Pseudomonas kirkiae sp. nov., a novel species isolated from oak in the United Kingdom, and phylogenetic considerations of the genera *Pseudomonas*, *Azotobacter and Azomonas*.

Victoria Bueno-Gonzalez[a*], Carrie Brady[a*], Sandra Denman[b], Joël Allainguillaume[a] and Dawn Arnold[a]

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

[b] Forest Research, Centre for Forestry and Climate Change, Farnham, United Kingdom

* Both authors contributed equally to this work

Correspondence: 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

Keywords: *Pseudomonas*, *Pseudomonas kirkiae*, Acute Oak Decline, *Azotobacter*, *Azomonas*.

Repositories: BCCM/LMG, Bacteria Collection, (Belgium). NCPPB, National Collection of Plant Pathogenic Bacteria, (United Kingdom).

The GenBank/EMBL/DDBJ accession numbers are as follows: MK159379 – MK159391 (16S rRNA), MN0044394 – MN0044406 (*rpoB*), MN044407 – MN044419 (*gyrB*), MN044420 – MN044432 (*rpoD*) and QJUO00000000 – QJUR00000000 (whole genome).

ABSTRACT

As the current episode of Acute Oak Decline (AOD) continues to affect native British oak in the United Kingdom, ongoing isolations from symptomatic and healthy oak have yielded a large *Pseudomonas* species population. These strains could be divided into taxa representing three potential novel species. Recently, two of these taxa were described as novel *Pseudomonas* species in the *P. fluorescens* lineage. Here, we demonstrate using a polyphasic approach that the third taxon represents another novel *Pseudomonas* species. 16S rRNA gene sequencing assigned the strains to the *P. aeruginosa* lineage, while multilocus sequence analysis (based on partial *gyrB*, *rpoB* and *rpoD* sequences) placed the 13 strains in a single cluster on the border of the *P. stutzeri* group. Whole genome intra-species comparisons (based on average nucleotide identity and *in silico* DNA-DNA hybridization) confirmed that the strains belong to a single taxon, while the inter-species comparisons with closest phylogenetic relatives yielded similarity values below the accepted species threshold. Therefore, we propose these strains as a novel species, namely *Pseudomonas kirkiae* sp. nov., with the type strain FRB 229^{T} (P4C^{T} = LMG 31089^{T} = NCPPB 4674^{T}). The phylogenetic analyses performed in this study highlighted the difficulties in assigning novel species to the genus *Pseudomonas* due to its polyphyletic nature and close relationship to the genus *Azotobacter*. We further propose that a thorough taxonomic re-evaluation of the genus *Pseudomonas* is essential and should be performed in the near future.

Over the last ten years, a total of two novel genera, 13 novel species and two novel subspecies have been classified from oak displaying symptoms of decline in the United Kingdom, Spain and the United States of America [1–7]. The majority of bacterial strains have been isolated in the UK from pedunculate oak (*Quercus robur*) exhibiting signs of Acute Oak Decline (AOD), with weeping bleeds, cracked bark plates and necrotic lesions in the underlying tissue [8]. The current episode of AOD in the UK is caused by a polymicrobial complex with *Brenneria goodwinii* and *Gibbsiella quercinecans* responsible for the necrosis, with possible insect involvement by *Agrilus biguttatus* [9]. The role of other species in symptom development, such as *Rahnella victoriana*, must still be elucidated.

Ongoing isolations from AOD-affected trees and a metagenome study of the oak microbiome have revealed a large *Pseudomonas* population present in both symptomatic and healthy oak [10, 11]. *Pseudomonas* is a ubiquitous genus, containing more than 190 species isolated from origins varying from human, animal or plant samples to the extreme environments of the desert and Antarctic [12]. Due to the heterogeneous sources *Pseudomonas* species are isolated from, their phylogeny is diverse and classification of novel strains can be difficult. Consequently, sequencing of the 16S rRNA and *gyrB* genes could not assign the *Pseudomonas* isolates from oak to a species with a high degree of confidence, but phylogenetic analysis revealed they could be divided into three clusters within the genus *Pseudomonas* suggesting the presence of three novel species. Strains from two of these clusters were recently described as novel species in the *Pseudomonas straminea* group, namely *Pseudomonas daroniae* and *Pseudomonas dryadis* [7]. In this study we use a polyphasic approach, based on genotypic, genomic, phenotypic and chemotaxonomic analyses, to demonstrate that the third of these *Pseudomonas* clusters constitutes another novel species for which the name *Pseudomonas kirkiae* sp. nov. is proposed.

Isolation

Symptomatic and non-symptomatic oak trees in three woodlands were sampled, Bisham Woods (51.54911° N, 0.77071° W), Stratfield Brake (51.80306° N, 001.28274° W) and Send (51.28760° N 0.52873° W). Bacteria were isolated from the inner bark and sapwood of

symptomatic oak, *Agrilus biguttatus* larval galleries in the phloem, as well as non-symptomatic sapwood. A list of strains used in this study are listed in Suppl. Table S1. Strains were initially isolated in potato yeast glucose agar (PYGA), and routinely cultured at 25 °C on King's B agar (KB) and in Luria Bertani (LB) broth.

