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Genome-Based Species Diversity Assessment in the *Pseudomonas chlororaphis* Phylogenetic Subgroup and Proposal of *Pseudomonas danubii* sp. nov. Isolated from Freshwaters, Soil, and Rhizosphere

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Abstract: The *Pseudomonas chlororaphis* phylogenetic subgroup of species, within the *Pseudomonas fluorescens* group, currently includes seven bacterial species, all of which have environmental relevance. Phylogenomic analyses help clarify the taxonomy of strains in the group and allow for precise identification. Thirteen antibiotic-resistant strains isolated in a previous study from nine different sampling sites in the Danube River were suspected to represent a novel species and are investigated taxonomically in the present study, together with four other strains isolated from the Woluwe River (Belgium) that were phylogenetically closely related in their *rpoD* gene sequences. The strains were characterized phenotypically, chemotaxonomically (fatty acid composition and main protein profiles), and phylogenetically. They could not be assigned to any known *Pseudomonas* species. Three genomes of representative strains were sequenced and analyzed in the context of the genome sequences of closely related strains available in public databases. The phylogenomic analysis demonstrates the need to differentiate new genomic species within the *P. chlororaphis* subgroup and that *Pseudomonas piscis* and *Pseudomonas aestus* are synonyms. This taxonomic study demonstrates that 14 of the characterized isolates are members of the Pseudomonas_E protegens_A species in the GTDB taxonomy and that they represent a novel species in the genus *Pseudomonas*, for which we propose the name *Pseudomonas danubii* sp. nov. with strain JDS02PS016T (=CECT 30214T = CCUG 74756T) as the type strain. The other three strains (JDS08PS003, rDWA16, and rDWA64) are members of the species Pseudomonas_E protegens_B in the GTDB taxonomy and need further investigation for proposal as a new bacterial species.

Keywords: *Pseudomonas*; *P. chlororaphis* subgroup; *P. danubii*; *rpoD*; Danube River; multidrug resistance; phylogenomics

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1. Introduction

Pseudomonas is a widely distributed bacterial genus with exceptional metabolic versatility and species diversity, occupying many ecological niches [1]. It is the genus of gram-negative bacteria with the highest number of species. In the current taxonomy, at least 282 validly described *Pseudomonas* species are considered in the List of Prokaryotic Names with Standing in Nomenclature (visited in February 2023) [2] The correct species

affiliation of new isolates is a prerequisite for accurate ecological studies, and species delineation within the genus has been revised recently by using phylogenomic approaches [3–5]. The genus has been phylogenetically divided into 14 groups [6] and some of them have been proposed recently as new genera [4,5,7]. The *Pseudomonas* genus has also been studied in a comparative analysis of the core proteome to find the major evolutionary groups [8]. The *Pseudomonas fluorescens* group contains the highest number of species and is subdivided into several subgroups. One of them, the *Pseudomonas chlororaphis* subgroup, is widely distributed and includes strains of species isolated from rhizosphere samples (*Pseudomonas sessilinigenes*) [9], others are known to be plant beneficial ("*Pseudomonas aestus*", *P. chlororaphis*, *Pseudomonas protegens*, *Pseudomonas sesami*), another is a xenobiotic degrader (*Pseudomonas saponiphila*), and others have been isolated from diseased freshwater fish (*Pseudomonas piscis*) or from the intestines of freshwater fish (*P. chlororaphis* subsp. *piscium*). *P. chlororaphis* subsp. *piscium* was suggested as a potential probiotic to control bacterial disease in freshwater fish. Recently, strains classified in *Pseudomonas fluorescens* subclades I and II have been isolated from human respiratory samples from a cystic fibrosis patient [10,11]. Clade II of Scales and collaborators coincides with the *P. chlororaphis* phylogenetic subgroup.

In a previous study screening for antibiotic-resistant environmental *Pseudomonas*, 611 strains were isolated from water samples along the course of the Danube River [12]. Thirteen isolates affiliated phylogenetically by their *rpoD* gene sequence were noted for belonging to the same phylospecies within the *P. chlororaphis* subgroup of species but could not be ascribed to any known *Pseudomonas* species [13]. These strains were provisionally classified as new phylospecies 6. In a previous study [14], we isolated two isolates closely related to phylospecies 6 from water samples of the Woluwe River (Belgium). The main objectives of the present study are the clarification of the taxonomy of strains in the *P. chlororaphis* subgroup of species from a phylogenomic perspective, the taxonomical characterization of the newly isolated strains, and to propose them as representatives of a new *Pseudomonas* species, for which the name *Pseudomonas danubii* is proposed. The phylogenomic approach also demonstrates that at least 12 other *Pseudomonas* strains, isolated mainly from freshwaters and whose genomes have been sequenced by other authors, also belong to *P. danubii*. The genome-based taxonomy of the whole *P. chlororaphis* subgroup is also discussed, and it is concluded that "*P. aestus*" [15] and *P. piscis* [16] are synonyms, and in addition to *P. danubii*, at least two other phylogenomic species can be delineated within the group.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

The 13 *Pseudomonas* isolates characterized taxonomically in the present study were isolated from freshwater samples taken in 9 different locations on the course of the Danube River, and 4 strains were isolated from one sampling point located near the source in the Woluwe River (Belgium) [12–14]. Details on the sampling sites, geographical origin, and references are summarized in Table 1 and Supplemental Table S1. Bacterial isolations were performed following standard procedures: 0.5 mL of the Danube River samples were plated on complex media (Endo Agar, Xylose Lysine Deoxycholate Agar and Chromocult Coliform Agar; all Merck, Rahway, NJ, USA) and incubated at 37 °C for 18–24 h. The Woluwe River sample was plated on *Pseudomonas*-selective CFC medium (Merck) and incubated overnight at 30 °C. The fact that different temperatures were used for isolation is due to the different initial objectives of the 2 studies: the search for antibiotic-resistant bacteria or the specific search for *Pseudomonas*. Isolated colonies were checked for purity on complex media. Species type strains are indicated by T. Species names that were published but not validated are marked within brackets.

The isolates were cultured routinely in LB medium (Lysogeny Broth, Difco, Forn El Chebbak, Lebanon) at 30 °C. For long-term storage, strains were kept in 20% glycerol at −70 °C.

Table 1. *P. danubii* strains analyzed and phylogenetically closely related strains included in this study.

Strain	Genome	Previous Identification in the GTDB Taxonomy or Corresponding Reference	NCBI Organism Name in the Assembly Information	NCBI Taxonomy: Species Check in the Assembly Information	Identification in This Study	Reference
JDS02PS006	-	*Pseudomonas* sp. PSNew6	-	-	*P. danubii*	[12,13]
JDS02PS016T	CP116502	*Pseudomonas* sp. PSNew6	-	-	*P. danubii*	[12,13]
JDS08PS001	-	*Pseudomonas* sp. PSNew6	-	-	*P. danubii*	[12,13]
JDS10PS002	-	*Pseudomonas* sp. PSNew6	-	-	*P. danubii*	[12,13]
JDS10PS014	CP120725	*Pseudomonas* sp. PSNew6	-	-	*P. danubii*	[12,13]
JDS22PS011	–	*Pseudomonas* sp. PSNew6	-	-	*P. danubii*	[12,13]
JDS22PS018	-	*Pseudomonas* sp. PSNew6	-	-	*P. danubii*	[12,13]
JDS28PS081	-	*Pseudomonas* sp. PSNew6	-	-	*P. danubii*	[12,13]
JDS28PS083	-	*Pseudomonas* sp. PSNew6	-	-	*P. danubii*	[12,13]
JDS36PS016	-	*Pseudomonas* sp. PSNew6	-	-	*P. danubii*	[12,13]
JDS63PS049	-	*Pseudomonas* sp. PSNew6	-	-	*P. danubii*	[12,13]
JDS67PS009	-	*Pseudomonas* sp. PSNew6	-	-	*P. danubii*	[12,13]
rDWA11	-	*Pseudomonas* sp.	-	-	*P. danubii*	[14]
rDWA138	-	*Pseudomonas* sp.	-	-	*P. danubii*	[14]
JDS08PS003	CP120724	*Pseudomonas* sp. PSNew6	-	-	*Pseudomonas* sp.	[12,13]
rDWA16	-	*Pseudomonas* sp.	-	-	*Pseudomonas* sp.	[14]
rDWA64	-	*Pseudomonas* sp.	-	-	*Pseudomonas* sp.	[14]
Closely related type strains in the Pseudomonas chlororaphis phylogenetic subgroup						
CMAA 1215 T	GCA_000474765.1	*"P. aestus"*	*P. aestus*	inconclusive	*P. piscis*	[15]
DSM 19603 T	GCA_003851835.1	*P. chlororaphis* ssp. *aurantiaca*	*P. chlororaphis* ssp. *aurantiaca*	OK; type material	*P. chlororaphis* ssp. *aurantiaca*	[17]
DSM 6698 T	GCA_003851905.1	*P. chlororaphis* ssp. *aureofaciens*	*P. chlororaphis* ssp. *aureofaciens*	OK; type material	*P. chlororaphis* ssp. *aureofaciens*	[17]
DSM 50083 T	GCA_016803445.1	*P. chlororaphis* ssp. *chlororaphis*	*P. chlororaphis* ssp. *chlororaphis*	OK; type material	*P. chlororaphis* ssp. *chlororaphis*	[17]
DSM 21509 T	GCF_001269555.1	*P. chlororaphis* ssp. *piscium*	*P. chlororaphis* ssp. *piscium*	OK; type material	*P. chlororaphis* ssp. *piscium*	[18]
DSM 19095 T	GCA_000397205.1	*P. protegens*	*P. protegens*	OK; type material	*P. protegens*	[19]
DSM 9751 T	GCA_900105185.1	*P. saponiphila*	*P. saponiphila*	OK; type material	*P. saponiphila*	[20]
KCTC 22518T	not available	*P. sesami*	*P. sesami*		*P. sesami*	[21]
CMR12a T	GCA_019139855.1	*P. sessilinigenes*	*P. sessilinigenes*	OK; type material	*P. sessilinigenes*	[9]
MC042 T	GCA_009380155.1	*P. piscis*	*P. piscis*	inconclusive	*P. piscis*	[16]

