Description of *Brenneria tiliae* sp. nov. isolated from symptomatic *Tilia x moltkei* and *Tilia x europaea* trees in the UK

1.1 Author names

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

1.2 Affiliation

[1] Centre for Research in Bioscience, Faculty of Health and Life Sciences, University of the West of England, Bristol, United Kingdom

[2] Harper Adams University, Newport, Shropshire, United Kingdom

[3] Centre for Ecosystems, Society and Biosecurity, Forest Research, Farnham, United Kingdom

1.3 Corresponding author

* Centre for Research in Bioscience, Faculty of Health and Life Sciences, University of the West of England, Bristol, BS16 1QY, United Kingdom

Tel: +44117 32 84225 email: carrie.brady@uwe.ac.uk

1.4 Keyword

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1.5 Repositories:

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).

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 (*is*DHH) values below the threshold for species delimitation, although the average nucleotide identity (ANI) 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 1990's, several bacterial phytopathogens belonging to the genus *Erwinia* were transferred to the novel genus *Brenneria* by Hauben *et al*. [1]. In the years following, 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* ssp. *roseae* have been isolated from oaks affected by acute oak decline (AOD) 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* x *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 United Kingdom, however the cause is currently unknown although *Phytophthora* species are suspected to play a role in some cases (S. Denman, pers. comm.). During a study to compare the cultivable bacterial microbiome of healthy and symptomatic *Tilia* hybrids [16], several strains 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 x moltkei* (WB1) located at Westonbirt Arboretum, Gloucestershire, one symptomatic *Tilia x europaea* (TW2) at Tidworth, Wiltshire, and two symptomatic *Tilia x europaea* (MC1 and MC11) at Minchinhampton, Gloucestershire (Suppl. Fig. S1). Sampling was undertaken in September 2020 and July 2021.

Strains were isolated from symptomatic tissue only, including swabs of exudate and inner bark panels (L 7-30 x W 5-15 x D 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) 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 Suppl. Table S1 for a list of strains used in this study.

Genotypic characterisation

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 v38.1 [20] followed by sequence alignment and trimming of overhangs using MEGA X v10.2.5 [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 reference in the phylogenetic analyses. Maximum likelihood phylogenetic analysis with 1000 bootstrap replicates was performed in PhyML 3.0 [22], following Smart Model Selection (SMS) [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 share the highest pairwise sequence similarity with *B. nigrifluens* (99.7 – 99.8 %) and *B. corticis* (98.7 %). In the 16S rRNA gene phylogenetic tree (Suppl. 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 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* ssp*. roseae* FRB 222^T and *B. rubifaciens* DSM 4483^T. The fingerprinting results (Suppl. 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 SPRI (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 hybridisation (*is*DHH) values were determined using the Genome-to-Genome Distance Calculator 3.0 (GGDC) [30, 31]. A phylogenomic tree was constructed using the Type (Strain) Genome Server (TYGS) [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 (SPR) 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 the 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 – 56.5 mol % for the three genomes. Genome features and assembly accession numbers are listed in Suppl. Table S2.

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 – 96.0 % and the *is*DDH values from 23.7 – 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 characterisation

To determine morphological features of the bacterial cells, a light microscope and the microscopy imaging software CellSens version 1.11 (Olympus Life Science, Tokyo, Japan) 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 3 times in distilled water, stained with 3 % uranyl acetate for 30 sec and washed again 3 times before wicking away excess liquid and air drying. To investigate colony morphology the bacteria were incubated aerobically for 48 hours at 28°C followed by characterisation. To assess growth at various temperatures, strains were plated onto Tryptic Soy Agar (TSA) and incubated for 48 hours 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 (TSB) to regulate the pH from 4 -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 hours 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 ranging from 0.7 – 1.1 x 1.5 – 2.8 µm, which are motile by means of peritrichous flagella (Fig. 4). On LB agar, colonies are 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 to 6 %. All strains are 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* sp. 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, D-cellobiose, potassium 2-ketogluconate, D-serine, glucuronamide, α-hydroxy-butyric acid, α-keto-butyric acid (differentiation from *B. corticis*); meliobiose, glycerol, D-arabinose, D-galactose, inositol, amgydalin, D-maltose, potassium gluconate, potassium 5-

ketogluconate dextrin, D-maltose, D-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 Inc.). 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 $C_{16:0}$, $C_{18:1}\omega7c$, $C_{17:0}$ cyclo, and summed features 2 (iso-$C_{16:1}$ and/or $C_{14:0}$ 3-OH) and 3 ($C_{16:1}$ ω7*c* and /or $C_{16:1}$ ω6*c*). The complete fatty acid profile is presented in Table 3.

