scientific reports

Check for updates

OPEN

Isolation and characterization of *Agrobacterium vacciniicorymbosi* sp. nov. originating from crown galls in blueberry (*Vaccinium corymbosum*) in Serbia

Renata Iličić[1], David J. Studholme[2]✉, Aleksandra Jelušić[3], Goran Barać[1], Ferenc Bagi[1] & Tatjana Popović Milovanović[4]✉

Seven bacterial isolates were obtained from crown gall in blueberry plants (*Vaccinium corymbosum*) growing in Serbia in 2020. Phylogenetic analysis of the novel isolates using four housekeeping genes – *atpD*, *dnaK*, *glnA*, and *rpoB*, as well as gene 16 S rRNA – revealed their distinct position, separate from other known *Agrobacterium* species, but closest to *A. vaccinii*, *A. rosae* and *A. rubi*. Genome sequencing of one of these blueberry isolates revealed less than 86% average nucleotide identity (ANI) between the blueberry isolate and type strains of *Agrobacterium* species, indicating that it does not belong to any previously described species. Although genome sequencing revealed absence of the tumor-inducing (Ti) plasmid, stabbing the crown region with bacterial cultures induced gall-like structures formed on blueberry plants (cultivar Duke). Phenotypically, these blueberry isolates were differentiated from closely related *Agrobacterium* species in their utilization of dulcitol, d-tagatose and xylitol. On the basis of these results, Serbian blueberry isolates are considered to represent a novel species of the genus *Agrobacterium*, for which the name *Agrobacterium vacciniicorymbosi* sp. nov. is proposed, with BA1120^T (NCPPB 4800 = CFBP 9290) as the type strain. This isolate and BA2520 (NCPPB 4801 = CFBP 9291) have been deposited in public collections of plant pathogenic bacteria.

Keywords Blueberry, Crown gall, *Agrobacterium* sp. nov

As global demand for blueberries (*Vaccinium corymbosum* L.) continues to rise, the crop has become a key player in modern fruit production, especially in highly developed countries. Highbush blueberry is a very popular commercial crop in Serbia[1], currently grown over an area of about 2,500 ha, with production tending to increase[2]. Intensive production, together with the trade and import of plant material, has led to increased emergence of new plant pathogens. Until now, the only known bacterial pathogen of blueberry in Serbia was *Pseudomonas syringae* pv. *syringae*[3].

Bacteria of the genera *Agrobacterium*, *Allorhizobium*, and *Rhizobium* are causal agents of crown gall, tumors and hairy root diseases on economically important crops[4]. These pathogens are widespread, causing significant yield losses, thereby reducing the profitability of crop production. *Agrobacterium* is used to introduce foreign genes into plants by transferring T-DNA from its Ti-plasmid (tumor-inducing) into plant cells, where it integrates into the plant chromosome and encodes the enzymes for the synthesis of auxin, cytokinin and opines[5,6]. Strains containing a Ti plasmid cause the disease known as crown gall, characterized by large tumor-like galls on the roots and stem. Those possessing a Ri (root-inducing) plasmid induce hairy root disease[7]. The Ti plasmid-borne *vir* genes encode a type IV secretion system (T4SS) and other elements necessary for processing and transporting the T-DNA into plant cells for transformation[8–10].

In blueberries, strains of *Agrobacterium* biovar 1 (*A. tumefaciens* species complex), *A. radiobacter* (formerly *R. radiobacter*), *R. rhizogenes* (*Agrobacterium* biovar 2/*Agrobacterium rhizogenes*), and *A. rubi* were reported as causal agents of crown and cane gall symptoms[7,11,12]. Recently, novel, non-tumorigenic *A. vaccinii* strains

[1]Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia. [2]Biosciences, University of Exeter, Exeter, UK. [3]University of Belgrade, Institute for Multidisciplinary Research, Kneza Višeslava 1, 11030 Belgrade, Serbia. [4]Institute for Plant Protection and Environment, Teodora Drajzera 9, 11000 Belgrade, Serbia. ✉email: d.j.studholme@exeter.ac.uk; tanjaizbis@gmail.com

were isolated from blueberry plants showing symptoms of galls on shoots in Poland[13]. According to Aloni and Ullrich[14], typical symptoms of *Agrobacterium* infection in blueberries include galls, in the form of ball-shaped or knob-like structures on roots, crowns and stems or even flowers and stems. Pulawska[15] stated that blueberry galls disease has significant impact on still-developing, young plants, while they do not cause serious problems in older plants. With new *Agrobacterium* species continuing to be discoveredworldwide[13,16–23], it is likely that this genus will continue to expand.

Our study focused on isolates obtained from blueberry plants showing symptoms of crown gall, grown under intensive production in the south of the Vojvodina region in Serbia. Initial analysis of 16 S rRNA sequence and MLSA indicated that they could represent a distinct and novel species of *Agrobacterium*. To investigate this, we used a polyphasic approach that involved genome sequence analysis, as well as phenotypic characterization.

Materials and methods

Collection of samples and bacterial isolation

Samples were collected from two-year-old blueberry plants (cultivar Duke) showing crown gall symptoms (Fig. 1), growing under intensive production in the southern part of Vojvodina (locality Temerin), Serbia, in 2020. Plants were collected from three different points of the observed plantation (1 ha in size).

Three samples, each consisting of one diseased blueberry plant, were taken for pathogen isolation. Galls were rinsed under tap water, then treated with a commercial solution of 10% bleach for 3 min, washed with sterilized distilled water (SDW) and dried. Small fragments taken from the interior of the gall tissue were macerated in SDW and plated onto D1 semi-selective medium[24] after 60 min. After 10 to 12 days of incubation at 26 °C, a total of seven bacterial colonies were purified on Nutrient Agar (NA) (coded as BA1120, BA1420, BA1920, BA2520, BA2720, BA2820, BA3320). Pure cultures were kept at − 20 °C in Luria Bertani (LB) broth containing 20% (v/v) glycerol.

Two representative isolates (BA1120 and BA2520) were chosen to be deposited in the National Collection of Plant Pathogenic Bacteria (NCPPB) and the French Collection for Plant Associated Bacteria (CFBP), with BA1120 designated as the type strain of the new species.

Phenotypic characterization

Pathogenicity

Pathogenicity tests were performed for seven Serbian blueberry isolates (BA1120, BA1420, BA1920, BA2520, BA2720, BA2820, BA3320) on one-year-old blueberry plants (cv. Duke) grown in pots. Bacterial isolates were grown on NA at 26 °C for 48 h. Prior to inoculation, blueberry plants were visually inspected for the presence of tumor/gall. In addition, collected cut pieces of plant tissue were tested using multiplex PCR with UF/B1R/B2R/AvR/ArR primers for detection of *Agrobacterium* spp. (biovars 1 and2, *A. vitis*, and *A. rubi*)[25]. Blueberry plants were inoculated by wounding the crown part with a sterile scalpel and coating wounds with pure culture of the bacterial isolate. The inoculation sites were covered with wet wool and aluminium foil for one week to ensure high-humidity conditions. Plants inoculated with SDW were used as control treatments. Pathogenicity was tested in two replicates, with one or two different inoculation sites on each plant. Inoculated plants were kept at a temperature of 25 ± 2 °C in a climate chamber and symptoms (gall formation) were recorded on a weekly basis.

Pathogenicity was checked on four-weeks-old tomato and sunflower seedlings by stabbing into the stem with a sterile needle dipped in isolates grown on NA medium at 26 °C for 48 hours[26]. Inoculated plants were maintained in a climate chamber at temperature 25 ± 2 °C. Control plants consisted of those treated with SDW. Inoculated plants were visually inspected for gall formation on a weekly basis.

