Systematic and Applied Microbiology 46 (2023) 126399

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.com/locate/syapm

Description of *Dryocola* gen. nov. and two novel species, *Dryocola boscaweniae* sp. nov. and *Dryocola clanedunensis* sp. nov. isolated from the rhizosphere of native British oaks

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ARTICLE INFO

Keywords:
AOD
Rhizosphere
Oak
Enterobacteriaceae
Soil

ABSTRACT

While investigating the role of the rhizosphere in the development of Acute Oak Decline, bacterial strains belonging to the family *Enterobacteriaceae* were isolated from rhizosphere soil following enrichment for the Enterobacterales. Partial sequencing of several housekeeping genes showed that these strains could not be assigned to an existing genus. Overall, 16 strains were investigated using a polyphasic approach to determine their taxonomic status. This involved phenotypic testing and fatty acid analysis paired with phylogenetic analyses of 16S rRNA and housekeeping gene sequences, as well as phylogenomic analysis of whole genome sequences. Phylogenomic and phylogenetic analyses consistently demonstrated that the 16 isolates could be separated into two distinct clusters in a monophyletic clade situated between the genera *Cedecea* and *Buttiauxella*. The two clusters could be genotypically and phenotypically differentiated from each other and from their closest neighbours. As such we propose the description of *Dryocola boscaweniae* gen. nov. sp. nov. (type strain H6W4^T = CCUG 76177^T = LMG 32610^T) and *Dryocola clanedunesis* sp. nov. (type strain H11S18^T = CCUG 76181^T = LMG 32611^T).

Introduction

Acute oak decline (AOD) is a decline disease originally identified on native British oak species, although cases on nine other species of oak have now been recorded in the UK (Crampton et al., 2022). AOD has also been reported on Persian oak in Iran (*Quercus brantii*); pedunculate oak (*Quercus robur*) in Spain and Poland; several oak species in Switzerland and cork oak (*Quercus suber*) in Portugal (Fernandes et al., 2022; González and Ciordia, 2019; Moradi-Amirabad et al., 2019; Ruffner et al., 2020; Tkaczyk et al., 2021). Decline diseases are complex multifactorial diseases involving abiotic and biotic elements (Manion, 1981; Sinclair, 1967; Sinclair and Hudler, 1988). Recently Denman *et al.* updated the original decline disease spiral model, which explains how decline disease systems operate. In this updated model the importance of the microbiome is emphasised, which includes feeder roots and the rhizosphere microbiomes (Denman et al., 2022). Healthy soils and feeder root function are essential and trees actively shape these environments in their response to stress (Vives-Peris et al., 2020; York et al., 2016). Thus, the rhizosphere, which is the first point of contact between plants and soil, is an essential feature of plant health. Both plant growth-promoting bacteria and phytopathogens colonise this region and play critical roles in nutrient mobilisation and protection, and these affect health/disease of plants (Lugtenberg and Kamilova, 2009; Philippot et al., 2013). The composition of the rhizosphere microbiome has already been shown to differ between healthy oaks and those suffering from AOD (Pinho et al., 2020), with an abundance of ammonia oxidising bacteria being significantly associated with healthy tree roots (Scarlett et al., 2021), but many of the bacterial species are unclassified and their function in tree health is not understood.

We designed a study to investigate the differences in rhizosphere microbiome composition and the role of rhizospheric *Enterobacteriaceae* in relation to AOD and oak health. Several bacterial strains were isolated from rhizosphere soil collected from symptomatic and healthy native oaks at Hatchlands Park, Guildford, UK that could not be assigned to an existing genus. Multilocus sequence analysis (MLSA) placed them in two monophyletic clusters that were phylogenetically related to both *Buttiauxella* and *Cedecea*.

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https://doi.org/10.1016/j.syapm.2023.126399
Received 21 September 2022; Revised 9 January 2023; Accepted 11 January 2023

The genus *Cedecea* was created for a set of 17 isolates from clinical sources designated 'Enteric Group 15′ at the Centre for Disease Control (CDC), with *Cedecea davisae* and *Cedecea lapagei* officially described in 1981 (Grimont', P.A.D., Grimont, F., Farmer, J.J., Asbury', M.A. , 1981). *Cedecea neteri* was classified one year later for strains isolated from blood samples taken from patients suffering with potential endocarditis (Farmer et al., 1982). Strains of this rarely isolated genus are reported as opportunistic human pathogens, and have also been isolated from water ticks and insects (Farmer, 2015). '*Cedecea colo*' (though not validly published) is the most recently described species, isolated from the hindgut (via fresh scat) of koala (*Phascolarctos cinereus*), and plays an important role in the breakdown of secondary plant metabolites that otherwise make eucalyptus leaves, the sole food source, non-toxic (Boath et al., 2020). The genus is mostly known for causing infection in humans, although it has also been seen to cause disease on a number of edible mushrooms in China (Farmer, 2015; Liu et al., 2021; Thompson and Sharkady, 2020; Yan et al., 2019).

Meanwhile, *Buttiauxella* is more commonly isolated from the wider environment, with a particular association to the intestines of snails and slugs worldwide (Kämpfer, 2015). The genus *Buttiauxella* was described in 1982 for strains belonging to the type species; *Buttiauxella agrestis,* isolated from water and unpolluted soils (Ferragut et al., 1981). In 1996; a further six species were validly published, with the majority isolated from molluscs and snails (Müller et al., 1996). Species of this geographically widespread genus can play a role as opportunistic pathogens in humans but are also noted for their plant growth-promoting properties; such as increased root length, surface area and number of root tips for plants grown under cadmium stress (Patra et al., 2018; Wu et al., 2018). Additionally; *B. agrestis* has been reported to help the acclimatisation of seedlings, stimulating growth of the plant including the roots and dry mass (de Araújo et al., 2021).

Due to both *Cedecea* and *Buttiauxella* species playing important roles in the environment it was decided that further investigation of the phylogenetically related monophyletic clade was warranted. The aim of this study was to use polyphasic taxonomic classification to define a novel genus and novel species.

Methods

Isolation

All strains in this paper were isolated from rhizosphere soil of 10 different oak trees, both symptomatic of AOD and healthy, at Hatchlands Park, Guildford, UK. Roots with rhizosphere soil still attached were collected from four cardinal points around each oak tree at a 2 m radius. Roots were confirmed to belong to oak using Loop-mediated Isothermal Amplification (LAMP) of the actin gene (Bridget Crampton, personal communication). Rhizosphere soil was gently removed from the roots by hand and passed through a 2 cm sieve to remove other debris. Ten grams of sieved wet soil was then placed in *Enterobacteriaceae* Enrichment broth (EE broth, Thermo Scientific) and incubated at 25 ˚C for 48 h. The EE suspension was diluted to 10^{-6} in ¼ Ringers solution (Oxoid) and spread-plated on Eosin Methyl Blue (EMB) agar (Merck), which was incubated both aerobically and anaerobically at 28 ˚C, allowing for the isolation of single colonies. Glycerol stocks of the pure cultures were made and stored at –80 ˚C. All strains were routinely cultured from stocks on Luria-Bertani (LB, Oxoid) agar or in LB broth (Oxoid) at temperatures between 28 and 37 ˚C. Suppl. Table S1 contains the full list of isolates investigated in this study.

