# *Pseudomonas daroniae* sp. nov. and *Pseudomonas dryadis* sp. nov. isolated from pedunculate oak affected by Acute Oak Decline in the United Kingdom.

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The GenBank/EMBL/DDBJ accession numbers are as follows: MK159357 - MK159378 (16S rRNA), MK293898 - MK293919 (*gyrB*), MK293920 - MK293941 (*rpoB*), MK293876-MK293897 (*rpoD*) and QJUH00000000 - QJUN00000000 (whole genome).

## ABSTRACT

Twenty-two cream-coloured bacterial strains were isolated from oak trees affected by acute oak decline (AOD) in Southern England. Isolates were Gram-negative, motile, slightly curved rods, aerobic, non-spore-forming, and catalase and oxidase positive. 16S rRNA gene sequence analysis placed the strains in two separate phylogenetic clusters in the *Pseudomonas straminea* group, with *Pseudomonas flavescens* as the closest phylogenetic relative. Multilocus sequence analyses (MLSA) of the genes *gyrB*, *rpoD* and *rpoB* supported the delineation of the strains into two separate taxa, which could be differentiated phenotypically and chemotaxonomically from each other, and their closest relatives. Average nucleotide identity (ANI) and *in silico* DNA-DNA hybridisation values revealed percentages of genome similarity below the species threshold (95% and 70% respectively) between the strains and the closest relatives, confirming their novel species status. Therefore, on the basis of this polyphasic approach we propose two novel *Pseudomonas* species, *Pseudomonas daroniae* sp. nov. (type strain FRB 228$^T$ = LMG 31087$^T$ = NCPPB 4672$^T$) and *Pseudomonas dryadis* sp. nov. (type strain FRB 230$^T$ = LMG 31087$^T$ = NCPPB 4673$^T$).

Bacteria are consistently isolated from pedunculate oak trees (*Quercus robur*) affected by acute oak decline (AOD) in the United Kingdom [1]. AOD has been described as a polymicrobial syndrome that causes vertical cracks in the bark, from which dark fluid emanates. The bacterial communities isolated from AOD-affected oaks at different sites in the UK show a significantly similar composition pattern, where *Brenneria goodwinii* and *Gibbsiella quercinecans* appear to be the predominant bacterial species [1]. In addition to these species, isolates identified as belonging to the genus *Pseudomonas* by 16S rRNA gene and *gyrB* sequencing have been consistently isolated from AOD-affected inner bark, and also in larval galleries created in the phloem by the buprestid beetle *Agrilus biguttatus* [2].

Species belonging to the genus *Pseudomonas* [3] are ubiquitous microorganisms commonly isolated from practically all environmental niches (such as water, soil and air), in addition to those coexisting, or causing disease in humans, plants and animals. They are aerobic, Gram-negative bacilli and motile by means of polar or peritrichous flagella. The genus *Pseudomonas* is a heterogeneous taxon with an increasing number of species [4]. The current classification approach for novel species of the genus *Pseudomonas* is polyphasic and includes genotypic, phenotypic, and chemotaxonomic characterisation of bacterial isolates. In the present study,

16S rRNA gene sequence analysis was performed, along with multilocus sequence analysis (MLSA) of the concatenated nucleotide sequences of the *gyrB*, *rpoD* and *rpoB* genes. The phylogenetic analyses were supported by whole genome relatedness values, and PCR-based fingerprinting methods were used to assess the genetic diversity of the bacterial isolates. Moreover, hypersensitivity reactions against a plant host were investigated.

Based on the data obtained from this polyphasic study, the AOD-associated bacterial strains constitute two novel species in the genus *Pseudomonas* for which we propose the names *Pseudomonas daroniae* sp. nov. and *Pseudomonas dryadis* sp. nov.

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## Isolation

Bacterial strains were isolated from the inner bark and sapwood, as well as galleries created in the phloem by *Agrilus biguttatus.* Oak trees were located in three sites in the United Kingdom: in two deciduous woodlands: Bisham Woods (51.54911°N, 0.77071°W) and Great Monk Wood (51.89955°N, 000.64606°E), and in the Royal Botanic Gardens, Kew. A list of strains used in this study are presented in Suppl. Table S1. Strains were initially isolated in potato yeast glucose agar, and routinely cultured at 25°C on King's B agar (KB) (Oxoid, UK) and in Luria Bertrani (LB) broth.

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## Genotypic characterisation

Genomic DNA was extracted following an alkali lysis method [5]. Amplification and sequencing of the 16S rRNA, *gyrB*, *rpoD* and *rpoB* genes for each of the 22 strains was as previously described [6–8]. After alignment and trimming, the final sequence lengths were as follows: 16S rRNA – 1399 bp, *gyrB* – 759 bp, *rpoD* – 636 bp, and *rpoB* – 750 bp. The 16S rRNA gene sequences for each of the 22 strains were queried against the EZBioCloud 16S rRNA database [9]. The pairwise similarities obtained for the strains were 98.73 – 99.23% to *P. flavescens*, 98.66 - 99.23% to *P. seleniipraecipitans*, 98.31 - 98.87% to *P. punonensis* 98.1 - 98.73% to *P. straminea* and 97.96 - 98.66% to *P. argentinensis*. Values of greater than 95% were observed to other members of the genus. For phylogenetic comparisons, the 16S rRNA gene sequences of the 36 closest related strains were downloaded from EzBioCloud. Sequences of *gyrB*, *rpoD* and *rpoB* from the closest phylogenetic neighbours, as determined by EzBioCloud and BLAST [10], were downloaded from the GenBank database (Suppl. Table S2) for analysis of the concatenated gene datasets. All sequences were aligned and trimmed in BioEdit 7.2.5 [11] and *gyrB*, *rpoD* and *rpoB* gene datasets were concatenated in

SequenceMatrix [12]. Nucleotide sequences were translated to amino acid sequences with BioEdit.