Table 1. *Cont.*

Strain	Genome	Previous Identification in the GTDB Taxonomy or Corresponding Reference	NCBI Organism Name in the Assembly Information	NCBI Taxonomy: Species Check in the Assembly Information	Identification in This Study	Reference
Closely related strains in public databases of genome sequences						
BIOMIG1BAC	GCA_001705995.1	Pseudomonas_E sp001705835	*Pseudomonas* sp.	OK	*P. sessilinigens*	NCBI web page
FD6	GCA_003363755.1	Pseudomonas_E protegens_A	*P. protegens*	inconclusive	*P. danubii*	NCBI web page
11	GCA_002891565.1	Pseudomonas_E protegens_A	*P. protegens*	inconclusive	*P. danubii*	NCBI web page
WS5414	GCA_012985795.1	Pseudomonas_E protegens_A	*Pseudomonas* sp.	inconclusive	*P. danubii*	NCBI web page
MB-090624	GCA_003205455.1	Pseudomonas_E protegens_A	*P. protegens*	inconclusive	*P. danubii*	NCBI web page
4	GCA_002891555.1	Pseudomonas_E protegens_A	*P. protegens*	inconclusive	*P. danubii*	NCBI web page
38G2	GCA_003731885.1	Pseudomonas_E protegens_A	*P. protegens*	inconclusive	*P. danubii*	NCBI web page
MB-090714	GCA_003205275.1	Pseudomonas_E protegens_A	*P. protegens*	inconclusive	*P. danubii*	NCBI web page
12H11	GCA_003731825.1	Pseudomonas_E protegens_A	*P. protegens*	inconclusive	*P. danubii*	NCBI web page
15H3	GCA_003731865.1	Pseudomonas_E protegens_A	*P. protegens*	inconclusive	*P. danubii*	NCBI web page
Go58	GCA_017347385.1	Pseudomonas_E protegens_A	*P. protegens*	inconclusive	*P. danubii*	NCBI web page
B2-1059	GCF_026016285.1	not found	*Pseudomonas* sp.	inconclusive	*P. danubii*	NCBI web page
PS1	GCA_019754235.1	not found	*P. protegens*	inconclusive	*P. danubii*	NCBI web page
14B2	GCA_003732485.1	Pseudomonas_E protegens_B	*P. protegens*	inconclusive	*Pseudomonas* sp.	NCBI web page
AU11706	GCA_001020715.1	Pseudomonas_E fluorescens_AP	*P. fluorescens*	inconclusive	*Pseudomonas* sp.	[11]
H1F5C	GCA_013407925.2	Pseudomonas_E fluorescens_AP	*P. protegens*	inconclusive	*Pseudomonas* sp.	NCBI web page
H1F10A	GCA_013409685.2	Pseudomonas_E fluorescens_AP	*P. protegens*	inconclusive	*Pseudomonas* sp.	NCBI web page
Pf-5	GCA_000012265.1	Pseudomonas_E protegens	*P. protegens*	OK	*P. protegens*	[22]
PF-1	GCA_005887595.1	Pseudomonas_E protegens	*P. protegens*	OK	*P. protegens*	NCBI web page
St29	GCA_001547915.1	Pseudomonas_E sp001547895	*Pseudomonas* sp.	inconclusive	*P. sesami*	NCBI web page
Os17	GCA_001547895.1	Pseudomonas_E sp001547895	*Pseudomonas* sp.	inconclusive	*P. sesami*	NCBI web page
XYZF4	GCA_004125385.1	Pseudomonas_E sp001547895	*P. protegens*	inconclusive	*P. sesami*	NCBI web page
BNJ-SS-45	GCA_003057655.1	Pseudomonas_E sp001547895	*P. protegens*	inconclusive	*P. sesami*	NCBI web page
BC42	GCA_021560055.1	not found	*Pseudomonas* sp.	inconclusive	*P. sesami*	NCBI web page

Table 1. *Cont.*

Strain	Genome	Previous Identification in the GTDB Taxonomy or Corresponding Reference	NCBI Organism Name in the Assembly Information	NCBI Taxonomy: Species Check in the Assembly Information	Identification in This Study	Reference
NFPP19	GCA_900110785.1	Pseudomonas_E protegens	*Pseudomonas* sp.	OK	*P. protegens*	NCBI web page
NFPP12	GCA_900103205.1	Pseudomonas_E protegens	*Pseudomonas* sp.	OK	*P. protegens*	NCBI web page
NFPP10	GCA_900109535.1	Pseudomonas_E protegens	*Pseudomonas* sp.	OK	*P. protegens*	NCBI web page
NFPP05	GCA_900114815.1	Pseudomonas_E protegens	*Pseudomonas* sp.	OK	*P. protegens*	NCBI web page
NFPP08	GCA_900113795.1	Pseudomonas_E protegens	*Pseudomonas* sp.	OK	*P. protegens*	NCBI web page
NFPP09	GCA_900119575.1	Pseudomonas_E protegens	*Pseudomonas* sp.	OK	*P. protegens*	NCBI web page
R26	GCF_002112545.1	Pseudomonas_E piscis	*Pseudomonas* sp.	OK	*P. piscis*	NCBI web page
B6(2017)	GCF_002112765.1	Pseudomonas_E piscis	*Pseudomonas* sp.	OK	*P. piscis*	NCBI web page
FW50712TSA	GCF_017350535.1	Pseudomonas_E piscis	*Pseudomonas* sp.	OK	*P. piscis*	NCBI web page
FW50714TSA	GCF_017350515.1	Pseudomonas_E piscis	*Pseudomonas* sp.	OK	*P. piscis*	NCBI web page

2.2. Phylogenetic Analyses of the 16S rRNA, gyrB, rpoB, and rpoD Gene Sequences and Multilocus Sequence Analysis (MLSA)

The template DNA for the PCRs was obtained with a Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) from an overnight culture of a fresh colony inoculated in 4 mL Lysogeny broth. The PCR amplification for the 16S rRNA, *gyrB*, *rpoB*, and *rpoD* genes, the primers used, the purification of the amplified products, the DNA sequencing conditions, and the sequence analysis procedures have been previously described [23]. Alternatively, the 16S rRNA gene sequences were retrieved from the corresponding whole genome sequences or from public databases. The GenBank/EMBL/DDBJ accession numbers of the gene sequences analyzed were obtained from public databases or were determined in the present study as indicated in Table 1 and Table S2 and in the phylogenetic trees.

The partial nucleotide gene sequences of the 16S rRNA (1350 nt), *gyrB* (RNA gyrase subunit B, 802 nt), *rpoD* (RNA polymerase subunit D, 689 nt), and *rpoB* (RNA polymerase beta subunit, 916 nt) genes were used to generate individual trees. The concatenation of the sequences of 4 genes, 3757 nucleotides, permitted the generation of a multilocus sequence analysis (MLSA) tree to locate the putative new *Pseudomonas* species in the described *Pseudomonas* phylogenetic groups of species. Distance matrices were generated by the Jukes and Cantor method (JC) [24], and the tree was reconstructed with neighbor-joining (NJ) [25]. Trees were also constructed by maximum likelihood (ML) and maximum parsimony (MP) methods as implemented in the MEGA package [26].

Additionally, a more robust MLSA was performed with the genome sequences obtained as described below by reconstructing a phylogenetic tree with the autoMLST web server (https://automlst.ziemertlab.com accessed on 21 November 2022) following the standard procedure described by Alanjary et al. [27]. Briefly, 100 housekeeping monocopy gene sequences with the lowest dN/dS ratio were automatically selected, and the sequences were concatenated and aligned using MAFFT. IQ-TREE implemented in the web server was used to infer the final species tree.

2.3. Genome Sequencing

Genomic DNA from the strains JDS02PS016T, JDS10PS014, and JDS08PS003 was isolated using the Nucleospin Microbial DNA Kit (Macherey-Nagel, Nordrhein-Westfalen, Germany) as described previously [28]. DNA quality and concentration were assessed by Nanodrop and Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) measurements. The sequencing library was prepared using the "Native Barcoding Kit 24 (Q20+)" (SQK-NBD112.24) (Oxford Nanopore Technologies, Ltd., Oxford, UK) according to the manufacturer's instructions. The library was sequenced on a MinION sequencer (Oxford Nanopore Technologies, Ltd., Oxford, UK) (www.nanoporetech.com) using an R10.4 flow cell and default parameters in MinKNOW, version 21.11.8. The FAST5 files containing the raw data were base-called with super accurate mode and demultiplexed with Guppy version 6.0.6.