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 (L 20-30 cm) from a healthy *Tilia x moltkei* tree were surface sterilised using 70 % ethanol and three longitudinal incisions (L 3-4 cm) per twig were made using a sterile scalpel to create bark flaps. 20 µL of bacterial suspension 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 (Suppl. 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 x 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, 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 utilisation 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, esculin ferric citrate, salicin, D-maltose, D-melibiose, D-saccharose, D-trehalose, D-raffinose, gentiobiose, D-arabitol, potassium gluconate and potassium 5-ketogluconate (API 50CHB/E). Strains can utilise the following carbon sources: dextrin, D-maltose, D-trehalose, gentiobiose, sucrose, D-turanose, D-raffinose, D-melibiose, β-methyl-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 D-cellobiose, tween 40 and sodium butyrate (Biolog GEN III).

Major fatty acids are $C_{16:0}$, $C_{18:1}\omega7c$, $C_{17:0}$ cyclo, and summed features 2 (iso-$C_{16:1}$ and/or $C_{14:0}$ 3-OH) and 3 ($C_{16:1}$ ω7*c* and /or $C_{16:1}$ ω6*c*).

The type strain is WC1b.1^T = LMG 32575^T = NCPPB 4697^T and was isolated from symptomatic tissue of a *Tilia x 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.

AUTHOR STATEMENTS

1.6 Authors and contributors

HK was involved in the conceptualisation, data curation, formal analysis, investigation, methodology, validation, visualisation and writing of the work. CB was involved in the conceptualisation, data curation, reviewing and editing of the manuscript. JA was involved in the reviewing and editing of the manuscript. DA and SD were responsible for funding acquisition, and reviewing and editing of the manuscript.

1.7 Conflicts of interest

The authors declare that there are no conflicts of interest.

1.8 Funding information

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1.9 Ethical approval

None

1.10 Consent for publication

None

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ABBREVIATIONS

WB1 = *Tilia x moltkei* located at Westonbirt Arboretum

TW2 = *Tilia x europaea* located at Tidworth Garrison

MC1 = *Tilia x europaea* located at Holy Trinity Church, Minchinhampton

MC11 = *Tilia x europaea* located at Holy Trinity Church, Minchinhampton

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FIGURES AND TABLES

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 hybridisation (*is*DDH – upper right). Percentages above cut-off value for species delimitation (>95 % for ANI and >70 % for *is*DDH) are shaded.

*is*DDH / FastANI	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

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463 1 = *Brenneria tiliae* WC1b.1^T (JAKPCB000000000), 2 = *Brenneria tiliae* Ex1a (JAKPCA000000000), 3 =
464 *Brenneria tiliae* MC1SB4.1 (JAKPBZ000000000), 4 = *Brenneria salicis* ATCC 15712^T (GCA_003315515),
465 5 = *Brenneria alni* NCPPB 3934^T (GCA_003666245), 6 = *Brenneria corticis* CFCC 11842^T
466 (GCA_003115855), 7 = *Brenneria goodwinii* FRB 141^T (GCA_002291445), 8 = *Brenneria nigrifluens* DSM
467 30175^T (GCA_005484965), 9 = *Brenneria roseae* ssp. *roseae* LMG 27714^T (GCA_003115845), 10 =
468 *Brenneria roseae* ssp. *americana* LMG 27715^T (GCA_003115815), 11 = *Brenneria rubrifaciens* 6D370
469 (GCA_005484945)

470 ^{T} = type strain.

471

472 **Table 2:** Phenotypic differences that can distinguish *Brenneria tiliae* sp. nov. from its closest
473 phylogenetic relatives

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475 1 = *Brenneria tiliae* sp. nov. (*n* = 6 APIs and *n* = *4* for Biolog), 2 = *Brenneria corticis* (*n*= 1) and 3 =
476 *Brenneria nigrifluens* (*n* = 5 for APIs and *n*= 4 for Biolog). Data for 2 and 3 taken from [36, 38]. *n* =
477 number of strains.

478

Characteristic	**1**	**2**	**3**
Acetoin production	-	+	-
Fermentation of (API 20E):			
Inositol	-	+	-
Sorbitol	-	+	-
Rhamnose	v	+	-
Saccharose	+	+	v
Melibiose	+	-	-
Acid production from (API 50 CBH/E):			
Glycerol	-	+	+
D-Arabinose	+	+	-

D-Galactose	+	+	-
D-Mannose	+	-	+
Inositol	-	+	+
Amygdalin	+	+	-
D-Cellobiose	-	+	-
D-Maltose	+	+	-
Potassium Gluconate	+	+	-
Potassium 2-Ketogluconate	-	+	-
Potassium 5-Ketogluconate	+	+	-
Utilisation of (Biolog):			
Dextrin	+	+	-
D-Maltose	+	nd	-
D-Cellobiose	v	nd	+
D-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	-	+	-
α-keto-butyric acid	-	+	-
Formic acid	+	+	-
Aztreonam	-	nd	+
Sodium butyrate	v	nd	-

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480 +, 90 – 100 % strains +; -, 91 – 100 % strains -; v, variable; nd, not determined

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

1 = *Brenneria tiliae* sp. nov. (*n* = 3), 2 = *Brenneria corticis* gBX10-1-2^T, 3 = *Brenneria nigrifluens* LMG 2695^T, 4 = *Brenneria populi* ssp. *populi* D9-5^T, 5 = *Brenneria populi* ssp. *brevivirga* D8-10-4-5^T

Data for 2 -5 taken from [36]. *n* = number of strains.