Phylogenetic analyses

Alkalic lysis (Niemann et al., 1997) was used for the extraction of DNA used in all PCR reactions, which was stored at −20 ˚C. Multilocus sequence analysis (MLSA) of the housekeeping genes *fusA, leuS, pyrG* and *rpoB* was performed for all strains using the primers and conditions as described (Delétoile et al., 2009). All additional sequences for the closest phylogenetic neighbours were downloaded from GenBank via BLAST against whole genome sequences (Benson et al., 2013). Sequences were aligned and trimmed in MEGA X v10.0 (Tamura et al., 2021) to the following lengths: *fusA* – 633 bp, *leuS* – 642 bp, *pyrG* – 306 bp, *rpoB* – 501 bp. The near complete 16S rRNA gene (1,346 bp) was sequenced for the type strains of the proposed novel species (H11S18^T and H6W4^T) using standard amplification cycles and an annealing temperature of 55 ˚C with the primers from Coenye *et al.* (Coenye et al., 1999). The EzBioCloud server was used to calculate the 16S rRNA gene pairwise similarity of the proposed type strains (Yoon et al., 2017).

Consensus sequence alignment was performed in UGENE V 38.1 (Okonechnikov et al., 2012) on all four housekeeping and 16S rRNA gene sequences to ensure coverage in both directions. A concatenated dataset was made from the four housekeeping genes and Smart Model Selection (SMS) (Lefort et al., 2017) performed on both datasets on the online PhyML server (Guindon and Gascuel, 2003). The output was then used to inform the Maximum Likelihood phylogenetic analysis in MEGA X. Reliability of the clusters generated in the phylogenetic tree were tested through 1000 bootstrap replications. Additionally, the housekeeping gene sequences were translated conceptually, and Maximum Likelihood phylogenetic analysis was performed on the resulting concatenated amino acid sequences in MEGA X using the JTT model and 1000 bootstrap replicates.

BOX-PCR

BOX PCR was performed on all isolated strains from both clusters to assess their genetic diversity using the BOX A1R primer and previously published protocol (Versalovic et al., 1991). Amplicons were separated in 1.5 % agarose at 50 V for ~ 3 h.

Genomic features

The genomes of strains from Cluster 1 (H11S18^T and H16N7) and Cluster 2 (H6W4^T, H18W14 and H20N1), as determined by MLSA, were sequenced by MicrobesNG (Birmingham, UK) on the Illumina HiSeq platform following DNA extraction by enzymatic cell lysis and DNA purification by Solid Phase Reversible Immobilization (SPRI) beads. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cut-off of Q15 (Bolger et al., 2014). *De novo* assembly was performed using SPAdes version 3.11.1 and the resulting contigs were annotated in Prokka 1.11 (Bankevich et al., 2012; Seemann, 2014). Whole genome sequence contamination was excluded by aligning the 16S rRNA gene sequences obtained via Sanger sequencing to the whole genome sequences in CodonCode v10.0.2 (CodonCode Corporation, USA).

Genome Blast Distance Phylogeny (GBDP) pairwise comparisons between genomes was used to infer the phylogenomic positions of the strains with the Type (Strain) Genome Server (TYGS) (Meier-Kolthoff and Göker, 2019). The algorithm 'trimming' and distance formula d_5 *was* used with 100 replicates to generate intergenomic distances against a number of reference genomes (Meier-Kolthoff et al., 2013). A genome caption tree was drawn with FastME 2.1.6.1 from the distances calculated from the genome sequences, with formula d_5 being used to scale the branch lengths, and the tree being rooted at the midpoint following Subtree Pruning and Regrafting (SPR) (Farris, 1972; Lefort et al., 2015).

In addition to the comparisons made using TYGS, whole genome comparisons were performed between the proposed novel species in this study and existing *Buttiauxella* and *Cedecea* species. Average nucleotide identity (ANI) with FastANI (Jain et al., 2018), average amino identity (AAI) through the Genome-based distance matrix calculator supported by Kostas lab (Rodriguez-R and Konstantinidis,

2016) and *in silico* DNA – DNA hybridisation (*is*DDH) d_5 values, calculated using the Genome-to-Genome Distance calculator (Goris et al., 2007) were used.

Comparative analysis of genomic traits

To assess the potential roles of strains from the proposed novel species, the PGAP-processed whole genome sequences of H6W4^T, H18W14, H20N1, H11S18^T and H16N7 were queried against several databases (Tatusova et al., 2016). The Virulence Factor Database (VFDB) (Liu et al., 2022), accessed on 29 December 2022, was queried using the DIAMOND v2.0.11.149 (Buchfink et al., 2021) BlastP command. A query cut-off of 97 % and a percentage identity of 50 % or higher were used to ensure that only high sequence identity alignments between genomes and the VFDB were made (Doonan et al., 2019). For the identification of type III secretion system (T3SS) effectors, open reading frames (ORFs) were identified using orfipy and exported as fasta files (Singh and Wurtele, 2021). The Effectidor web server was then used to predict T3SS effectors from the ORF fasta files (Wagner et al., 2022). Finally, the 'plant bacterial only interaction factors' (PIFAR-Pred) database accessed through the PLant-associated BActeria web resource (PLaBAse) was used to assess the microbe-plant interaction potential of each strain by querying the genomes against the database using the BlastP + HMMER Aligner/Mapper (Martínez-García et al., 2016; Patz et al., 2021).

Cell imaging and physiology

Cell size, morphology and motility were determined using light microscopy and the microscopy imaging software CellSens version 1.11 with Olympus SC180 (Olympus Life Science, Tokyo, Japan). Flagella arrangement for both novel species was observed by transmission electron microscopy (FEI Tecnai 12 120 kV BioTwin Spirit TEM) following negative staining. For negative staining, grids were floated on mid-log phase bacterial suspensions for 2 min, washed in distilled water three times and stained with 3 % uranyl acetate for 30 sec before repeating the wash step, wicking excess liquid and air drying.

The growth temperature range was assessed on tryptone soy agar (TSA, Sigma) in triplicate and incubated at 4, 10, 25, 28, 30, 37 and 41 ˚C, with colony morphology observed from the 28 ˚C plates after 48 h. Tolerance to changes in pH was tested by inoculation of tryptone soya broth (TSB, Sigma) where the range varied from 4 to 10 (in increments of 1) after adjusting with sodium acetate/acetic acid and carbonate/bicarbonate buffers. Salt tolerance at 1 – 7 % was tested in saline-free nutrient broth (3 g l^{-1} beef extract, 5 g l^{-1} peptone) with the salt concentration adjusted by the addition of 1 % w/v NaCl. Both salt and pH tests were inoculated with each isolate in triplicate and incubated overnight at 30 ˚C with shaking at 180 rpm.

Antibiotic resistance

Antibiotic resistance of the proposed novel species to penicillin G 10 μg, penicillin V 10 μg, tetracycline 30 μg, ampicillin 10 μg, chloramphenicol 30 μg and colistin sulphate 10 μg was tested. Strains were grown overnight in TSB at 30 ˚C with shaking at 180 rpm. 100 μL of mid-log range overnight culture was spread plated on TSA and six antibiotic discs were applied at equal distances using a disc dispenser (Oxoid). Plates were inspected for a zone of clearance surrounding the antibiotic discs after incubation at 30 ˚C for 24 h. Type strains of *C. davisae* LMG 7862^T, *C. lapegei* LMG 7863^T and *C. neteri* LMG 7864^T were included as positive controls.