Fig. 1. Crown galls on naturally infected blueberry plants (cv. Duke).

Biochemical characterization

Biochemical properties of Blueberry bacterial isolates were examined using the API 50 CH according to the manufacturer's instructions (bioMérieux, France). Isolates were additionally analyzed for the following characteristics: Gram reaction, presence of catalase and oxidase, production of fluorescent pigment on King's B medium, and 3-ketolactose production[27].

Genotypic characterization

Total bacterial DNA extraction

Genomic DNA from all tested blueberry isolates was extracted, from 48-hour-old pure cultures grown on NA, using a genomic DNA isolation kit (DNeasy Plant Mini Kit, Qiagen, Germany) according to the manufacturer's instructions. The obtained DNA was stored at − 20 °C until use.

Multi-locus sequence analysis (MLSA)

All seven Serbian blueberry isolates (BA1120, BA1420, BA1920, BA2520, BA2720, BA2820, and BA3320) were characterised based on the partial sequences of four housekeeping genes: *atpD*, *dnaK*, *glnA*, and *rpoB*, encoding ATP synthase F1 beta subunit, molecular chaperone DnaK, glutamine synthetase type I, and RNA polymerase beta subunit, respectively, as well as partial sequences of 16S rRNA gene. For DNA amplification of the above-mentioned housekeeping genes, we used primers proposed by Aujoulat et al.[28], i.e. 800 F (5'- GGCCAGGACG TTCTGTTCTT-3')/1350R (5′-CTTGAAGCCCTTGATCGTGT-3′) for *atpD*; 720 F (5′-GAAGACTTCGACA TGCGTCT-3′)/1400R (5′-GCCGAGCAGCTTGTTGTC-3′) for *dnaK*; 144 F (5′-GTCATGTTCGACGGCTC CT-3′)/900R (5′-CCTTGGCATGCTTGATGAT-3′) for *glnA*, and 2040 F (5′-GAAAACGACGACGCCAAC-3′)/2718R (5′-GCGCAGAAGCTTTTCTTCC-3′) for *rpoB*. Additionally, primers 27 F (5′-GAGAGTTTGAT CCTGGCTCAG-3′) and 1495R (5′-CTACGGCTACCTTGTTACGA-3′), proposed by Caccamo et al.[29], were used for the amplification of the 16 S rRNA gene.

The DNA of the isolates was amplified in a PCR mix (25 µL), consisting of 12.5 µL of FastGene Taq 2x Ready Mix (Nippon Genetics Europe GmbH), ultrapure DNases/RNase-free water (9.5 µL), 10 µM primers (1 µL forward plus 1 µL reverse), and sample DNA (1 µL). The programs used for PCR amplification were as specified previously[30].

The obtained PCR products were checked for the presence of amplicons of the expected size (*atpD*: 465 bp, *dnaK*: 480 bp, *glnA*: 474 bp, *rpoB*: 534 bp, and 16 S rRNA: 1550 bp) on a 1.5% agarose gel stained with ethidium bromide and sent for sequencing to the Eurofins Genomics sequencing service (Hamburg, Germany). The obtained sequences were manually checked for quality using BioEdit software v.7.2.

For preliminary exploration of the gene sequences from blueberry isolates, we performed nucleotide Basic Local Alignment Search Tool (BLASTn) searches against the nucleotide sequence collection at the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), using default options and settings. This helped to gather related sequences to be used in the subsequent phylogenetic analysis.

A multi-locus phylogenetic analysis was performed for identification of seven blueberry isolates based on the five sequenced loci. For each isolate, we concatenated the five partial gene sequences prior to alignment and tree building and included sequences from 21 *Agrobacterium* and five *Rhizobium* strains retrieved from GenBank via the NCBI (Table 1).

Prior to the construction of the neighbor-joining (NJ) phylogenetic tree, sequences of the tested and comparative isolates/strains were aligned using the ClustalW multiple alignment tool in BioEdit v.7.2, resulting in an alignment with total concatenated sequence length of 3213 nt. The phylogenetic tree was generated with MEGA7 software using the Kimura two-parameter model[31] with *Mesorhizobium huakuii* strain NZP2235 serving as an out-group (Table 1). Since no differences were observed between the isolates for any of the sequenced genes, the sequences of all five genes from two selected Serbian isolates (BA1120 and BA2520) were deposited in GenBank and accession numbers obtained.

Detection of virulence genes

We tested for the presence of four virulence genes (*virD2*, *ipt* and *virC*) in the blueberry isolates, as has been done in previous studies in *Agrobacterium* spp[32,33]. Duplex-PCR analysis was performed with primers A/C' (*virD2*) and CYT/CYT' (*ipt*)[32] for the first, and VCF3/VCR3 (*virC2-virC1*)[33] for the second PCR reaction. PCR reactions were performed in a volume of 25 µl containing 1× DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA), 0.4 µM each primer and 2.5 µl of template DNA. Amplification protocols are outlined by Kuzmanović et al.[34]. The amplification profile for primer combination A/C' and CYT/CYT' consisted of an initial denaturation at 94 °C for 1 min followed by 40 cycles of denaturation at 94 °C, annealing at 50 °C and elongation at 72 °C for 1 min. Thermal-cycler program for primer combination VCF3/VCR3 consisted of an initial denaturation at 94 °C for 1 min followed by 35 cycles of denaturation at 94 °C, annealing at 56 °C and elongation at 72 °C for 1 min. The final extension was carried out at 72 °C for 5 min. The PCR products were separated by electrophoresis using a 2% agarose gel, stained with Midori Green Advance, and visualized using UV light. Fragment sizes were estimated via comparison with the GeneRuler Low Range DNA Ladder (Thermo Scientific™).

Whole-genome sequencing, assembly and annotation

Extracted DNA was shipped to Macrogen Europe (Amsterdam, The Netherlands) and whole-genome sequencing was performed using NovaSeq (Illumina Inc., San Diego, CA, USA). The whole-genome library was prepared using the TruSeq Nano DNA (Illumina Inc., San Diego, CA, USA) library kit. After trimming and filtering the sequence reads using TrimGalore[35] (command-line options: "-q 30 –paired"), we assembled the reads *de novo*