Fatty acid	**1**	**2**	**3**	**4**	**5**
Saturated fatty acids					
$C_{12:0}$	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 fatty acids					
$C_{17:1}$ ω8*c*	-	5.8	-	-	1.2
$C_{18:1}$ ω7*c*	11.4 (± 0.3)	12.1	9.9	21.4	21.2
Cyclopropane fatty acids					
$C_{17:0}$	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

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

a)

b)

Figure 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

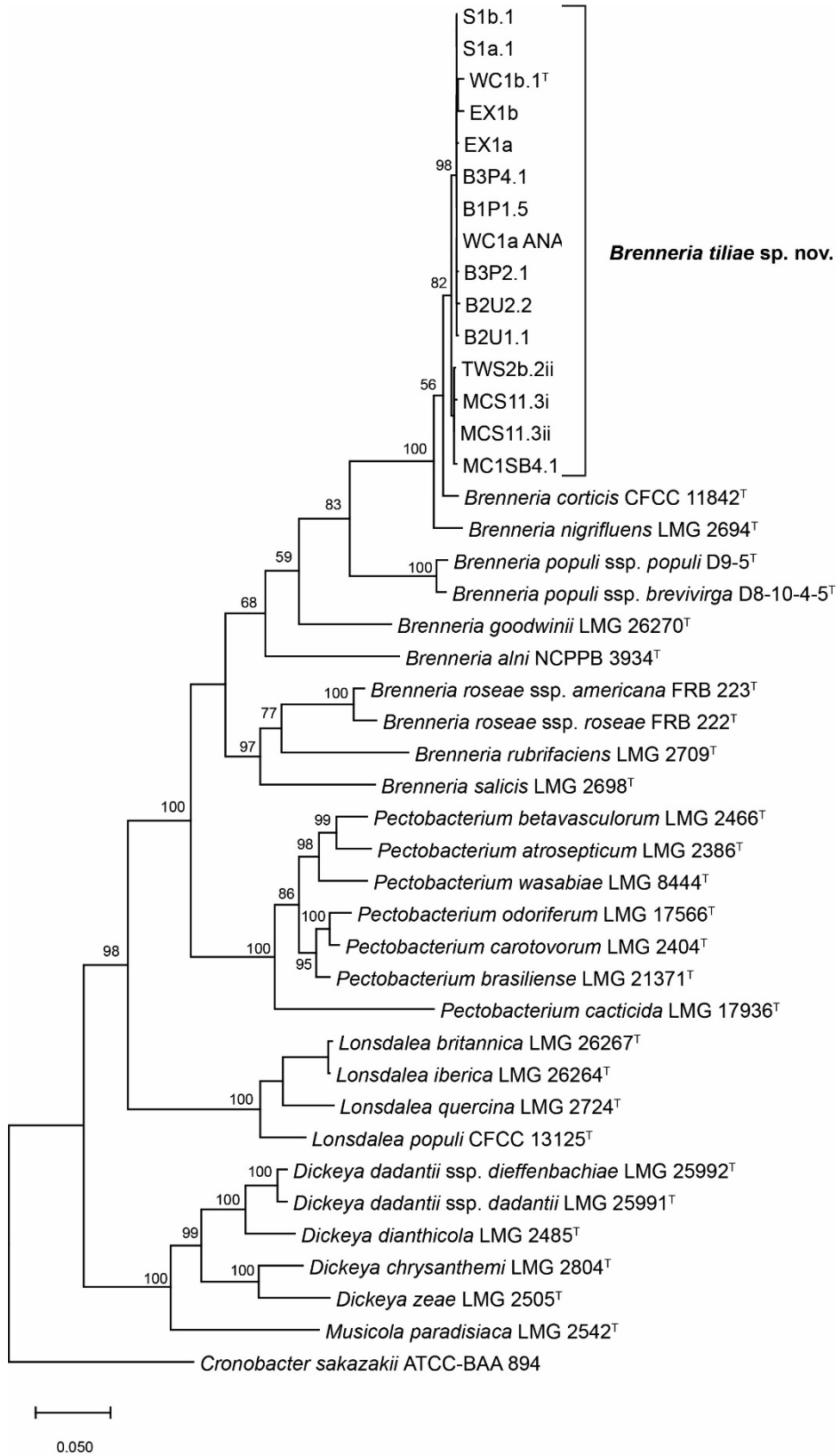


Figure 3: Phylogenomic tree of *Brenneria tiliae* sp. nov., existing *Brenneria* species and the closest phylogenetic relatives. GBDP pseudo-bootstrap support values > 60 % shown at the nodes (from 100 replicates), with an average branch support of 85.4 %. The branch lengths are scaled in terms of GBDP distance formula d_5 and the tree is rooted at the midpoint. ^{T} = type strain