Phenotypic assays and FAMES analysis

Phenotypic testing was performed on a selection of strains from MLSA Clusters 1 (H11S18^T and H16N7) and 2 (H6W4^T, H16W4 and H20N1) with the commercial assays API 20 E, API 50 CHB/E, ID 32 E (bioMérieux) and GEN III GN/GP microplates (Biolog) all used according to the manufacturer's instructions. API 20 E and ID 32 E galleries were scored after 24 h incubation at 37 ˚C. Following incubation at 30 ˚C, API 50 CHB/E galleries and GEN III plates were both scored twice, after 24 and 48 h, and 18 h and 24 h, respectively. Type strains of *Cedecea* species (LMG 7862^T, LMG 7863^T and LMG 7864^T) were included in the above assays for comparison. Information for comparison to *Buttiauxella* species was taken from published literature (Kämpfer, 2015; Zgheib et al., 2022). Catalase and oxidase activity were also tested, with 3 % v/v H_2O_2 to test for bubble production and staining with Kovács reagent (1 % tetra-methyl-p-phenylenediamine dihydrochloride) to see if a purple colour formed using the donor electron acceptor, respectively.

Fatty acid methyl ester (FAME) analysis was performed on strains from MLSA Cluster 1 (H11S18^T and H16N7), MLSA Cluster 2 (H6W4^T, H16W4 and H20N1) and the three type strains of *Cedecea* species (LMG 7862^T, LMG 7863^T and LMG 7864^T) by FERA Science Ltd. All strains were grown on TSA at 30 ˚C for 24 h after which the protocol for Sherlock Microbial Identification System Version 6.4 (MIDI Inc.) was followed. Comparison library RTSBA6 6.21 was used as a reference for comparison of the results. The FAMES profiles for *Buttiauxella* were obtained from published data (Kämpfer et al., 1997).

Results and discussion

Genotypic characterisation

In the Maximum Likelihood phylogenetic tree based on the concatenated MLSA nucleotide sequences (Fig. 1), the strains isolated from oak rhizosphere soil were divided into two strongly supported clusters descended from the same node, with no validly published species present in the clade. The first cluster (Cluster 1) contained five isolates that showed little sequence variation, while the second cluster (Cluster 2) contained eight isolates with minor sequence variation, but both with 100 % bootstrap support suggesting two potential novel species. A recently described, but not yet validly published species, '*Cedecea colo*' (Boath et al., 2020) was included in the clade on a separate branch on the border of Cluster 1, but with low bootstrap support. The position of both potential novel species clusters, in relation to their closest phylogenetic neighbours, suggests they belong to a novel genus. The inclusion of '*Cedecea colo*' in the potential novel genus clade suggests that this species may not belong to *Cedecea*, as the three validly published *Cedecea* species are contained in a separate monophyletic clade with 99 % bootstrap support. The topology of the concatenated MLSA amino acid phylogenetic tree was congruent with that of the concatenated MLSA nucleotide tree, with the novel strains divided into two strongly supported clusters (Suppl. Fig. S1).

H11S18^T (Cluster 1) demonstrated highest 16S rRNA pairwise sequence similarity to *C. neteri* (98.7 %), while H6W4^T (Cluster 2) exhibited highest similarity to *Buttiauxella izardii* (98.6 %). Many publications have observed and commented on the poor taxonomic resolution gained through 16S rRNA gene sequencing within the order Enterobacterales due to the observed high homogeneity (Naum et al., 2008). As such, the taxonomic position of the proposed novel genus and species was not reliably represented by either the Neighbour Joining or Maximum Likelihood 16S rRNA gene phylogenetic trees (Suppl. Fig. S2 and Suppl. Fig. S3). In both trees the isolates clustered loosely with species of *Buttiauxella* and *Cedecea*, but with no, or very low, bootstrap support.

The results from the BOX PCR demonstrated that although some isolates were clonal regardless of sampling location, a degree of genetic diversity exists within both proposed novel species (Suppl. Fig. S4).

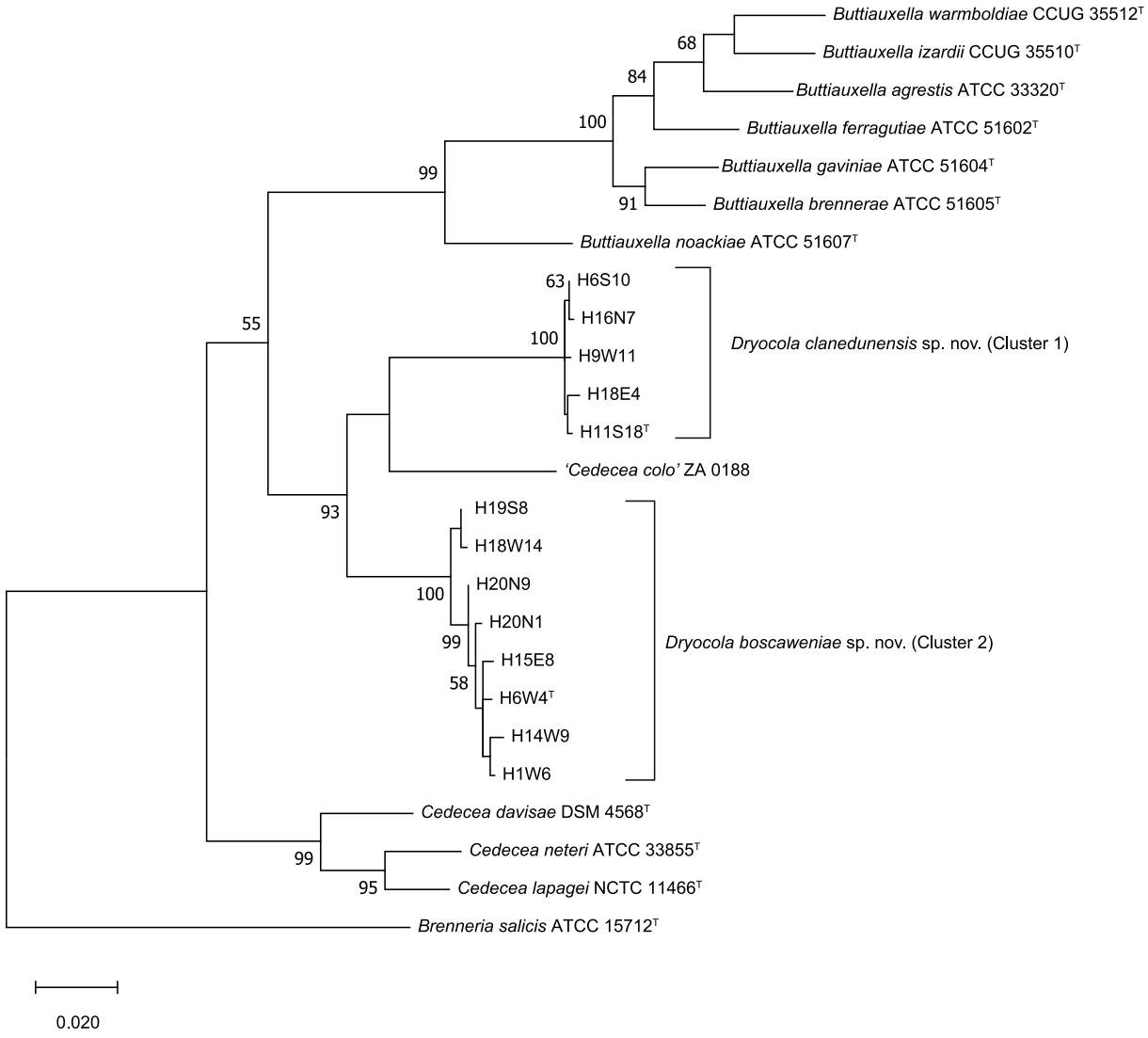

Fig. 1. Maximum likelihood tree based on the concatenated partial nucleotide sequences of *fusA, leuS, pyrG* and *rpoB* from species of the proposed genus *Dryocola* gen. nov., and its closest phylogenetic neighbours *Cedecea* and *Buttiauxella*. *Brenneria salicis* (ATCC 15712^T) was included as the outgroup. Percentages for bootstrap values exceeding 50 % following 1000 replicates are shown. The scale bar represents the number of substitutions per site. ^{T} denotes type strains.

