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Pararhizobium polonicum sp. nov. isolated from tumors on stone fruit rootstocks☆

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ABSTRACT

Five Gram-negative, rod-shaped, non-spore-forming bacteria were isolated from galls on different stone fruit rootstocks in Poland: strains F5.1^T and F5.3 from *Prunus avium* F12/1, strains CP3.5 and CP17.2.1 from *Prunus avium* and strain AL5.1.8 from *Prunus cerasifera*. On the basis of 16S rDNA phylogeny, the strains cluster together and belong to the genus *Pararhizobium* with type strain of *Pararhizobium herbae* (99.6–99.8%) as their closest relative. Phylogenetic analysis of the novel strains using housekeeping genes *atpD*, *recA* and *rpoB* revealed their distinct position separate from other known *Rhizobium* species and confirmed their relation to *P. herbae*. DNA–DNA hybridization of strains F5.1^T, with the type strain of *P. herbae* LMG 25718^T and *Pararhizobium giardinii* R-4385^T revealed 28.3% and 27.9% of DNA–DNA relatedness, respectively. Phenotypic and physiological properties differentiate the novel isolates from other closely related species.

On the basis of the results obtained, the five isolates are considered to represent a novel species of the genus *Pararhizobium*, for which the name *Pararhizobium polonicum* sp. nov. (type strain F5.1^T = LMG 28610^T = CFBP 8359^T) is proposed.

Crown gall is one of the most important bacterial diseases of fruit trees, grapevines and roses. Bacteria causing this disease were classified earlier in the genus *Agrobacterium* and since 2001 in the revised genus *Rhizobium* together with plant symbiotic, nitrogen fixing species [26,27]. At present, tumorigenic bacteria are allocated in three genera: *Agrobacterium*, *Allorhizobium* and *Rhizobium* [15]. Besides well-defined species, several genomovars in the genus *Agrobacterium* were distinguished based on *recA* gene sequence [5]. During an infection process a fragment of tumor-inducing (Ti) plasmid, called T-DNA, is transferred to the plant cell, and afterwards incorporated into the plant genome. The gall is formed as a result of the expression of genes encoding the synthesis of auxin and cytokinin located on T-DNA. Apart from genes coding for plant hormone synthesis, the T-DNA also contains regions responsible for synthesis of opines, a class of specific amino acid derivatives [6]. Opines are not utilized by plants but they serve as selective nutrients for pathogenic *Agrobacterium* strains that possess opine utilization genes located in the Ti plasmid, outside the T-DNA. The utilization of opines is not a unique feature of virulent agrobacteria and closely related strains, but bacteria of several other genera possess this ability and are well adapted to live in the gall tissue [23]. However, to our knowledge members of newly described *Pararhizobium* genus comprising bacteria isolated worldwide from legume plants or fresh water [15] have never been reported as opine-catabolizing bacteria.

In the years 2008–2011, during a study on the etiology of crown gall on stone fruits in Poland, eighteen strains were isolated from the inner tissue of galls and, on the basis of 16S rDNA sequence analysis, assigned to the genus *Pararhizobium* [19]. None of the strains was pathogenic. Five non-clonal strains of this group are

☆ *Note*: The GenBank/EMBL/DDBJ accession numbers for the partial 16S rDNA sequences of studied strains are: CP17.2.1 – LN845899, AL5.1.8 – LN845890, CP3.5 – LN845903, F5.1^T – LN845906, F5.3 – LN845907. Accession numbers for the partial *recA* gene sequences are: CP17.2.1 – LN812140, AL5.1.8 – LN812129, CP3.5 – LN812144, F5.1^T – LN812148, F5.3 – LN812149. Accession numbers for the partial *atpD* gene sequences are: CP17.2.1 – LN876257, AL5.1.8 – LN876256, CP3.5 – LN876258, F5.1^T – LN876259, F5.3 –LN876260. Accession numbers for the partial *rpoB* gene sequences are: CP17.2.1 – LN876262, AL5.1.8 – LN876261, CP3.5 – LN876263, F5.1^T – LN876264, F5.3 – LN876265. Accession number of genome sequence of strain F5.1^T is LGLV01000000.

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reported here. Strains were isolated from galls on *Prunus avium* F12/1 (strains F5.1 and F5.3), *Prunus avium* (strains CP 3.5 and CP 17.2.1) and *Prunus cerasifera* (strain AL5.1.8). The strains F5.1, F5.3, CP3.5, AL5.1.8 were recovered from tumors on mannitol agar supplemented with 70 mg l^{-1} of potassium tellurite [14] while CP17.2.1 was isolated from tumors on 1A + 2E medium [18] after incubation at 27 °C for 5 days.

The 16S rDNA sequencing was performed with primers fD1 and rP2 [25]. The obtained 16S rDNA sequences (1276 bp) were aligned and a phylogenetic tree was constructed using MEGA 6 software package with the Maximum likelihood method and the Tamura-3 parameter as the best nucleotide substitution model [22]. Bootstrap analysis with 500 replicate datasets was performed to assess the support of the clusters (Fig. 1, an extended tree containing a larger number of reference sequences is available as Supplementary Fig. S1). The strains F5.1^T, F5.3, CP3.5, CP17.2.1 and AL5.1.8 possessed almost identical 16S rDNA sequences and were grouped within the genus *Pararhizobium*. Their closest relatives are type strains of *Pararhizobium herbae* (99.6–99.8% sequence similarity), *Pararhizobium giardinii* (98.6–98.7%) and *Pararhizobium sphaerophysae* (97.1–97.3%), with which they form a cluster with 96% bootstrap support (Fig. 1).