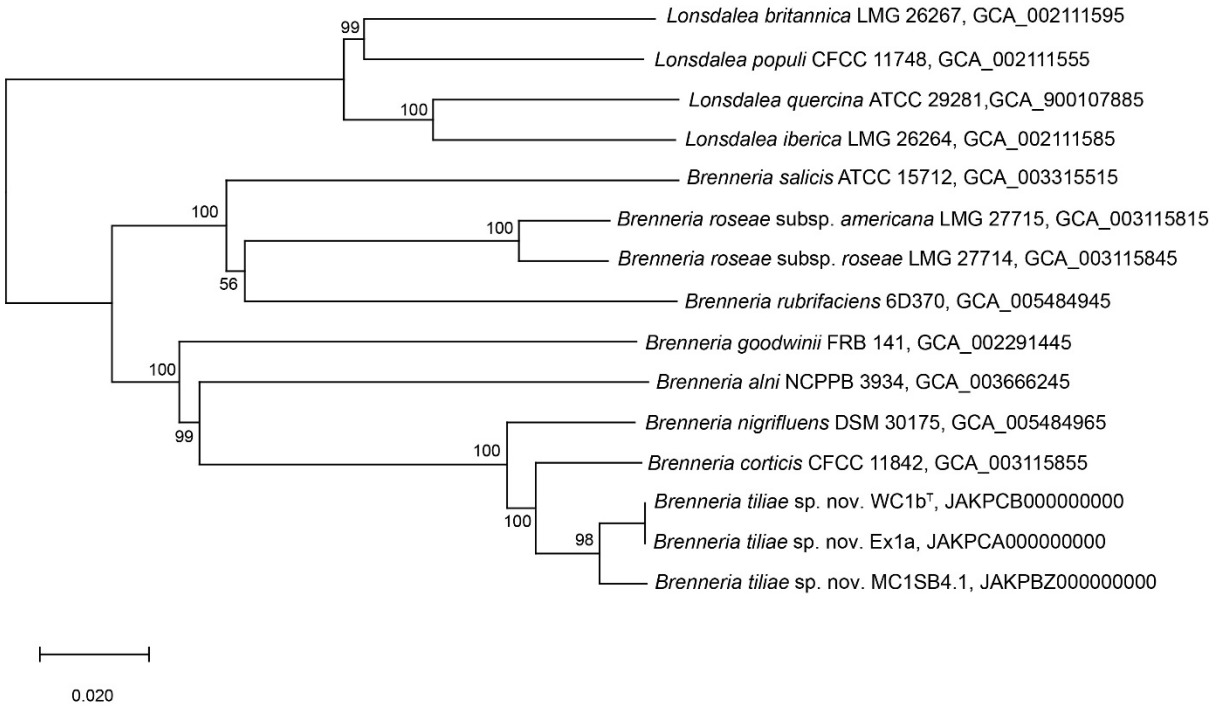
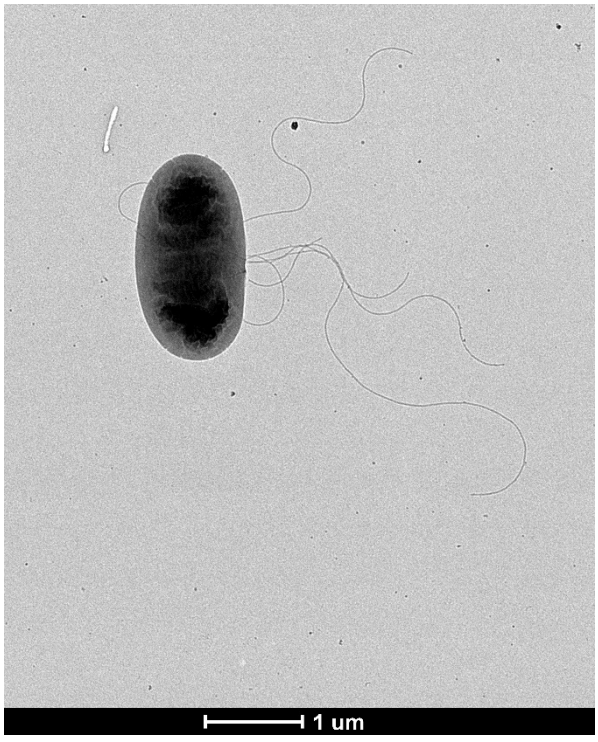


Figure 4: Transmission electron microscopy of *Brenneria tiliae* sp. nov. displaying the peritrichous flagella arrangement. Scale bar, 1 µm.