Genomic features

Genome sizes of the two potential novel species varied, with strains of Cluster 1 ranging from 4.82 to 5.23 Mbp and strains of Cluster 2 being 4.4 – 4.45 Mbp. The DNA G + C content was 53.0–53.9 mol % across strains from both potential novel species. Genomes sequences were submitted to GenBank under the BioProject number PRJNA814476, and genome features and assembly accessions are listed in Suppl. Table. S2.

The resulting phylogenomic tree (Fig. 2) from the TYGS analysis demonstrated both potential novel species clusters formed a robust clade with '*Cedecea colo*', with 100 % bootstrap support. The clade is positioned between the *Buttiauxella* and *Cedecea* genus clades also with 100 % support, reflecting the position observed in the MLSA phylogenetic tree (Fig. 1) and further supporting the description of a novel genus, two novel species and the possible transfer of '*Cedecea colo*' to the novel genus.

The genome comparison values demonstrated that strains from Cluster 1 shared ANI values of 99.1 % and AAI values of 99 % to each other, and 81.7 – 82.4 % ANI and 82 – 86 % AAI to species of *Buttiauxella* and *Cedecea*. Cluster 2 strains demonstrated 99.1 – 100 % similarity through ANI values and 99.0 % AAI values with each other, and 81.9 – 82.8 % ANI and 83 – 87 % AAI to species of *Buttiauxella* and *Cedecea*. The intra-species values exceed the suggested 95 % similarity required to delimit species for ANI and 96 % for AAI (Konstantinidis and Tiedje, 2005), confirming that both clusters constituted single taxa as shown in Fig. 2. The conclusions drawn from the ANI analysis were confirmed by *in silco* DNA-DNA hybridisation (*is*DDH) with both clusters exceeding the cut-off value of > 70 % indicating a different species while showing high similarity to each other (Goris et al., 2007). Both the ANI values and *is*DDH values for each cluster can be seen in Table 1.

Comparative analysis of genomic traits

Queries against the VFDB using the suggested BlastP parameters revealed the presence of 201 – 202 and 199 virulence genes for strains from Cluster 1 and Cluster 2, respectively. Of these, the three most prevalent categories of genes were related to Motility, Nutritional and Metabolic factors, and Immune modulation. Strains from both clusters contained a number of genes related to effector delivery systems, with strains from Cluster 1 containing 10 – 11, and strains from

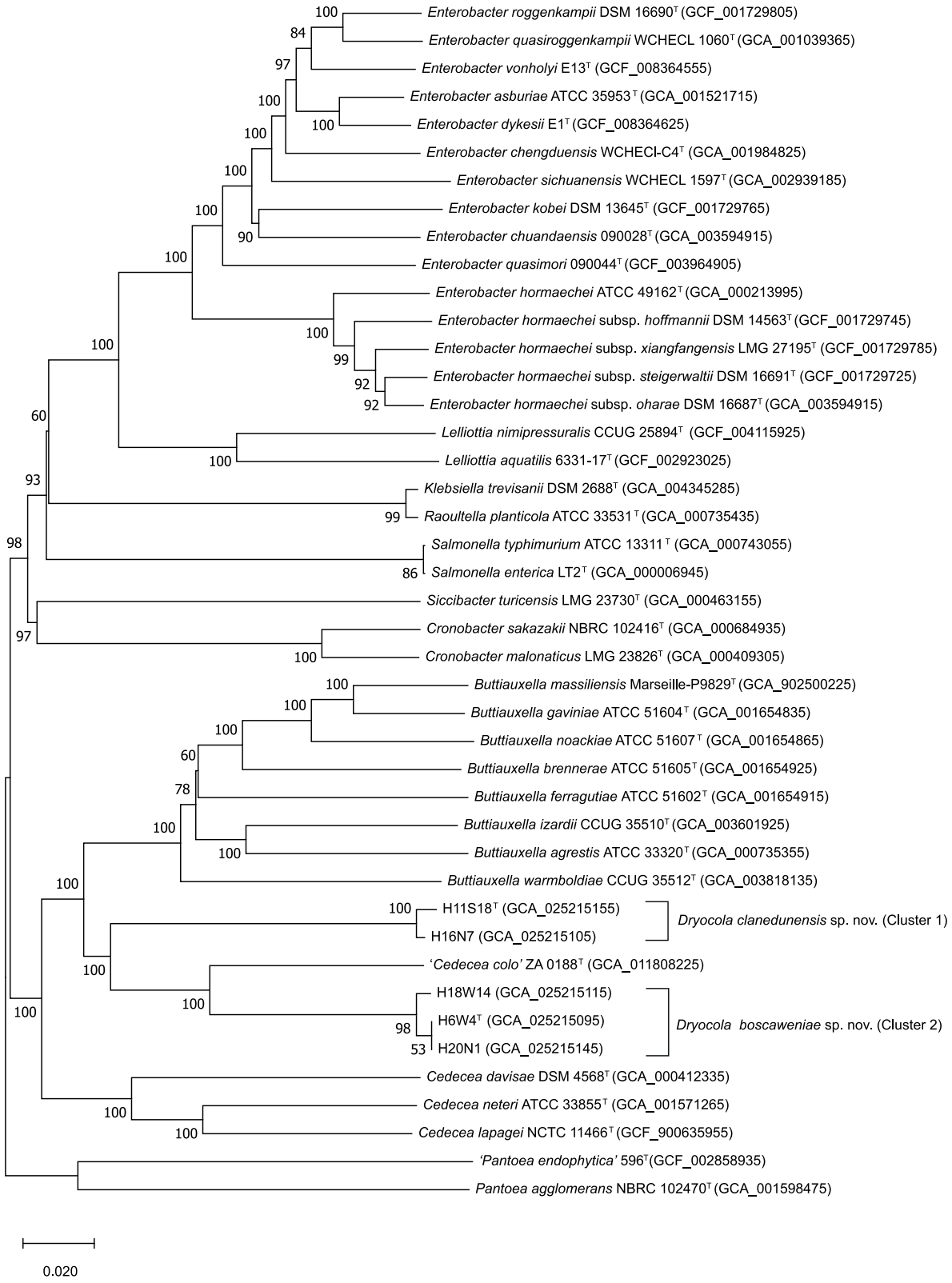

Fig. 2. Phylogenomic tree of the proposed genus *Dryocola* gen. nov., and its closest phylogenetic neighbours. GBDP pseudo-bootstrap support values from 100 replicates exceeding 50 % are shown at the nodes, with an average branch support of 94.4 %. Branch lengths are scaled from the d_5 GBDP distance formula and the tree is rooted at the midpoint. ^{T} signifies the type strain and GenBank assembly numbers are shown in parentheses.

