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Pseudomonas cerasi sp. nov. (non Griffin, 1911) isolated from diseased tissue of cherry☆

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ABSTRACT

Eight isolates of Gram-negative fluorescent bacteria (58^T, 122, 374, 791, 963, 966, 970a and 1021) were obtained from diseased tissue of cherry trees from different regions of Poland. The symptoms resembled those of bacterial canker. Based on an analysis of 16S rDNA sequences the isolates shared the highest over 99.9% similarity with *Pseudomonas ficuserectae* JCM 2400^T and *P. congelans* DSM 14939^T. Phylogenetic analysis using housekeeping genes *gyrB*, *rpoD* and *rpoB* revealed that they form a separate cluster and confirmed their closest relation to *P. syringae* NCPPB 281^T and *P. congelans* LMG 21466^T. DNA–DNA hybridization between the cherry isolate 58^T and the type strains of these two closely related species revealed relatedness values of 58.2% and 41.9%, respectively. This was further supported by Average Nucleotide Identity (ANIb) and Genome-to-Genome Distance (GGDC) between the whole genome sequences of strain LMG 28609^T and closely related *Pseudomonas* species. The major cellular fatty acids are 16:0 and summed feature 3 (16:1 ω7c/15:0 iso 2OH). Phenotypic characteristics differentiated the novel isolates from other closely related species. The G+C content of the genomic DNA of strain 58^T was 59%. The diversity was proved by PCR MP and BOX PCR, eliminating the possibility that they constitute a clonal population. Based on the evidence of this polyphasic taxonomic study the eight strains are considered to represent a novel species of the genus *Pseudomonas* for which the name *P. cerasi* sp. nov. (non Griffin, 1911) is proposed. The type strain of this species is 58^T (=LMG 28609^T = CFBP 8305^T).

Eight isolates (58^T, 122, 374, 791, 963, 966, 970a and 1021) belonging to the genus *Pseudomonas* were obtained from diseased tissue of different organs of cherry trees, collected in orchards located in various geographical regions of Poland (Table 1). Symptoms were similar to those typical for bacterial canker, including necrotic spots on leaves and fruits, dying of the flowers as well as necrotic areas on shoots as described by Cameron [9]. Since many years the causal agents of bacterial canker have been assigned to the *Pseudomonas syringae* complex which comprises plant pathogens of 60 pathovars [56]. On King's B medium, most of them produce fluorescent pigment visible under UV light [26]. As determined by DNA–DNA hybridization [14] bacteria causing bacterial canker on stone fruit trees were classified into three genomospecies: (1) *P. syringae* pv. *syringae* (*Psy*), (2) *P. syringae* pv. *morsprunorum* race 1 (*Pmp* race 1) and (3) *P. syringae* pv. *morsprunorum* race 2 (*Pmp* race 2), *P. syringae* pv. *avii* (*Psa*) and *P. syringae* pv. *persicae* (*Ppe*). The last two pathogens caused similar symptoms but only on wild cherry and peach, respectively [13,33,38,49,53,55]. Recently, Multi Locus Sequence Analysis (MLSA of genes *cts*, *gapA*, *rpoD* and *gyrB*) has been used to redefine the phylogenetic relationships within the *P. syringae* species complex revealing the presence of 13 phylogroups (PG) [5] largely corresponding to the genomospecies defined by DNA–DNA hybridization [14].

☆ *Note*: The GenBank/EMBL/DDBJ accession numbers for the partial 16S rDNA sequences of strains 58^T, 122, 374, 791, 963, 966, 970a and 1021 are: LN713316–LN713323, respectively. Accession numbers for the partial *gyrB* gene sequences of strains 58^T, 122, 374, 791, 963, 966, 970a and 1021 are: HG000215, HG000224, HG000252, HG000269, LN713306, LN713307, HG000272, HG000273, respectively. Accession numbers for the partial *rpoB* gene sequences of strains 58^T, 122, 374, 791, 963, 966, 970a and 1021 are: LN713308–LN713315, respectively. Accession numbers for the partial *rpoD* gene sequences of strains 58^T, 122, 374, 791, 963, 966, 970a and 1021 are: HG000020, HG000029, HG000057, HG000074, LN713304, LN713305, HG000077, HG000078, respectively. Accession numbers of genome sequence of strain 58^T are LT222313–LT222319.

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Table 1
Strains used in this study.