599 **Supplementary Table S1:** Strains of *Brenneria tiliae* sp. nov. investigated in this study

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Strain	**Location**	**Year of isolation**	**Source**	***gyrB****	***rpoB****	***infB****	***atpD****
WC1b.1^{T}	Westonbirt Arboretum, Gloucestershire, UK	2020	*Tilia* x *moltkei*, outer bark wood chip bleeding lesion	OM523122	OM523152	OM523137	OM523107
Ex1a	Westonbirt Arboretum, Gloucestershire, UK	2020	*Tilia* x *moltkei*, exudate from bleeding lesion	OM523119	OM523149	OM523134	OM523104
Ex1b	Westonbirt Arboretum, Gloucestershire, UK	2020	*Tilia* x *moltkei*, exudate from bleeding lesion	OM523120	OM523150	OM523135	OM523105
WC1aANA	Westonbirt Arboretum, Gloucestershire, UK	2020	*Tilia* x *moltkei*, outer bark wood chip bleeding lesion	OM523121	OM523151	OM523136	OM523106
S1a.1	Westonbirt Arboretum, Gloucestershire, UK	2020	*Tilia* x *moltkei*, swab from bleeding lesion	OM523123	OM523153	OM523138	OM523108
S1b.1	Westonbirt Arboretum, Gloucestershire, UK	2020	*Tilia* x *moltkei*, swab from bleeding lesion	OM523124	OM523154	OM523139	OM523109
B1P1.5	Westonbirt Arboretum, Gloucestershire, UK	2021	*Tilia* x *moltkei*, inner bark necrotic lesion	OM523125	OM523155	OM523140	OM523110
B2U1.1	Westonbirt Arboretum, Gloucestershire, UK	2021	*Tilia* x *moltkei*, inner bark necrotic lesion	OM523126	OM523156	OM523141	OM523111
B2U2.2	Westonbirt Arboretum, Gloucestershire, UK	2021	*Tilia* x *moltkei*, inner bark necrotic lesion	OM523127	OM523157	OM523142	OM523112

B3P2.1	Westonbirt Arboretum, Gloucestershire, UK	2021	*Tilia* x *moltkei*, inner bark necrotic lesion	OM523128	OM523158	OM523143	OM523113
B3P4.1	Westonbirt Arboretum, Gloucestershire, UK	2021	*Tilia* x *moltkei*, inner bark necrotic lesion	OM523129	OM523159	OM523144	OM523114
TWS2b.2ii	Tidworth, Wiltshire, UK	2021	*Tilia* x *europaea*, swab from bleeding lesion	OM523132	OM523162	OM523147	OM523117
MC1SB4.1	Minchinhampton, Gloucestershire, UK	2021	*Tilia* x *europaea*, swab from bleeding lesion	OM523133	OM523163	OM523148	OM523118
MCS11.3i	Minchinhampton, Gloucestershire, UK	2021	*Tilia* x *europaea*, swab from bleeding lesion	OM523130	OM523160	OM523145	OM523115
MCS11.3.ii	Minchinhampton, Gloucestershire, UK	2021	*Tilia* x *europaea*, swab from bleeding lesion	OM523131	OM523161	OM523146	OM523116