Cluster 2 containing 25 – 27. In Cluster 1 strains, the effector delivery systems were predominantly type VI secretion systems (T6SS), although one type IV protein was identified. However, Cluster 2 strains contained the same T6SS proteins, but also coded for several T3SS proteins. T3SS proteins are highly associated with bacterial pathogenesis of plants (Niu et al., 2013). Furthermore, the T3SS is crucial for

Table 1
In silico DNA-DNA Hybridisation (*is*DDH – top right) and Average Nucleotide Identities matrix (fastANI – bottom left) percentage values for *Dryocola boscaweniae* sp. nov. and *Dryocola clanedunensis* sp. nov. and all species of *Cedecea* and *Buttiauxella*, the closest phylogenetic neighbours. Values in bold exceed the cut off point for species delimitation (>70 % *is*DDH and > 95 % for ANI).

isDDH / fastANI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	**100**	**94**	**94**	26.4	26.4	35.7	22.8	22.7	22.8	23.7	23.8	23.6	23.6	24.2	23.3	24.1
2	**99.1**	**100**	**100**	26.5	26.5	35.8	22.8	22.7	22.6	23.9	23.7	23.6	23.6	24.2	23.3	24.1
3	**100**	**99.1**	**100**	26.4	26.4	35.8	22.8	22.7	22.6	23.7	23.7	23.6	23.5	24.2	23.3	24.1
4	84.2	84.2	84.2	**100**	**89.9**	26.4	22.7	22.7	22.6	22.9	23	22.9	22.9	23.3	22.7	23.7
5	84.1	84.1	84.1	**99.1**	**100**	26.5	22.7	22.8	22.7	23.9	24.5	23.7	26.4	23.9	24.5	24.2
6	88.5	88.6	88.6	83.9	88.7	**100**	22.9	22.8	22.8	23.6	23.5	23.5	23.3	24.1	23.2	24.1
7	81.9	81.8	81.9	81.7	81.8	81.9	**100**	28.9	28.9	21.6	21.5	21.5	21.3	22	21.4	22.3
8	81.6	81.6	81.6	81.8	81.8	81.6	85.9	**100**	28.9	21.8	21.4	21.5	21.3	21.9	21.3	22.2
9	81.7	81.9	81.7	81.7	81.8	81.7	86.1	89.1	**100**	21.6	21.6	21.6	21.3	21.9	21.5	22.3
10	82.4	82.5	82.4	82.1	82.6	82.3	81	81.1	81.1	**100**	31.3	30.8	31	35.8	30.3	31.5
11	82.3	82.3	82.3	81.7	82.9	81.9	80	80.4	80.7	87.5	**100**	30.8	39.1	30.1	32.7	29.3
12	82.2	82.1	82.2	81.8	82.2	82	81	80.7	80.9	86.6	86.8	**100**	30.2	29.8	29.8	28.9
13	82.2	82.2	82.2	81.7	84.1	81.8	80.9	80.6	80.7	87.3	91	86.3	**100**	29.8	44.6	29.4
14	82.6	82.6	82.6	82.1	82.6	82.3	81.3	80.9	81.2	89.2	86.7	86.3	86.5	**100**	29.6	31.3
15	82.1	82.9	82.1	81.5	82.9	81.9	80.9	80.7	80.9	86.7	87.9	86	91.9	86.2	**100**	29.5
16	82.9	82.8	82.8	82.4	82.8	82.6	81.5	81.2	81.2	87	85.6	85.3	86.7	86.7	85.5	**100**

(1) *Dryocola boscaweniae* H6W4^T (GCA_025215095), (2) *Dryocola boscaweniae* H18W14 (GCA_025215115), (3) *Dryocola boscaweniae* H20N1 (GCA_025215145), (4) *Dryocola clandunensis* H11S18^T (GCA_025215155), (5) *Dryocola clandunensis* H16N7 (GCA_025215105), (6) '*Cedecea colo*' ZA 0188^T (GCA_011808225), (7) *Cedecea davisae* DSM 4562^T (GCA_000412335), (8) *Cedecea lapagei* ATCC 33855^T (GCA_001571265), (9) *Cedecea neteri* NCTC 11466^T (GCF_900635955), (10) *Buttiauxella agrestis* ATCC 33320^T (GCA_000735355), (11) *Buttiauxella brennerae* ATCC 51605^T (GCA_001654925), (12) *Buttiauxella ferragutiae* ATCC 51602^T (GCA_001654915), (13) *Buttiauxella gaviniae* ATCC 51604^T (GCA_001654835), (14) *Buttiauxella izardii* CCUG 35510^T (GCA_003601925), (15) *Buttiauxella noackiae* ATCC 51607^T (GCA_001654865), (16) *Buttiauxella warmboldiae* CCUG 35512^T (GCA_003818135).

causing galls, wilting, soft rot and necrosis in plants by members of the family *Enterobacteriaceae* (Xu et al., 2021). As such, these proteins were further investigated using Effectidor to predict the T3SS effectors and core proteins from each strain's genome ORF collection. The results likewise showed a collection of the core proteins required for a function T3SS as well as several known effectors. More importantly

