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1 **_Pseudomonas kirkiae_ sp. nov., a novel species isolated from oak in the United Kingdom, and**
2 **phylogenetic considerations of the genera _Pseudomonas_, _Azotobacter and Azomonas_.**
3

4 Victoria Bueno-Gonzalez[a*], Carrie Brady[a*], Sandra Denman[b], Joël Allainguillaume[a] and Dawn
5 Arnold[a]
6
7 [a] Centre for Research in Bioscience, Faculty of Health and Life Sciences, University of the West
8 of England, Frenchay Campus, Bristol, United Kingdom
9 [b] Forest Research, Centre for Forestry and Climate Change, Farnham, United Kingdom
10 * Both authors contributed equally to this work
11
12 Correspondence: Centre for Research in Bioscience, Faculty of Health and Life Sciences,
13 University of the West of England, Bristol, BS16 1QY, United Kingdom
14 Tel: +44117 32 84225 email: carrie.brady@uwe.ac.uk
15

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17

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20

21 The GenBank/EMBL/DDBJ accession numbers are as follows: MK159379 – MK159391 (16S
22 rRNA), MN0044394 – MN0044406 (_rpoB_), MN044407 – MN044419 (_gyrB_), MN044420 –
23 MN044432 (_rpoD_) and QJUO00000000 – QJUR00000000 (whole genome).
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34 ## ABSTRACT

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

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66 Over the last ten years, a total of two novel genera, 13 novel species and two novel subspecies
67 have been classified from oak displaying symptoms of decline in the United Kingdom, Spain
68 and the United States of America [1–7]. The majority of bacterial strains have been isolated
69 in the UK from pedunculate oak (*Quercus robur*) exhibiting signs of Acute Oak Decline (AOD),
70 with weeping bleeds, cracked bark plates and necrotic lesions in the underlying tissue [8]. The
71 current episode of AOD in the UK is caused by a polymicrobial complex with *Brenneria*
72 *goodwinii* and *Gibbsiella quercinecans* responsible for the necrosis, with possible insect
73 involvement by *Agrilus biguttatus* [9]. The role of other species in symptom development,
74 such as *Rahnella victoriana*, must still be elucidated.
75
76 Ongoing isolations from AOD-affected trees and a metagenome study of the oak microbiome
77 have revealed a large *Pseudomonas* population present in both symptomatic and healthy oak
78 [10, 11]. *Pseudomonas* is a ubiquitous genus, containing more than 190 species isolated from
79 origins varying from human, animal or plant samples to the extreme environments of the
80 desert and Antarctic [12]. Due to the heterogeneous sources *Pseudomonas* species are
81 isolated from, their phylogeny is diverse and classification of novel strains can be difficult.
82 Consequently, sequencing of the 16S rRNA and *gyrB* genes could not assign the *Pseudomonas*
83 isolates from oak to a species with a high degree of confidence, but phylogenetic analysis
84 revealed they could be divided into three clusters within the genus *Pseudomonas* suggesting
85 the presence of three novel species. Strains from two of these clusters were recently
86 described as novel species in the *Pseudomonas straminea* group, namely *Pseudomonas*
87 *daroniae* and *Pseudomonas dryadis* [7]. In this study we use a polyphasic approach, based on
88 genotypic, genomic, phenotypic and chemotaxonomic analyses, to demonstrate that the
89 third of these *Pseudomonas* clusters constitutes another novel species for which the name
90 *Pseudomonas kirkiae* sp. nov. is proposed.
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93 Isolation

94 Symptomatic and non-symptomatic oak trees in three woodlands were sampled, Bisham
95 Woods (51.54911° N, 0.77071° W), Stratfield Brake (51.80306° N, 001.28274° W) and Send
96 (51.28760° N 0.52873° W). Bacteria were isolated from the inner bark and sapwood of

97 symptomatic oak, *Agrilus biguttatus* larval galleries in the phloem, as well as non-
98 symptomatic sapwood. A list of strains used in this study are listed in Suppl. Table S1. Strains
99 were initially isolated in potato yeast glucose agar (PYGA), and routinely cultured at 25 °C on
100 King's B agar (KB) and in Luria Bertani (LB) broth.
101
102

