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Occurrence and Identification of *Pectobacterium carotovorum* subsp. *brasiliensis* and *Dickeya dianthicola* Causing Blackleg in Some Potato Fields in Serbia

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Abstract

Blackleg outbreaks were noticed on three fields (about 100 ha total) in 2 consecutive years (2018, 2019) in one of the main potato growing areas in Serbia (Bačka region, Vojvodina). The percentage of infected plants reached 40 to 70%, with 10.5 to 44.7% yield reductions. From the three fields, out of 90 samples *Pectobacterium carotovorum* subsp. *brasiliensis* was most frequently identified and diagnosed as causal agent of potato blackleg in Serbia for the first time (29 isolates). *Dickeya dianthicola* was a less frequently causative bacterium, which was also noticed for the first time (nine isolates). A total of 38 isolates were characterized based on their phenotypic and genetic features, including a pathogenicity test on potato. The repetitive element PCR (rep-PCR) using BOX, REP, and ERIC primer pairs differentiated five genetic profiles among 38 tested isolates. Multilocus sequence analysis (MLSA) of four housekeeping genes, *acnA*, *gapA*, *icdA*, and *mdh*, revealed the presence of three so far unknown *P. c.* subsp. *brasiliensis* multilocus genotypes and confirmed clustering into two main genetic clades as determined in other studies. MLSA also revealed the presence of a new genotype of *D. dianthicola* in Serbia.

Keywords: characterization, *Dickeya*, disease development and spread, epidemiology, MLSA, pathogen detection, potato, *Pectobacterium*, prokaryotes, yield loss and economic impacts

Potato (*Solanum tuberosum* L.) is one of the major cultivated world crops, with a production rate of 368 million tons per year (FAO 2018). Deviation in potato yield significantly depends on the presence of potato pests and diseases, climate, and agricultural standards (Oerke 2006). One of the most important and widely distributed diseases is potato blackleg, caused by plant pathogenic pectolytic bacteria belonging to the genera *Pectobacterium* and *Dickeya* (formerly *Erwinia*) (Charkowski et al. 2020; Czajkowski et al. 2015). *Dickeya* spp. cause losses of ≤25% (Tsror et al. 2009). In temperate zones, *Pectobacterium atrosepticum* is the primary causative agent of blackleg, whereas *Pectobacterium carotovorum* subsp. *carotovorum* is less significant, and only virulent strains of this bacterium can cause true blackleg symptoms under favorable conditions (De Haan et al. 2008). *P. carotovorum* subsp. *brasiliensis*, as well as *Dickeya* spp., occur mainly in regions with warm climate or in warm growing seasons in temperate climates (Duarte et al. 2004; Nasaruddin et al. 2019; Oulghazi et al. 2017; Toth et al. 2011; van der Merwe et al. 2010; van der Wolf et al. 2017; Wright et al. 2018).

Potato blackleg caused by the *P. c.* subsp. *brasiliensis* has been reported worldwide (Duarte et al. 2004; Ma et al. 2007, 2018; van der Merwe et al. 2010). *Dickeya* sp. (formerly *Erwinia chrysanthemi*) causes diseases in a wide range of economically important crops, including potato (Degefu et al. 2013; Toth et al. 2011). *Pectobacterium* and *Dickeya* species are regulated nonquarantine pests for potato seed and potato consumption in the European Union (Commission Implementing Regulation [EU] 2019/2072).

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The development of potato blackleg disease depends on inoculum concentration in seed tubers and volunteers, susceptibility of the potato cultivar, environmental conditions, and soil moisture content (Pérombelon 2002). The main symptom in aboveground plant parts is black discoloration at the base of potato stems, in the beginning often appearing only on one stem per plant (Ma et al. 2018; van der Merwe et al. 2010). The pathogen starts to develop symptoms in infected mother tubers at various times during the vegetative season and spreads through the vascular system into the stem, followed by stem tissue maceration and pith necrosis. Wilting first appears on top of leaves, which roll and become necrotic on the margins, then the wilting progresses to lower leaves. Infected stems are soft and slimy in wet conditions, and in drought lesions are dry and brittle. Under favorable environmental conditions, the disease leads to plant decay (De Boer et al. 2012; Pérombelon 2002; van der Merwe et al. 2010). One major source of inoculum for blackleg infection is (latently) infected potato seed tubers (Czajkowski et al. 2009; Toth et al. 2011; van der Merwe et al. 2010).