Additionally, for strain JDS02PS016T, paired-end library reads were generated using the Illumina HiSeq 2000 platform. The quality of the Illumina paired-end reads was analyzed with FastQC, version 0.11.3 (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Nanopore reads were split with Duplex Tools 0.2.9 (ONT) de novo assembled with Flye 2.9 software ("–nano-hq" parameter) [29], and polished with racon 1.3.1 ("-m 8 -x -6 -g -8 -w 500") and medaka 1.5.0 ("-m r104_e81_sup_g5015" parameter). For strain JDS02PS016T, hybrid polishing of the Flye assembly using the Illumina reads was performed with Polypolish version 0.5.0, after trimming Illumina reads with Trimmomatic, v.0.39 ("PE HEADCROP:10 SLIDINGWINDOW:5:20").

The draft genomes obtained were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). These whole-genome shotgun sequences have been deposited at DDBJ/ENA/GenBank under accession numbers CP116502 (JDS02PS016T), CP120725 (JDS10PS014), and CP120724 (JDS08PS003). The version described in this paper is the first version. The three genomes were also annotated with PROKKA on the KBase website (https://docs.kbase.us/ accessed on 21 November 2022) and with RAST (Rapid Annotation using Subsystem Technology; https://rast.nmpdr.org/rast.cgi, accessed on 1 March 2023) [30].

2.4. Phylogenomic Analyses

The genome relatedness of the 3 isolates, JDS02PS016T, JDS10PS014, and JDS08PS003, to the whole-genome shotgun sequences of all species type strains and closely related strains in the *P. chlororaphis* phylogenetic group of species available in public databases was determined based on the average nucleotide identity determined with the BLASTN algorithm (ANIb). It was calculated using the JSpecies software tool available at http://jspecies.ribohost.com/jspeciesws/ accessed on 21 November 2022 [31]. Additionally, digital DNA–DNA hybridization between the selected strains was performed by the genome-to-genome distance (GGDC) method. GGDC was calculated using a web service (http://ggdc.dsmz.de accessed on 21 November 2022) [32] and the recommended BLAST method. The presented GGDC results are based on the recommended Formula 2. The similarity to the genomes of closely related species type strains was also calculated using the Type Strain Genome Server (TYGS), a free bioinformatics platform (https://tygs.dsmz.de accessed on 21 November 2022) [33].

Orthologous genes in the genomes of the three sequenced isolates were analyzed using the MICROBIALIZER web server (https://microbializer.tau.ac.il/index.html accessed on 21 November 2022) [34] together with the genome sequences of the species type strains in the *P. chlororaphis* group and closely related strains. Briefly, the server extracts the orthologous sets of genes in each genome and analyzes the gene presence–absence patterns. The default settings were used: maximal e-value cutoff: 0.01; identity minimal percent cutoff: 70.0%; minimal percentage for core: 100.0%. The Jaccard similarity index implemented in the PAST package of programs was used as a measure of similarity in pairwise comparisons. The percentage of shared genes was calculated pairwise with the Jaccard index and represented in a dendrogram with the PAST program [35]. The Jaccard index of similarity was calculated as SJ = a/(a + b + c + d), in which a is the number of genes that were present in both genomes

of each pair, b and c are the number of genes present in one strain but absent in the other, and d is the number of orthologs absent in both strains. The final matrix was represented in a UPGMA dendrogram with PAST [35]. The orthologous genes shared by the pairs of strains were also represented in a split tree decomposition as discussed by Huson and Bryant [36] as well as in a heatplot using the same program. SplitsTree (version 5) software was used for computing unrooted phylogenetic networks from molecular sequence data. A Venn diagram was constructed on the web page https://bioinfogp.cnb.csic.es/tools/venny/ accessed on 21 November 2022 [37] to differentiate the three genome-sequenced strains.

2.5. Genome Insights

The presence of antibiotic resistance genes was screened on the CARD website [38] (https://card.mcmaster.ca; accessed on February 2023). The presence of prophages in the genomes was screened with the PHASTER web server (PHAge Search Tool Enhanced Release; http://phaster.ca/ accessed on 21 November 2022) [39].

Analysis and comparison of the functional annotations were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Automatic Annotation Server [40].

2.6. Morphological, Biochemical, and Physiological Tests

The bacteria were cultured routinely on LB medium at 30 °C. Production of fluorescent pigments was tested on King B medium (Pseudomonas agar F, Difco), and pyocyanin production was tested on King A medium (Pseudomonas agar P, Difco). The strains were phenotypically characterized using API 20 NE strips (bioMérieux, Marcy l'Etoile, France) and Biolog GEN III MicroPlates (Biolog, Hayward, CA, USA) following the manufacturer's recommendations. Different growth temperatures (4, 10, 25, 30, 37, and 42 °C) and the pH range that allowed for bacterial growth (4–11) were tested in LB medium. Growth in the presence of NaCl (0–10% *w*/*v*) was tested in NB medium (nutrient broth, Difco).

Cell size, morphology, and flagellum insertion were determined by transmission electron microscopy of cells from the exponential growth phase in LB medium. A Hitachi Model H600 electron microscope was used at 75 kV. The samples were negatively stained with phosphotungstic acid (1%, pH 7.0) as previously described [41].

Antibiotic resistance was tested in duplicate following standard procedures that were described previously [12].

2.7. Chemotaxonomic Analysis

The main protein profiles of the isolates, together with their closely related species type strains, were obtained by WC-MALDI-TOF MS. Data were obtained by the Scientific-Technical Services of the University of Balearic Islands (Spain) with a Bruker instrument. Sample treatment and analysis with the Biotyper program were previously described in detail [42].

Whole-cell fatty acid methyl ester (FAME) analyses were performed at the Spanish Type Culture Collection (CECT) in Valencia, Spain (http://cect.org/identificaciones accessed on 21 November 2022). Fatty acids were extracted and prepared according to highly standardized protocols as described for the MIDI Microbial Identification System. The cellular fatty acid content was analyzed by gas chromatography with an Agilent 6850 with the MIDI Microbial Identification System using the RTSBA6 method and the Microbial Identification Sherlock software package version 6.1 [43].

3. Results

3.1. 16S rRNA and rpoD Gene Phylogenies

Figure 1 shows the phylogenetic relationships of the 16S rRNA gene of the 17 isolates, together with their closely related strains available in public databases. All 17 sequences were grouped in the same branch, together with the *P. protegens* type strain, at similarities ranging from 99.9 to 100%. *P. sesami* was the next closest type strain (similarity 99.1–99.2%), followed by *P. saponiphila* (98.7–98.8%), although they appeared in distant branches of

the tree. The 16S rDNA sequence clearly included the 17 isolates in the *P. fluorescens* group within the *P. chlororaphis* phylogenetic subgroup of species. Another 10 nontype strains assigned in the Genome Taxonomy Data Base (GTDB; [44]) to the putative species Pseudomonas_E protegens_A and Pseudomonas_E protegens_B clustered in the same branch and were included in the phylogenetic analyses.

The analysis of the *rpoD* gene sequence is a powerful tool for species differentiation in the genus *Pseudomonas*, with a higher discrimination power than the 16S RNA gene sequence [6,35,45–47]. The corresponding phylogenetic tree is depicted in Figure S1. All strains were located in two separated branches of the *P. chlororaphis* subgroup of species, close to *P. saponiphila* and *P. protegens* type strains, but, as shown in Table S3, at distances higher than the 96% species threshold established for the *rpoD* gene partial sequence in the genus *Pseudomonas* [6]. Other type strains affiliated in the *P. protegens-P. saponiphila* phylogenetic branch in the 16S rRNA analysis, such as *P. sesami*, were separated in the *rpoD* tree.

3.2. Multilocus Sequence Analysis (MLSA)

The concatenated partial sequences of the four housekeeping genes were analysed, and the results confirmed the groupings based on the *rpoD* gene sequences previously observed (Figure 2). The closest related type strains were *P. saponiphila* and *P. protegens*. The phylogenetic distances of the 17 isolates under study to *P. saponiphila* were below the 97% species threshold established for the four-gene concatenated multilocus sequence analysis [6]. The distances to *P. protegens* range from 96.6 to 97.1%, the borderline for species differentiation in the four-gene MLSA (Figure 2 and Table S3). The distances among the 14 isolates of the major group (A) range from 99.2 to 100%. Two isolates from the Woluwe River (rDWA16, rDWA64) and one isolate from the Danube River (JDS08PS003) clustered separately from the other 14 isolates at phylogenetic distances at the borderline for species differentiation in the four-gene analysis. These three isolates formed a second homogeneous Group B (distances of 99.9–100% among them, 97.6–97.9% with the other 14 strains studied, and 96.6–96.7% with the *P. protegens* type strain). *P. protegens* and *P. saponiphila* type strains are at a distance of 96.8%. The distances among the four *P. chlororaphis* subspecies range from 98.2–98.6% in the four-gene MLSA. These results indicate that the 17 isolates cannot be ascribed to their closest related species *P. protegens* or to *P. saponiphila* in the *P. chlororaphis* subgroup of species, and this assumption was corroborated in this investigation by genomic comparisons. The strains XY2F4, Os17, St29, BC42, and BNJ-SS-45, whose identification was considered inconclusive in the NCBI species taxonomy check, clustered together with the *P. sesami* type strain (Figure 2).

3.3. Genome Characteristics

Two isolates from the main group A and one strain of group B were selected for genome sequencing. The genome characteristics and assembly metrics of the three sequenced isolates of the present study are given in Table 2. The genome sizes range from 6.7 Mbp to 7.1 Mbp (6.6–7.6 in the group), the GC content ranges from 62.1% to 62.2% (61.8–63.8% in the group), and the numbers of coding sequences are in the range of the other strains in the group.