103 **Genotypic characterisation**

104 Alkalic lysis was used to extract genomic DNA for all subsequent PCR reactions [13].
105 Amplification and sequencing of the 16S rRNA, *gyrB*, *rpoD* and *rpoB* genes were performed
106 on the 13 *Pseudomonas* strains isolated from oak as previously described [14–16]. The 16S
107 rRNA gene sequence pairwise similarities were obtained by comparison with reference strains
108 in EZBioCloud [17], and were all >96.3 % similar to *P. nosocomialis*. Values of 95.2 – 95.7 %
109 similarity *to P. xanthomarina*, *P. aeruginosa*, *P. stutzeri*, *P. furukawaii*, *P. composti*, *P.*
110 *balearica* and *P. mangiferae*, as well as *Azotobacter chroococcum* were observed. The 16S
111 rRNA gene sequences for the closest phylogenetic neighbours to the oak strains were
112 downloaded from EzBioCloud for phylogenetic analyses. Additional sequences of the *gyrB*,
113 *rpoD* and *rpoB* genes for the closest phylogenetic neighbours, as determined by EzBioCloud
114 and BLAST [18], were downloaded from the GenBank database (Suppl. Table S2). These
115 included species from both *Pseudomonas* and *Azotobacter*. All four gene sequences were
116 aligned and trimmed in BioEdit 7.2.5 [19], final sequence lengths were as follows: 16S rRNA –
117 1367 bp, *gyrB* – 771 bp, *rpoB* – 753 bp and *rpoD* – 651 bp. Protein-encoding gene sequences
118 were conceptually translated in BioEdit. Following concatenation of the *gyrB*, *rpoB* and *rpoD*
119 gene datasets in SequenceMatrix [20], Smart Model Selection (SMS) [21] was applied to both
120 the 16S rRNA gene and concatenated datasets to determine the best-fit nucleotide
121 substitution model for construction of maximum likelihood (ML) and neighbour joining (NJ)
122 trees, respectively.
123
124 Phylogenetic tree construction was performed using PhyML [22] for ML trees and MEGA X
125 10.1.7 [23] for NJ trees, applying the selected evolutionary models and parameters. The ML
126 16S rRNA and MLSA nucleotide trees were computed using the general time reversible model
127 with invariable sites and gamma distribution (GTR+I+G), while the ML MLSA amino acid tree
128 was computed using LG+G+F. The NJ 16S rRNA and MLSA nucleotide trees were constructed

129 using the Kimura 2-parameter model with gamma distribution (K2P+G). Bootstrap support
130 with 1000 replicates was generated to assess the confidence of the clusters. Bootstrap values
131 less than 50 % are not included in the phylogenetic trees. *Endozoicomonas elysicola* DSM
132 22380^T was selected as an outgroup.
133
134 In the ML 16S rRNA gene phylogenetic tree (Fig. 1) the type strain of the proposed species,
135 FRB 229^T, is positioned on a separate branch closely on the border of the *P. aeruginosa* lineage
136 also containing *Azotobacter*, *Azorhizophils* and *Azomonas* species [12, 24, 25] but with no
137 significant bootstrap support. The topology of the NJ 16S rRNA tree (Suppl. Fig. S1) is
138 different, with FRB 229^T positioned on a separate branch on the border of a clade containing
139 *P. thermotolerans*, *P. mangiferae*, *Azotobacter* species and *Azorhizophilus paspali*, within the
140 *P. aeruginosa* lineage. Again, there is no bootstrap support for this clade, suggesting the
141 taxonomic position of FRB 229^T could be easily changed by the addition of further novel
142 *Pseudomonas* species. It is not possible to determine which *Pseudomonas* group [12] the
143 proposed novel species belongs to, based on 16S rRNA gene sequencing, as inclusion of
144 *Azotobacter* and *Azorhizophilus* species disrupts the currently defined groups. Species of
145 *Azotobacter*, *Azomonas* and *Azorhizophilus* were included in our 16S rRNA gene sequence
146 analysis following the results of the EZBioCloud comparison.
147
148 In both the ML and NJ phylogenetic trees (Fig. 2 and Suppl. Fig. S2), based on concatenated
149 protein-encoding genes (*gyrB*, *rpoD* and *rpoB*), strains from the proposed novel species form
150 a single robust cluster with 100 % bootstrap support. This cluster is positioned on the border
151 of the *P. stutzeri* group, within the *P. aeruginosa* lineage [12, 25]. The ML tree based on the
152 concatenated amino acid sequences is congruent with the trees constructed from nucleotide
153 sequences (Suppl. Fig. S3).
154
155 As with the 16S rRNA gene phylogeny, the concatenated multigene phylogeny demonstrates
156 a division in the *Pseudomonas* genus clade by *Azotobacter* species. In recent years, there has
157 been an increasing number of studies highlighting the close relationship between
158 *Pseudomonas* and the genera known as the '*Azotobacter*' group, namely *Azotobacter*,
159 *Azorhizophilus* and *Azomonas*. The phylogenetic predicament of the genus *Pseudomonas* and
160 the '*Azotobacter*' group is discussed in detail following the species protologue.