Maximum likelihood (ML) and Bayesian Inference (BI) trees were constructed in PhyML [13] and MrBayes [14], respectively. The best-fit nucleotide substitution model – based on the Akaike information criterion (AIC) – was selected by the software Smart Model Selection (SMS) [15] in ML trees, and by JModelTest [16] in BI trees. The general time reversible model with invariable sites and gamma distribution (GTR+I+G) was the best substitution model for the 16S rRNA gene ML and BI phylogenetic trees, as well as for the concatenated (*gyrB, rpoD* and *rpoB*) ML tree. The best-fit nucleotide substitution model for each individual protein-encoding gene alignment and the concatenated alignment for the BI tree was the transitional model with invariable sites and gamma distribution (TIM2+I+G). The best substitution model for the ML tree based in amino acid concatenated sequences was: LG +G+I+F. "+F" indicates the use of the empirical amino acid distribution in the analysed alignment, instead of the model default distribution; and being "LG" the name of the model, given after the authors [17].

Phylogenetic inference was tested 1,000 times by the bootstrap method in all ML trees, and bootstrap values less than 50% were not included in the trees. The branch-support values in BI, or posterior probabilities (PP) [18, 19] were determined by Markov Chain Monte Carlo sampling. Four chains were run simultaneously 10,000,000 times, one in every 1,000 trees was sampled, and the first 1,000 trees were discarded whilst the other 9,000 were used to calculate the PP. Values of PP lower than 0.5 are not included in the BI trees. *Acinetobacter baumannii* DSM 30007$^{T}$ was chosen as outgroup in both 16S rRNA analyses, and *P. pertucinogena* LMG 1874$^{T}$ was the outgroup for all concatenated nucleotide and amino acid phylogenetic analyses.

The topology of both the ML and BI 16S rRNA phylogenetic trees was mostly congruent (Fig. 1 and Suppl. Fig. S1). The type strains of both proposed novel species, FRB 228$^{T}$ and FRB230$^{T}$, formed their own cluster within the *P. straminea* group on the border of the *P. fluorescens* lineage [20] with 83% bootstrap support in the ML tree, but lacking support in the BI tree. The closest relative, according to the 16S rRNA phylogenetic analyses, is *P. flavescens* although without significant bootstrap or PP support.

MLSA based on the protein-coding genes *gyrB, rpoD* and *rpoB* have proven useful for examining phylogenetic relationships within the genus *Pseudomonas* [21, 22]. To further

clarify the taxonomic position of the *Pseudomonas* strains isolated from AOD-affected oak, MLSA of the *gyrB, rpoD* and *rpoB* genes was performed. Both phylogenetic tree constructions (ML and BI) clearly separated the 22 AOD-associated strains into two clusters (MLSA cluster A and MLSA cluster B) with 100% bootstrap and PP support, constituting each a potential novel species (Fig. 2 and Suppl. Fig. S2). MLSA cluster A (*P. daroniae* sp. nov.), containing strains only from Bisham Woods, formed an independent branch with *P. seleniipraecipitans* and *P. flavescens* as the closest phylogenetic relatives, although without significant bootstrap or PP values. MLSA cluster B (*P. dryadis* sp. nov.) contained strains from both Bisham and Great Monk Woods as well as Kew Gardens. The closest phylogenetic neighbours to MLSA cluster B, without bootstrap support in ML trees but with good PP values in BI trees, are *P. argentinensis, P. punonensis, P. flavescens, P. seleniipraecipitans* and *P. straminea*, as well as MLSA cluster A. The ML tree based on the concatenated amino acid sequences was congruent with the trees constructed from nucleotide sequences (Suppl. Fig. S3).

Three PCR-based DNA fingerprinting techniques including BOX, ERIC (enterobacterial repetitive intergenic consensus) and RAPD (random amplification of polymorphic DNA) were used to investigate the genetic diversity of the AOD-associated *Pseudomonas*. Amplification was performed on all 22 strains, and closest related strains, according to the published protocols [23–25]. Primers BOX-A1R, ERIC-1R and ERIC-2, and OPA-04 were used for the BOX, ERIC, and RAPD PCR reactions, respectively.

RAPD-PCR was found to be the most discriminative fingerprinting method for the *Pseudomonas* strains. The RAPD patterns for MLSA cluster A showed differences between strain P18A and the remaining strains (Suppl. Fig. S4), while four different band patterns were observed for strains belonging to MLSA cluster B: one for strains isolated in Great Monk Wood (FRB 230$^{T}$, P26B, GM38b, GM48c and GM50b), one for Bisham Woods (P6B), and two for strains isolated in Kew Gardens (one pattern for strain I151 and another pattern for I160, I163 and I166). These results suggest that there are two clonal populations within MLSA cluster A and four clonal populations within MLSA cluster B.

## Genome Features

Representative strains from each MLSA cluster were selected for whole genome sequencing. FRB 228$^{T}$, P9A, P18A, P23A, FRB 230$^{T}$, P6B and P26B were sequenced by MicrobesNG

(Birmingham, UK) using the Illumina HiSeq platform. The reads were trimmed using Trimmomatic [26], and their quality was assessed using in-house scripts combined with the following software: Samtools, BedTools and bwa-mem [27–29].