Species	Strain	Isolation source	Country	Accession number				
				atpD	dnaK	glnA	rpoB	16 S rRNA
A. tumefaciens	DA5	Tobacco	Serbia	PP471554	PP471555	PP471556	PP471557	PP464228
	HAMBI 105	Soil	USA	CP139997	CP139997	CP139997	CP139997	CP139997
	Gle002	Walnut	USA	CP048564	CP048564	CP048564	CP048564	CP048564
	12D1	-	-	CP033031	CP033031	CP033031	CP033031	CP033031
	O54/95	Cherry	-	CP124967	CP124967	CP124967	CP124967	CP124967
	BIM B-1315G	Root endosphere (soybean)	Belarus	CP061003	CP061003	CP061003	CP061003	CP061003
A. fabrum	C58^{T}	-	-	AE007869	AE007869	AE007869	AE007869	AE007869
	1D132	*Prunus* sp.	USA	CP033022	CP033022	CP033022	CP033022	CP033022
A. pusense	76	*Fusarium oxysporum* f. sp. *cucumerinum*	China	CP053856	CP053856	CP053856	CP053856	CP053856
	CFBP5496	-	-	CP116735	CP116735	CP116735	CP116735	CP116735
A. larrymoorei	AF3.44	*Ficus benjamina*	USA	CP072167	CP072167	CP072167	CP072167	CP072167
	CFBP5477	-	-	CP124733	CP124733	CP124733	CP124733	CP124733
	CFBP5473^{T}	-	-	CP039691	CP039691	CP039691	CP039691	CP039691
A. arsenijevicii	KFB 330^{T}	Raspberry		KP835697	JWIT01000001	KP835703	KP835709	NR_178777
A. cucumeris	O132^{T}	Cucumber hairy roots	Poland	CP080387	CP080387	CP080387	CP080387	CP080387
A. leguminum	CFBP4996	-	-	CP120211	CP120211	CP120211	CP120211	CP120211
A. salinitolerans	CFBP5507	-	-	CP109968	CP109968	CP109968	CP109968	CP109968
A. vaccinii	B7.6^{T}	Blueberry	Poland	CP054150	CP054150	CP054150	CP054150	CP054150
A. rosae	NCPPB 1650^{T}	*Rosa* hybrid cultivar	South Africa	MF447443	NXEJ01000001	NXEJ01000004	MF447448	NR_178307
A. rubi	W2/73	Raspberry	-	CP049206	CP049206	CP049206	CP049206	CP049206
A. vitis	CG412	-	-	CP055265	CP055265	CP055265	CP055265	CP055265
R. rhizogenes	LBA9402	*Rosa* sp.	-	CP044122	CP044122	CP044122	CP044122	CP044122
	CA75/95	Raspberry	-	CP049213	CP049213	CP049213	CP049213	CP049213
	A4	*Rosa* sp.	-	CP073110	CP073110	CP073110	CP073110	CP073110
R. tumorigenes	1078^{T}	*Rubus* sp.	Serbia	CP117255	CP117255	CP117255	CP117255	CP117255
	932	*Rubus* sp.	Serbia	CP117261	CP117261	CP117261	CP117261	CP117261
M. huakuii[a]	NZP2235	*Lotus japonicus*	New Zealand	CP139858	CP139858	CP139858	CP139858	CP139858

Table 1. Gene sequences of *Agrobacterium* and *Rhizobium* strains obtained from GenBank for inclusion in the multi-locus phylogenetic analysis. ^{T} type strain of a certain species; [a] Outgroup strain.

using SPAdes v. 3.13.1[36] with default settings and using the "--careful" switch. We submitted the assembly to GenBank[37], via the NCBI, under BioProject accession PRJNA1126269. The genome assembly was annotated by the NCBI's Prokaryotic Genome Annotation Pipeline (PGAP)[38] (June 24, 2024 version).

Average nucleotide identity

To calculate pairwise average nucleotide identities (ANIs) between genome assemblies, we used FastANI[39] version 1.1. The FastANI command line was: "fastANI --ql query_list.txt --rl ref_ list.txt -o all-versus-fastANI. short.out -t 6 --visualize –matrix".

Phylogenomics analysis

We generated a phylogenomic tree from genome sequence assemblies using PhaME[40]. This software can use several different tree-building software packages; here, we selected FastTree 2[41]. To identify the root of the tree, we used the genome of *Rhizobium leguminosarum* as an outgroup. We performed 1000 bootstraps. We generated a graphical depiction of the resulting phylogenetic tree using the web-based Interactive Tree of Life (iTOL)[42] version 6.9.1.

Results

Phenotypic characterization

Isolation from blueberry samples with the crown gall symptoms produced circular, convex, light blue to dark olive-green bacterial colonies on D1 medium after 10 days of growth. Inoculations on blueberry plants (cv. Duke) resulted in development of spherical, white, spongy gall-like structures at the inoculation sites after 24 days, which enlarged over the duration of seven weeks of development (Fig. 2). No symptoms were observed on the negative-control blueberry plants. Pathogenicity was not observed on tomato and sunflower seedlings during five weeks following inoculation.

Table 2 presents biochemical features for Serbian blueberry isolates and closely related *Agrobacterium* spp. for comparison[13,19,22,30,43,44]. All blueberry isolates from this study showed identical reactions. They were Gram negative, catalase positive, oxidase negative; they did not produce fluorescent pigment on King's B medium, but produced 3-ketolactose from lactose. According to results of API tests (API 50 CH) isolates were positive for

Fig. 2. Gall-like structures formed on inoculated blueberry plants (cv. Duke) using Serbian blueberry bacterial isolates.

fermentation of glycerol, d-arabinose, l-arabinose, d-ribose, d-xylose, d-adonitol, methyl-β-d-xylopyranoside, d-galactose, d-glucose, d-fructose, d-mannose, l-rhamnose, inositol, d-mannitol, d-sorbitol, aesculin ferric citrate, salicin, d-cellobiose, d-maltose, d-lactose, d-melibiose, d-sucrose, d-trehalose, d-raffinose, xylitol, d-turanose, d-lyxose, d-fucose, l-fucose, d-arabitol and l-arabitol, and negative for erythritol, l-xylose, l-sorbose, dulcitol, methyl α-d-mannopyranoside, methyl α-d-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, inulin, d-melezitose, amidon (starch), glycogen, gentiobiose, d-tagatose, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate.

Genotypic characterization

Partial gene sequences were PCR-amplified and sequenced from two of the Serbian blueberry bacterial isolates. Sequences from isolates BA1120 and BA2520 respectively were deposited in GenBank under the following accession numbers: 16 S rRNA (PQ897764 and PQ897765), *atpD* (PV010960 and PV010961), *dnaK* (PV010964 and PV010965), *glnA* (PV010962 and PV010963), and *rpoB* (PV010966 and PV010967).

The BLASTn results for five loci from seven isolates (BA1120, BA1420, BA1920, BA2520, BA2720, BA2820, and BA3320) did not consistently match any single previously-described species. These BLASTn results are presented in Table 3. The 16 S rRNA sequence was identical to GenBank accessions KM253034.1 and MH669265.1 from "*Rhizobium* sp." strains DR 7 – 06 and H90. Strain DR 7 – 06 originated from the root endophytic microbiome of an unspecified plant. Strain H90 was a putative root endophyte isolated from *Pinus* sp. in Korea[45]. The closest 16 S rRNA match from a bacterial type strain was RefSeq accession NR_117203 from *A. nepotum* strain 39/7[46], which was 99.42% identical. In contrast to the 16 S rRNA results, for genes *dnaK* and *rpoB*, the blueberry isolates showed the highest identity with *A. larrymoorei* strains CFBP5473 and AF3.44, i.e. 93.60% and 96.73%, respectively. For genes *atpD* and *glnA*, the blueberry isolates showed the highest identity with species *A. rosae* (strain NCPPB 1650) and *A. vaccinii* (strain B19.1.4), respectively.

All seven Serbian blueberry isolates shared identical sequences at the five loci. We constructed a phylogenetic tree for the seven Serbian blueberry isolates along with related strains from *Agrobacterium* and *Rhizobium* genera; the tree is presented in Fig. 3. They fell within a clade that included the type strains of species *A. rosae*, *A. rubi* and *A. vaccinii*. Interestingly, the *A. vaccinii* type strain B7.6 was also isolated from blueberry (in Poland)[13]. Consistent with previous studies, species belonging to genera *Agrobacterium* and *Rhizobium* were clearly separated in two distinct clades. Clear separation could also be observed between different species within each genus.

Three primer pairs used in PCR to detect virulence genes (*virD2*, *ipt*, *virC1-virC2*) failed to amplify fragments of expected length in blueberry isolates (224 bp, 427 bp, and 414 bp, respectively).

We sequenced the genome of strain BA1120 and *de-novo* assembled 34 contigs with an N_{50} length of 323 kb. The genome assembly is deposited in GenBank as accession GCA_040427045.1. The total length of the assembled sequence was 4,963,342 bp, which is similar to that of *A. vaccinii* B7.6, at 4,872,905 bp. The G + C content of the BA1120 genome sequence was 57.5%, the same as for *A. vaccinii* B7.6.