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

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172 Genome Features

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

179

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

187

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

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

204 The morphology, physiology and biochemistry of strains belonging to the novel species were
205 examined using a range of tests. Cell size and motility were measured using light microscopy
206 and the microscopy imaging software CellSens version 1.11 (Olympus Life Science, Tokyo,
207 Japan). Cells of the type strain of *P. kirkiae* sp. nov. were imaged by transmission electron
208 microscopy (TEM) as previously described [7]. Oxidase tests were performed according to the
209 manufacturer's instructions (bioMérieux), while catalase activity was observed by gas bubble
210 formation following suspension of the bacteria in 3 % w/w hydrogen peroxide. Strains were
211 grown on KB agar at 25 °C for fluorescent pigment production analysis [38] and on tryptone
212 soy agar (TSA) under anaerobic conditions at 35 °C to determine respiration conditions.
213 Tolerance to temperature was tested by measuring growth on KB agar and TSA at 4 - 10 °C,
214 33 °C, 37 °C, 39 °C and 41 °C. Plates were incubated and monitored for bacterial growth for 6
215 days. Growth in the presence of salt was studied by supplementing tryptone soy broth (TSB)
216 with NaCl (0.5 – 4.5 % w/v in 1 % increments), while tolerance to pH (5.0 – 9.0) was tested in
217 (TSB). Both were incubated at 25 °C for 48 h and monitored for growth.
218

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

225 The strains tested are tolerant to media supplemented with 0.5 – 3.0 % NaCl, but FRB 229^T
226 can grow at a NaCl concentration of 3.5 %. All strains form colonies in the pH range of 6.0 to
227 8.0 (*Pseudomonas* do not typically grow under acidic conditions [39]).
228
229 Physiological and biochemical tests were performed on representative strains from the novel
230 species (FRB 229^T, P28C and P30C), as well as type strains from the closest phylogenetic
231 relatives (*Pseudomonas stutzeri* LMG 11199^T, *Pseudomonas azotifigens* LMG 23662^T,
232 *Pseudomonas balearica* LMG 18376^T and *Pseudomonas xanthomarina* LMG 23572^T). API 20
233 NE and API 50 CH galleries (bioMérieux), and GN2 Microplates (Biolog) were performed
234 according to the manufacturer's instructions and results recorded after 24 and 48 h, and again
235 after five days (API 50 CH galleries only). *P. kirkiae* sp. nov. is biochemically weak but strains
236 can be distinguished from *P. stutzeri*, *P. azotifigens*, *P. balearica*, *P. xanthomarina* and *P.*
237 *nosocomialsis* by their inability to assimilate potassium gluconate, D-mannitol and citrate,
238 their ability to weakly ferment potassium 5-ketogluconate and to oxidize 2-aminoethanol.
239 The most distinguishing characteristics are listed in Table 1, while detailed results of the
240 phenotypic assays are available in Suppl. Table S4.
241
242 Fatty acid methyl ester (FAME) analysis was performed by Fera Science Ltd. (York, UK) on
243 strains of *P. kirkiae* sp. nov. (FRB 229^T, P17C, P28C and P30C) grown in TSA medium at 28 °C
244 for 24 h. The protocol was based on the Sherlock Microbial Identification System Version 6.2
245 (MIDI Inc.) and the results obtained were compared against the library TSBA6 6.10. The
246 whole-cell fatty acid methyl ester composition of *P. kirkiae* sp. nov. is similar to that of
247 members of the *P. stutzeri* group [40, 41]. Major fatty acids include $C_{12:0}$, $C_{16:0}$, $C_{17:0}$ cyclo, $C_{19:0}$
248 cyclo ω8c and summed features 3 ($C_{16:1}$ ω6c and/or $C_{16:1}$ ω7c) and 8 ($C_{18:1}$ ω7c and/or $C_{18:1}$
249 ω6c). The complete fatty acid profiles for *P. kirkiae* sp. nov. and the closest phylogenetic
250 relatives are displayed in Table 2.
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258 ## Protologue