De novo assembly was performed on samples using SPAdes version 3.11.1 [30] and the resulting contigs were annotated in Prokka 1.11 [31]. Assembly of strain FRB 228$^T$ yielded 56 contigs > 1,000 bp with a total length of 5.43 Mbp, while FRB 230$^T$ generated 71 contigs > 1,000 bp with a total length of 5.76 Mbp. The genome sequences of strains FRB 228$^T$, P9A, P18A, P23A, FRB 230$^T$, P6B and P26B were submitted to GenBank and received the accession numbers QJUH00000000, QJUI00000000, QJUJ00000000, QJUK00000000, QJUN00000000, QJUL00000000 and QJUM00000000, respectively. The DNA G + C mol% content for strains FRB 228$^T$, P9A, P18A and P23A ranged from 61.97 – 62.02 mol%, and 64.94 – 64.97 mol% for strains FRB 230$^T$, P6B and P26B.

The average nucleotide identity (ANI) was calculated using JSpecies [32]. Whole genomes of the potential novel *Pseudomonas* species and the closest related species available from GenBank were uploaded and compared. ANI values of 98.3 – 100% were obtained when strains FRB 228$^T$, P9A, P18A and P23A (MLSA cluster A) were compared to each other, while strains FRB 230$^T$, P6B and P26B (MLSA cluster B) exhibited 98.6 – 100%. The threshold for species delimitation of 95% ANI has been shown to correspond to the DNA-DNA hybridisation value of 70% [33], confirming that each of the MLSA species clusters corresponds to a single taxon. When the whole genomes of the closest relatives (including *P. flavescens* and *P. seleniipraecipitans*) were compared to strains belonging to each novel species, ANI values ranging from 74.2 to 87.2% were obtained. In addition to ANI, *in silico* DNA-DNA hybridisation (*is*DDH) using the genome to genome distance calculator (GGDC), was performed on the genomes of the novel species and closest related species [34]. The values generated by *is*DDH were in agreement with the ANI values, with strains in each novel species sharing > 87.9% whole genome similarity, and <39.2% to their closest relatives. The percentages generated by ANI and *is*DDH analysis indicate that the *Pseudomonas* isolates are well below the cut-off limit when compared to the closest related species, supporting their classification as two novel species: *Pseudomonas daroniae* sp. nov. (MLSA cluster A) and *Pseudomonas dryadis* sp. nov. (MLSA cluster B). A detailed summary of the pairwise whole genome comparisons is listed in Suppl. Table S3a and S3b.

## Phenotypic and chemotaxonomic characterisation

Morphological, physiological and biochemical characteristics were determined for representative strains from the two novel species (*P. daroniae* sp. nov.: FRB 228$^T$, P9A, P18A, P23A and *P. dryadis* sp. nov.: FRB 230$^T$, P6B and P26B), as well as type strains from the closest phylogenetic relatives. Cell size and motility were measured using light microscopy and the microscopy imaging software CellSens version 1.11 (Olympus Life Science, Tokyo, Japan). Strains were grown on KB agar for fluorescent pigment production analysis [35] and Gram reaction was determined following growth on LB agar [36]. Oxidase tests were performed according to the manufacturer's instructions (bioMérieux), while catalase activity was monitored by spreading fresh bacterial colonies in 3% w/w hydrogen peroxide ($H_2O_2$) and recording gas bubbles formation. Tolerance ranges of temperature (4-10°C, 33°C, 37°C, 39°C and 41°C) were studied on KB agar and tryptone soy agar (TSA), while NaCl tolerance (3.5%, 4.5%, 5.5%, 6.5%, 7.5%, 8.5% and 9.5%) and pH (6.0, 7.0, 8.0 and 9.0) were studied only on TSA at 25°C. Plates were incubated and monitored for bacterial growth for 6 days. To test if they were able to grow anaerobically, *P. daroniae* sp. nov. and *P. dryadis* sp. nov. strains were incubated in TSA for 4 days at 35°C in anaerobic conditions.

Transmission electron microscopy (TEM) was used to image cells of *P. daroniae* FRB 228$^T$ and *P. dryadis* FRB 230$^T$ (Fig. 3). The strains were grown in tryptone soy broth in shaking conditions. Cell cultures with an optical density of 1.3 were centrifuged 10 minutes at 3000 g. Cell pellets were resuspended in 200 µL of Tris-saline buffer (50mM Tris pH 8.0, 130mM NaCl). This washing step was performed twice. Five µL of the samples were applied to glow-discharged carbon coated copper grids (Cu 300 mesh). Grids were washed and stained for a minute with 2% (w/v) uranyl acetate. Digital images were acquired in a Tecnai T12 TEM microscope with a CETA 16M camera (ThermoFisher Scientific) at a nominal magnification of 2900.

Cells from both *P. daroniae* sp. nov., and *P. dryadis* sp. nov. were slightly curved, motile rods, with average sizes of 1.7 µm x 0.4 µm and 2.3 µm x 0.4 µm, respectively. Cells from both species had a single polar flagellum, observed by TEM. All strains were Gram-negative, and positive for catalase and oxidase activity. All isolates grew in visible, cream-coloured, 2 mm diameter colonies on KB agar after incubation at 25°C for 48 hours. Only strain P18A from MLSA cluster A produced a fluorescent pigment, whereas all strains belonging to MLSA

cluster B produced fluorescent pigments. All strains of cluster A grew at 33°C but failed to grow at or above 37°C, whereas strains of cluster B grew well at 33°C, 37°C and 39°C, but failed to grow at 41°C. Only strain P18A from cluster A grew at 4-10°C, while all strains of cluster B could grow at 4–10°C. However, compared to typical growth at 25°C all strains produced relatively smaller colonies at these lower temperatures. All strains from both novel species grew at pH 6.0, 7.0, and 8.0. However, after 6 days, only small colonies of P18A appeared on pH 9.0 plates. All isolates from both species grew at a NaCl concentration of 3.5% (w/v) but only FRB 228$^T$ grew on 5.5% (w/v) NaCl plates. No growth from either species was observed on plates containing a higher concentration of NaCl.