We found no evidence of virulence plasmids in the BA1120 genome sequence. Most of the BA1120 genome sequence (29 of 34 contigs) was alignable against the chromosome or megaplasmid of *Agrobacterium vaccinii*

Reaction	A. vacciniicorymbosi sp. nov. BA1120, BA1420, BA1920, BA2520, BA2720, BA2820, BA3320	A. vaccinii CFBP8740^T	A. rubi LMG17935^T, NCPPB1854^T, TR3^T, F266, ICPB TR2	A. rosae NCPPB1650^T, AE.1.	A. tumefaciens biovar 1 DA5	A. fabrum C58^T = ATCC33970 =LMG287
Gram reaction	-	-	-	-	-	-
catalase	+	nd	+	nd	+	+
oxidase	-	+	-	+	-	+
production of fluorescent pigment on King's B medium	-	nd	-	nd	-	-
3-ketolactose production	+	-	-	-/+	+	+
Fermentation of:						
glycerol	+	nd	+	+	+	-
erythritol	-	nd	-	nd	-	-
d-arabinose	+	nd	+	nd	+	+
l-arabinose	+	nd	+/-	+	+	+
d-ribose	+	nd	nd	nd	+	+
d-xylose	+	nd	nd	nd	+	+
l-xylose	-	nd	nd	nd	-	-
d-adonitol	+	nd	nd	-	+	+
methyl-ßd-xylopyranoside	+	nd	nd	nd	+	-
d-galactose	+	+	nd	nd	+	-
d-glucose	+	+	+/-	+	+	+
d-fructose	+	+	nd	+	+	+
d-mannose	+	+	+	nd	+	+
l-sorbose	-	nd	nd	nd	-	+
l-rhamnose	+	nd	nd	+	+	+
dulcitol	-	nd	nd	+	+	+
inositol	+	nd	-	+	+	+
d-mannitol	+	+	+	+	+	+
d-sorbitol	+	+	nd	-/+	+	+
methyl -αd-mannopyranoside	-	nd	nd	nd	-	-
methyl-αd-glucopyranoside	-	nd	nd	nd	-	-
n-acetylglucosamine	-	+	+	+	-	-
amygdalin	-	nd	nd	nd	-	-
arbutin	-	nd	nd	nd	-	-
aesculin ferric citrate	+	nd	-	nd	+	+
salicin	+	+	nd	+	-	+
d-cellobiose	+	+	nd	+	+	+
d-maltose	+	+	+	+	+	+
d-lactose (bovine origin)	+	nd	nd	nd	+	+
d-melibiose	+	+	+/-	nd	+	+
d-saccharose (d-sucrose)	+	+	+	+	+	+
d-trehalose	+	+	+	+	+	+
inulin	-	nd	nd	nd	-	-
d-melezitose	-	nd	nd	-	-	-
d-raffinose	+	nd	+	+	+	+
amidon (starch)	-	nd	-	nd	-	-
glycogen	-	nd	-	nd	-	-
xylitol	+	nd	-	nd	-	-
gentiobiose	-	nd	nd	nd	-	-
d-turanose	+	+	nd	+	+	+
d-lyxose	+	nd	nd	nd	+	+
d-tagatose	-	nd	nd	nd	+	+
d-fucose	+	+	+	+	+	-
l-fucose	+	+	+	+	+	-
d-arabitol	+	+	nd	+	+	-
l-arabitol	+	nd	nd	nd	+	+
potassium gluconate	-	nd	nd	nd	-	-
Continued						

Reaction	A. vacciniicorymbosi sp. nov. BA1120, BA1420, BA1920, BA2520, BA2720, BA2820, BA3320	A. vaccinii CFBP8740^T	A. rubi LMG17935^T, NCPPB1854^T, TR3^T, F266, ICPB TR2	A. rosae NCPPB1650^T, AE.1.	A. tumefaciens biovar 1 DA5	A. fabrum C58^T = ATCC33970 =LMG287
potassium 2-ketogluconate	-	nd	nd	nd	-	-
potassium 5-ketogluconate	-	nd	nd	nd	-	-

Table 2. Biochemical characteristics of Serbian blueberry isolates (*A. vacciniicorymbosi* sp. nov.) and related species of the genus *Agrobacterium*. * + positive; - negative; nd - no data; ^{T}- type strain. Data source: **A. vaccinii** (CFBP8740^T) - Puławska et al.[13]; **A. rubi** (LMG17935^T) - Puławska et al.[13], **A. rubi** (NCPPB1854^T) - Kuzmanović, et al.[19], **A. rubi** (TR3^T) - Mafakheri et al.[22], **A. rubi** (F266) - Alippi et al.[44], **A. rubi** (ICPB TR2) - Bouzar et al.[43]; **A. rosae** (NCPPB1650^T) - Kuzmanović et al.[19], (AE.1.) - Mafakheri et al.[22]; **A. tumefaciens biovar 1** (DA5), **A. fabrum** (C58) - Iličić et al.[30].

Gene	Highest percent identity
atpD	*Agrobacterium rosae* strain NCPPB 1650 (96.32%)
dnaK	*Agrobacterium larrymoorei* strains CFBP5473 and AF3.44 (93.60%)
glnA	*Agrobacterium vaccinii* strain B19.1.4 (93.63%)
rpoB	*Agrobacterium larrymoorei* strains CFBP5473 and AF3.44 (96.73%)
16 S rRNA	*Rhizobium sp.* DR 7 – 06 and H90 (100%)

Table 3. Preliminary identification of seven tested isolates based on the partial sequences of genes *atpD*, *dnaK*, *glnA*, *rpoB*, and 16 S rRNA according to NCBI BLASTn analysis.

B7.6[13]. The five unalignable contigs were short, ranging in size between 501 and 1322 bp, with a total length of 3537 bp, too small to represent a virulence plasmid. Virulence plasmids in *Agrobacterium* are typically around 200 kb[48] and can be as large as around 600 kb[49].

Consistent with the MLSA, phylogenetic reconstruction using genome sequences (Fig. 4) confirmed that strain B1120 falls within a clade that also contains *A. vaccinii*, *A. rubi* and *A. rosae*. The closest genome sequence was that of a strain designated as "*Rhizobium* sp. Leaf262", which was isolated from the leaf of *Arabidopsis*[47]. Despite this designation, strain Leaf262 clearly falls within the *Agrobacterium* clade rather than *Rhizobium*, but is not close to the type strain of any named species.

On the basis of genomic sequence similarities, the TYGS[50] concluded that strain BA1120 represents a potential new species, not showing close affinity with type strains of any other bacterial species. This status is supported by our calculations of genomic average nucleotide identities (ANI). The genome of strain BA1120 shared less than 86% average nucleotide identity (ANI) with genomes of species type strains (Table 4); this is well below the commonly applied threshold of 95% ANI used to delineate bacterial species.

Discussion

Over the past 20 years, several new species have been described in genus *Agrobacterium* (*larrymoorei*, *arsenijevicii*, *rosae*, "*deltaense*" (name not validly published), *salinitolerans*, *cavarae*, *leguminum*, *burrii*, *shirazense*, *cucumeris*)[16–23]. Most *Agrobacterium* species produce tumors in various plant species[12,18,44]. Nevertheless, the number of non-pathogenic species has been increasing in recent years[20]. In blueberry, strains of *Agrobacterium* (*A. tumefaciens* species complex), *A. radiobacter* (formerly *R. radiobacter*), *R. rhizogenes* and *A. rubi* were reported as causal agents of crown and cane galls[7,11,12]. The last described species, *A. vaccinii* isolated from the galls of blueberry plants in Poland, was found to be non-pathogenic to blueberry plants and sunflower seedlings[13].