259

260 ### Description of *Pseudomonas kirkiae* sp. nov.

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

264

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

270

271 Strains are negative for indole production, arginine dihydrolase, urease, β-glucosidase,
272 gelatinase and β -galactosidase. Nitrate cannot be reduced to nitrite or nitrogen. Only caprate
273 and malate are assimilated. Acid is weakly produced from potassium 5-ketogluconate by all
274 strains (API 50 CH). Tween 80, L-arabinose, pyruvic acid methyl ester, succinic acid mono-
275 methyl ester, acetic acid, α-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, D,L-
276 lactic acid, propionic acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide,
277 D-alanine, L-alanine, L-aspargine, L-aspartic acid, L-glutamic acid, L-proline and 2-
278 aminoethanol are oxidised (Biolog GN2). Variable reactions are observed for fermentation of
279 L-arabinose, D-ribose, D-galactose, D-glucose and D-lactose, and oxidation of D-serine, γ-
280 aminobutyric acid, dextrin, glycogen and tween 40. Major fatty acids included $C_{12:0}$, $C_{16:0}$, $C_{17:0}$
281 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
282 and/or $C_{18:1}$ ω6c).

283

284 The type strain FRB 229^T (P4C^T = LMG 31089^T = NCPPB 4674^T) was isolated from AOD
285 symptomatic inner bark of *Quercus robur*, in the United Kingdom. The G+C content of the type
286 strain (QJUO00000000) is 63.7 mol %.

287

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

289 Several studies over the past 19 years have indicated a close relationship between the genera
290 *Pseudomonas* and *Azotobacter*, *Azomonas* and *Azorhizophilus* (known collectively as the
291 '*Azotobacter*' group) [42–44], with possible synonymy of *Azotobacter* and *Azomonas*, as well
292 as *Azotobacter* and *Pseudomonas* suggested. Studies of protein-encoding genes and pan-
293 genome analyses have demonstrated that species of *Azotobacter* are phylogenetically related
294 to species in the *P. aeruginosa* clade, and share a third of protein families with *Pseudomonas*
295 [43–45]. This has led researchers to conclude that *Azotobacter* species could be transferred
296 to *Pseudomonas*, although the genus description would have to be amended to reflect the
297 phenotypic discrepancies.