Metabolic profiles, carbohydrate fermentation and carbon utilisation data were obtained using API 20 NE strips, (bioMérieux,), API 50 CH galleries (bioMérieux,) and Biolog GN2 Microplates (Biolog), respectively. All tests were performed according to the manufacturer's instructions and strains *P. flavescens* LMG 18387$^T$, *P. seleniipraecipitans* LMG 25475$^T$, *P. argentinensis* LMG 22563$^T$, *P. straminea* LMG 21615$^T$ and *P. punonensis* LMG 26839$^T$ were included as reference strains using the same experimental conditions.

The phenotypic features that can distinguish *P. daroniae* sp. nov. from its closest relative, *P. flavescens*, are the inability to ferment D-maltose, D-saccharose (sucrose) and D-trehalose (API 50 CH), the inability to oxidise sucrose, D-trehalose and urocanic acid, and the ability to oxidise D-galacturonic acid, D-glucuronic acid, glucuronamide and D-alanine (Biolog GN2). *P. dryadis* sp. nov. can be phenotypically differentiated from its closest neighbours (*P. flavescens*, *P. argentinensis*, *P. punonensis*, *P. seleniipraecipitans*, *P. straminea* and *P. daroniae* sp. nov.) by the inability to assimilate L-arabinose (API 20 NE), the inability to produce acid from L-arabinose and D-galactose (API 50 CH), as well as the lack of oxidation of D-galactose and D-gluconic acid (Biolog GN2).

Interestingly, all strains of *P. daroniae* sp. nov. and *P. dryadis* sp. nov. share an exclusive feature, not present in any of the closest neighbours. This is the ability to weakly ferment D-lyxose, a very rare sugar in nature, that can be found as part of bacterial glycolipids [37]. A summary of the most distinguishing characteristics is presented in Table 1, while detailed results of the phenotypic assays are available in Suppl. Table S4.

Fatty acid methyl ester (FAME) profiles were determined by Fera Science Ltd. (York, UK) for strains of *P. daroniae* sp. nov. (FRB 228$^T$, P9A, P18A, P23A) and *P. dryadis* sp. nov. (FRB 230$^T$, P6B P26B) using the MIDI microbial identification system Sherlock Version 6.2. Strains were grown in TSA medium at 28°C for 24 h for this analysis. The results obtained were compared against the library TSBA6 6.10. Similar profiles were obtained for strains of both novel species with summed features 3 ($C_{16:1}$ ω6c and/or $C_{16:1}$ ω7c) and 8 ($C_{18:1}$ ω7c and/or $C_{18:1}$ ω6c) as the major fatty acids. As expected, both proposed novel species possess $C_{10:0}$ 3-OH, $C_{12:0}$ 3-OH and $C_{12:0}$ which are characteristic for members of the genus *Pseudomonas* [38]. Furthermore *P. daroniae* sp. nov. and *P. dryadis* sp. nov. display these fatty acids in similar amounts to other members of the *P. straminea* group, along with $C_{16:0}$ and summed features 3 and 8. The fatty acid profiles for *P. daroniae* sp. nov., *P. dryadis* sp. nov. and their closest phylogenetic relatives are displayed in Table 2. Other chemotaxonomic characteristics, which could be considered for differentiation of species, are the presence of polar lipids and quinones. However, there is no information available for polar lipids for the five species of the *P. straminea* group (*P. straminea*, *P. seleniipraecipitans*, *P. flavescens*, *P. argentinensis* and *P. punonensis*) [39–43], and the presence of Q9 has only been reported for two species, namely *P. straminea* and *P. punonensis* [39, 40, 41, 42, 43]. Based on the results provided here from genotypic, phenotypic, genomic and chemotaxonomic data, we propose the classification of two novel species, *P. daroniae* sp. nov. and *P. dryadis* sp. nov.

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## Protologue

### Description of *Pseudomonas daroniae* sp. nov.

*Pseudomonas daroniae* (da.ron.i'ae. N.L. fem. adj. *daroniae* from Daron, the Celtic goddess of oak).

Cells are Gram-negative, slightly curved rods (1.7 µm x 0.4 µm), motile by a polar flagellum, strictly aerobic, non-spore forming, and positive for catalase and oxidase activity. Colonies on KB agar are cream-coloured, circular and convex, with entire margins and measure 2 mm in diameter after 48 hours of incubation at 25°C. Only strain P18A produces fluorescent pigment. Growth is optimum at 28°C and pH 6.0 - 8.0, but strains can grow at 33°C and weakly at 37°C. Only strain P18A is able to grow small colonies on KB at refrigeration temperatures (4-10°C) and at pH 9.0. All strains grow on 3.5% (w/v) NaCl supplemented TSA plates, and only FRB 228$^T$ grows on 5.5% (w/v) NaCl.