In the present work, we characterized isolates obtained from crown galls on blueberry plants from the southern part of Serbia in 2020. The biochemical characteristics of the blueberry isolates were generally consistent with the profiles of other related *Agrobacterium* species, based on available data from the literature[13,16,19,22,30,44]. The blueberry isolates differed from other species in utilization of dulcitol (negative), d-tagatose (negative) and xylitol (positive). Deviations from at least one comparative *Agrobacterium* species were observed in specific reactions, including production of oxidase and 3-ketolactose, utilization of d-adonitol, methyl-ßd-xylopyranoside, d-galactose, inositol, n-acetylglucosamine, aesculin ferric citrate, l-sorbose, salicin, d- and l-fucose and d-arabitol. These phenotypic differences support the distinctiveness of the blueberry isolates as a new species.

Based on a phylogenetic tree constructed on the concatenated sequences of genes *atpD*, *dnaK*, *glnA*, *rpoB* and 16 S rRNA, the blueberry isolates were most closely related to *A. vaccinii*, isolated from blueberry in Poland[13], as well as to *A. rosae* and *A. rubi*. Based on the partial sequences of *atpD*, *recA* and *rpoB*, Puławska et al.[13] showed that *A. vaccinii* belongs to the 'rubi' sub-clade of the *Agrobacterium* genus and is most closely related to *A. rubi* and *A. bohemicum*. Therefore, the Serbian blueberry isolates should also be considered to belong to this 'rubi' group.

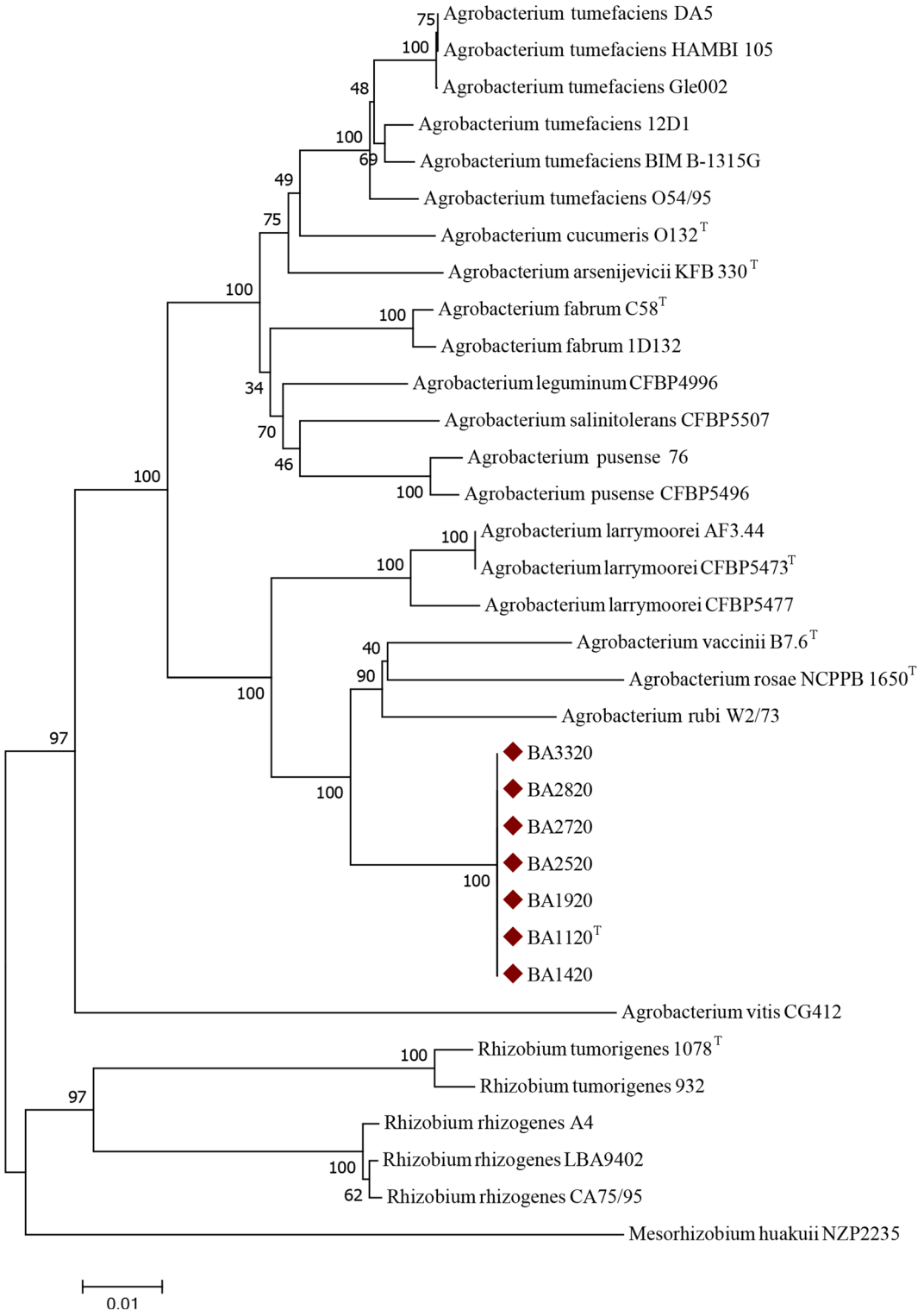

Fig. 3. Multi-locus phylogenetic analysis of the blueberry bacterial isolates. The neighbor-joining phylogenetic tree was based on the concatenated sequences of genes *atpD*, *dnaK*, *glnA*, *rpoB* and 16 S rRNA. The Serbian blueberry isolates are highlighted with red diamonds. We included sequences from 26 strains of *Agrobacterium* and *Rhizobium*, all obtained from the NCBI. *Mesorhizobium huakuii* strain NZP2235 was included as an out-group, to determine the root of the tree.