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602 ^{T} = type strain

603 * GenBank accession numbers

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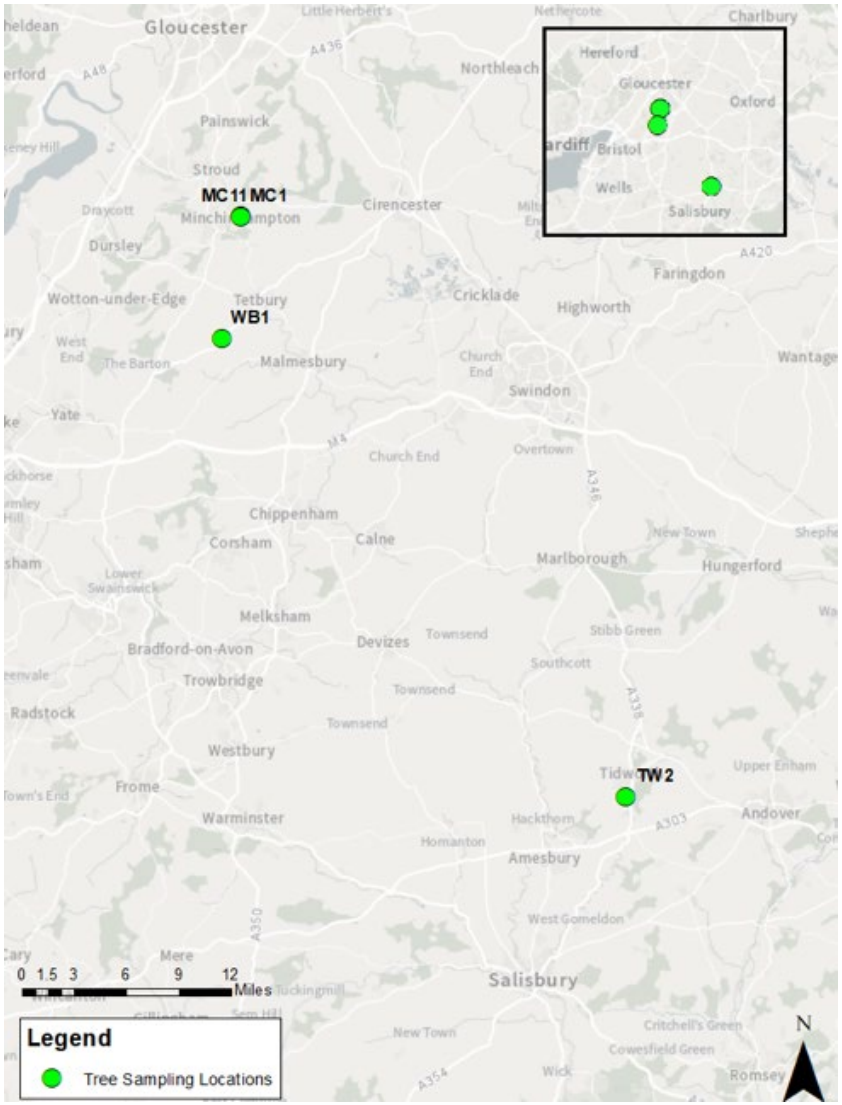
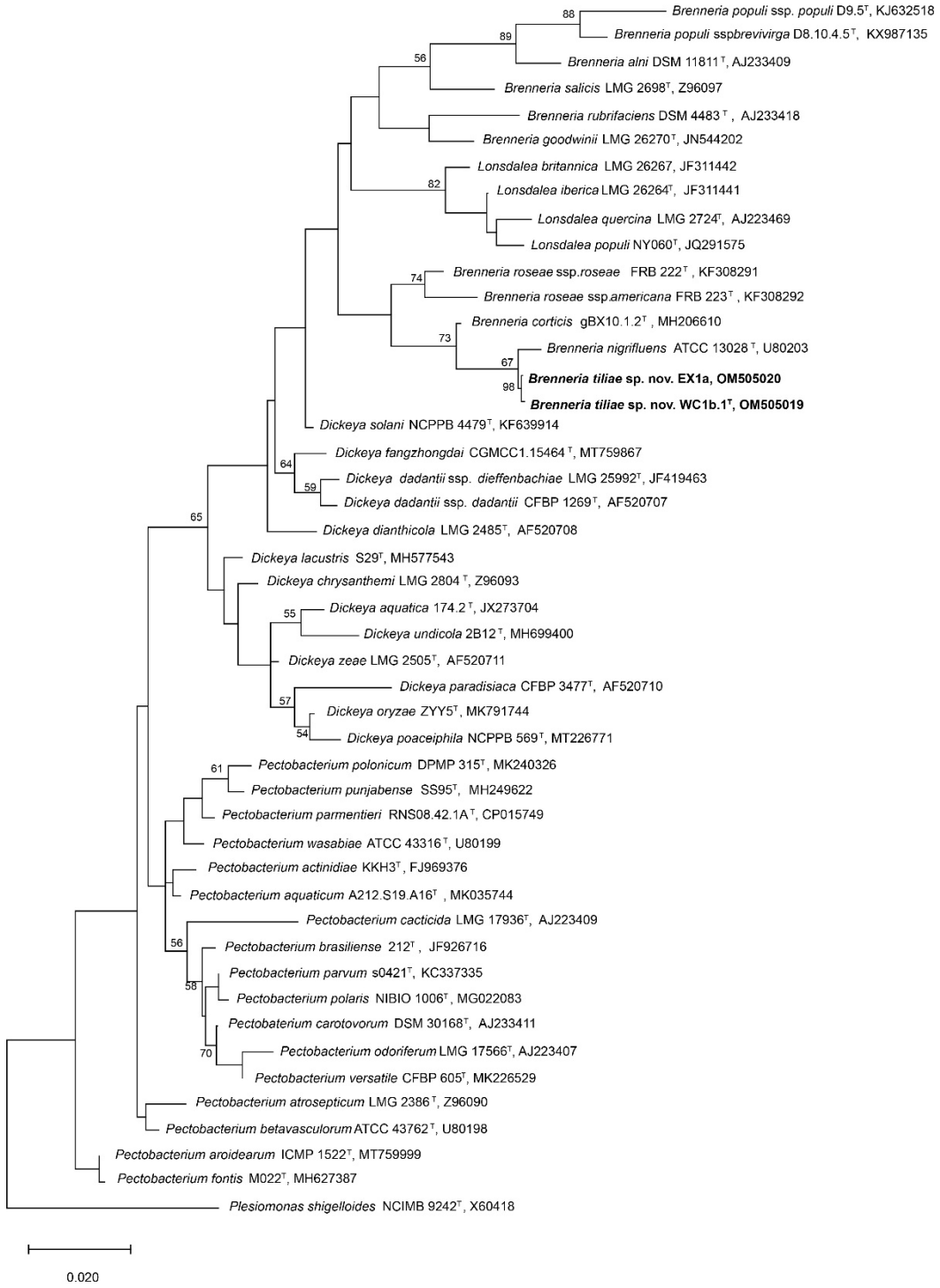
Supplementary Table S2: Whole genome sequence information of strains of *Brenneria tiliae* sp. nov. investigated in this study