Strains are negative for arginine dihydrolase, indole production, urease, β-glucosidase, gelatinase and β-galactosidase. There is no evidence of denitrification or nitrification activity. D-glucose, L-arabinose, D- mannose, D-mannitol, potassium gluconate, caprate, malate and citrate are assimilated. Acid is produced from: glycerol, L-arabinose, D, galactose, D-glucose, D-fructose, D-mannose, D-mannitol, and weakly from D-lyxose and D-arabitol (API 50 CHB/E). L-arabinose, D-fructose, D-galactose, α-D-glucose, D-mannitol, D-mannose, pyruvic acid methyl ester, succinic acid mono-methyl ester, acetic acid, *cis*-aconitic acid, citric acid, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, γ-hydroxybutyric acid, itaconic acid, α-ketoglutaric acid, D,L-lactic acid, quinic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, L-alanine, L-aspargine, L-aspartic acid, L-glutamic acid, hydroxy-L-proline, L-proline, L-pyroglutamic acid, L-serine, γ-amino butyric acid, 2-aminoethanol and glycerol are oxidised (Biolog GN2). Reactions for D-arabitol, dextrin, formic acid, glycogen, D-psicose, turanoseα-hydroxybutyric acid, β-hydroxybutyric acid, α-ketobutyric acid, α-ketovaleric acid, propionic acid, D-saccharic acid, D,L-carnitine, putrescine, 2,3-butanediol , and D,L,α-glycerol phosphate are variable. Major fatty acids are $C_{12:0,}$ $C_{16:0}$ and summed features 3 ($C_{16:1}$ ω6c and/or $C_{16:1}$ ω7c) and 8 ($C_{18:1}$ ω7c and/or $C_{18:1}$ ω6c).

The type strain FRB 228$^T$ (LMG 31087$^T$ = NCPPB 4672$^T$) was isolated from *Quercus robur* stem tissue displaying symptoms of acute oak decline in the United Kingdom. The DNA G+C content of the type strain (QJUH00000000) is 62.01 mol%.

## Description of *Pseudomonas dryadis* sp. nov.

*Pseudomonas dryadis* (dry.a'dis. L. gen. n. of a Dryad, the oak tree nymph)

Cells are Gram-negative, slightly curved rods (2.3 µm x 0.4 µm), motile by a polar flagellum, strictly aerobic, non-spore forming, and positive for catalase and oxidase activity. Colonies on KB agar are cream-coloured, circular and convex, with entire margins and measure 2 mm in diameter after 48 hours of incubation at 25°C. All strains produce fluorescent pigment. Growth is optimum at 28°C but is observed up to 39°C, while weak growth occurs at 4 to 10°C. Growth occurs in the pH range 6.0 to 8.0, and on 3.5% (w/v) NaCl supplemented TSA plates.

Negative for urease, indole production, arginine dihydrolase, β-glucosidase, gelatinase and β-galactosidase. D-glucose, D-mannose, D-mannitol, potassium gluconate, caprate, malate and citrate are assimilated (API 20 NE). Acid is produced from glycerol, D-glucose, D-fructose, D-mannose, D-mannitol, and weakly from D-lyxose, D-fucose and D-arabitol (API 50 CHB/E). L-arabinose, D-arabitol, α-D-glucose, D-mannitol, pyruvic acid methyl ester, succinic acid mono-methyl ester, acetic acid, *cis*-aconitic acid, citric acid, γ-hydroxybutyric acid, itaconic acid, α-ketoglutaric acid, D,L-lactic acid, quinic acid, succinic acid, bromosuccinic acid, L-aspartic acid, L-glutamic acid, hydroxy-L-proline, L-proline, γ-aminobutyric acid and glycerol are oxidised (Biolog GN2). Reactions for dextrin, glycogen, tween 40, tween 80, D-fructose, D-galactose, D-mannose, turanose, formic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, α-ketobutyric acid, propionic acid, succinamic acid, L-aspargine, L-pyroglutamic acid, L-serine, 2-aminoethanol, 2,3-butanediol are variable. Major fatty acids are $C_{12:0,}$ $C_{16:0}$ and summed features 3 ($C_{16:1}$ ω6c and/or $C_{16:1}$ ω7c) and 8 ($C_{18:1}$ ω7c and/or $C_{18:1}$ ω6c).

The type strain FRB 230$^T$ (LMG 31087$^T$ = NCPPB 4673$^T$) was isolated from inner bark and beetle galleries in the phloem of *Quercus robur* in the United Kingdom. The G+C content of the type strain (QJUN00000000) is 64.97 mol%.

## AUTHOR STATEMENTS

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The authors have no conflicts of interest to declare.

## ABBREVIATIONS

AIC: Akaike information criterion

ANI: average nucleotide identity

AOD: acute oak decline

BI: Bayesian inference

ERIC-PCR: enterobacterial repetitive intergenic consensus

FAME: Fatty acid methyl ester

GGDC: genome to genome distance calculator

GTR+I+G: general time reversible model with invariable sites and gamma distribution