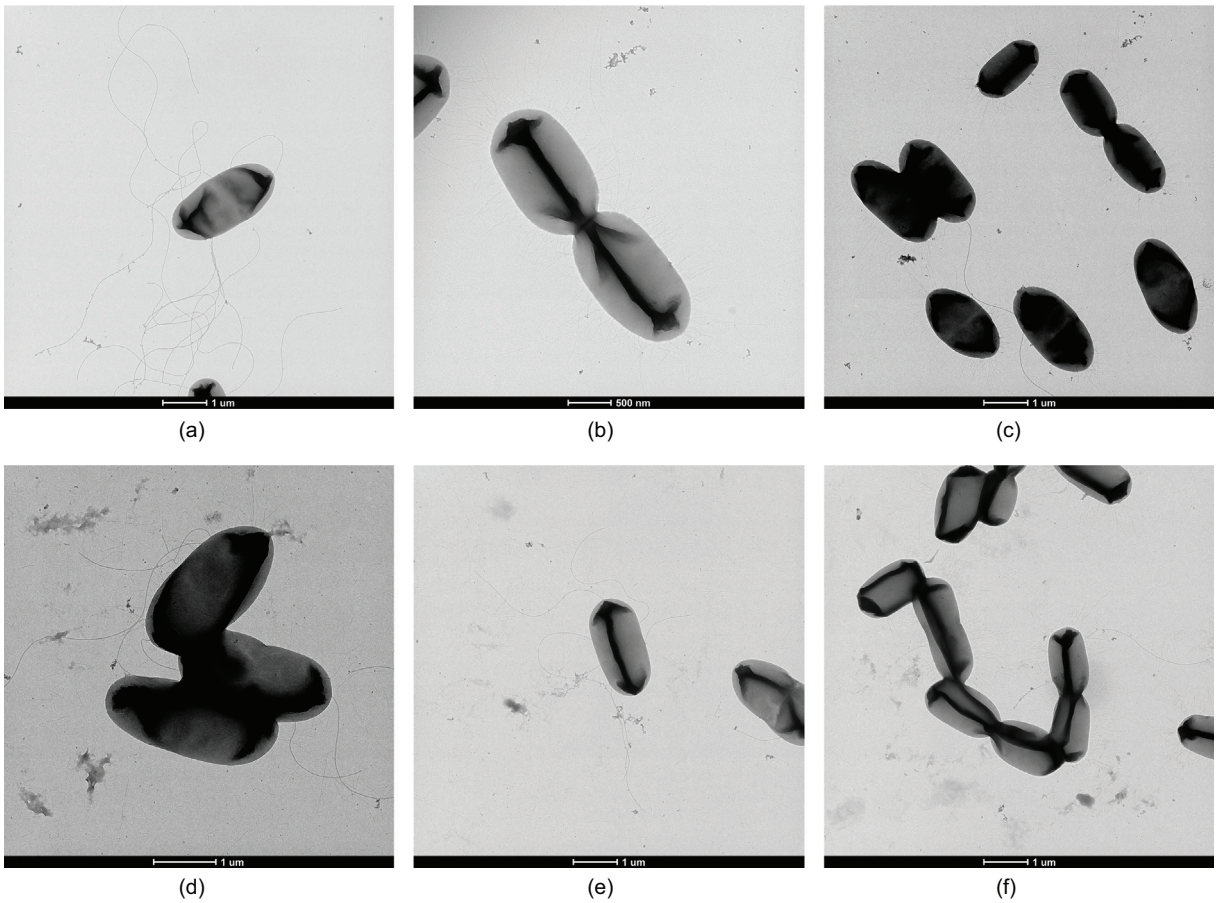

Fig. 3. Transmission electron microscopy of *Dryocola boscaweniae* sp. nov. H6W4^T (a - c) and *Dryocola clanedunesis* sp. nov. (d - f) displaying the peritrichous flagella, fimbriae and cell arrangement. Scale bar, 1 μm.

Table 2
Key phenotypic characteristics that allow for the differentiation between *Dryocola boscaweniae* sp. nov. and *Dryocola clanedunesis* sp. nov. from each other, and existing species of *Cedecea* and *Buttiauxella*. (1) *Dryocola boscaweniae* (*n* = 3), (2) *Dryocola clanedunensis* (*n* = 2), (3) *Cedecea davisae* LMG 7682^T, (4) *Cedecea lapagei* LMG 7863^T, (5) *Cedecea neteri* LMG 7864^T, (6) *Buttiauxella agrestis*, (7) *Buttiauxella brennerae*, (8) *Buttiauxella ferragutiae*, (9) *Buttiauxella gavinae*, (10) *Buttiauxella izardii*, (11) *Buttiauxella noackiae*, (12) *Buttiauxella warmboldiae*. Data for *Buttiauxella* (6–12)* taken from (Kämpfer, 2015).

Reaction	1	2	3	4	5	6	7	8	9	10	11	12
arginine dihydrolase	+	+	–	+	+	–	–	–	–	–	v	–
ornithine decarboxylase	–	v	+	–	–	+	v	+	–	+	–	–
Fermentation of:												
glycerol	+	v	–	+	–	v	–	–	–	–	–	–
D-arabinose	–	–	–	–	–	v	–	–	v	+	v	+
L-arabinose	+	+	–	–	–	+	+	+	+	+	+	+
D-adonitol	+	–	–	–	–	–	v	–	v	–	–	–
L-rhamnose	+	+	–	–	–	+	+	+	+	+	+	+
methyl-α-D-glucopyranoside	+	+	–	–	–	–	v	–	–	–	–	–
D-lactose	+	–	+	+	+	+	+	–	–	+	–	–
D-melibiose	+	–	–	+	–	v	+	+	–	v	–	–
D-raffinose	+	–	–	+	–	v	+	–	–	–	–	–
D-lyxose	+	–	–	–	–	ND	ND	ND	ND	ND	ND	ND
L-fucose	–	v	–	–	–	+	–	–	v	+	–	+
D-arabitol	+	–	+	+	+	–	v	–	v	–	–	–
potassium gluconate	+	+	+	–	–	ND	ND	ND	ND	ND	ND	ND
potassium 2-ketogluconate	+	–	–	–	–	ND	ND	ND	ND	ND	ND	ND
potassium 5-ketogluconate	+	–	–	–	–	–	+	–	+	v	+	–
phenol red	+	+	–	–	–	ND	ND	ND	ND	ND	ND	ND
palatinose	+	–	+	+	–	+	+	+	+	V	+	–
α-glucosidase	+	+	–	–	–	ND	ND	ND	ND	ND	ND	ND
α-galactosidase	+	–	–	–	–	ND	ND	ND	ND	ND	ND	ND
N-acetyl-D-galactosamine	–	–	ND	ND	ND	+	v	+	+	+	+	+
Utilisation of:												
citrate	–	–	+	+	+	v	v	–	v	+	+	–
D-melibiose	+	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D-arabitol	+	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
L-arginine	–	+	ND	ND	ND	–	–	–	–	–	–	–
L-pyroglutamic acid	+	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
α-hydroxy-butyric acid	+	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Growth temperature range (°C)	4–41	4–37	15–37	15–37	15–37	4–37	4–37	4–37	4–37	4–37	4–37	4–37

+, 90– 100 % strains +; -, 91– 100 % strains -; v, variable; ND, not determined.
* Data for the genus *Buttiauxella* were generated using standard biochemical tests.

Table 3
The average percentage of peak areas making up the fatty acid methyl ester composition of *Dryocola* gen. nov., *Cedecea* and *Buttiauxella* species. (1) *Dyrocola boscaweniae* (*n* = 3), (2) *Dryocola clandunensis* (*n*= 2), (3) *Cedecea davisae* (LMG 7862^T), (4) *Cedecea lapagei* (LMG 7863^T), (5) *Cedecea neteri* (LMG 7864^T), (6) *Buttiauxella agrestis* (*n*= 13), (7) *Buttiauxella ferragutiae* (*n* = 5), (8) *Buttiauxella gavinae* (*n* = 11), (9) *Buttiauxella brennerae* (*n* = 7), (10) *Buttiauxella izardii* (*n* = 12), (11) *Buttiauxella noackiae* (*n* = 14), (12) *Buttiauxella warmboldiae* (*n*= 5). Data for 6 – 12 taken from (Kämpfer et al., 1997).