Strain	Genbank accession number	Biosample	Size (Mbp)	Number of contigs (with PEGs)	N50	Number of coding sequences	Number of RNAs	GC content (mol %)
WC1b.1^T	JAKPCB000000000	SAMN25749586	5.17	152	273 546	5008	88	56.5
Ex1a	JAKPCA000000000	SAMN25749587	5.16	132	314 842	4992	90	56.5
MC1SB4.1	JAKPBZ000000000	SAMN25749588	5.25	118	313 598	5128	93	56.3

Supplementary Figure S1: Location of the symptomatic *Tilia* trees from where the novel strains were isolated. The four trees were located at Westonbirt Arboretum (WB1), Minchinhampton (MC1 and MC11) and Tidworth (TW2). The map was created using ArcGIS Desktop 10.5.



Supplementary Figure S2: Maximum likelihood tree based on the almost complete 16S rRNA gene sequences (1344 bp) of *Brenneria tiliae* sp. nov., existing *Brenneria* species and closest phylogenetic relatives**.** Bootstrap support values higher than 50 % after 1000 replicates are shown next to the branches and *Plesiomonas shigelloides* was used as an outgroup. The fraction of substitutions per site is indicated by the scale bar.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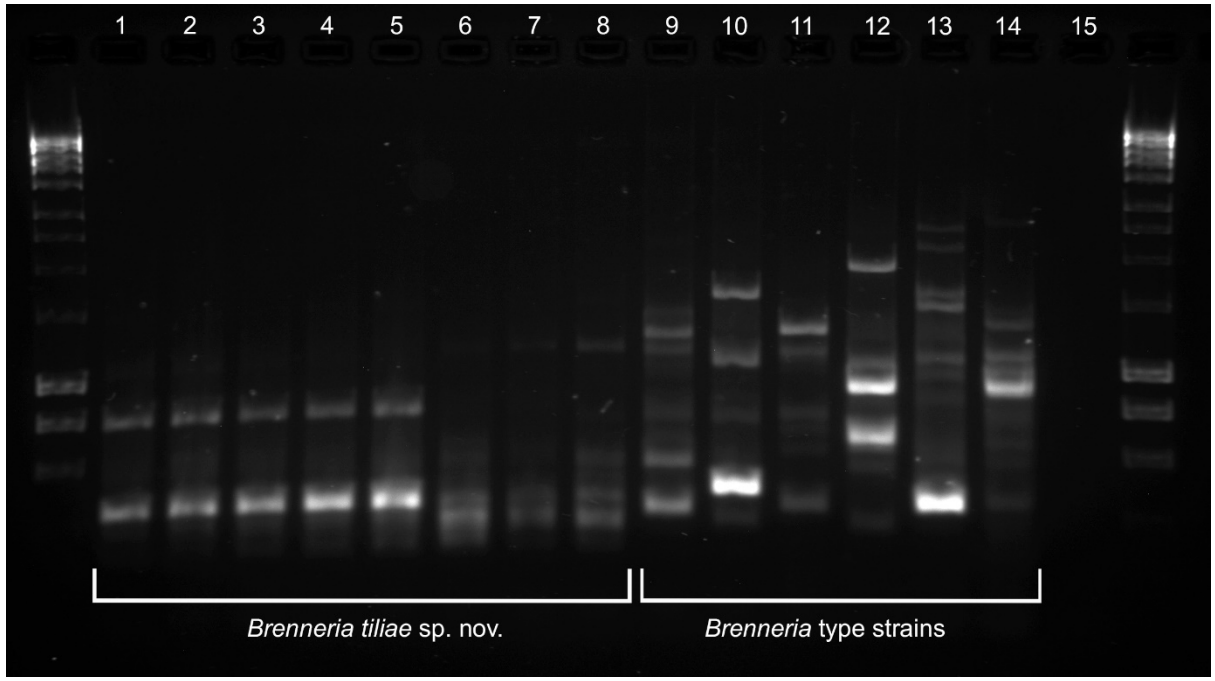
Brenneria populi ssp. populi D9.5T, KJ632518
Brenneria populi sspbrevivirga D8.10.4.5T, KX987135
Brenneria alni DSM 11811T, AJ233409
Brenneria salicis LMG 2698T, Z96097
Brenneria rubrifaciens DSM 4483T, AJ233418
Brenneria goodwinii LMG 26270T, JN544202
Lonsdalea britannica LMG 26267, JF311442
Lonsdalea iberica LMG 26264T, JF311441
Lonsdalea quercina LMG 2724T, AJ223469
Lonsdalea populi NY060T, JQ291575
Brenneria roseae ssp.roseae FRB 222T, KF308291
Brenneria roseae ssp.americana FRB 223T, KF308292
Brenneria corticis gBX10.1.2T, MH206610
Brenneria nigrifluens ATCC 13028T, U80203
Brenneria tiliae sp. nov. EX1a, OM505020
Brenneria tiliae sp. nov. WC1b.1T, OM505019
Dickeya solani NCPPB 4479T, KF639914
Dickeya fangzhongdai CGMCC1.15464T, MT759867
Dickeya dadantii ssp. dieffenbachiae LMG 25992T, JF419463
Dickeya dadantii ssp. dadantii CFBP 1269T, AF520707
Dickeya dianthicola LMG 2485T, AF520708
Dickeya lacustris S29T, MH577543