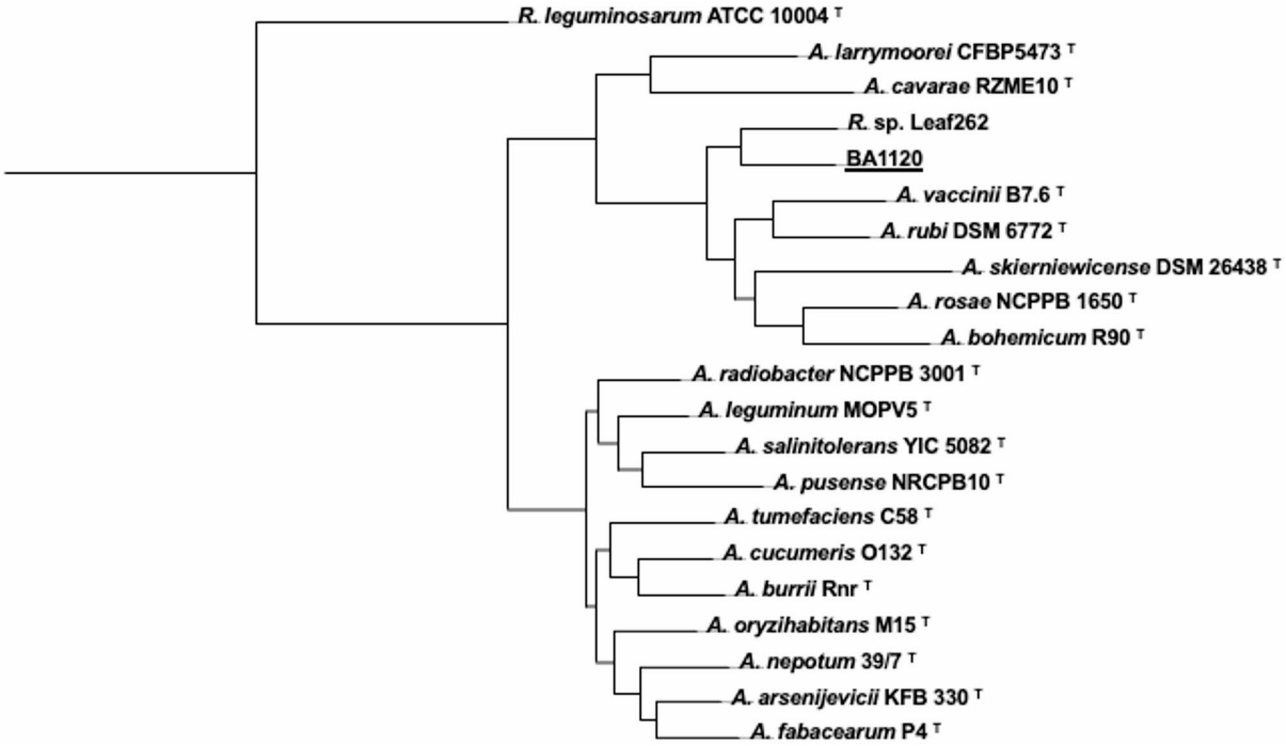

Fig. 4. Genome-based phylogeny of strain BA1120. The maximum-likelihood tree was generated using PhaME as described in the Methods section. GenBank accession numbers for the genome assemblies are provided in Table 4. We included the type strain of *R. leguminosarum* as an outgroup to determine the root of the tree.

Strain	GenBank accession	ANI (%)
R. sp. Leaf262	GCA_001422245.1	87.60
A. rosae NCPPB 1650^T	GCA_002915175.1	85.48
A. rubi DSM 6772^T	GCA_017873055.1	85.37
A. vaccinii B7.6^T	GCA_021310995.1	85.10
A. bohemicum R90^T	GCA_002896715.1	83.77
A. skierniewicense DSM 26,438^T	GCA_014196515.1	83.48
A. larrymoorei CFBP5473^T	GCA_005145045.1	82.13
A. leguminum MOPV5^T	GCA_015704895.1	80.95
A. salinitolerans YIC 5082^T	GCA_002008225.1	80.78
A. burrii Rnr^T	GCA_017313405.1	80.76
A. radiobacter NCPPB 3001^T	GCA_001541305.1	80.75
A. cavarae RZME10^T	GCA_004310285.1	80.73
A. cucumeris O132^T	GCA_030036535.1	80.70
A. tumefaciens C58^T	GCA_000092025.1	80.59
A. oryzihabitans M15^T	GCA_010669145.1	80.59
A. nepotum 39/7^T	GCA_000949865.1	80.58
A. arsenijevicii KFB 330^T	GCA_000949895.1	80.54
A. pusense NRCPB10^T	GCA_002008275.1	80.32
A. vitis NCPPB 3554^T	GCA_001541345.2	78.21
A. rhizogenes NBRC 13,257^T	GCA_000696095.1	78.19
R. leguminosarum ATCC 10,004^T	GCA_033842645.1	78.15
R. alvei TNR-22^T	GCA_030551875.1	77.56

Table 4. Average nucleotide identity (ANI) between the genome sequence of strain BA1120 and genomes of related strains. Type strains are marked with 'T'.

In pathogenicity tests, blueberry isolates produced gall-like structures at the inoculation site (crown area) on blueberry plants within 24 days post-inoculation. In contrast, inoculated tomato and sunflower seedlings showed no disease symptoms, indicating a lack of host response to the pathogen. In the study by Puławska et al.[13], *A. vaccinii* — one of the species most similar to the isolates in this study — was classified as non-pathogenic, as it showed no ability to cause gall formation, neither in sunflower seedlings nor in host blueberry plants. Our PCR assays indicated absence of three key genes commonly associated with virulence in *Agrobacterium* spp.: *virD2*, *ipt* and *virC1-virC2*. This strongly suggests that the isolates do not carry the tumor-inducing (Ti) plasmid, which is considered indispensable for the pathogenicity and gall formation in agrobacteria[51]. Further analysis of the annotated genes and sequence similarity searches in the whole-genome sequence of isolate BA1120 (GenBank: GCA_040427045.1) confirmed not only the absence of the tested virulence genes, but also the complete absence of a tumor-inducing (Ti) plasmid, further supporting the non-pathogenic nature of these blueberry isolates. Considering that, in general, *Agrobacterium* species are unable to induce typical tumor formation in plants without the presence of the tumor-inducing (Ti) plasmid — which carries the essential virulence genes and the T-DNA region responsible for transferring oncogenic sequences into the plant genome, leading to gall formation — the paradoxical occurrence of gall-like structures observed in pathogenicity tests on blueberry in this study may be attributed to the presence of unidentified genes causing this behavior not yet characterized, plant stress responses that mimic tumor symptoms, or horizontal transfer events[6,9,52].

Genome annotation of isolate BA1120 revealed the presence of three genes potentially associated with virulence. Those genes encode an AcvB/VirJ family lysyl-phosphatidylglycerol hydrolase, a YihY/BrkB family virulence factor protein, and a plant virulence effector HPE1-like domain-containing protein. These genes have also been annotated in *A. vaccinii* strain B7.6[13]. In *A. tumefaciens* strain C58, the *acvB* gene is situated on the circular chromosome directly downstream of the *lpiA* gene, which encodes a low pH-inducible protein A. The *lpiA* gene product functions as an aminoacyl-phosphatidylglycerol synthase, with a specific role in the biosynthesis of lysyl-phosphatidylglycerol (L-PG), as shown through expression in *Escherichia coli*. The *acvB* gene, in contrast, produces a periplasmic enzyme that acts as a hydrolase, degrading L-PG and thereby regulating its concentration in the bacterial membrane. When *acvB* is disrupted, L-PG accumulates excessively within the membrane, interfering with the bacterium's ability to transfer T-DNA to host plant cells, ultimately preventing tumor formation[53]. Effector proteins play a key role in bacterial virulence, and in *'Candidatus* Liberibacter solanacearum', the Sec-translocon-dependent hypothetical protein effector 1 (Lso-HPE1) has been shown to contribute to the suppression of programmed cell death in host plants[54]. This suppression is likely linked to interference with the ubiquitin-proteasome system, potentially through direct binding to the ubiquitin-like domain found in RAD23 proteins[55]. The function of the YihY/BrkB family virulence factor protein in *Agrobacterium* remains uncharacterized and has not yet been the focus of detailed research.

Given that *A. vaccinii*, a closely related species, has been described as non-pathogenic[13], the origin of the gall-like structures observed on inoculated blueberry plants remains unclear and is as an open question that warrants further investigation. Horizontal gene transfer allows *Agrobacterium* species to acquire new traits, including virulence factors, from other bacteria. This can enable strains to exhibit pathogenicity without the classic Ti plasmid, and strains could induce symptoms such as gall formation or other plant responses, potentially through the acquisition of genes related to plant manipulation, stress response, or effector proteins. This highlights the adaptability of *Agrobacterium* and its capacity to evolve new pathogenic behaviors through genetic exchange with other organisms.

Description of *Agrobacterium vacciniicorymbosi* sp. nov

Agrobacterium vacciniicorymbosi (vac.ci.ni.i.co.rym.bo′si. N.L. n. *Vaccinium corymbosum*, the highbush blueberry; N.L. gen. n. *vacciniicorymbosi*, of *Vaccinium corymbosum*, referring to the plant from which the bacterium was isolated).