3.4. Genome-Based Phylogeny and Species Circumscription

The genome sequences of the three isolates under study were compared among themselves and to publicly available genome sequences of strains in the *P. chlororaphis* subgroup of species, including the type strains of 10 species or subspecies in the subgroup. Nine strains classified as Pseudomonas protegens_A and one strain classified as Pseudomonas protegens_B in the GTDB taxonomy were included in two types of phylogenomic analyses, autoMLST and core-gene phylogeny. Genome sequences were retrieved from the NCBI database, and their accession numbers are given in Table 1.

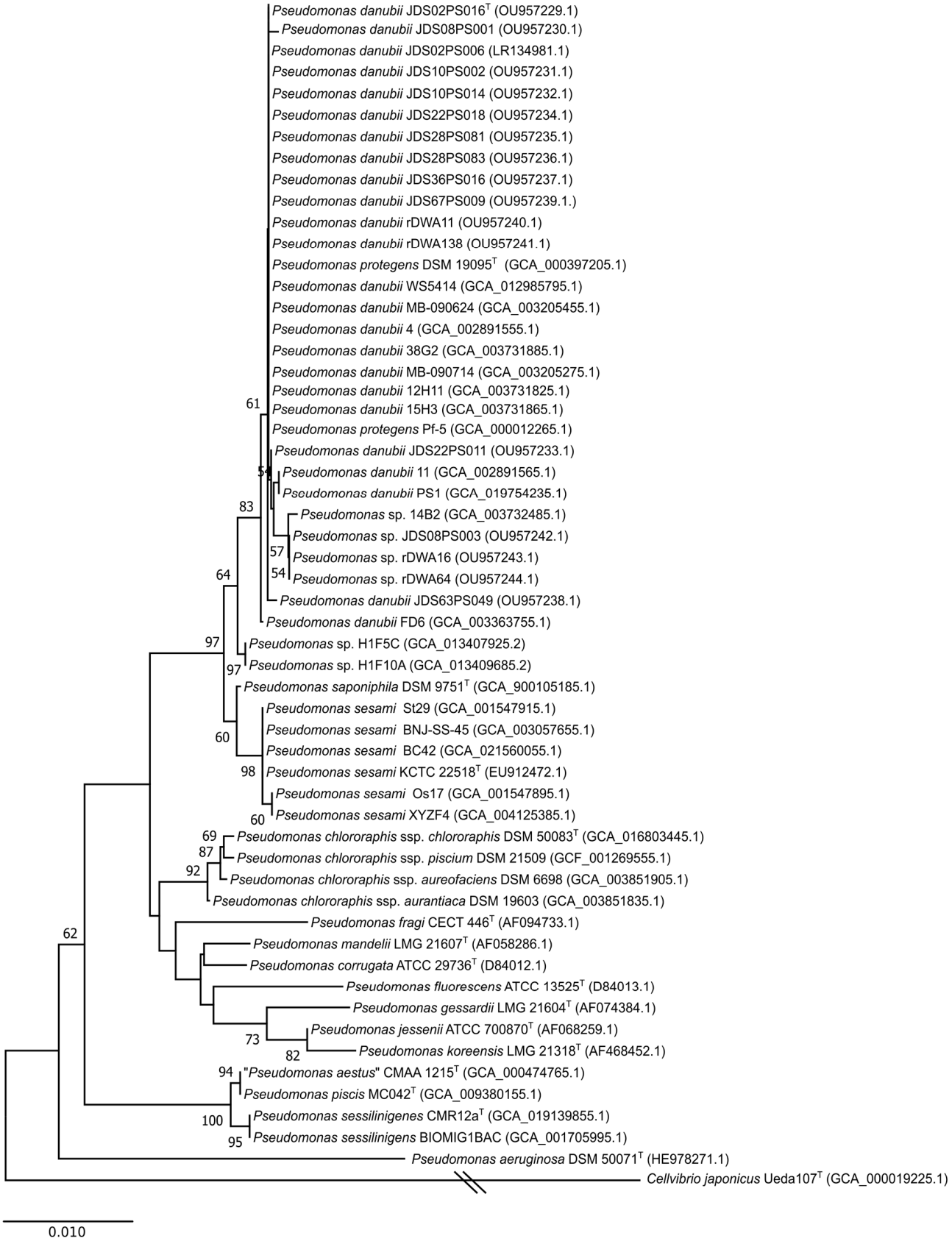

Figure 1. Phylogenetic tree based on the 16S RNA gene sequence (Jukes–Cantor, Maximum Likelihood). Bootstrap values higher than 50 are indicated in the nodes. Bar indicates sequence divergence.

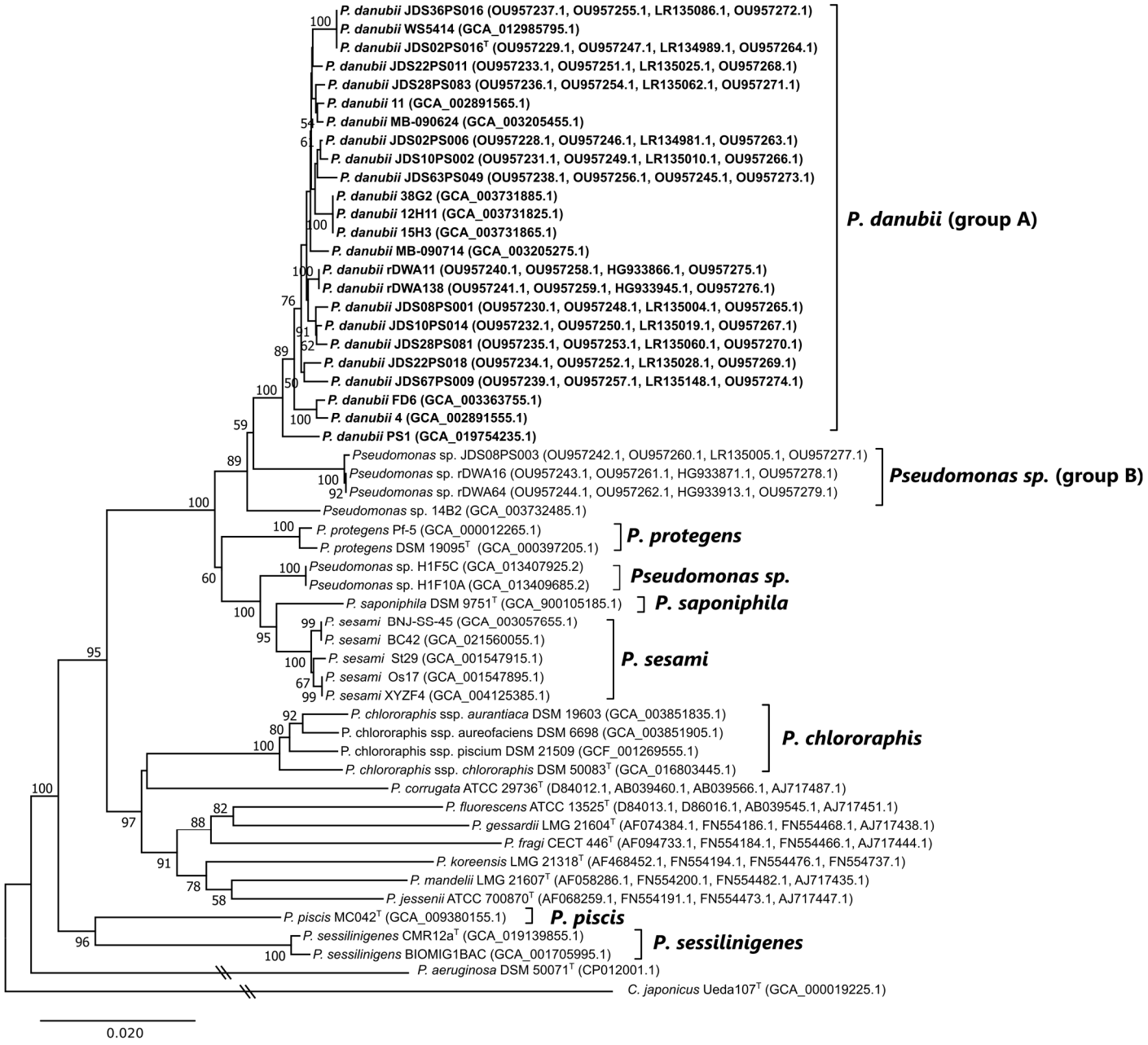

Figure 2. Phylogenetic tree based on the 4-gene concatenated sequences (Jukes–Cantor, Maximum Likelihood). Bootstrap values higher than 50 are indicated in the nodes. Bar indicates sequence divergence. Strains assigned to the new species *P. danubii* are highlighted in bold.

Figure 3 represents the analysis based on the concatenated sequences of 100 housekeeping genes (Table S4) selected by the autoMLST program (96,596 nt in total). The topology of the tree was supported by high bootstrap values, and it was highly coincident with the four-gene phylogenetic tree in the *P. chlororaphis* phylogenetic subgroup. Eight clusters of strains can be distinguished. The three isolates under study are monophyletic and clearly separated from the known species. Isolates JDS02PS016 and JDS10PS014 are located in the same branch, together with the strain classified as Pseudomonas_E protegens_A. Isolate JDS08PS003 is close to the strain classified as Pseudomonas_E protegens_B. "*P. aestus*" and *P. piscis* type strains are closely related in the analysis, suggesting that they conform to a single species.