Saturated fatty acids	1	2	3	4	5	6	7	8	9	10	11	12
$C_{12:0}$	3.9 (±0.6)	4.2 (±1.2)	4.8	2.3	3.9	2.9 (±1.4)	1.0 (±1.1)	3.0 (±0.6)	2.6 (±0.7)	2.6 (±3.0)	2.9 (±1.0)	3.3 (±0.8)
$C_{14:0}$	5.8 (±0.7)	6.9 (±0.4)	4.6	2.6	3.9	6.1 (±1.8)	6.5 (±1.2)	6.9 (±1.2)	6.7 (±0.8)	6.4 (±0.8)	6.6 (±1.7)	5.0 (±0.4)
$C_{16:0}$	34.0 (±0.8)	31.3 (±1.3)	35.9	33.1	29.6	26.0 (±8.77)	27.1 (±2.1)	26.1 (±3.1)	24.9 (±3.1)	26.8 (±5.0)	28.3 (±6.6)	25.6 (±2.1)
$C_{17:0}$	0.4 (±0.1)	0.5 (±0.1)	–	0.7	1.6	1.1 (±3.4)	–	1.4 (±2.4)	1.3 (±2.3)	1.4 (±2.9)	1.3 (±1.6)	1.0 (±0.1)
Unsaturated fatty acids												
$C_{18:1}$ ω7c	11.3 (±1.8)	16.0 (±0.4)	11.6	20.2	21.2	15.8 (±4.2)	15.4 (±2.2)	14.0 (±2.3)	15.3 (±2.9)	15.5 (±3.6)	14.9 (±3.1)	18.0 (±2.6)
Cyclopropane fatty acids												
$C_{17:0}$ cylco	13.6 (±3.9)	12.6 (±3.9)	10	11.07	9.34	10.75 (±9.68)	7.64 (±2.01)	12.67 (±4.34)	10.59 (±7.47)	11.12 (±4.96)	14.03 (±8.76)	8.75 (±1.73)
$C_{19:0}$ ω8c	0.93 (±0.76)	2.1 (±1.0)	0.9	2.5	0.9	–	–	–	–	–	–	–
Summed features												
2: $C_{14:0}$ 3-OH and/or *iso*-$C_{16:1}$	9.3 (±1.0)	8.8 (±1.5)	10.1	9.6	8.5	9.1 (±3.4)	9.1 (±1.1)	9.4 (±1.5)	9.3 (±1.5)	9.1 (±2.5)	8.5 (±2.1)	8.8 (±0.5)
3: $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c	19.9 (±2.9)	15.8 (±1.8)	14.3	11.3	15.8	22.5 (±12.3)	30.4 (±4.9)	20.8 (±5.6)	21.7 (±6.1)	20.3 (±5.3)	17.8 (±8.0)	25.6 (±3.1)
5: $C_{18:2}$ ω6,9c and/or $C_{18:0}$ ante	0.7 (±0.4)	0.5 (±0.4)	1.4	0.6	0.5	–	–	–	–	–	–	–

Table 5
Protologue description of *Dryocola* gen. nov., *Dryocola boscaweniae* sp. nov. *and Dryocola clanedunensis* sp. nov.

Genus name	*Dryocola*	-	-
Species name	–	*Dryocola boscaweniae*	*Dryocola clanedunensis*
Genus status	gen.nov.	–	–
Genus etymology	**Dry.o'co.la. Gr. fem. n. *drys*, an oak; L. suff. –*cola* (from L. masc. n. *incola*), inhabitant; N.L. masc. n. *Dryocola*, an inhabitant of oaks**		
Type species of the genus	***Dryocola boscaweniae***	–	–
Specific epithet	–	*boscaweniae*	*clanedunensis*
Species status	–	sp.nov.	sp.nov.
Species etymology	–	**bos.ca.we'ni.ae. N.L. gen. n. *boscaweniae*, of Boscawen, named to honour Lady Frances Boscawen, the first lady of the Hatchlands Park estate, Guildford, UK**	**cla.ne.dun.en'sis. M.L. masc. adj. *clanedunensis*, pertaining of Clanedun, the medieval name of Clandon where Hatchlands Park, the origin of isolation for the original strains, is located**
Description of the new taxon and diagnostic traits	Gram-negative rods (0.96 – 1.34 × 1.87 – 2.49 μm), facultatively anaerobic, oxidase negative and catalase positive. Cells occur singly, in pairs, groups of three and occasionally form chains, are motile by peritrichous flagella and can produce fimbriae. Colonies are cream coloured, with a darker convex centre and uneven margins on TSA. Major fatty acids are $C_{12:0}$, $C_{14:0}$, C $_{16:0}$, $C_{18:1}$ ω7*c*, summed feature 2 ($C_{14:0}$ 3-OH and/or *iso*-$C_{16:1}$) and summed feature 3 ($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c). The DNA G + C content ranges from 53.0 to 53.9 mol %.	The description is as given for the genus with the following additions. Cells are short rods (0.8–1.3 × 1.8–2.4 μm), that occur singly, as pairs and in chains. Strains grow well between 4 and 41 °C and exhibit strong growth from pH 6 – 9. Acid is produced from melibiose, glycerol, D-adonitol, arbutin, salicin, D-lactose, D-raffinose, D-lyxose, D-arabitol, potassium 2-ketogluconate and potassium 5-ketogluconate (API 20 and 50 CHB/E). Positive for acidification of palatinose and production of α-galactosidase (ID32). Utilises the additional carbon sources: β-methyl-D-glucoside, D-lactic acid methyl ester, tween 40 and α-hydroxy-butyric acid. Variable for acid production from D-turanose and galacturonate and utilisation of bromo-succinic acid. Resistant to penicillin G and V, but susceptible to tetracycline, ampicillin, chloramphenicol and colistin sulphate. The DNA G + C content of the type strain is 53.0 mol %.	The description is as given for the genus with the following additions. Cells are longer rods (1 – 1.4 × 2.3 – 2.7 μm) that occur singly, in clusters of three and in chains. Strains grow well between 4 and 37 °C and exhibit strong growth at pH 6 – 8. Utilises the additional carbon sources: L-arginine, citric acid and bromo-succinic acid. Variable for ornithine decarboxylase, acid production from glycerol, arbutin, salicin, L-fucose and utilisation of sucrose, β-methyl-D-glucoside, D-aspartic acid, D-lactic acid methyl ester, D-malic acid and tween 40. Resistant to penicillin G and V, but susceptible to tetracycline, ampicillin, chloramphenicol and colistin sulphate. The DNA G + C content of the type strain is 53.8 mol %.
Country of origin	United Kingdom	United Kingdom	United Kingdom
Region of origin	Guildford	Guildford	Guildford
Date of isolation (dd/mm/yyyy)	11/2020	11/2020	11/2020
Source of isolation	*Quercus robur* (healthy) rhizosphere soil	*Quercus robur* (healthy) rhizosphere soil	*Quercus robur* (AOD) rhizosphere soil
Sampling date (dd/mm/yyyy)	10/2020	10/2020	10/2020
Latitude (xx°xx′xx″N/S)	51°15′25.9'' N	51°15′25.9'' N	51°15′25.9'' N
Longitude (xx°xx′xx″E/W)	0°28′17.7'' W	0°28′17.7'' W	0°28′17.7'' W
Altitude (meters above sea level)	55 *m*	55 *m*	55 *m*
16S rRNA gene accession nr.	–	OM971056	OM971055
Genome accession number [RefSeq; EMBL; …]	–	RefSeq = JALHAP000000000	RefSeq = JALHAM000000000
Genome status	–	Incomplete	Incomplete
Genome size	–	4.41	5.23
GC mol%	–	53.0	53.8
Number of strains in study	–	8	5
Source of isolation of non-type strains	*Quercus robur* (healthy and AOD) rhizosphere soil		
Information related to the Nagoya Protocol	N/A	N/A	N/A
Designation of the Type Strain	–	H6W4^T	H11S18^T
Strain Collection Numbers	–	CCUG 76177^T = LMG 32610^T	CCUG 76181^T = LMG 32611^T

both clusters, but with particular focus on strains from Cluster 2, appear to contain a large reservoir of potentially interesting T3SS effectors which may give them significant pathogenic potential. Although it should be noted that a satisfying classifier could not be trained due to a small positive set for strain H18W14 through Effectidor and as such no results were obtained for that genome.