298

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

306

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

316

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

319 indicating that these two species are phylogenetically related and supporting the re-inclusion
320 of *Azorhizophilus paspasli* in the genus *Azotobacter*. Conversely, these phylogenies show a
321 more distant relationship between species of *Azotobacter* and *Azomonas*. In both 16s rRNA
322 phylogenetic trees, *Azomonas* species are on separate lineages to *Azotobacter* species. This
323 arrangement is also observed in the phylogenetic trees based on MLSA, both nucleotide and
324 amino acid, (Supp. Figs. S6a, S6b and S6c) with species of *Azotobacter* and *Azomonas* forming
325 separate, well-supported clades on separate lineages negating the possible synonymy of
326 these two genera. Although the positions of these clades are not stable and appear to change
327 when additional species are added to the analyses. However, the *Azotobacter* and *Azomonas*
328 clades do cause clear divisions within the genus *Pseudomonas*, specifically in the *P.*
329 *aeruginosa* lineage, creating polyphyletic taxa. To correct this, either all species of
330 *Azotobacter* and *Azomonas* would have to be transferred to *Pseudomonas* or these two
331 genera are left unchanged and species of *Pseudomonas* are divided among several novel
332 genera to reflect the polyphyletic nature of the genus. There is currently no consensus view
333 or opinion on how best to resolve this complicated taxonomic issue.
334
335 Data from the different studies examining the relationship between *Pseudomonas* and
336 *Azotobacter* does confirm a close phylogenetic and evolutionary relationship between the *P.*
337 *aeruginosa* lineage and *Azotobacter* species. If *Azotobacter* species were transferred to
338 *Pseudomonas*, their relationship to *P. aeruginosa* (the type species of the genus) would signify
339 them as 'true' *Pseudomonas* species, which would necessitate a thorough amendment of the
340 genus as suggested by Young and Park [43], to implement a much broader *Pseudomonas*
341 genus description. Following this option would partially resolve the polyphyly in
342 *Pseudomonas*. However, there is still the issue of the genus *Azomonas*, whose phylogenetic
343 position is unstable, and the *Pseudomonas* outliers. The genus *Pseudomonas* is expanding
344 rapidly every year and currently includes over 190 validated species [12]. The majority of the
345 more recently described species are assigned to one of the three lineages (*P*. *aeruginosa*, *P.*
346 *fluorescens* or *P. pertucinogena*). Those species which cannot be assigned to a defined
347 *Pseudomonas* group or lineage are considered outliers and, when included along with species
348 of *Azotobacter* and *Azomonas* in the phylogenetic analysis, add to the disruption of the
349 *Pseudomonas* phylogeny. This serves to emphasise the fact that the genus *Pseudomonas* does
350 require a taxonomic re-evaluation.

351 A recent phylogenomic study of *Pseudomonas* species has highlighted the substantial
352 genomic diversity, as well as vast difference in genome size and G + C content between validly
353 described members of this genus [48]. The smallest genome observed is *P. caeni* (3.03 Mbp)
354 while the largest is *P. saponiphila* (7.38 Mbp), and the G + C content varies from 48 to 68 mol
355 %, which is not taxonomically viable when the genus description lists the G + C content range
356 as 58 – 69 mol % [39]. The study by Hesse *et al*. [48], detected 189 potential novel
357 *Pseudomonas* species after including more than 1200 available genomes in their analyses.
358 With such varying degrees of genome size and G + C content, it is highly unlikely that the
359 current *Pseudomonas* genus description can sustain further inclusions of novel species.
360 Indeed, Peix *et al*. [12] predict that as further novel *Pseudomonas* species are described, the
361 genus will be forced to split into several genera.
362
363 Rather than amending the genus description to accommodate *Azotobacter* species and
364 adjusting the C + C content range to span a difference of 20 mol % across the validly described
365 species, we recommend that *Azotobacer* and *Azomonas* remain separate genera and that
366 *Pseudomonas* be divided to reflect the phylogenomic diversity amongst species, groups and
367 lineages. It is probable that the 'true' *Pseudomonas* genus will retain only a handful of species
368 with *P. aeruginosa* remaining the type species, while species belonging to the *P. fluorescens*
369 and *P. pertucinogena* lineages will be transferred to novel genera. This will be an enormous
370 undertaking, but the re-evaluation must be a thorough examination of all *Pseudomonas*,
371 *Azotobacter* and *Azomonas* species based on phylogenetic analyses of multiple core protein
372 sequences, phenotypic and morphological characteristics; incorporating a more modern
373 taxonomic approach while still acknowledging the original genus description.
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381 ## AUTHOR STATEMENTS

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387

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390

391 The authors have no conflicts of interest to declare.

392

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393 ## ABBREVIATIONS

394 ANI: average nucleotide identity
395 AOD: acute oak decline
396 BI: Bayesian inference
397 ERIC-PCR: enterobacterial repetitive intergenic consensus
398 FAME: Fatty acid methyl ester
399 GGDC: genome to genome distance calculator
400 GTR+I+G: general time reversible model with invariable sites and gamma distribution
401 *is*DDH: *in silico* DNA-DNA hybridisation
402 ML: Maximum likelihood
403 MLSA: multilocus sequence analysis
404 PP: Posterior probabilities
405 PYGA: potato yeast glucose agar
406 RAPD-PCR: random amplification of polymorphic DNA

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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.