Genotypic characterisation

Alkalic lysis was used to extract genomic DNA for all subsequent PCR reactions [13]. Amplification and sequencing of the 16S rRNA, *gyrB*, *rpoD* and *rpoB* genes were performed on the 13 *Pseudomonas* strains isolated from oak as previously described [14–16]. The 16S rRNA gene sequence pairwise similarities were obtained by comparison with reference strains in EZBioCloud [17], and were all >96.3 % similar to *P. nosocomialis*. Values of 95.2 – 95.7 % similarity *to P. xanthomarina*, *P. aeruginosa*, *P. stutzeri*, *P. furukawaii*, *P. composti*, *P. balearica* and *P. mangiferae*, as well as *Azotobacter chroococcum* were observed. The 16S rRNA gene sequences for the closest phylogenetic neighbours to the oak strains were downloaded from EzBioCloud for phylogenetic analyses. Additional sequences of the *gyrB*, *rpoD* and *rpoB* genes for the closest phylogenetic neighbours, as determined by EzBioCloud and BLAST [18], were downloaded from the GenBank database (Suppl. Table S2). These included species from both *Pseudomonas* and *Azotobacter*. All four gene sequences were aligned and trimmed in BioEdit 7.2.5 [19], final sequence lengths were as follows: 16S rRNA – 1367 bp, *gyrB* – 771 bp, *rpoB* – 753 bp and *rpoD* – 651 bp. Protein-encoding gene sequences were conceptually translated in BioEdit. Following concatenation of the *gyrB*, *rpoB* and *rpoD* gene datasets in SequenceMatrix [20], Smart Model Selection (SMS) [21] was applied to both the 16S rRNA gene and concatenated datasets to determine the best-fit nucleotide substitution model for construction of maximum likelihood (ML) and neighbour joining (NJ) trees, respectively.

Phylogenetic tree construction was performed using PhyML [22] for ML trees and MEGA X 10.1.7 [23] for NJ trees, applying the selected evolutionary models and parameters. The ML 16S rRNA and MLSA nucleotide trees were computed using the general time reversible model with invariable sites and gamma distribution (GTR+I+G), while the ML MLSA amino acid tree was computed using LG+G+F. The NJ 16S rRNA and MLSA nucleotide trees were constructed

using the Kimura 2-parameter model with gamma distribution (K2P+G). Bootstrap support with 1000 replicates was generated to assess the confidence of the clusters. Bootstrap values less than 50 % are not included in the phylogenetic trees. *Endozoicomonas elysicola* DSM 22380^T was selected as an outgroup.

In the ML 16S rRNA gene phylogenetic tree (Fig. 1) the type strain of the proposed species, FRB 229^T, is positioned on a separate branch closely on the border of the *P. aeruginosa* lineage also containing *Azotobacter*, *Azorhizophils* and *Azomonas* species [12, 24, 25] but with no significant bootstrap support. The topology of the NJ 16S rRNA tree (Suppl. Fig. S1) is different, with FRB 229^T positioned on a separate branch on the border of a clade containing *P. thermotolerans*, *P. mangiferae*, *Azotobacter* species and *Azorhizophilus paspali*, within the *P. aeruginosa* lineage. Again, there is no bootstrap support for this clade, suggesting the taxonomic position of FRB 229^T could be easily changed by the addition of further novel *Pseudomonas* species. It is not possible to determine which *Pseudomonas* group [12] the proposed novel species belongs to, based on 16S rRNA gene sequencing, as inclusion of *Azotobacter* and *Azorhizophilus* species disrupts the currently defined groups. Species of *Azotobacter*, *Azomonas* and *Azorhizophilus* were included in our 16S rRNA gene sequence analysis following the results of the EZBioCloud comparison.

In both the ML and NJ phylogenetic trees (Fig. 2 and Suppl. Fig. S2), based on concatenated protein-encoding genes (*gyrB*, *rpoD* and *rpoB*), strains from the proposed novel species form a single robust cluster with 100 % bootstrap support. This cluster is positioned on the border of the *P. stutzeri* group, within the *P. aeruginosa* lineage [12, 25]. The ML tree based on the concatenated amino acid sequences is congruent with the trees constructed from nucleotide sequences (Suppl. Fig. S3).

As with the 16S rRNA gene phylogeny, the concatenated multigene phylogeny demonstrates a division in the *Pseudomonas* genus clade by *Azotobacter* species. In recent years, there has been an increasing number of studies highlighting the close relationship between *Pseudomonas* and the genera known as the '*Azotobacter*' group, namely *Azotobacter*, *Azorhizophilus* and *Azomonas*. The phylogenetic predicament of the genus *Pseudomonas* and the '*Azotobacter*' group is discussed in detail following the species protologue.