Table 2. Genomic characteristics and assembly metrics of *Pseudomonas danubii* sp. nov. JDS02PS016T, JDS10PS014, and *Pseudomonas* sp. JDS08PS003 strains.

	***P. danubii* JDS02PS016T**	***P. danubii* JDS10PS014**	***Pseudomonas* sp. JDS08PS003**
GeneBank ID	CP116502	CP120725	CP120724
BioProject	PRJNA922378	PRJNA922378	PRJNA922378
BioSample	SAMN32652947	SAMN33777683	SAMN33777684
Genome size (bp)	6,722,326	6,746,028	7,107,113
GC-content (%)	62.3	62.3	62.1
Total genes	6021	6055	6357
Protein-coding genes (CDS)	5931	5965	6267
RNA genes (clusters)	90	90	90
tRNAs	70	70	70
Pseudogenes	58	56	65

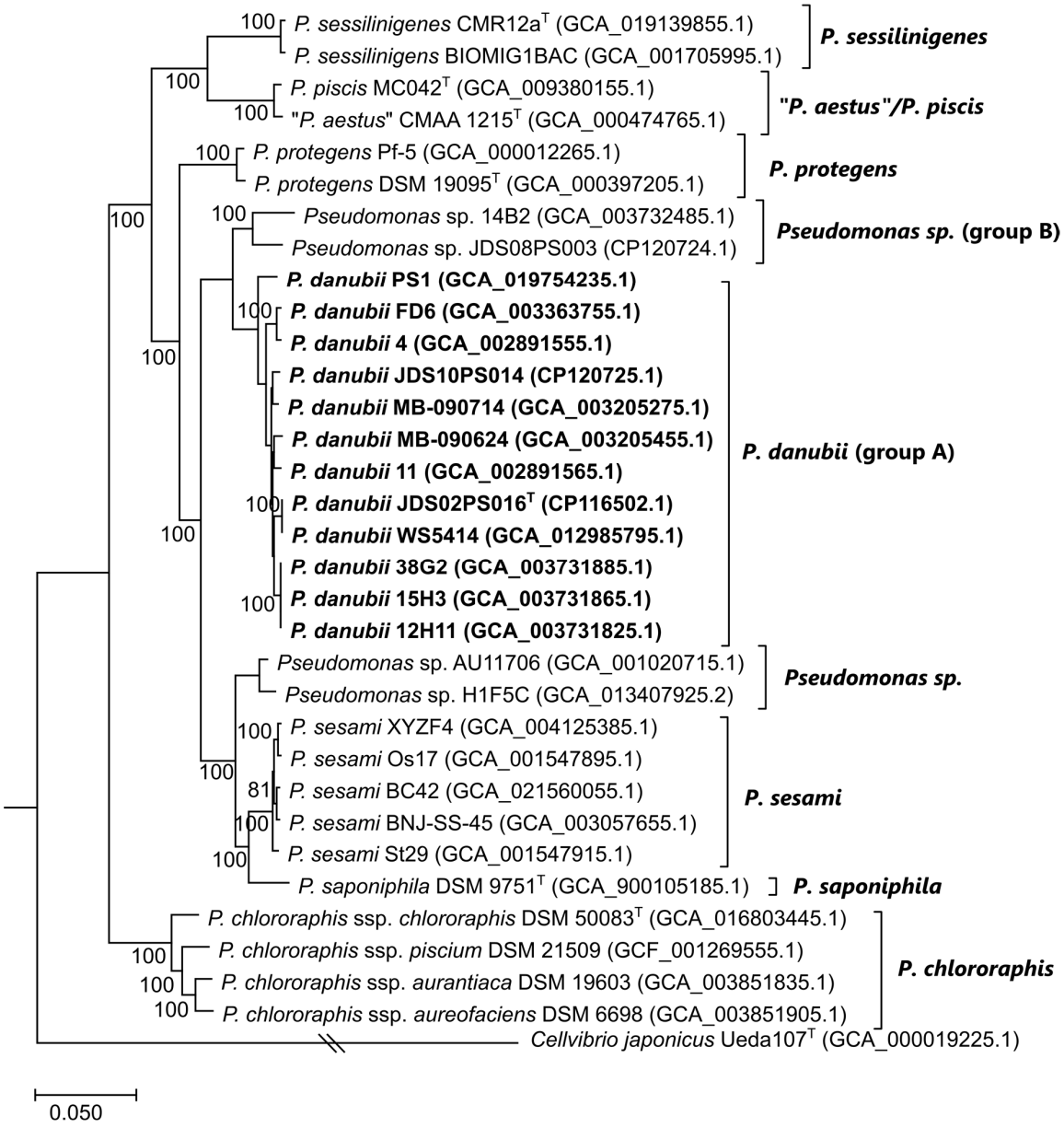

Figure 3. ML phylogenetic tree based on the 100 selected genes in the autoMLST analysis. Bootstrap values higher than 50 are indicated in the nodes. Bar indicates sequence divergence. Strains assigned to the new species *P. danubii* are highlighted in bold.

The MICROBIALIZER web server (https://microbializer.tau.ac.il/index.html accessed on 21 November 2022) [34] was utilized for a deeper phylogenomic analysis of the set of genomes selected in the *P. chlororaphis* subgroup of species. Orthologous genes were analyzed in the sequenced genomes of the three isolates under study and in twenty additional strains selected. A total of 11,081 orthologous genes were found among the 34 genomes analyzed, and 3471 of them conformed to the core proteome (i.e., genes shared among all strains) of the *P. chlororaphis* phylogenetic subgroup of species. The final species tree is a maximum-likelihood phylogenetic tree reconstructed based on the analysis of the aligned sequences of the core proteome with a length of 1,230,882 nucleotides (Figure 4). The tree was highly concordant with the four-gene and one-hundred-gene phylogenetic trees previously obtained, confirming that the three strains under study are monophyletic. Their phylogeny was also assessed, including the *Cellvibrio japonicum* type strain as an outgroup with the same settings in the program. The number of core genes was then limited to 249, but the groupings were identical, and the topology of the tree was almost identical, with one exception: the branch of the *P. protegens* strains separated earlier from the *P. danubi* strains (Figure S2). All trees were supported with high bootstrap values.

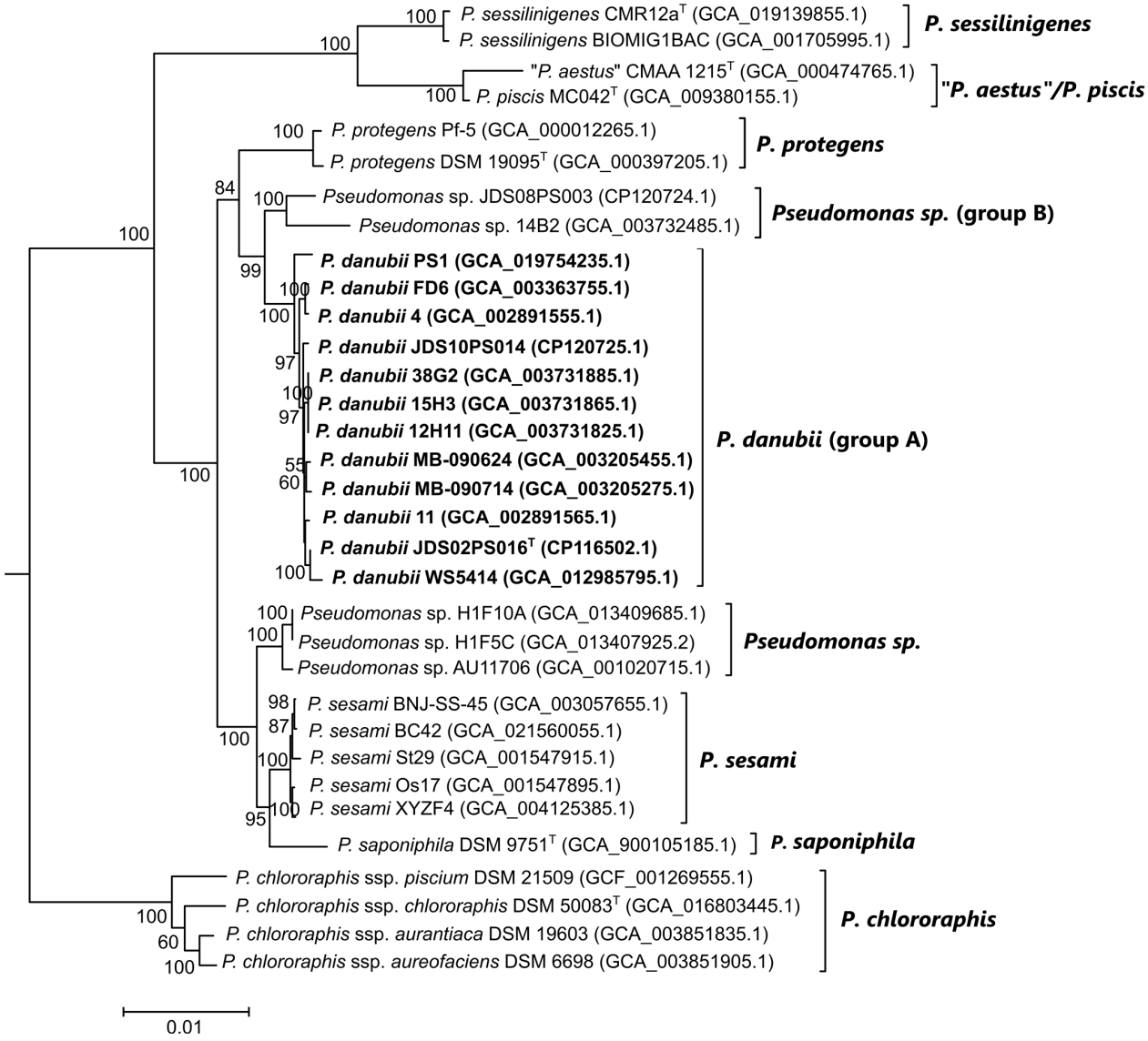

Figure 4. ML phylogenetic tree based on the 3471 concatenated gene sequences of the core genome. Bootstrap values higher than 50 are indicated in the nodes. Bar indicates sequence divergence. Strains assigned to the new species *P. danubii* are highlighted in bold.