Colonies of the bacterium are circular, convex, light blue to dark olive-green on D1 medium; on NA they are whitish to cream, circular. The bacterium is Gram negative, catalase positive and oxidase negative; negative production of fluorescent pigment on King's B medium and positive production of 3-ketolactose from lactose. Fermentation is positive for glycerol, d-arabinose, l-arabinose, d-ribose, d-xylose, d-adonitol, methyl-β-d-xylopyranoside, d-galactose, d-glucose, d-fructose, d-mannose, l-rhamnose, inositol, d-mannitol, d-sorbitol, aesculin ferric citrate, salicin, d-cellobiose, d-maltose, d-lactose, d-melibiose, d-sucrose, d-trehalose, d-raffinose, xylitol, d-turanose, d-lyxose, d-fucose, l-fucose, d-arabitol and l-arabitol and negative for erythritol, l-xylose, l-sorbose, dulcitol, methyl α-d-mannopyranoside, methyl α-d-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, inulin, d-melezitose, amidon (starch), glycogen, gentiobiose, d-tagatose, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate.

The draft genome size is 4.96 Mbp with a DNA G + C content of 57.5 mol%. Phylogenomic analysis based on core genes places the strain within the genus *Agrobacterium*, clustering closely with *Agrobacterium vaccinii*, *Agrobacterium rubi* and *Agrobacterium rosae*, but separated at the species level. ANI value between strain BA1120^T and its closest relative, *A. rosae*, is 85.48%, below the species delineation threshold.

The type strain is BA1120^T (NCPPB 4800 = CFBP 9290) was isolated from blueberry in Serbia in 2020.

Data availability

The genome sequence data generated during the current study are available in GenBank under accession number GCA_040427045.1 and in RefSeq under accession number GCF_040427045.1 [https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_040427045.1].The partial gene sequences 16 S rRNA, *atpD*, *dnaK*, *glnA* and *rpoB* are available in GenBank under accession numbers:- PQ897764 [https://www.ncbi.nlm.nih.gov/nuccore/PQ897764.1],- PQ897765 [https://www.ncbi.nlm.nih.gov/nuccore/PQ897765],- PV010960 [https://www.ncbi.nlm.nih.gov/nuccore/PV010960],- PV010961 [https://www.ncbi.nlm.nih.gov/nuccore/PV010961],- PV010964

[https://www.ncbi.nlm.nih.gov/nuccore/PV010964],- PV010965 [https://www.ncbi.nlm.nih.gov/nuccore/PV010965],- PV010962 [https://www.ncbi.nlm.nih.gov/nuccore/PV010962],- PV010963 [https://www.ncbi.nlm.nih.gov/nuccore/PV010963],- PV010966 [https://www.ncbi.nlm.nih.gov/nuccore/PV010966],- PV010967 [https://www.ncbi.nlm.nih.gov/nuccore/PV010967].

Received: 19 June 2025; Accepted: 3 October 2025
Published online: 10 November 2025

References

1. Milivojević, J. M., Radivojević, D. D., Maksimović, V. M. & Dragišić, M. J. J. Variation in health promoting compounds of blueberry fruit associated with different nutrient management practices in a soilless growing system. *J. Agricultural Sci.* **65**, 175–185 (2020).
2. Živković, S., Vasić, T., Jevremović, D. & Leposavić, A. Identification of mycopopulation on american highbush blueberry in Serbia. Proceedings of the XII International Scientific Agricultural Symposium Agrosym 2021, Jahorina, Bosnia and Herzegovina, October 7–10, 577–582. (2021).
3. Popović Milovanović, T. et al. Genetic diversity and virulence traits of Pseudomonas syringae pv. syringae isolated from various hosts in Serbia. *Ann. Appl. Biol.* https://doi.org/10.1111/aab.12972 (2025).
4. Hooykaas, M. J. G. & Hooykaas, P. J. J. Complete genomic sequence and phylogenomics analysis of *Agrobacterium* strain AB2/73: a new *Rhizobium* species with a unique mega-Ti plasmid. *BMC Microbiol.* **21**, 295 (2021).
5. Pan, S. Q. et al. An *Agrobacterium* virulence factor encoded by a Ti plasmid gene or a chromosomal gene is required for T-DNA transfer into plants. *Mol. Microbiol.* **17**, 259–269 (1995).
6. Dessaux, Y., Petit, A., Farrand, S. K. & Murphy, P. J. Opines and opine-like molecules involved in plant-Rhizobiaceae interactions. In *The Rhizobiaceae: Molecular Biology of Model plant-associated Bacteria* 173–197 (Springer Netherlands, 1998).
7. Millas, P. et al. First report of *Rhizobium rhizogenes* causing crown gall on blueberry and Cherry in Chile. *New. Disease Rep.***49**, 1–3, e12253. (2024).
8. Lassalle, F. et al. Genomic species are ecological species revealed by comparative genomics in *Agrobacterium tumefaciens*. *Genome Biol. Evol.* **3**, 762–781 (2011).
9. Lacroix, B. & Citovsky, V. Genetic factors governing bacterial virulence and host plant susceptibility during *Agrobacterium* infection. Adv Genet. 2022; 110, 1–29. (2022).
10. Weisberg, A. J., Wu, Y., Chang, J. H., Lai, E. M. & Kuo, C. H. Virulence and Ecology of Agrobacteria in the Context of Evolutionary Genomics. Annu Rev Phytopathol 2023, 61. (2023).
11. Alippi, A. M., Ana, C., López & Balatti, P. A. Diversity among agrobacteria isolated from diseased plants of blueberry (*Vaccinium corymbosum*) in Argentina. *Eur. J. Plant Pathol.* **134**, 415–430 (2012).
12. Kuzmanović, N. et al. A novel group of *Rhizobium tumorigenes*-like agrobacteria associated with crown gall disease of rhododendron and blueberry. *Phytopathology* **109**, 1840–1848 (2019).
13. Puławska, J., Kuzmanović, N. & Trzciński, P. *Agrobacterium vaccinii* sp. nov. Isolated from galls on blueberry plants (*Vaccinium corymbosum*). *Syst. Appl. Microbiol.* **45**, 126319 (2022).
14. Aloni, R. & Ullrich, C. I. in *In Biology of Crown Gall Tumors BT - Agrobacterium: from Biology To Biotechnology*. 565–591 (eds Tzfira, T. & Citovsky, V.) (Springer), 2008).
15. Pulawska, J. Crown gall of stone fruits and nuts, economic significance and diversity of its causal agents: tumorigenic *Agrobacterium* spp. *J. Plant. Pathol.* **92**, S87–S98 (2010).
16. Bouzar, H. & Jones, J. B. *Agrobacterium Larrymoorei* sp. nov., a pathogen isolated from aerial tumours of *Ficus Benjamina*. *Int. J. Syst. Evol. Microbiol.* **51**, 1023–1026 (2001).
17. Yan, J. et al. *Agrobacterium deltaense* sp. nov., an endophytic bacteria isolated from nodule of *Sesbania Cannabina*. *Arch. Microbiol.* **199**, 1003–1009 (2017).
18. Kuzmanović, N., Smalla, K., Gronow, S. & Puławska, J. *Rhizobium tumorigenes* sp. nov., a novel plant tumorigenic bacterium isolated from cane gall tumors on thornless blackberry. *Sci. Rep.* **8**, 9051 (2018a).
19. Kuzmanović, N., Puławska, J., Smalla, K. & Nesme, X. *Agrobacterium Rosae* sp. nov., isolated from galls on different agricultural crops. *Syst. Appl. Microbiol.* **41**, 191–197 (2018b).
20. Flores-Félix, J. D. et al. *Agrobacterium Cavarae* sp. nov., isolated from maize (*Zea Mays* L.) roots. *Int. J. Syst. Evol. MicroBiol.* **70**, 5512–5519 (2020).
21. Castellano-Hinojosa, A. et al. *Agrobacterium leguminum* sp. nov., isolated from nodules of *Phaseolus vulgaris* in Spain. *Int. J. Syst. Evol. MicroBiol.* **71**, 5120 (2021).
22. Mafakheri, H., Taghavi, S. M., Khezerpour, K., Kuzmanović, N. & Osdaghi, E. Genomic analyses of Rose crown gall-associated bacteria revealed two new *Agrobacterium species*: *Agrobacterium burrii* sp. nov. And *Agrobacterium Shirazense* sp. nov. *Phytopathology* **112**, 1208–1213 (2022).
23. Warabieda, M., Kuzmanović, N., Trzciński, P. & Puławska, J. *Agrobacterium Cucumeris* sp. nov. Isolated from crazy roots on cucumber (*Cucumis sativus*). *Syst. Appl. Microbiol.* **46**, 126402 (2023).
24. Kado, C. I. & Heskett, M. G. *Selective Media for Isolation of Agrobacterium, Corynebacterium, Erwinia, Pseudomonas and Xanthomonas*60969–976 (Phytopathology, 1970).
25. Puławska, J., Willems, A. & Sobiczewski, P. Rapid and specific identification of four *Agrobacterium* species and Biovars using multiplex PCR. *Syst. Appl. Microbiol.* **29**, 470–479 (2006).
26. Lamovšek, J., Zidarič, I., Pleško, I. M., Gregor, U. R. E. K. & Trdan, S. Comparative study of diagnostic methods used for monitoring of common grape vine (*Vitis vinifera* L.) crown gall (*Agrobacterium vitis* Ophel & Kerr) in Slovenia. *Acta Agriculturae Slov.* **103**, 313–321 (2015).
27. Schaad, N. W., Jones, J. B. & Chun, W. *Laboratory Guide for Identification of Plant Pathogenic Bacteria* (The American Phytopathological Society, 2001).
28. Aujoulat, F., Jumas-Bilak, E., Masnou, A., Sallé, F., Faure, D., Segonds, C., … Teyssier,C. (2011). Multilocus sequence-based analysis delineates a clonal population of Agrobacterium (Rhizobium) radiobacter (Agrobacterium tumefaciens) of human origin. Journal of Bacteriology, 193, 2608–2618.
29. Caccamo, D., Di Cello, F., Fani, R., Gugliandolo, C. & Maugeri, T. L. Polyphasic approach to the characterisation of marine luminous bacteria. *Res. Microbiol.* **150**, 221–230 (1999).
30. Iličić, R. et al. In-Depth characterization of crown gall disease of tobacco in Serbia. *Agronomy* **14**, 851 (2024).
31. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**, 111–120 (1980).
32. Haas, J. H., Moore, L. W. & Ream, W. Universal PCR primers for detection of phytopathogenic *Agrobacterium* strains. *Appl. Environ. Microbiol.* **61**, 2879–2884 (1995).
33. Suzaki, K., Yoshida, K. & Sawada, H. Detection of tumorigenic *Agrobacterium* strains from infected Apple saplings by colony PCR with improved PCR primers. *J. Gen. Plant. Pathol.* **70**, 342–347 (2004).
34. Kuzmanović, N., Ivanović, M., Ćalić, A., Gašić, K. & Obradović, A. Differentiation of phytopathogenic *Agrobacterium* spp. *Pesticides Phytomedicine*. **26**, 245–253 (2011).