The genetic diversity of strains of the proposed novel *Pseudomonas* species was examined using three PCR-based DNA fingerprinting techniques, including BOX, ERIC (enterobacterial repetitive intergenic consensus) and RAPD (random amplification of polymorphic DNA) as previously published [26–28]. Primers BOX-A1R, ERIC-1R and ERIC-2, and OPA-04 were used for the BOX, ERIC, and RAPD PCR reactions, respectively. BOX-PCR provided the most discriminative fingerprints, with strains from the proposed novel species exhibiting different banding patterns for each of the three woodlands (Suppl. Fig. S4). The difference in banding patterns can be attributed to isolation location, indicating that the populations of the proposed novel *Pseudomonas* species at each woodland could be clonal.

Genome Features

Four strains from the proposed novel species were selected for whole genome sequencing, based on differing BOX-PCR patterns linked to geographical location. FRB 229^T (= P4C^T), P17C (= PW164bi)a), P28C (= S40) and P30C (= SB60b) were sequenced by MicrobesNG (Birmingham, UK) on the Illumina HiSeq platform. The reads were trimmed using Trimmomatic [29], and their quality was assessed using in-house scripts combined with the following software: Samtools, BedTools and bwa-mem [30–32].

De novo assembly was performed on samples using SPAdes version 3.11.1 [33] and the resulting contigs were annotated in Prokka 1.11 [34]. The GenBank accession numbers for the whole genome sequences of strains FRB 229^T, P17C, P28C and P30C are QJUO00000000, QJUP00000000, QJUQ00000000 and QJUR00000000, respectively. Assembly of the strains FRB 229^T, P17C, P28C and P30C yielded genome sizes of 4.38 Mbp (74 contigs), 4.38 Mbp (61 contigs), 4.32 Mbp (68 contigs) and 4.35 Mbp (46 contigs), respectively. The DNA G + C content for strains of the proposed species ranged from 63.6 to 63.7 mol %.

Average nucleotide identity (ANI) and *in silico* DNA-DNA hybridisation (*is*DDH) were performed on the proposed novel species and closest phylogenetic relatives using JSpecies and the genome to genome distance calculator (GGDC), respectively [35, 36]. Whole genome sequences of the closest related species were downloaded from GenBank for the comparison. ANI values of 99.16 – 99.98 %, and *is*DDH values of 95.2 – 100 %, were obtained when strains

FRB 229^T, P17C, P28C and P30C were compared to each other, confirming they belong to a single taxon. When strains from the novel species were compared to the closest relative species from *Pseudomonas* and *Azotobacter*, ANI values ranged from 75.5 to 77.68 % while *is*DDH values were 21.7 to 23.4 %. As these genome similarity values are well below the accepted thresholds of 95 % ANI [37] and 70 % *is*DDH [36] for species delineation and provide support for the classification of a novel species, we propose the name *Pseudomonas kirkiae* sp. nov. for these isolates from symptomatic oak in the UK. A complete list of pairwise whole genome similarity values can be found in Suppl. Table S3.

Phenotypic and chemotaxonomic characterisation

The morphology, physiology and biochemistry of strains belonging to the novel species were examined using a range of tests. Cell size and motility were measured using light microscopy and the microscopy imaging software CellSens version 1.11 (Olympus Life Science, Tokyo, Japan). Cells of the type strain of *P. kirkiae* sp. nov. were imaged by transmission electron microscopy (TEM) as previously described [7]. Oxidase tests were performed according to the manufacturer's instructions (bioMérieux), while catalase activity was observed by gas bubble formation following suspension of the bacteria in 3 % w/w hydrogen peroxide. Strains were grown on KB agar at 25 °C for fluorescent pigment production analysis [38] and on tryptone soy agar (TSA) under anaerobic conditions at 35 °C to determine respiration conditions. Tolerance to temperature was tested by measuring growth on KB agar and TSA at 4 - 10 °C, 33 °C, 37 °C, 39 °C and 41 °C. Plates were incubated and monitored for bacterial growth for 6 days. Growth in the presence of salt was studied by supplementing tryptone soy broth (TSB) with NaCl (0.5 – 4.5 % w/v in 1 % increments), while tolerance to pH (5.0 – 9.0) was tested in (TSB). Both were incubated at 25 °C for 48 h and monitored for growth.

Cells of *P. kirkiae* sp. nov. are slightly curved rods, with average sizes of 1.5 µm x 0.4 µm. Cells are motile by means of amphitrichous flagella, observed by TEM (Fig. 3) and positive for catalase and oxidase activity. All strains grow in visible, cream-coloured, 1 mm diameter colonies on KB agar after incubation at 25 °C for 48 h and produce fluorescent pigments, but none are able to grow under anaerobic conditions. Growth is visible on both KB and TSA media from 4 - 10 °C to 39 °C while tolerance to 41 °C incubation is variable according to the strain.

The strains tested are tolerant to media supplemented with 0.5 – 3.0 % NaCl, but FRB 229^T can grow at a NaCl concentration of 3.5 %. All strains form colonies in the pH range of 6.0 to 8.0 (*Pseudomonas* do not typically grow under acidic conditions [39]).