For the species circumscription, two indices were calculated, ANIb and GGDC. The results are shown in Table S5 and in Figure S3. Both similarity values of the three strains under study in comparison with the type strains of the species in the *P. chlororaphis* phylogenetic group are below the species cutoff established for each method (95–96% for ANIb and 70% for GGDC), confirming that isolates JDS02PS016T, JDS10PS014, and JDS08PS003 do not belong to any known species. Isolates JDS02PS016T and JDS10PS014, together with the nine isolates classified as Pseudomonas_E protegens_A, belong to the same genomic species. Isolate JDS08PS003 and the strain classified as Pseudomonas_E protegens_B belong to a different genomic species related to the other group at ANIb values of 93.35–93.84% and GGDC values of 54–56%. However, the calculated indices between isolates JDS08PS003 and 14B2 are 94.56–94.52% (for ANIb) and 61.90% (for GGDC), values considered on the borderline for genomic species differentiation. A similar situation could be observed in the *P. chlororaphis* species: three subspecies are recognized, and the lowest ANI values among them range between 93.95 and 94.42%. The results confirm that "*P. aestus*" and *P. piscis* are members of the same phylogenomic species and have to be considered synonyms, with an ANI of 98%. The cluster of five strains identified as *P. sesami* in the MLSA analysis also conforms to a homogenous genomic group, with ANI values higher than 98.64%, separated from *P. saponiphila* but with identities of 94.03–94.42% in ANI. Strains AU11706, H1F10A, and H1F5C constitute a potential new species, yet to be described, which is close to *P. sesami* and *P. saponiphila*. The phylogram obtained in the TYGS web server was the same as the tree constructed with the GGDC values and proposes the same genomic species in the group.

3.5. Gene Content Comparisons

In an attempt to clarify the possible species status of those strains on the borderline of species circumscription, we analyzed the genes shared by all strains in the group. The 11,081 orthologous genes detected in the MICROBIALIZER analysis were studied by three different approaches. The Jaccard index matrix among the pairwise comparisons of the strains is depicted in the dendrogram represented in Figure 5. The clustering of strains in the same species is maintained. Strains from each of the nine phylogenomic species share at least 85% of the orthologous genes, but the Jaccard index for strains of the above-mentioned closely related groups is higher than 80%, and a clear threshold cannot be delineated. Similar results were observed in the split tree decomposition (Figure S4a) obtained with SplitsTree. The presence–absence of the orthologous genes was also represented in a heatmap (Figure S4b) that clearly shows the combinations of orthologous genes that differentiate the species in the group.

A more detailed comparison was performed among two genomes of strains in the group of *P. danubii* (isolates JDS02PS016T and JDS10PS014) and the closely related isolates JDS08PS003 and 14B2 assigned to Pseudomonas_E protegens_B in the GTDB taxonomy. A Venn diagram was constructed on the web page https://bioinfogp.cnb.csic.es/tools/venny/ accessed on 21 November 2022 [37], as shown in Figure S5. Only 321 genes (4%) were shared by strains JDS08PS003 and 14B2 and were not present in the other two genomes. However, most of these 321 genes were also present in the genomes of other members of the *P. chlororaphis* phylogenetic group. Only 22 genes were exclusive to this potential species, Pseudomonas_E protegens_B, and no characteristic differential trait was encoded by these genes.

3.6. Genome Insights

The KEGG analysis of the proteome of the sequenced three isolates of the proposed new species reveals the presence of genes of metabolic pathways that are relevant for their ecological potential. Some traits that have been considered in the taxonomy of *Pseudomonas* were found. Starch can be hydrolyzed by means of alpha-amylase and iso-amylase, and the degradative products can then be metabolized through glycolysis or the pentose phosphate cycle; benzoate can be metabolized through catechol, which is cleaved in the ortho position

and followed by the ortho pathway enzymes. All the necessary enzymes for the assimilatory reduction of nitrate to ammonium were detected, as well as those for the synthesis of type II and type VI secretion systems. The type VI secretion system might be related to the alginate biosynthesis potential and secretion for biofilm formation. The strains can be beta-lactam resistant due to the presence of RND efflux pumps and the synthesis of a class C beta-lactamase.

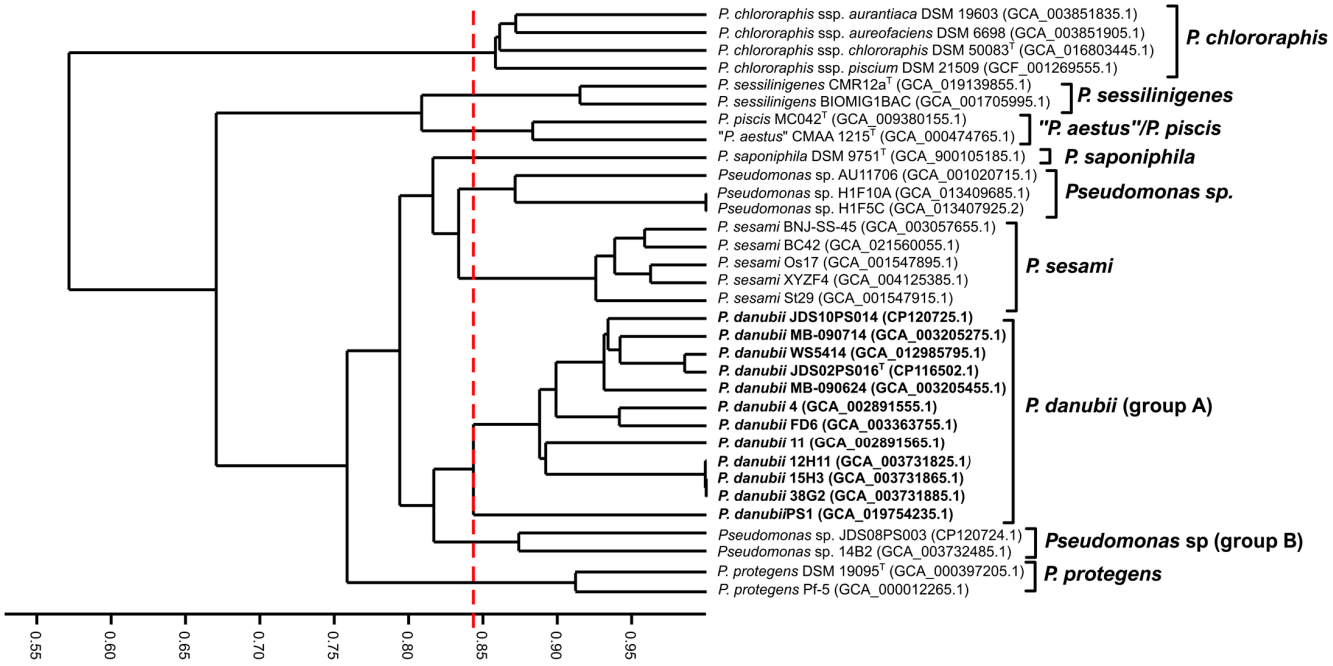

Figure 5. UPGMA dendrogram based on the Jaccard index calculated from the phyletic pattern. Strains assigned to the new species *P. danubii* are highlighted in bold.

The presence of antibiotic resistance genes was screened on the CARD website. Table 3 summarizes the genes and the potential resistance against antibiotics found. Seven strict hits were detected by protein homology in the three genomes. Following the Antibiotic Resistance Ontology ("ARO") terminology, three to four adeF determinants were identified, as well as *fosA* and *fosA8* and *vanG*, *vanW*, and *yajC*. The potential resistances determined were related to antibiotic efflux pumps and antibiotic target inactivation.

Prophages are important modulators of the characteristics of their host and are species- or strain-specific [48]. As specified in Table S6, three, four, or five sequence regions in each genome contained phage genes. A region containing 40 to 56 protein-coding sequences in the four genomes was considered intact and was most similar to the reference *Salmonella* phage 118970_sal3 selected by the server. A nucleotide BLAST search in the NCBI database revealed that at least some of these sequences are also present in at least 50 isolates of the *P. chlororaphis* phylogenetic subgroup of strains comprising most species in the group, suggesting the presence of this phage in their common ancestor. The significance of this phage needs further investigation but was not the main subject of this study. Another intact phage (48 protein-coding sequences) was found in strain 14B2, and other incomplete regions differentiated the isolates studied.