The *atpD* and *rpoB* gene fragments of the new isolates and reference strains were amplified with the primers and protocols described by Martens et al. [10] while the *recA* gene was amplified with the primers of Costechareyre et al. [5]. Phylogenetic analyses were performed on individual gene sequences (*atpD* – 401 bp, *recA* – 340 bp, *rpoB* – 505 bp) as well as on the concatenated data set (1246 bp). The genetic distances between the sequences were estimated by Tamura 3-parameter for *atpD* gene and General time reversible for *recA* and *rpoB* genes and Maximum likelihood trees were generated in MEGA 6 [22]. The significance of internal branches of the phylogenetic trees was estimated with 500 bootstrap replicates. The novel isolates F5.1^T, F5.3, CP3.5, CP17.2.1 and AL5.1.8 possessed very similar sequences for each of the protein-encoding genes studied and formed a separate phylogenetic lineage (Supplementary Figs. S2, S3, S4, and Fig. 2). The similarity between their sequences of *atpD*, *recA* and *rpoB* genes and the sequence of the same genes of the closest relative, type strain of *P. herbae*, was: 94–95.3%, 95–95.6% and 94.2–94.8%, respectively.

DNA–DNA hybridizations (DDH) were performed at 42 °C with photobiotin-labeled probes in microplate wells as described before [7], using an HTS7000 Bio Assay Reader (PE Applied Biosystems) for the fluorescence measurements. DDH of F5.1^T, AL5.1.8 and CP3.5 with the type strains of *P. herbae* LMG 25718^T and *P. giardinii* R-4385^T, their closest relatives, revealed a DNA–DNA relatedness lower than 33% (Table 1). The DNA G+C content of novel strains was determined by HPLC as described previously [12] and was found to be 60.3–60.7 mol% (Table 1).

Total genomic DNA of bacterial strains was extracted according to the protocol described by Aljanabi and Martinez [2]. Using the Illumina MiSeq platform, paired-end libraries (insert size 300–500 bp) and mate-pair libraries (insert sizes 1.5–3 and 6–9 kb) were sequenced with 2× 250 bp paired end reads, generating 3,587,710 sequences in pairs from the paired-end library and 4,421,198 sequences in pairs from the mate-pair libraries (Genomed SA, Warsaw, Poland).

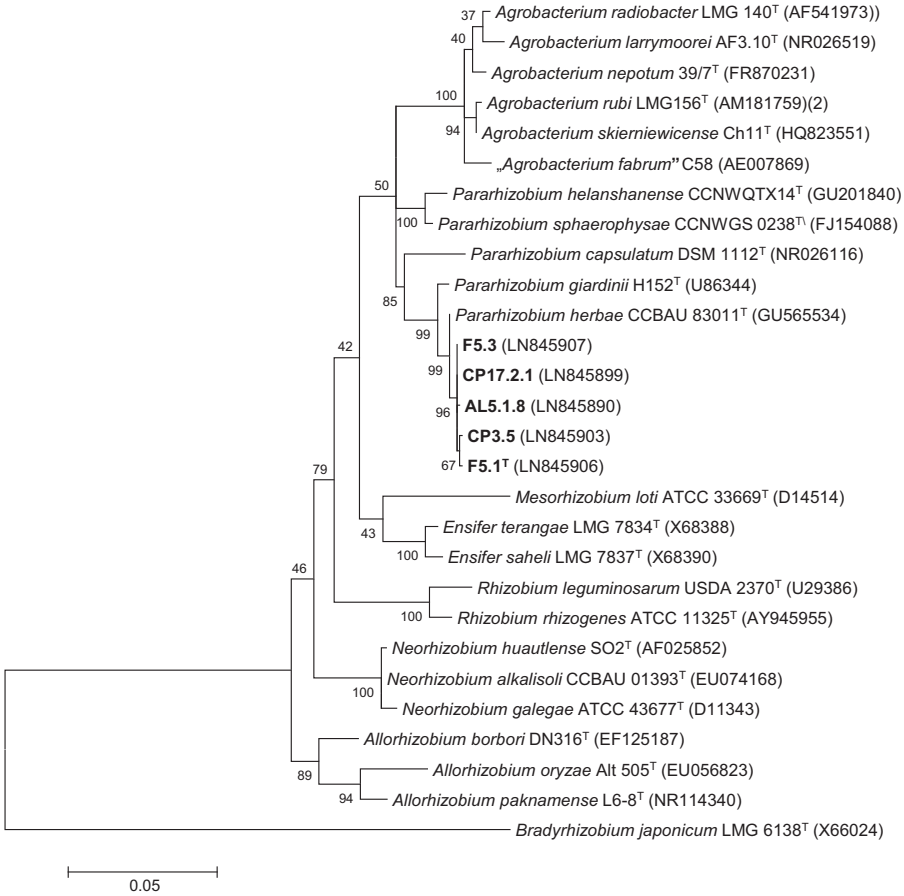

Fig. 1. Maximum likelihood tree showing the phylogenetic relationship between strains F5.1^T, F5.3, C3.5, AL5.1.8 and CP17.2.1 and related species based on 16S rDNA sequences. Bootstrap values (expressed as percentages of 500 replications) are given at the nodes. GenBank accession numbers are given in parentheses. Bar – estimated nucleotide substitutions per site.