Physiological and biochemical tests were performed on representative strains from the novel species (FRB 229^T, P28C and P30C), as well as type strains from the closest phylogenetic relatives (*Pseudomonas stutzeri* LMG 11199^T, *Pseudomonas azotifigens* LMG 23662^T, *Pseudomonas balearica* LMG 18376^T and *Pseudomonas xanthomarina* LMG 23572^T). API 20 NE and API 50 CH galleries (bioMérieux), and GN2 Microplates (Biolog) were performed according to the manufacturer's instructions and results recorded after 24 and 48 h, and again after five days (API 50 CH galleries only). *P. kirkiae* sp. nov. is biochemically weak but strains can be distinguished from *P. stutzeri*, *P. azotifigens*, *P. balearica*, *P. xanthomarina* and *P. nosocomialsis* by their inability to assimilate potassium gluconate, D-mannitol and citrate, their ability to weakly ferment potassium 5-ketogluconate and to oxidize 2-aminoethanol. The most distinguishing characteristics are listed in Table 1, while detailed results of the phenotypic assays are available in Suppl. Table S4.

Fatty acid methyl ester (FAME) analysis was performed by Fera Science Ltd. (York, UK) on strains of *P. kirkiae* sp. nov. (FRB 229^T, P17C, P28C and P30C) grown in TSA medium at 28 °C for 24 h. The protocol was based on the Sherlock Microbial Identification System Version 6.2 (MIDI Inc.) and the results obtained were compared against the library TSBA6 6.10. The whole-cell fatty acid methyl ester composition of *P. kirkiae* sp. nov. is similar to that of members of the *P. stutzeri* group [40, 41]. Major fatty acids include $C_{12:0}$, $C_{16:0}$, $C_{17:0}$ cyclo, $C_{19:0}$ cyclo ω8c 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 complete fatty acid profiles for *P. kirkiae* sp. nov. and the closest phylogenetic relatives are displayed in Table 2.

Protologue

Description of *Pseudomonas kirkiae* sp. nov.

Pseudomonas kirkiae (kirk.i'ae. N.L. gen. fem. n. *kirkiae*, of Kirk, named after Susan Kirk MBE in recognition of her technical contribution to research on the pathology of serious tree diseases in the UK between 1976 and 2015).

Cells are Gram-negative, slightly curved rods (1.5 µm x 0.4 µm), motile by amphitrichous flagella, non-spore forming, strictly aerobic, and positive for catalase and oxidase activity. Colonies are fluorescent, cream-coloured, circular and convex, with entire margins and measure 1 mm in diameter after 48 hours of incubation at 25 °C on KB agar. Optimum growth occurs at 28 °C and pH 6.0 - 8.0, all strains grow from 4 - 10 °C to 39 °C.

Strains are negative for indole production, arginine dihydrolase, urease, β-glucosidase, gelatinase and β -galactosidase. Nitrate cannot be reduced to nitrite or nitrogen. Only caprate and malate are assimilated. Acid is weakly produced from potassium 5-ketogluconate by all strains (API 50 CH). Tween 80, L-arabinose, pyruvic acid methyl ester, succinic acid mono-methyl ester, acetic acid, α-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, D,L-lactic acid, propionic acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, D-alanine, L-alanine, L-aspargine, L-aspartic acid, L-glutamic acid, L-proline and 2-aminoethanol are oxidised (Biolog GN2). Variable reactions are observed for fermentation of L-arabinose, D-ribose, D-galactose, D-glucose and D-lactose, and oxidation of D-serine, γ-aminobutyric acid, dextrin, glycogen and tween 40. Major fatty acids included $C_{12:0}$, $C_{16:0}$, $C_{17:0}$ cyclo, $C_{19:0}$ cyclo ω8c 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 229^T (P4C^T = LMG 31089^T = NCPPB 4674^T) was isolated from AOD symptomatic inner bark of *Quercus robur*, in the United Kingdom. The G+C content of the type strain (QJUO00000000) is 63.7 mol %.

Phylogenetic considerations of *Pseudomonas* and the '*Azotobacter*' group

Several studies over the past 19 years have indicated a close relationship between the genera *Pseudomonas* and *Azotobacter*, *Azomonas* and *Azorhizophilus* (known collectively as the '*Azotobacter*' group) [42–44], with possible synonymy of *Azotobacter* and *Azomonas*, as well as *Azotobacter* and *Pseudomonas* suggested. Studies of protein-encoding genes and pan-genome analyses have demonstrated that species of *Azotobacter* are phylogenetically related to species in the *P. aeruginosa* clade, and share a third of protein families with *Pseudomonas* [43–45]. This has led researchers to conclude that *Azotobacter* species could be transferred to *Pseudomonas*, although the genus description would have to be amended to reflect the phenotypic discrepancies.

A further complication is that *Azotobacter vinelandii* consistently clusters with *Azorhizophilus paspasli* (basonym *Azotobacter paspali*), which causes a division in the *Azotobacter* clade. Despite appearing on a Validation List in the International Journal of Systematic Bacteriology (Validation List no. 6. *Int. J. Syst. Bacteriol.*, 1981, 31, 215-218), the classification of *Azorhizophilus paspasli* [46] is not widely accepted. Consequently, the species was included under the genus *Azotobacter* as its basonym in the 2005 edition of Bergey's Manual of Systematics of Archaea and Bacteria [47].

