reviewing and editing of the manuscript. J.A. was involved in the reviewing and editing of the manuscript. D.A. and S.D. were responsible for funding acquisition, and reviewing and editing of the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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