Strain number	Place and year of isolation		Host plant	Organ	Gene presence based on PCR amplification			
					cfl	*irp1*	*syrB*	*syrD*
Pseudomonas amygdali LMG 1318^T	Greece	1967	*Prunus amygdalus*	No data	–[a]	–	–	–
Pseudomonas avellanae LMG 2166^T	Greece	1976	*Corylus avellana*	No data	–	–	–	–
Pseudomonas cannabina LMG 509^T	Hungary	1957	*Cannabis sativa*	No data	+	–	–	–
Pseudomonas caricapapayae LMG 2152^T	Brazil	1966	*Carica papaya*	No data	–	+	–	–
Pseudomonas congelans LMG 21466^T	Germany	1994	Grasses, phyllosphere	No data	–	–	–	–
Pseudomonas meliae LMG 222^T	Japan	1974	*Melia azedarach*	No data	–	–	–	–
Pseudomonas ficuserectae LMG 569^T	Japan	1983	*Ficus erecta Inubiwa*	No data	–	–	–	–
Pseudomonas savastanoi LMG 220^T	Former Yugoslavia	No data	*Olea europaea*	No data	–	–	–	–
Pseudomonas syringae LMG 1247^T	United Kingdom	1950	*Syringa vulgaris*	No data	–	–	+	+
Pseudomonas tremae LMG 2212^T	Japan	1979	*Trema orientalis*	No data	–	–	–	–
Pseudomonas cerasi 58^T	Poland, Studzianek near Babsk	2007	Sour cherry	Leaf	–	–	–	–
Pseudomonas cerasi 122	Poland, Pamiętna	2007	Sour cherry	Shoot	–	–	–	–
Pseudomonas cerasi 374	Poland, Dębowa Góra	2008	Sour cherry	Flower	–	–	–	–
Pseudomonas cerasi 791	Poland	2001	Sour cherry	Fruit	–	–	–	–
Pseudomonas cerasi 963	Poland, Piaski near Lublin	2009	Sweet cherry	Leaf	–	–	–	–
Pseudomonas cerasi 966	Poland, Strojno near Lublin	2009	Sour cherry	Flower	–	–	–	–
Pseudomonas cerasi 970a	Poland, Strojno near Lublin	2009	Sour cherry	Flower	–	–	–	–
Pseudomonas cerasi 1021	Poland, Nowy Dwór	2009	Sour cherry	Flower	–	–	–	–

[a] +, positive-presence of features; –, negative results-lack of features.

In this study the taxonomic status of all eight novel cherry isolates was investigated using the polyphasic approach including phenotypic characterization, genome sequencing, ANIb, GGDC, DNA–DNA hybridization, sequence analysis of 16S rDNA and three housekeeping genes – MLSA, PCR MP, rep-PCRs, determination of G+C content, FAME and MALDI-TOF MS analysis.

Small pieces from the margin between healthy and diseased tissue of samples collected were macerated in 500 μl of sterile phosphate-buffered saline (PBS: 0.27% Na_2HPO_4, 0.04% NaH_2PO_4, 0.8% NaCl). The resulting mixture was transferred by loop onto NSA medium (Difco Nutrient Agar 2.3%; sucrose 5%) [29] and King's B [26], and incubated at 27 °C for 2 days. Selected colonies were purified on King's B medium and subjected to further studies. Cells are Gram-negative, strictly aerobic, non-spore-forming and rod-shaped (0.5× 2–3.5 μm).

The pathogenicity test performed on immature cherry fruitlets according to the method and conditions described by Kałużna and Sobiczewski [24] showed that the isolates produced the brownish, water soaked superficial lesions similar to those caused by strains of both races of *P. syringae* pv. *morsprunorum* but they were less virulent.

Genomic DNA of bacteria was isolated according to the method of Aljanabi and Martinez [2] with modification as described in Kałużna et al. [22] and used for amplification with all applied primers.

For all novel isolates almost complete 16S rDNA (1398 bp) with primers fD1 and rP2 [52] was amplified and sequenced. The BLASTn analysis of 16S rDNA sequence revealed that the novel cherry isolates are members of the *Pseudomonas* genus and belong to the *P. syringae* group [3]. Multiple sequence alignments and phylogenetic analysis of 16S rDNA sequences of the cherry isolates with sequences of the type strains of other *Pseudomonas* species obtained from GenBank were performed using MEGA v.5.0 [47]. Maximum Likelihood phylogenetic trees of closely related species (Fig. 1) and all species of the *Pseudomonas* genus (Fig. S1) were constructed using Tamura 3 parameter evolutionary model with gamma distribution and by assuming that a certain fraction of sites are evolutionarily invariable (+I) (found as the best substitution model). The significance of the internal branches of the dendrograms was estimated with bootstrap values expressed as percentages of 1000 replications. Analyses of sequence similarity were done using MegAlign software of the Lasergene v. 8 package (DNASTAR, Madison, WI, USA). This analysis showed that the isolates 58^T, 122, 374, 791, 963, 966, 970a and 1021 have identical 16S rDNA sequences (100% sequence similarity) and they share 99.9% sequence similarity with of *P. ficuserectae* JCM 2400^T and *P. congelans* DSM 14939^T, 99.8% sequence similarity with *P. syringae* NCPPB 281^T/*P. tremae* CFBP 6111^T, 99.7% sequence similarity with *P. caricapapayae* ATCC 33615^T/*P. amygdali* CFBP 3205^T, 99.6% sequence similarity with *P. cannabina* CFBP 2341^T and 99.5% sequence similarity with *P. savastanoi* ATCC 13522^T.

Although 16S rDNA sequence analysis is a useful tool for genus assignments, due to the low resolution of this gene at the intrageneric level it is not sufficient for discrimination of species and pathovars [12,15,54]. Indeed, the application of 16S rDNA sequence in classification of bacteria, its validity as a marker for phylogenetic inferences and taxonomic studies has been questioned by many authors [1,16,25,37,42]. On the other hand, use of housekeeping core genes was proved to provide an accurate approach for the phylogenetic analysis and for a correct phylogenetic affiliation of the genus *Pseudomonas* [16,25,27,37]. MLSA including *gyrB*, *rpoB* and *rpoD* compared with indices based on the analysis of whole-genome sequences revealed their strongest correlation with ANIb [16].