To provide a simplified overview of the relationship amongst the four genera, 16S rRNA gene and MLSA (based on *gyrB*, *rpoB* and *rpoD* gene sequences) phylogenetic trees (maximum likelihood and Bayesian inference) were constructed as described earlier. The dataset included two representative species from each of the *Pseudomonas* groups according to Gomila et al. [25], *Pseudomonas* outliers (species which cannot be assigned to an existing *Pseudomonas* group) as well as all available *Azotobacter, Azomonas* and *Azorhizophilus* species (Suppl. Table S5). Due to a lack of available sequences, a limited number of *Azotobacter* and *Azomonas* species could be incorporated in the MLSA analysis, while *Azorhizophilus paspali* could not be included at all.

Both 16S rRNA ML and BI phylogenies demonstrate the clustering of *Azorhizophilus paspasli* with *Azotobacter vinelandii* with high bootstrap and PP support (Suppl. Fig. S5a and S5b),

indicating that these two species are phylogenetically related and supporting the re-inclusion of *Azorhizophilus paspasli* in the genus *Azotobacter*. Conversely, these phylogenies show a more distant relationship between species of *Azotobacter* and *Azomonas*. In both 16s rRNA phylogenetic trees, *Azomonas* species are on separate lineages to *Azotobacter* species. This arrangement is also observed in the phylogenetic trees based on MLSA, both nucleotide and amino acid, (Supp. Figs. S6a, S6b and S6c) with species of *Azotobacter* and *Azomonas* forming separate, well-supported clades on separate lineages negating the possible synonymy of these two genera. Although the positions of these clades are not stable and appear to change when additional species are added to the analyses. However, the *Azotobacter* and *Azomonas* clades do cause clear divisions within the genus *Pseudomonas*, specifically in the *P. aeruginosa* lineage, creating polyphyletic taxa. To correct this, either all species of *Azotobacter* and *Azomonas* would have to be transferred to *Pseudomonas* or these two genera are left unchanged and species of *Pseudomonas* are divided among several novel genera to reflect the polyphyletic nature of the genus. There is currently no consensus view or opinion on how best to resolve this complicated taxonomic issue.

Data from the different studies examining the relationship between *Pseudomonas* and *Azotobacter* does confirm a close phylogenetic and evolutionary relationship between the *P. aeruginosa* lineage and *Azotobacter* species. If *Azotobacter* species were transferred to *Pseudomonas*, their relationship to *P. aeruginosa* (the type species of the genus) would signify them as 'true' *Pseudomonas* species, which would necessitate a thorough amendment of the genus as suggested by Young and Park [43], to implement a much broader *Pseudomonas* genus description. Following this option would partially resolve the polyphyly in *Pseudomonas*. However, there is still the issue of the genus *Azomonas*, whose phylogenetic position is unstable, and the *Pseudomonas* outliers. The genus *Pseudomonas* is expanding rapidly every year and currently includes over 190 validated species [12]. The majority of the more recently described species are assigned to one of the three lineages (*P. aeruginosa*, *P. fluorescens* or *P. pertucinogena*). Those species which cannot be assigned to a defined *Pseudomonas* group or lineage are considered outliers and, when included along with species of *Azotobacter* and *Azomonas* in the phylogenetic analysis, add to the disruption of the *Pseudomonas* phylogeny. This serves to emphasise the fact that the genus *Pseudomonas* does require a taxonomic re-evaluation.

A recent phylogenomic study of *Pseudomonas* species has highlighted the substantial genomic diversity, as well as vast difference in genome size and G + C content between validly described members of this genus [48]. The smallest genome observed is *P. caeni* (3.03 Mbp) while the largest is *P. saponiphila* (7.38 Mbp), and the G + C content varies from 48 to 68 mol %, which is not taxonomically viable when the genus description lists the G + C content range as 58 – 69 mol % [39]. The study by Hesse *et al*. [48], detected 189 potential novel *Pseudomonas* species after including more than 1200 available genomes in their analyses. With such varying degrees of genome size and G + C content, it is highly unlikely that the current *Pseudomonas* genus description can sustain further inclusions of novel species. Indeed, Peix *et al*. [12] predict that as further novel *Pseudomonas* species are described, the genus will be forced to split into several genera.

Rather than amending the genus description to accommodate *Azotobacter* species and adjusting the C + C content range to span a difference of 20 mol % across the validly described species, we recommend that *Azotobacer* and *Azomonas* remain separate genera and that *Pseudomonas* be divided to reflect the phylogenomic diversity amongst species, groups and lineages. It is probable that the 'true' *Pseudomonas* genus will retain only a handful of species with *P*. *aeruginosa* remaining the type species, while species belonging to the *P. fluorescens* and *P. pertucinogena* lineages will be transferred to novel genera. This will be an enormous undertaking, but the re-evaluation must be a thorough examination of all *Pseudomonas*, *Azotobacter* and *Azomonas* species based on phylogenetic analyses of multiple core protein sequences, phenotypic and morphological characteristics; incorporating a more modern taxonomic approach while still acknowledging the original genus description.