Dickeya chrysanthemi LMG 2804T, Z96093
Dickeya aquatica 174.2T, JX273704
Dickeya undicola 2B12T, MH699400
Dickeya zeae LMG 2505T, AF520711
Dickeya paradisiaca CFBP 3477T, AF520710
Dickeya oryzae ZYY5T, MK791744
Dickeya poaceiphila NCPPB 569T, MT226771
Pectobacterium polonicum DPMP 315T, MK240326
Pectobacterium punjabense SS95T, MH249622
Pectobacterium parmentieri RNS08.42.1AT, CP015749
Pectobacterium wasabiae ATCC 43316T, U80199
Pectobacterium actinidiae KKH3T, FJ969376
Pectobacterium aquaticum A212.S19.A16T, MK035744
Pectobacterium cacticida LMG 17936T, AJ223409
Pectobacterium brasiliense 212T, JF926716
Pectobacterium parvum s0421T, KC337335
Pectobacterium polaris NIBIO 1006T, MG022083
Pectobaterium carotovorum DSM 30168T, AJ233411
Pectobacterium odoriferum LMG 17566T, AJ223407
Pectobacterium versatile CFBP 605T, MK226529
Pectobacterium atrosepticum LMG 2386T, Z96090
Pectobacterium betavasculorum ATCC 43762T, U80198
Pectobacterium aroidearum ICMP 1522T, MT759999
Pectobacterium fontis M022T, MH627387
Plesiomonas shigelloides NCIMB 9242T, X60418
0.020

Supplementary Figure S3: ERIC-PCR fingerprinting band patterns of *Brenneria tiliae* sp. nov. compared to the type strains of existing *Brenneria* species. Lanes: (1) Ex1a, (2) WC1b.1^{T}, (3) S1a.1, (4) B3P2.1, (5) B2U2.2, (6) TWS2b.2ii, (7) MC1SB4.1, (8) MCS11.3i, (9) *B. salicis* DSM 30166^{T}, (10) *B. alni* DSM 11811^{T}, (11) *B. goodwinii* FRB 14I^{T}, (12) *B. nigrifluens* DSM 30175^{T}, (13) *B. roseae* ssp. *roseae* FRB 222^{T}, (14) *B. rubifaciens* DSM 4483^{T} and (15) negative control. Hyperladder 1kb (Bioline)



Supplementary Figure S4: Pathogenicity testing of *Brenneria tiliae* sp. nov. isolated from a symptomatic *Tilia x moltkei.* Twigs of a healthy *Tilia x moltkei* at Westonbirt Arboretum were inoculated with the potential pathogen by creating a bark flap followed by sealing of the wound after inoculation. a) Necrosis of the innerbark of twigs inoculated with strains of *B. tiliae*. b) Wound response of the innerbark of twigs inoculated with sterile water.

a)

b)