The novel genus also appears virulent based on the PIFAR-Pred results with a number of genes 'related to plant interaction and virulence' identified as the plant only interaction factors. Overall, 30 – 38 genes related to plant interaction and virulence were identified. These included volatiles, exo- and lipo-polysaccharides, and plant cell wall degrading enzymes. Suppl. Table S3 details the virulence genes identified from the different comparisons. All identified features indicated that both species within the proposed novel genus demonstrate a large pathogenic potential with genes that allow for the adhesion, invasion, infection and degradation of plant cells. However, these predictions are based off computational comparison and further work performing infection studies would be required to determine if this potential is effectively expressed within their phenotype.

Phenotypic and chemotaxonomic characterisation

All strains were straight rods with an average size of 1.17 × 2.28 μm. Cells appeared as single units, in groups of two or three and in chains. All strains were motile by peritrichous flagella (Fig. 3 a-f). This is in keeping with the genome annotation of the sequenced strains, which revealed the presence of 55 genes that control the synthesis and motility of flagella in prokaryotes. Both new species also show the ability to produce fimbriae.

The appearance of colonies on TSA after 48 h incubation at 28 °C were cream coloured, with a darker convex centre, uneven margins and 1–2 mm in diameter. Strains from Cluster 1 grew at 4, 10, 25, 28, 30 and 37 °C, but not 41 °C, while strains from Cluster 2 grew at 4, 10, 25, 28, 30, 37 and 41 °C. All strains grew in a pH range of 6 – 8, although strains from Cluster 2 were also capable of weak growth at pH 9. Growth for all strains was also observed in TSB supplemented with up to 6 % NaCl, with some strains from both clusters exhibited weak growth at 7 and 8 %. Antibiotic resistance to penicillin G and V was recorded, with susceptibility to tetracycline, ampicillin, chloramphenicol and colistin sulphate observed for all strains of the novel species as well as the type strains of *Cedecea* species.

Both species from the proposed novel genus were clearly differentiated from each other based on the phenotypic data as shown in Table 2 (by their fermentation of D-adonitol, D-lactose, D-melibiose, D-raffinose, D-lyxose, D-arabitol, potassium 2-ketogluconate, potassium 5-ketogluconate and palatinose; and utilisation of D-melibiose, D-arabitol, L-arginine and α-hydroxy-butyric acid), and from *Cedecea* species (differing reactions to fermentation of L-arabinose, L-rhamnose, methyl-αD-glucopyranoside, phenol red and α-glucosidase and citrate utlisation) as shown in Suppl. Table S3. Differentiation from *Buttiauxella* based on the information from other studies was less clear, although no single feature is known to be able to differentiate *Buttiauxella* from other members of *Enterobacteriaceae* (Kämpfer, 2015). The most useful phenotypic characteristics for differentiation at the species and genus level are listed in Table 2 and Suppl. Table S4. All strains tested were observed as both oxidase negative and strongly catalase positive.

The major fatty acids for both novel species were $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:1}$ ω7*c*, summed features 2 ($C_{14:0}$ 3-OH and/or *iso*-$C_{16:1}$) and 3 ($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c). The FAMES profiles were mostly consistent between genera, with little differences between the novel genus, *Cedecea* and *Buttiauxella*. The FAME profiles for all the strains including *Cedecea* and *Buttiauxella* can be seen in Table 3, *Buttiauxella* results were obtained from (Kämpfer et al., 1997).

Conclusion

The order Enterobacterales has changed much in recent years with the restructuring from one large family, *Enterobacteriaceae* containing 33 genera, into six families (Adeolu et al., 2016) comprising approx. 54 genera (Janda and Abbott, 2021). This has included greater awareness of the global nature of the order, ranging from clinical disease, insect vectored diseases and plant growth-promoting bacteria. The complete phenotypic, phylogenetic, and genomic data presented here, has further increased our understanding and knowledge of the clinically and environmentally important order Enterobacterales. The proposed novel genus and two novel species will help to bridge our collective insight to the taxonomic relationship between the genus *Buttiauxella*, which is frequently isolated from a wide range of environmental niches, and the rarely isolated species of *Cedecea*. Investigation of the rhizosphere of oak has already yielded interesting information on the relationship between bacteria and the health status of oak (Pinho et al., 2020; Scarlett et al., 2021). This work furthers our understanding of the oak rhizosphere microbiome through the isolation of a novel genus and two novel species. Additionally, whole genome sequencing has provided a genetic baseline from which functional data can be extracted, as such the genomic data determined in this study will contribute to understanding the role these bacteria play in oak health. Additionally, the data presented here indicate the '*C. colo*' does not belong to the genus *Cedecea* and should be transferred to the proposed genus *Dryocola*. However, as '*C. colo*' is yet to be validly published, this transfer cannot take place at this time.

The genotypic, genomic, phenotypic, and chemotaxonomic data generated in this study support the proposal of a novel genus, *Dryocola*, with two novel species, *Dryocola boscaweniae* gen. nov. sp. nov. (type strain H6W4^T = CCUG 76177^T = LMG 32610^T) and *Dryocola clanedunesis* sp. nov. (type strain H11S18^T = CCUG 76181^T = LMG 32611^T) for strains isolated from the oak rhizosphere. The full protologue descriptions for the novel genus and species are presented in Table 5.

Funding information

This research was supported by Woodland Heritage (Charity no. 1041611) and the University of the West of England. CB, SD and DA received support from the UK Research and Innovation's (UKRI) Strategic Priorities Fund (SPF) programme on Bacterial Plant Diseases (grant BB/T010886/1) funded by the Biotechnology and Biological Sciences Research Council (BBSRC), the Department for Environment, Food and Rural Affairs (Defra), the Natural Environment Research Council (NERC) and the Scottish Government.

CRediT authorship contribution statement

Daniel Maddock: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Carrie Brady:** Conceptualization, Writing – review & editing. **Sandra Denman:** Funding acquisition, Writing – review & editing. **Dawn Arnold:** Funding acquisition, Writing – review & editing.

Data availability

All data is publicly available in research repositories as indicated in the data availability statement.

Acknowledgements

The authors would like to thank the Tree Survey Unit (TSU, Forest Research) especially Harry Kaluszniak for his assistance in the

collection of the rhizosphere samples in this study; Susan Streeter and the National Trust for permission and assistance in the collection of samples from Hatchlands Park. We gratefully acknowledge Prof. Aharon Oren for his advice on suitable names and for providing the etymology of these and the Wolfson Bioimaging Facility, especially Dr Lorna Hodgson, for assistance with the electron microscopy. Genome sequencing was provided by MicrobesNG (http://www.microbesng.uk) which is supported by the BBSRC (grant number BB/L024209/1).

Repositories

The GenBank/EMBL/DDBJ accession numbers are as follows: OM971055 and OM971056 (16S rRNA); ON124952 - ON124964 (*fusA*); ON124965 - ON124977 (*leuS*); ON124978 - ON124990 (*pyrG*); ON124991 - ON125003 (*rpoB*); JALHAL000000000 - JALHAP000000000 (Whole genome).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2023.126399.

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