AUTHOR STATEMENTS

This study was funded by the University of the West of England, The Forestry Commission and Woodland Heritage. The authors would like to thank Dr Sara Alvira-de-Celis for providing the TEM images of the cells. We would also like to acknowledge Dr Maria Chuvochina for assisting with the etymology of the novel species, and Dr Javier Bobo-Pinilla for his help with the program MrBayes.

Genome sequencing was provided by MicrobesNG (http://www.microbesng.uk), which is supported by the BBSRC (grant number BB/L024209/1).

The authors have no conflicts of interest to declare.

ABBREVIATIONS

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

PYGA: potato yeast glucose agar

RAPD-PCR: random amplification of polymorphic DNA

REFERENCES

1. **Brady C, Denman S, Kirk S, Venter S, Rodríguez-Palenzuela P, *et al.*** Description of *Gibbsiella quercinecans* gen. nov., sp. nov., associated with Acute Oak Decline. *Syst Appl Microbiol* 2010;33:444–450.

2. **Brady CL, Cleenwerck I, Denman S, Venter SN, Rodríguez-Palenzuela P, *et al.*** Proposal to reclassify *Brenneria quercina* (Hildebrand and Schroth 1967) Hauben et al. 1999 into a new genus, *Lonsdalea* gen. nov., as *Lonsdalea quercina* comb. nov., descriptions of *Lonsdalea quercina* subsp. *quercina* comb. nov., *Lonsdalea quercina* subsp. *ib*. *Int J Syst Evol Microbiol* 2012;62:1592–1602.

3. **Denman S, Brady C, Kirk S, Cleenwerck I, Venter S, *et al.*** *Brenneria goodwinii* sp. nov., associated with acute oak decline in the UK. *Int J Syst Evol Microbiol* 2012;62:2451–2456.

4. **Brady C, Hunter G, Kirk S, Arnold D, Denman S**. Description of *Brenneria roseae* sp. nov. and two subspecies, *Brenneria roseae* subspecies *roseae* ssp. nov and *Brenneria roseae* subspecies *americana* ssp. nov. isolated from symptomatic oak. *Syst Appl Microbiol* 2014;37:396–401.

5. **Brady C, Hunter G, Kirk S, Arnold D, Denman S**. *Gibbsiella greigii* sp. nov., a novel species associated with oak decline in the USA. *Syst Appl Microbiol* 2014;37:417–422.

6. **Brady C, Hunter G, Kirk S, Arnold D, Denman S**. *Rahnella victoriana* sp. nov., *Rahnella bruchi* sp. nov., *Rahnella woolbedingensis* sp. nov., classification of *Rahnella* genomospecies 2 and 3 as *Rahnella variigena* sp. nov. and *Rahnella inusitata* sp. nov., respectively and emended description of the genus R. *Syst Appl Microbiol* 2014;37:545–552.

7. **Bueno-Gonzalez V, Brady C, Denman S, Plummer S, Allainguillaume J, *et al.*** *Pseudomonas daroniae* sp. nov. and *Pseudomonas dryadis* sp. nov., isolated from pedunculate oak affected by acute oak decline in the UK. *Int J Syst Evol Microbiol* 2019;159378:1–9.

8. **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.

9. **Denman S, Doonan J, Ransom-Jones E, Broberg M, Plummer S, *et al.*** Microbiome and infectivity studies reveal complex polyspecies tree disease in Acute Oak Decline. *ISME J* 2018;12:386–399.

10. **Denman S, Plummer S, Kirk S, Peace A, McDonald JE**. Isolation studies reveal a shift in the cultivable microbiome of oak affected with Acute Oak Decline. *Syst Appl Microbiol* 2016;39:484–490.

11. **Sapp M, Lewis E, Moss S, Barrett B, Kirk S, *et al.*** Metabarcoding of bacteria associated with the Acute Oak Decline syndrome in England. *Forests* 2016;7:95.

12. **Peix A, Ramírez-Bahena MH, Velázquez E**. The current status on the taxonomy of *Pseudomonas* revisited: An update. *Infection, Genetics and Evolution* 2018;57:106–116.

13. **Niemann S, Pühler 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.

14. **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.

15. **Mulet M, Bennasar A, Lalucat J, Garcı E**. An *rpo*-based PCR procedure for the identification of *Pseudomonas* species and for their detection in environmental samples. 2009;23:140–147.

16. **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.

17. **Yoon S, Ha S, Kwon S, Lim J, Kim Y, *et al.*** Introducing EzBioCloud : a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. 2019;1613–1617.

18. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ**. Basic local alignment search tool. *J Mol Biol* 1990;215:403–10.

19. **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.

20. **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.

21. **Lefort V, Longueville J-E, Gascuel O**. SMS: Smart Model Selection in PhyML. *Mol Biol Evol* 2017;34:2422–2424.

22. **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.