*Is*DDH: *in silico* DNA-DNA hybridisation

ML: Maximum likelihood

MLSA: multilocus sequence analysis

PP: Posterior probabilities

RAPD-PCR: random amplification of polymorphic DNA

TIM2+I+G: transitional model with invariable sites and gamma distribution

## REFERENCES


1. **Denman S, Brown N, Kirk S, Jeger M, Webber J**. A description of the symptoms of Acute Oak Decline in Britain and a comparative review on causes of similar disorders on oak in Europe. *Forestry* 2014;87:535–551.
2. **Brown N, Inward DJG, Jeger M, Denman S**. A review of *Agrilus biguttatu*s in UK forests and its relationship with acute oak decline. *Forestry* 2015;88:53–63.
3. **Migula W**. Über ein neues System der Bakterien. *Arb aus dem Bakteriol Inst der Tech Hochschule zu Karlsruhe* 1894;235–238.
4. **Peix A, Ramírez-Bahena M-H, Velázquez E**. The current status on the taxonomy of *Pseudomonas* revisited: An update. *Infect Genet Evol* 2018;57:106–116.
5. **Niemann S, Puhler A, Tichy H-V, Simon R, Selbitschka W**. Evaluation of the resolving power of three different DNA fingerprinting methods to discriminate among isolates of a natural *Rhizobium meliloti* population. *J Appl Microbiol* 1997;82:477–484.
6. **Beiki F, Busquets A, Gomila M, Rahimian H, Lalucat J, *et al.*** New *Pseudomonas* spp. are pathogenic to citrus. *PLoS One* 2016;11:1–16.
7. **Mulet M, Bennasar A, Lalucat J, García-Valdés E**. An *rpoD*-based PCR procedure for the identification of *Pseudomonas* species and for their detection in environmental samples. *Mol Cell Probes* 2009;23:140–147.
8. **Ait Tayeb L, Ageron E, Grimont F, Grimont PAD**. Molecular phylogeny of the genus *Pseudomonas* based on *rpoB* sequences and application for the identification of isolates. *Res Microbiol* 2005;156:763–773.
9. **Yoon S-H, Ha S-M, Kwon S, Lim J, Kim Y, *et al.*** Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int J Syst Evol Microbiol* 2017;67:1613–1617.
10. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ**. Basic local alignment search tool. *J Mol Biol* 1990;215:403–10.
11. **Hall TA**. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In: *Nucleic acids symposium series*. 1999. pp. 95–98.
12. **Vaidya G, Lohman DJ, Meier R**. SequenceMatrix: concatenation software for the fast assembly of multi-gene datasets with character set and codon information. *Cladistics* 2011;27:171–180.
13. **Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, *et al.*** New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance

of PhyML 3.0. *Syst Biol* 2010;59:307–321.

14. **Huelsenbeck JP, Ronquist F**. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 2001;17:754–755.
15. **Lefort V, Longueville JE, Gascuel O**. SMS: Smart Model Selection in PhyML. *Mol Biol Evol* 2017;34:2422–2424.
16. **Darriba D, Taboada GL, Doallo R, Posada D**. jModelTest2: more models, new heuristics and parallel computing. *Nat Methods* 2012;9:772.
17. **Le SQ, Gascuel O**. An Improved General Amino Acid Replacement Matrix. *Mol Biol Evol* 2008;25:1307–1320.
18. **Rannala B, Yang Z**. Probability distribution of molecular evolutionary trees: A new method of phylogenetic inference. *J Mol Evol* 1996;43:304–311.
19. **Zhaxybayeva O, Gogarten JP, Woese C, Fox G, Woese C, *et al.*** Bootstrap, Bayesian probability and maximum likelihood mapping: exploring new tools for comparative genome analyses. *BMC Genomics* 2002;3:4.
20. **Gomila M, Peña A, Mulet M, Lalucat J, García-Valdés E**. Phylogenomics and systematics in *Pseudomonas*. *Front Microbiol* 2015;6:214.
21. **Mulet M, Lalucat J, García-Valdés E**. DNA sequence-based analysis of the *Pseudomonas* species. *Environ Microbiol* 2010;12:1513–1530.
22. **Yamamoto S, Kasai H, Arnold DL, Jackson RW, Vivian A, *et al.*** Phylogeny of the genus *Pseudomonas*: Intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. *Microbiology* 2000;146:2385–2394.
23. **Versalovic J, Schneider M, de Bruijn F, Lupski JR**. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* 1994;5:25–40.
24. **Versalovic J, Koeuth T, Lupski JR**. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* 1991;19:6823–6831.
25. **Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey S V.** DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 1990;18:6531–6535.
26. **Bolger AM, Lohse M, Usadel B**. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;30:2114–2120.
27. **Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, *et al.*** The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 2009;25:2078–2079.

28. **Quinlan AR, Hall IM**. BEDTools: A flexible suite of utilities for comparing genomic features. *Bioinformatics* 2010;26:841–842.
29. **Li H, Durbin R**. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* 2010;26:589–595.
30. **Nurk S, Bankevich A, Antipov D, Gurevich A, Korobeynikov A, *et al.*** Assembling genomes and mini-metagenomes from highly chimeric reads. In: *Lecture Notes in Computer Science (including subseries Lecture Notes in Artificial Intelligence and Lecture Notes in Bioinformatics)*. 2013. pp. 158–170.
31. **Seemann T**. Prokka: Rapid prokaryotic genome annotation. *Bioinformatics* 2014;30:2068–2069.
32. **Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J**. JSpeciesWS: A web server for prokaryotic species circumscription based on pairwise genome comparison. *Bioinformatics* 2015;32:929–931.
33. **Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, *et al.*** DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *Int J Syst Evol Microbiol* 2007;57:81–91.
34. **Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M**. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 2013;14:60.
35. **King EO, Ward MK, Raney DE**. Two simple media for the demonstration of pyocyanin and fluorescin. *The Journal of laboratory and clinical medicine* 1954;44:301–307.
36. **Doetsch RN**. Determinative Methods of Light Microscopy. *Man Methods Gen Bacteriol* 1981;21–33.
37. **Khoo KH, Suzuki R, Dell A, Morris HR, McNeil MR, *et al.*** Chemistry of the lyxose-containing mycobacteriophage receptors of Mycobacterium phlei/Mycobacterium smegmatis. *Biochemistry* 1996;35:11812–11819.
38. **Palleroni NJ**. Pseudomonas. In: *Bergey's Manual of Systematics of Archaea and Bacteria*. Chichester, UK: John Wiley & Sons, Ltd. pp. 1–1.
39. **Uchino M, Kosako Y, Uchimura T, Komagata K**. Emendation of Pseudomonas straminea Iizuka and Komagata 1963. *Int J Syst Evol Microbiol* 2000;50:1513–1519.
40. **Hunter WJ, Manter DK**. *Pseudomonas seleniipraecipitatus* sp. nov.: A selenite reducing γ-Proteobacteria Isolated from Soil. *Curr Microbiol* 2011;62:565–569.
41. **Hildebrand DC, Palleroni NJ, Hendson M, Toth J, Johnson JL**. *Pseudomonas*

*flavescens* sp. nov., isolated from walnut blight cankers. *Int J Syst Bacteriol* 1994;44:410–415.