3.7. Phenotypic Traits: Morphology, Physiology, Biochemical Traits, and Chemotaxonomy

The colonies were round or oval, flat, opaque, and beige colored with regular margins, and measured 2–3 mm in diameter after incubation on LB agar plates (Luria-Bertani, Difco) at 30 °C for 48 h. The cells were short rods (2.5–1.9 µm long and 1.3–0.5 µm wide), motile by a single or two polar-inserted flagella (Figure S6) and stained gram-negative. *P. protegens*, *P. saponiphila*, *P. chlororaphis*, *P. koreensis*, and *P. fluorescens* type strains were included in the

analysis as controls and for comparative purposes. The results are detailed in Table 4 and Table S7. The metabolism was strictly aerobic and non-fermentative, and the strains were not able to reduce nitrate to nitrite. Catalase and oxidase tests were positive. Growth was observed in liquid LB medium at temperatures between 4 and 37 °C, and the strains were able to grow at pH values between 5 and 9 or 10. All isolates tolerated NaCl concentrations up to 6% (*w*/*v*). Only four of the fourteen strains in the *P. danubii* proposed species were able to grow in the presence of 8% NaCl in the GENIII galleries. Fluorescent pigments were produced on King B (Difco) but not on King A (Difco) media. Many of the sugars and organic acids tested were assimilated. The 17 strains were not efficient in the use of Tween 40, a characteristic that differentiates them from their closest relatives. The 17 isolates under study were phenotypically homogeneous, and few biochemical tests could differentiate them from their closest related *P. protegens*, *P. saponiphila*, and *P. chlororaphis* type strains. Table 4 shows the differences found in the biochemical tests. The use of adipate, arabitol, sucrose, and aspartic acid differentiates *P. danubii* from *P. protegens*, *P. saponiphila*, and *P. chlororaphis* type strains. Substrates used by these type strains, such as serine, saccharic acid, and formic acid, are not used by *P. danubii* strains.

Five of the seventeen Danube isolates were considered multidrug resistant in a previous study [12] because they revealed resistance to three (MDR3) or four (MDR4) antibiotic classes. The antibiotic resistance patterns were analyzed for the Woluwe strains and are reported in Table S8 together with the previously obtained results. The five MDR isolates from the Danube River were JDS02PS016T (TZP (piperacillin/tazobactam), CAZ (ceftazidime), GM (gentamicin), MEM (meropenem), and CIP (ciprofloxacin); JDS02PS020 to CAZ, MEM, and CIP; JDS22PS018 to CAZ, IPM (imipenem), and MEM; JDS28PS083 to CAZ, FEP (cefepime), IPM, and MEM; and JDS28PS113 to CAZ, IPM, and MEM. Isolate JDS10PS014 was only resistant to MEM, and isolate JDS08PS003 was sensitive to all antibiotics tested. The four Woluwe River isolates showed the same antibiotic sensitivity profile, being resistant to carbapenems only (MEM and IPM). The three strains classified in Pseudomonas_E protegens_B were more sensitive to antibiotics than the *P. danubii* strains.

The total fatty acid methyl ester composition (FAME) and the main protein profiles were determined for phenotypic chemotaxonomic characterization. The FAME results were compared with the profiles of the three closest related type strains and are shown in Table S9. As in other *Pseudomonas*, the major fatty acid components were C16:0 (30.93%) and summed feature three (27.84%). The major difference from *P. saponiphila*, *P. protegens*, and *P. chlororaphis* is the absence of dodecanoic acid (C12:0).

The mass to charge (m/z) data dendrogram of the whole cells is shown in Figure S7. As reported in a previous study, the 17 investigated strains clustered together in the dendrogram close to *P. saponiphila*, *P. protegenes*, and *P. chlororaphis* type strains at a distance level of 100 arbitrary units (Figure S7a), confirming our previous results indicating the difficulty in differentiating the species in this phylogenetic group by their major protein profile in the context of more distant strains [13]. However, a detailed analysis of the strains in the *P. chlororaphis* phylogenetic group alone demonstrated clear differences (Figure S7b) and the consistency of the newly proposed species.

Table 3. Genomes of the three sequenced strains were analyzed for the presence of antibiotic resistance genes in the CARD website (https://card.mcmaster.ca/analyze/rgi accessed on 21 November 2022). Seven strict hits were detected by protein homology in the three genomes.

				Strain					
				JDS02PS016T		JDS10PS014		JDS08PS003	
ARO Term	**AMR Gene Family**	**Drug Class**	**Resistance Mechanism**	**% Identity of Matching Region**	**% Length of Reference Sequence**	**% Identity of Matching Region**	**% Length of Reference Sequence**	**% Identity of Matching Region**	**% Length of Reference Sequence**
adeF	resistance-nodulation-cell division (RND) antibiotic efflux pump	fluoroquinolone antibiotic, tetracycline antibiotic	antibiotic efflux	41.48	98.87	41.48	98.87	41.38	98.87
adeF				44.02	97.45	44.02	97.45	44.59	97.45
adeF				67.36	100.00	67.36	100.00	67.36	100.00
adeF								67.79	100.76
FosA8	fosfomycin thiol transferase	phosphonic acid antibiotic	antibiotic inactivation	61.76	97.87				
FosA						73.13	102.22		
vanG	glycopeptide resistance gene cluster, Van ligase	glycopeptide antibiotic	antibiotic target alteration	36.91	104.30	36.91	104.30	36.91	104.30
vanW gene in *vanG* cluster		glycopeptide antibiotic	antibiotic target alteration	30.57	100.71	30.57	100.71	28.83	102.91
YajC	resistance-nodulation-cell division (RND) antibiotic efflux pump	fluoroquinolone antibiotic, cephalosporin, glycylcycline, penam, tetracycline antibiotic, oxazolidinone antibiotic, glycopeptide antibiotic, rifamycin antibiotic, phenicol antibiotic, disinfecting agents, and antiseptics	antibiotic efflux	90.18	100.00	90.18	100.00	89.29	100.00

Table 4. Characteristics differentiating *Pseudomonas danubii* from the type strains of the most related species. +, positive; −, negative; w, weak. Strains: *Pseudomonas danubii* sp. nov. (1. JDS02PS016T, 2. JDS02PS006, 3. JDS08PS001, 4. JDS10PS002, 5. JDS10PS014, 6. JDS22PS011, 7. JDS22PS018, 8. JDS28PS081, 9. JDS28PS083, 10. JDS36PS016, 11. JDS63PS049, 12. JDS67PS009, 13. rDWA11, and 14. rDWA138), *Pseudomonas* sp. (15. JDS08PS003, 16. rDWA16, and 17. rDWA64), 18. *P. protegens* DSM 19095T, 19. *P. saponiphila* DSM 9751T, 20. *P. chlororaphis* subsp. *chlororaphis* DSM 50083T, 21. *P. koreensis* LMG 21318T, and 22. *P. fluorescens* ATCC 13525T.

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
NaCl (%) (/v)	0–6	0–6	0–6	0–6	0–6	0–6	0–6	0–6	0–6	0–6	0–6	0–6	0–5	0–5	0–6	0–6	0–6	0–6	0–5	0–6	0–6	0–8
pH	5–10	5–10	5–10	5–9	5–9	5–10	5–10	5–9	5–10	5–9	5–9	5–10	5–9	5–9	5–9	5–9	5–9	5–10	5–10	5–9	5–9	5–9
API 20 NE test:																						
Reduction of nitrate to nitrite	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
Reduction of nitrite to N2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
Hydrolysis of gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Assimilation of:																						
Arabinose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
Adipate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−
Phenylacetate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
BIOLOG GENIII test:																						
Carbon source utilization assays																						
D-Sorbitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
p-Hydroxy-Phenylacetic Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Tween 40	w	w	w	w	−	w	+	−	−	w	w	w	w	+	+	−	−	+	+	+	+	+
glycyl-L-proline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+
D-Galacturonic Acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Methyl Pyruvate	w	w	w	w	−	w	w	−	−	−	−	−	−	−	w	−	−	w	w	w	+	w
D-Arabitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+
alfa-Hydroxy Butyric Acid	w	−	−	−	−	w	w	−	−	w	−	w	−	w	−	−	w	w	w	w	w	+
D-Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
D-Galactose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
myo-Inositol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
D-Glucuronic Acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
alfa-Keto-Butyric Acid	w	−	−	−	−	w	w	−	−	w	−	w	−	+	−	−	−	−	w	+	w	+

Table 4. *Cont.*

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
D-Fucose	w	w	–	–	–	w	w	–	–	w	–	–	–	–	w	–	w	w	w	w	+	+
D-glucose-6-PO4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–
Glucuronamide	+	+	+	+	w	w	w	w	w	w	w	–	w	–	+	w	w	+	w	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+
D-fructose-6-PO4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w	–	w
Mucic Acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
D-Malic Acid	–	–	–	–	–	–	–	–	–	–	–	w	–	+	–	–	+	–	–	+	+	+
L-Rhamnose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
D-Aspartic Acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
D-Serine	–	w	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+	w	+	+	+
D-Saccharic Acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
Bromo-Succinic Acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	w	w	+	+
Formic Acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+
Chemical sensitivity assays																						
Sodium Butyrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	w	+
8% NaCl	w	w	w	+	w	w	w	+	–	w	+	+	w	–	+	+	+	+	–	–	–	+

4. Discussion

The *P. chlororaphis* subgroup in the *Pseudomonas fluorescens* phylogenetic group of species is a well-defined phylogenomic branch within the proposed Pseudomonas_E genus in the GTDB taxonomy. It is worth highlighting that all studied genomic indices are concordant and that the *rpoD* alone was predictive of the new species. The species included in the P. chlororaphis subgroup are "*P. aestus*" (not validly published) [15], *P. chlororaphis* (with four subspecies) [17,18], *P. piscis* [16], *P. protegens* [19], *P. saponiphila* [20], *P. sesami* [21], and *P. sessilinigenes* [9]. The group as a whole is widely distributed, with relevant ecological properties, including interactions with plants and fish. Only isolate AU11706 was isolated from a clinical sample [11]. The phylogenetic analysis and genomic indices obtained in the present study indicate that *P. piscis* and "*P. aestus*" are synonyms, as suggested in previous studies, and that the 17 isolates taxonomically characterized in this study conform to a monophyletic branch that represents one or two new species. Fifteen out of seventeen strains belong to the proposed species Pseudomonas_E protegens_A in the GTDB taxonomy, which contains nine isolates isolated from diverse habitats and geographical locations (Table 1). Ten strains classified as *P. protegens* and considered inconclusive in the NCBI taxonomy check are reclassified in the species *P. danubii* in the present study.