35. J Krueger, F. et al. TrimGalore: v0.6.10. *Zenodo* https://doi.org/10.5281/zenodo.7598955 (2023).
36. Prjibelski, A., Antipov, D., Meleshko, D., Lapidus, A. & Korobeynikov, A. Using spades *De Novo* assembler. *Curr. Protocols Bioinf.* **70**, e102 (2020).
37. Sayers, E. W. et al. *GenBank Nucleic Acids Res.*, **47**, D94–D99. (2019).
38. Tatusova, T. et al. NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res.* **44**, 6614–6624 (2016).
39. Jain, C., Rodriguez-R, L. M., Phillippy, A. M., Konstantinidis, K. T. & Aluru, S. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat. Commun.* **9**, 5114 (2018).
40. Shakya, M. et al. Standardized phylogenetic and molecular evolutionary analysis applied to species across the microbial tree of life. *Sci. Rep.* **10**, 1723 (2020).
41. Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree 2–approximately maximum-likelihood trees for large alignments. *PloS One*. **5**, e9490 (2010).
42. Letunic, I. & Bork, P. Interactive tree of life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* **49**, W293–W296 (2021).
43. Bouzar, H., Jones, J. B. & Hodge, N. C. Differential characterization of *Agrobacterium* species using carbon-source utilization patterns and fatty acid profiles. *Phytopathology* **83**, 733–739 (1993).
44. Alippi, A. M., López, A. C. & Balatti, P. A. First report of Agrobacterium Rubi and A. rhizogenes causing crown and root gall and hairy root on blueberry in Argentina. *Plant Dis.* **94** (8), 1064–1064 (2010).
45. Liu, Y. et al. Distribution and diversity of bacterial endophytes from four *Pinus* species and their efficacy as biocontrol agents for devastating pine wood nematodes. *Sci. Rep.* **9**, 12461 (2019).
46. Puławska, J., Willems, A., De Meyer, S. E. & Süle, S. *Rhizobium nepotum* sp. nov. Isolated from tumors on different plant species. *Syst. Appl. Microbiol.* **35**, 215–220 (2012).
47. Bai, Y. et al. Functional overlap of the *Arabidopsis* leaf and root microbiota. *Nature* **528**, 364–369 (2015).
48. Gordon, J. E. & Christie, P. J. The *Agrobacterium* Ti plasmids. *Microbiol. Spectr.* **2** (6). https://doi.org/10.1128/microbiolspec.plas-0010-2013 (2014).
49. Hooykaas, P. J. The Ti plasmid, driver of *Agrobacterium* pathogenesis. *Phytopathology* **113** (4), 594–604 (2023).
50. Meier-Kolthoff, J. P. & Göker, M. TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. *Nat. Commun.* **10**, 2182 (2019).
51. Kuzmanović, N. et al. *Diversity And Evolutionary History of Ti Plasmids of Tumorigenes Clade of Rhizobium spp. And their Differentiation from Other Ti And Ri Plasmids*15p.evad133 (Genome Biology and Evolution, 2023).
52. Schierstaedt, J. et al. Role of plasmids in plant-bacteria interactions. *Curr. Issues. Mol. Biol.* **30**, 17–38 (2019).
53. Groenewold, M. K. et al. Virulence of *Agrobacterium tumefaciens* requires lipid homeostasis mediated by the lysyl-phosphatidylglycerol hydrolase AcvB. *Mol. Microbiol.* **111**, 269–286 (2019).
54. Levy, J. G. et al. Lso-HPE1, an effector of '*Candidatus* liberibacter solanacearum', can repress plant immune response. *Phytopathology* **110**, 648–655 (2020).
55. Kan, C. C. et al. HPE1, an effector from zebra chip pathogen interacts with tomato proteins and perturbs ubiquitinated protein accumulation. *Int. J. Mol. Sci.* **22**, 9003 (2021).

Author contributions
R.I. and T.P.M. conceived and designed the experiments, performed experimental work, and wrote the original draft. D.J.S., A.J., G.B. and F.B. performed experimental work and participated in writing the original draft. All authors reviewed and approved the final manuscript.

Funding
This work was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia [contract numbers 451-03-136/2025-03/200117, 451-03-136/2025-03/200053, 451-03-136/2025-03/200010]. D.J.S. was supported by UKRI grant BB/W018853/1.

Declarations

Competing interests
The authors declare no competing interests.

Consent for publication
All authors have reviewed the manuscript and approved the final version submitted to the journal.

Additional information
Correspondence and requests for materials should be addressed to D.J.S. or T.P.M.