42. **Peix A, Berge O, Rivas R, Abril A, Velázquez E**. *Pseudomonas argentinensis* sp. nov., a novel yellow pigment-producing bacterial species, isolated from rhizospheric soil in Córdoba, Argentina. *Int J Syst Evol Microbiol* 2005;55:1107–1112.
43. **Ramos E, Ramirez-Bahena M-H, Valverde A, Velazquez E, Zuniga D, *et al.*** *Pseudomonas punonensis* sp. nov., isolated from straw. *Int J Syst Evol Microbiol* 2013;63:1834–1839.

**Figure 1**: Maximum likelihood phylogenetic tree of the 16S rRNA gene sequences obtained from the AOD-associated type strains of *Pseudomonas daroniae* sp. nov., *Pseudomonas dryadis* sp. nov. and the closest phylogenetic neighbours. A final dataset of 38 partial 16S rRNA gene sequences of 1399 nucleotides long was compared to infer the evolutionary relationships of the taxa, using the ML method in PhyML. Bootstrap support values higher than 50% (from 1,000 replicates) are shown next to the branches. The closest phylogenetic neighbour to both *Pseudomonas daroniae* sp. nov. and *Pseudomonas dryadis* sp. nov. (in bold) is *Pseudomonas flavescens* with low bootstrap support. The scale indicates the nucleotide substitutions per site. *Acinetobacter baumannii* DSM 30007$^T$ (X811660) was used as an outgroup. Species names are followed by the strain number and the GenBank accession number. $^T$ = type strain.

**Figure 2**: Maximum likelihood phylogenetic tree of the concatenated *gyrB, rpoB* and *rpoD* gene sequences obtained from 12 AOD-associated strains of *Pseudomonas daroniae* sp. nov., 10 strains of *Pseudomonas dryadis* sp. nov. and the closest phylogenetic neighbours. A final dataset of 58 gene sequences of 2172 nucleotides long was compared to infer the evolutionary relationships of the taxa using the ML method in PhyML. Bootstrap support values higher than 50% (from 1,000 replicates) are shown next to the branches. AOD-associated *Pseudomonas* strains grouped in two well-defined clusters (100% bootstrap support): MLSA cluster A (*Pseudomonas daroniae* sp. nov.) and MLSA cluster B (*Pseudomonas dryadis* sp. nov.), in bold. The scale indicates the nucleotide substitutions per site. *Pseudomonas pertucinogena* LMG 1874$^T$ was used as an outgroup. Species names are followed by the strain number. $^T$ = type strain.

**Figure 3**: Transmission electron microscopy images of cells from AOD-associated bacteria *Pseudomonas daroniae* FRB 228$^T$ (left) and *Pseudomonas dryadis* FRB 230$^T$ (right). Scale bar: 1 µm.

**Table 1**. Distinguishing phenotypic features of *Pseudomonas daroniae* sp. nov., *Pseudomonas dryadis* sp. nov. and closest phylogenetic neighbours. Data were generated in this study using the commercial tests API 20 NE, API 50 CH and Biolog GN2. For complete phenotypic profile results see Suppl. Table S4. All strains tested were negative for indole production, acidification from D-glucose, esculin hydrolysis, gelatin hydrolysis, presence of arginine dihydrolase, urease and β-galactosidase; and assimilation of N-acetyl-glucosamine, D-maltose, adipate and phenyl-acetate. All strains failed to ferment erythritol, D-arabinose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-lactose, inulin, D-melezitose, D, raffinose, amidon, glycogen, xylitol, D-turanose, D-tagatose, L-fucose, L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. None of the strains oxidised α-cyclodextrin, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, D-cellobiose, i-erythritol, L-fucose, gentiobiose, m-inositol, α-D-lactose, lactulose, maltose, D-melibiose, D-raffinose, L-rhamnose, D-sorbitol, xylitol, D-glucosaminic acid, p-hydroxy-phenylacetic acid, sebacic acid, glycyl-L-aspartic acid, L-histidine, L-leucine, L-ornithine, L-phenylalanine, D-serine, inosine, uridine, thymidine, phenylethylamine and α-D-glucose-1-phosphate. α-Ketovaleric acid was not oxidised by any of the *Pseudomonas dryadis* sp. nov. or the reference strains tested. All strains were able to assimilate D-glucose, D-mannose, D-mannitol, potassium gluconate, caprate, malate and citrate. All strains displayed cytochrome oxidase activity, and were able to ferment D-glucose, D-fructose, D-mannose and D-mannitol. Strains were positive for oxidation of L-arabinose, D-mannitol, pyruvic acid methyl ester, cis-aconitic acid, citric acid, itaconic acid, α-ketoglutaric acid, D,L-lactic acid, bromosuccinic acid, L-aspartic acid, L-glutamic acid, L-proline, and γ-aminobutyric acid. All strains except for *Pseudomonas dryadis* sp. nov. ferment L-arabinose and D-galactose; assimilate L-arabinose and D-galactose; and oxidise D-gluconic acid, L-aspargine, L-pyroglutamic acid.