The other three taxonomically studied strains (JDS08PS003, rDWA11, and rDWA64) belong to the proposed species Pseudomonas_E protegens_B represented thus far by a single strain (14B2) isolated from the Missouri River. These last four strains are on the borderline for species differentiation with the main group represented by Pseudomonas_E protegens_A strains. In our opinion, more isolates and/or more genomes are needed to decide if Pseudomonas_E protegens_B constitute one or two different species or if both have to be considered subspecies of *P. danubii* because the phylogenomic indices are at the same level as those among strains of the four *P. chlororaphis* subspecies. The difficulty in differentiating species or subspecies in *P. chlororaphis* has been discussed previously [3].

The phylogenomic analysis also demonstrates that strains XY2F4, Os17, St29, BC42, and BNJ-SS-45 are members of *P. sesami*, a species on the borderline for genomic differentiation with *P. saponiphila*. These strains were not classified previously at the species level, or were identified as *P. protegens* strains, but their species assignation was considered inconclusive in the NCBI taxonomy check or were considered different species in the GTDB taxonomy. The five strains are monophyletic, and the genomic indices between strains are higher than the species thresholds. Their ANIb values range from 94.18 to 94.35 with the *P. saponiphila* type strain, and the corresponding GGDC values range from 58.3 to 58.6. The genome sequence of the *P. sesami* type strain is needed to clarify the species status. Experimental DNA–DNA hybridization of *P. sesami* with *P. saponiphila* was reported to be lower than 70%, which justified the separation into two species [21].

The strains *P. fluorescens* AU11706 and *P. protegens* H1F10A and H1F5C are members of a different phylogenomic species, classified as P. fluorescens_AP in the GTDB taxonomy. These strains were isolated from two quite different habitats: strains thus far classified as *P. protegens* (H1F10A and H1F5C) were isolated from samples taken in Yellowstone National Park (USA) together with other strains, and strain AU11706 was isolated from cystic fibrosis sputum in Michigan (USA) and initially classified as *Pseudomonas fluorescens* [10]. The ecological and potential pathogenicity characteristics of these strains raise interesting questions that merit specific investigations.

Genome analysis also revealed the metabolic and ecological potential of the new species. However, it must be kept in mind that the presence of genes for a specific trait may not always indicate expression. Two examples are assimilatory nitrate reduction or resistance to antibiotics. Genes for the reduction of nitrate to nitrite and nitrite to ammonium are present in the genomes, but the phenotypic tests rendered negative results. Likewise, genes for the synthesis of efflux pumps might confer resistance to many antibiotics, but strains with this gene content were experimentally found to be antibiotic sensitive. All isolates investigated for their antibiotic resistance were of environmental, aquatic origins. Those isolated from a sample taken in the Woluwe River near the source were only resistant

to carbapenems and sensitive to the other antibiotics tested. These waters are not considered contaminated [14], and thus, carbapenem resistance must be intrinsic to the strains. However, the MDR strains of the new species have most likely acquired resistance and are in habitats in which resistance can provide a selective advantage.

On the basis of the phylogenetic, genomic, phenotypic, and chemotaxonomic properties described in the present investigation, we propose that fourteen strains of our culture collection isolated from nine freshwater samples of two rivers (the Danube and Woluwe), and nine strains classified Pseudomonas_E protegens_A in the GTDB taxonomy, belong to a new species for which the name *Pseudomonas danubii* is proposed, with strain JDS02PS01T (=CECT 30214 T = CCUG 74756 T) as the type strain. A list of the 23 *P. danubii* strains is given in Table 1. Genetic and phenotypic analyses indicate that a simple distinction between species can be obtained on the basis of the *rpoD* gene sequence and their main protein profiles obtained by MALDI TOF mass spectrometry. Adipate assimilation, a weak utilization of tween 40 and serine, and a lack of assimilation of formic acid differentiates *P. danubii* isolates from the other closely related species.

5. Protologue

Description of Pseudomonas danubii sp. nov.

P. danubii sp. nov. (da.nu'bi.i. L. gen. n. danubii, of the Danube, referring to the sampling site of the type strain).

Rod-shaped cells, 2.5–1.9 µm long and 1.3–0.5 µm wide, forming colonies after 24 h incubation at 30 °C on LB. The cells are gram-negative, motile by means of one or two polar-inserted flagella, and exhibit growth at pH of 5 to 9 and in the range of 4 to 37 °C (optimum 30 °C); no growth detected at 42 °C. NaCl concentrations up to 6% (*w*/*v*) are tolerated. Fluorescent pigments were produced on King B but not on King A medium. Strictly aerobic, not fermentative. Catalase and oxidase positive. Nitrate is not reduced. The API 20NE test was positive for arginine dihydrolase and hydrolysis of gelatin but not esculin and was negative for urease and beta-galactosidase; it was positive for the assimilation of glucose, mannose, mannitol, N-acetyl-D-glucosamine, gluconate, caprate, adipate, malate, citrate, and phenylacetate but negative for arabinose and maltose. In Biolog GENIII testing, plates were positive for assimilation of alfa-D-glucose, p-hydroxy-phenylacetic acid, D-mannose, D-mannitol, glycyl-L-proline, gamma-amino-butyric acid, D-fructose, D-arabitol, L-alanine, D-trehalose, myo-inositol, L-arginine, D-gluconic acid, L-lactic acid, beta-hydroxy-D,L butyric acid, D-cellobiose, and D-salicin. Adipate assimilation, weak utilization of tween 40 and serine, and lack of assimilation of formic acid differentiate it from the other closely related species. The major fatty acids are C16:0 and summed feature 3 (C15:0 iso 2OH and C16:1 w7c). The absence of C12:0 differentiates it from closely related species. Simple distinction between the species can be obtained on the basis of the *rpoD* gene sequence.

The type strain is JDS02PS016T (=CECT 30214T = CCUG 74756T). The GenBank accession number for the rRNA gene sequence is OU957229.1, and that for the genome is CP116502. The G+C content of the type strain is 62.3 mol% based on the draft genome sequence, and it ranges from 62.0 to 62.5 mol% in 11 strains of the species.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d15050617/s1. Figure S1. Phylogenetic tree based on the *rpoD* gene sequence. Figure S2. Maximum likelihood phylogenetic tree of strains studied in the *P. chlororaphis* subgroup of species based on the concatenated sequences of 249 core genes. Figure S3. (a) Dendrograms of the aggregated ANIb values. (b) Dendrogram of the GGDC similarities among the studied strains. Figure S4. Graphical representation of the distribution of the 11,081 orthologous genes among the strains: (a) The phyletic pattern is represented in an unrooted network by the split decomposition method. (b) Heat plot of the phyletic pattern representing the presence (red) or absence (blue) of the orthologous genes. Figure S5. Venn diagram of the shared orthologous genes of two sequenced strains of *P. danubii* (JDS02PS016T and JDS10PS014) and two closely related strains of Pseudomonas_E protegens_B in the GTDB taxonomy (JDS08PS003 and 14B2). Figure S6. Electron microscopy of negatively stained flagellated cells of strain JDS02PS016T in the exponential growth

phase (left). Colony morphology of strain JDS02PS016T after incubation at 30 °C for 48 h on LB agar (right). Figure S7. Dendrogram of the main proteins obtained by MALDI-TOF mass spectrometry. (A) with outgroups; (B) only species in the *P. chlororaphis* subgroup. Table S1. Sampling sites, geographical origins, and references of the strains included in the present study. Table S2. GenBank accession numbers of sequences used in this study. Table S3. Pairwise sequence similarities of the 16S rRNA, concatenated genes (16S rRNA, *gyrB*, *rpoB*, and *rpoD*) and *rpoD* genes between *Pseudomonas danubii* and the strains of the *Pseudomonas fluorescens* group included in this study. Table S4. One hundred housekeeping genes (filtered from 450 total entries) were selected for the autoMLST analysis. Table S5: Genomic indices among the studied strains. Table S6. Prophage regions detected by the PHASTER server in the genome sequences of the four strains sequenced in the present study. Table S7. Physiological and biochemical characteristics of *Pseudomonas danubii* and related *Pseudomonas* strains and species type strains. Table S8. Antibiotic susceptibility tests of the studied strains. (S: sensitive, R: resistant). Table S9. Cellular fatty composition (%) derived from FAME analysis of *Pseudomonas danubii* JDS02PS016T and the type strains of closely related *Pseudomonas* species [49].

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Abbreviations

ANI	genome-aggregated average nucleotide identity
autoMLST	automated multilocus species tree
FAME	fatty acid methyl ester analysis
GGDC	genome to genome distance calculator
MALDI-TOF MS	matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry
MLSA	multilocus sequence analysis

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