23. **Kumar S, Stecher G, Li M, Knyaz C, Tamura K**. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 2018;35:1547–1549.

24. **Mulet M, Lalucat J, García-Valdés E**. DNA sequence-based analysis of the *Pseudomonas* species. *Environ Microbiol* 2010;12:1513–1530.

25. **Gomila M, Peña A, Mulet M, Lalucat J, García-Valdés E**. Phylogenomics and systematics in *Pseudomonas*. *Front Microbiol* 2015;6:1–13.

26. **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.

27. **Versalovic J, Koeuth T, Lupski JR**. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* 1991;19:6823–31.

28. **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.

29. **Bolger AM, Lohse M, Usadel B**. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;30:2114–2120.

30. **Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, *et al.*** The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 2009;25:2078–2079.

31. **Quinlan AR, Hall IM**. BEDTools: A flexible suite of utilities for comparing genomic

features. *Bioinformatics* 2010;26:841–842.

32. **Li H, Durbin R**. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* 2010;26:589–595.

33. **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.

34. **Seemann T**. Prokka: Rapid prokaryotic genome annotation. *Bioinformatics* 2014;30:2068–2069.

35. **Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J**. JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. *Bioinformatics* 2016;32:929–931.

36. **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.

37. **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.

38. **King EO, Ward MK, Raney DE**. Two simple media for the demonstration of pyocyanin and fluorescin. *J Lab Clin Med* 1954;44:301–7.

39. **Palleroni NJ**. *Pseudomonas*. In: *Bergey's Manual of Systematics of Archaea and Bacteria*. 2015. pp. 58–69.

40. **Romanenko LA, Uchino M, Falsen E, Lysenko AM, Zhukova N V, *et al.*** *Pseudomonas xanthomarina* sp. nov., a novel bacterium isolated from marine ascidian. *J Gen Appl Microbiol* 2005;51:65–71.

41. **Anwar N, Rozahon M, Zayadan B, Mamtimin H, Abdurahman M, *et al.*** *Pseudomonas tarimensis* sp. nov., an endophytic bacteria isolated from *Populus euphratica*. *Int J Syst Evol Microbiol* 2017;67:4372–4378.

42. **Anzai Y, Kim H, Park JY, Wakabayashi H, Oyaizu H**. Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *Int J Syst Evol Microbiol* 2000;50:1563–

1589.

43. **Young JM, Park DC**. Probable synonymy of the nitrogen-fixing genus *Azotobacter* and the genus *Pseudomonas*. *Int J Syst Evol Microbiol* 2007;57:2894–2901.

44. **Özen AI, Ussery DW**. Defining the *Pseudomonas* genus: where do we draw the line with *Azotobacter*? *Microb Ecol* 2012;63:239–248.

45. **Rediers H, Vanderleyden J, De Mot R**. *Azotobacter vinelandii*: A *Psedomonas* in disguise? *Microbiology* 2004;150:1117–1119.

46. **Thompson JP, Skerman VBD**. *Azotobacteraceae*: the taxonomy and ecology of the aerobic nitrogen-fixing bacteria. In: *Azotobacteraceae: the taxonomy and ecology of the aerobic nitrogen-fixing bacteria.* London: Academic Press. p. 417 pp.

47. **Kennedy C, Rudnick P, MacDonald ML, Melton T**. *Azotobacter*. In: *Bergey's Manual of Systematics of Archaea and Bacteria*. 2015. pp. 1–33.

48. **Hesse C, Schulz F, Bull CT, Shaffer BT, Yan Q, *et al.*** Genome-based evolutionary history of *Pseudomonas* spp. *Environ Microbiol* 2018;20:2142–2159.

49. **Mulet M, Gomila M, Ramírez A, Lalucat J, Garcia-Valdes E**. *Pseudomonas nosocomialis* sp. nov., isolated from clinical specimens. *Int J Syst Evol Microbiol* 2019;69:3392–3398.

Table 1. Distinguishing phenotypic features of strains of *Pseudomonas kirkiae* sp. nov. and closest phylogenetic neighbours. Data was generated in this study using the commercial tests API 20 NE, API 50 CH (6 days incubation) and Biolog GN2, except for *Pseudomonas nosocomialis* [49]. For complete phenotypic profiles see Suppl. Table S4. All *P. kirkiae* strains tested were able to assimilate caprate and malate, displayed cytochrome oxidase activity and were positive for oxidation of tween 80, L-arabinose, D-mannitol, pyruvic acid methyl ester, succinic acid mono-methyl ester, acetic acid, α-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, D,L-lactic acid, propionic acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, D-alanine, L-alanine, L-aspargine, L-aspartic acid, L-glutamic acid, L-proline and 2-aminoethanol.