(1) *Pseudomonas daroniae* sp. nov. (*n*=3) (2) *Pseudomonas dryadis* sp. nov. (*n*=3) (3) *Pseudomonas flavescens* LMG 18387$^{T}$ (4) *Pseudomonas argentinensis* LMG 22563$^{T}$ (5) *Pseudomonas punonensis* LMG 26839$^{T}$ (6) *Pseudomonas seleniipraecipitans* LMG 25475$^{T}$ (7) *Pseudomonas straminea* LMG 21615$^{T}$. $^{T}$ = type strain.

| **Characteristic** | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
|---|---|---|---|---|---|---|---|
| **API 20 NE** | | | | | | | |
| Nitrate reduction to nitrites (nitrification) | - | - | - | + | - | + | - |
| Nitrate reduction to nitrogen (denitrification) | - | - | - | - | - | + | - |
| Assimilation L-arabinose | + | - | + | + | + | + | + |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **(API 50 CH) Acid from:** | | | | | | | |
| L-Arabinose | + | - | + | + | + | + | + |
| D-Xylose | - | - | - | + | - | - | - |
| D-Galactose | + | - | + | + | + | + | + |
| D-Maltose | - | - | + | + | - | - | - |
| D-Melibiose | - | - | - | + | - | - | - |
| D-Saccharose (sucrose) | - | - | + | - | - | - | - |
| D-Trehalose | - | - | + | + | - | - | - |
| Gentiobiose | - | - | - | + | - | - | - |
| D-Lyxose | w+ | w+ | - | - | - | - | - |
| **(Biolog GN2) Oxidation of:** | | | | | | | |
| D-Galactose | + | - | + | + | + | + | + |
| Sucrose | - | - | + | - | - | - | - |
| D-Trehalose | - | - | + | + | - | - | - |
| Succinic acid mono-methyl ester | + | + | + | + | + | + | w+ |
| Acetic acid | + | + | + | - | w+ | + | - |
| D-Galacturonic acid | + | - | - | + | + | + | + |
| D-Gluconic acid | + | - | + | + | + | + | + |
| D-Glucuronic acid | + | - | - | + | + | + | + |
| γ-hydroxybutyric acid | + | + | + | w+ | + | + | w+ |
| Malonic acid | - | - | w+ | - | + | - | - |
| Quinic acid | + | + | + | + | + | - | + |
| Succinic acid | + | + | + | - | + | + | + |
| Bromosuccinic acid | + | + | + | + | + | + | + |
| Glucuronamide | + | - | - | - | + | + | + |
| L-alaninamide | w+ | - | - | - | w+ | w+ | + |
| D-alanine | + | w+ | - | - | w+ | + | w+ |
| L-alanine | + | w+ | + | - | + | + | + |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| L-alanyl-glycine | - | - | - | - | w+ | w+ | w+ |
| Hydroxy-L-proline | + | + | + | - | + | + | + |
| Urocanic acid | - | - | + | - | w+ | - | + |
| Glycerol | + | + | + | w+ | w+ | w+ | w+ |

+: 100 % of strains positive; -: 100 % of strains negative; w+: 100 % of strains weakly positive.

**Table 2**: Percentages of cell fatty acid methyl esters (FAMEs) of *Pseudomonas daroniae* sp. nov, and *Pseudomonas dryadis* sp. nov. Summed features are sets of two or more fatty acids, which the Microbial Identification System (MIDI) did not manage to separate. Summed feature 3 is $C_{16:1}$ ω6c and/or $C_{16:1}$ ω7c and summed feature 8 is $C_{18:1}$ ω7c and/or $C_{18:1}$ ω6c. Data was generated in this study, except for the profiles of reference strains, which were obtained from [43]. Values are displayed as average percentage per species investigated, with the standard deviation shown in parentheses. *Pseudomonas daroniae* sp. nov. displayed traces (<1%) of $C_{12:0}$ 2-OH, $C_{10:0}$, $C_{14:0}$, $C_{17:0}$, $C_{18:0}$, $C_{17:1}$ ω8c and $C_{19:0}$ cyclo ω8c. Traces (<1%) of the fatty acids $C_{10:0}$, $C_{14:0,}$ $C_{18:0}$ and $C_{17:0}$ iso were found in *Pseudomonas dryadis* sp. nov.

(1) *Pseudomonas daroniae* sp. nov (*n*=4) (2) *Pseudomonas dryadiae* sp. nov. (*n*=3) (3) *Pseudomonas flavescens* LMG 18387$^T$ (4) *Pseudomonas argentinensis* LMG 22563$^T$ (5) *Pseudomonas punonensis* LMT03$^T$ (6) *Pseudomonas straminea* IAM 1598$^T$. - = not detected. $^T$ = type strain.

| **Fatty acid** | **1** | **2** | **3** | **4** | **5** | **6** |
|---|---|---|---|---|---|---|
| **$C_{10:0}$ 3-OH** | 3.34 (±0.21) | 2.8 (±0.20) | 3.74 | 2.4 | 4.83 | 3.91 |
| **$C_{12:0}$ 3-OH** | 3.76 (±0.20) | 3.10 (±0.45) | 3.55 | 2.58 | 4.54 | 3.57 |
| **$C_{12:0}$** | 9.88 (±0.60) | 9.29 (±0.51) | 9.23 | 7.88 | 8.31 | 9.58 |
| **$C_{16:0}$** | 20.2 (±0.63) | 22 (±0.37) | 19.75 | 19.69 | 15.2 | 17.63 |
| **$C_{17:0}$ cyclo** | 2.88 (±1.11) | 2.74 (±0.42) | - | - | - | - |
| **Summed feature 3** | 22.15 (±2.13) | 17.9 (±0.61) | 22.4 | 21.3 | 23.7 | 22.4 |
| **Summed feature 8** | 34.45 (±0.90) | 39.68 (±0.29) | 38.51 | 41.52 | 40.82 | 39.73 |