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
API 20 NE (Assimilation of):								
D-mannitol	-	-	-	-	+	-	+	+
potassium gluconate	-	-	-	+	+	+	+	+
citrate	-	-	-	+	+	-	-	+
API 50 CH (Acid from):								
D-Lactose	-	-	+	-	-	-	-	ND
potassium 5-ketogluconate	w+	w+	w+	-	-	-	-	ND
Biolog GN2 (Oxidation of):								
dextrin	-	-	+	+	+	+	+	+

D-fructose	-	-	-	+	-	w+	-	+
citric acid	-	-	-	+	+	+	-	+
D-gluconic acid	-	-	-	+	+	+	-	+
tween 40	-	+	+	+	+	+	-	+
D-serine	-	-	+	-	-	-	-	+
2-aminoethanol	+	+	+	-	-	w+	-	ND
DNA G+C content, mol %	63.7	63.5	63.6	63.9	66.7	64.7	60.3	65.5

(1) *Pseudomonas kirkiae* sp. nov. FRB 229^T (2) *Pseudomonas kirkiae* sp. nov. P28C (3) *Pseudomonas kirkiae* sp. nov. P30C (4) *Pseudomonas stutzeri* LMG 11199^T (5) *Pseudomonas azotifigens* LMG 23662^T (6) *Pseudomonas balearica* LMG 18376^T (7) *Pseudomonas xanthomarina* LMG 23572^T (8) *Pseudomonas nosocomialis* A31/70^T.

^{T} = type strain. +: 100 % of strains positive; -: 100 % of strains negative; w+: 100 % of strains weakly positive; ND: not determined

Table 2: Percentages of cell fatty acid methyl esters (FAMEs) in strains of *Pseudomonas kirkiae* sp. nov. Summed features are sets of two or more fatty acids, which the Microbial Identification System (MIDI) could not 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: *Pseudomonas stutzeri* and *Pseudomonas xanthomarina* [40], *Pseudomonas azotifigens* and *Pseudomonas balearica* [41] and *Pseudomonas nosocomialis* [49]. Values are displayed as average percentage per species investigated, with the standard deviation shown in parentheses. *Pseudomonas kirkiae* sp. nov. displayed trace amounts (<1 %) of $C_{10:0}$ and $C_{18:0}$.

Fatty acid	**1**	**2**	**3**	**4**	**5**	**6**
$C_{10:0}$ 3-OH	2.9 (± 0.4)	-	3.4	3.4	-	3.5
$C_{12:0}$	8.4 (± 0.6)	6.8	10.0	9.6	7.8	8.4
$C_{12:0}$ 3-OH	3.4 (± 0.3)	<1	2.8	3.4	<1	<1
$C_{16:0}$	24.0 (± 0.8)	22	18.6	20.2	21.8	22.5
$C_{16:1}$ ω9c	-	24.5	-	-	30.4	-
$C_{17:0}$ cyclo	13.7 (± 4.0)	0.4	1.4	4.0	2.9	-
$C_{18:1}$	-	41.3	-	-	28.9	-
$C_{19:0}$ cyclo ω8c	8.5 (± 3.0)	-	2.5	3.6	-	-
Summed feature 3 ($C_{16:1}$ ω6c and/or $C_{16:1}$ ω7c)	9.8 (± 3.9)	-	26.6	23.3	-	20.5
Summed feature 8 ($C_{18:1}$ ω7c and/or $C_{18:1}$ ω6c)	27.4 (± 2.3)	-	32.0	28.8	-	38.6

(1) *Pseudomonas kirkiae* sp. nov. (*n* = 3) (2) *Pseudomonas stutzeri* CIP 103022^{T} (3) *Pseudomonas azotifigens* JCM 12708^{T} (4) *Pseudomonas balearica* DSM 6083^{T} (5) *Pseudomonas xanthomarina* CCUG 46543^{T} (6) *Pseudomonas nosocomialis* A31/70^{T}. ^{T} = type strain, - = not detected.

Figure 1: Maximum likelihood phylogenetic tree of the 16S rRNA gene sequences of *Pseudomonas kirkiae* sp. nov. and the closest phylogenetic neighbours. A dataset of 42 almost complete 16S rRNA gene sequences (1367 bp) was compared to infer the evolutionary relationships of the taxa using PhyML. The nucleotide substitution model used was GTR+I+G. Bootstrap values >50 % are shown next to the branches. The scale indicates the nucleotide substitutions per site. Species names are followed by the strain number and the GenBank accession number. Outgroup*:* *Endozoicomonas elysicola* DSM 22380^{T}. ^{T} = type strain.

Figure 2: Maximum likelihood phylogenetic tree of the concatenated *gyrB* – 771 bp, *rpoB* – 753 bp, *rpoD* – 651 bp gene sequences obtained from 13 strains of *Pseudomonas kirkiae* sp. nov. and the closest phylogenetic neighbours. A dataset of 41 gene sequences (2175 bp) was compared to infer the evolutionary relationships of the taxa using PhyML. Bootstrap values >50 % are shown next to the branches. The scale indicates the nucleotide substitutions per site. The scale indicates the nucleotide substitutions per site. Species names are followed by the strain number. Outgroup: *Endozoicomonas elysicola* DSM 22380^{T}. ^{T} = type strain.

Figure 3: Transmission electron microscopy image of *Pseudomonas kirkiae* sp. nov. FRB 229^{T}. Bar, 1 µm.