In Serbia, *P. atrosepticum* and *P. c.* subsp. *carotovorum* were reported as potato blackleg pathogens in the 1990s (Arsenijević et al. 1994; Obradović 1996), and since that time the disease is not known to have occurred. Recently, potato blackleg outbreaks (in cultivar 'Lady Claire') were observed in 2 consecutive years (2018 and 2019) in three potato fields in the Bačka region, one of the major potato production areas in Serbia. The field area covered by the disease was >100 ha. Therefore, the main aim of the present study was to determine and characterize the causal agents of the recent potato blackleg outbreak in Serbia by using conventional bacteriological methods and molecular characterization tools as an aid to efficiently prevent and control the disease in the future.

Materials and Methods

Potato field monitoring. In 2018 an outbreak of potato blackleg was recorded in one field coded as T-N1/2, with 58 ha of plot size (GPS 45°21′05.0" N, 19°22′47.8" E), located in Obrovac. In 2019 the disease appeared in two fields coded as T-25, with 30 ha of plot size (GPS 45°37′11.7" N, 19°52′06.3" E) and T-28, 14 ha of plot size (GPS 45°38′85.6" N, 19°52′65.5" E), located in Maglić and Kulpin, respectively (Table 1). All fields were in the Bačka region (Vojvodina), the main potato growing area in Serbia. In all monitored fields,

the potato cultivar was 'Lady Claire'. The previous crops were seed corn in T-N1/2, corn in T-25, and wheat in T-28. The type of soil in this region is chernozem, and a linear move sprinkler system was used for irrigation. Potato was planted in the middle of April 2018 and beginning of May 2019. Monitoring, visual inspection, and disease development assessment were performed biweekly during the potato vegetation period from the beginning of June onward. The percentages of infection in the fields were determined three times during vegetative seasons by counting diseased plants (i.e., 100 plants from 10 diagonally selected points). From each point (within a 3-m radius) 10 plants were rated. Two diagonals were chosen, from the bottom left to upper right corner, and vice versa, because the fields were rectangular. The distance between points was calculated and measured in the field with a measuring rope. These points were marked by reflective field flags and used when the counting was repeated and for sampling. Weather conditions (temperature and rainfall) were obtained from the nearest meteorological station (https://web.archive.org/web/20200215225052/http://www.hidmet.gov.rs/podaci/agro/godina.pdf) for the locality of Novi Sad. Rainfall was measured in the fields under observation with a rain gauge. For both years, irrigation was reduced (for three or four applications with about 70 mm less water) compared with that of healthy potato crops. Commonly used pesticide treatments were performed in the observed fields. Potato was harvested with a potato harvester in early September in dry and warm weather.

Assessment of yield loss. To determine the impact of the disease on yield, potato mass was weighed and yield was expressed in tons per hectare for each year and field. The yields obtained from the diseased crops in 2018 and 2019 were compared separately, with the average yield obtained from potato crops (cultivar 'Lady Claire') in a 7-year period (2011 to 2017) in the Bačka region (Ž. Bijelić, *personal communication*). Yield losses of the diseased crops were calculated according to the following formula:

$$\text{Yield loss } (\%) = \frac{(\text{Ya} - \text{Yd})}{\text{Ya}} \times 100,$$

where Ya is the average yield achieved in a 7-year period (Bačka) and Yd is the yield determined in the diseased field.

Sample collection and bacterial isolation. In June, potato plants with characteristic blackleg symptoms, such as stem necrotic lesions, blackness at the stem base, wilting, and rotting of tubers, were collected from 10 different points in each of the three observed fields, for a total of 90 plants. The samples were first washed in tap water and dried on filter paper at room temperature. Plant segments were isolated, taken from the margins of healthy and diseased tissue, kept in a sterile phosphate buffer for 30 to 60 min, and then plated onto crystal violet pectate (CVP) media (Hélias et al. 2011). Thereafter, Petri dishes were incubated at 26°C for 48 h. The bacterial colonies

Table 1. Serbian potato *Pectobacterium carotovorum* subsp. *brasiliensis* and *Dickeya dianthicola* isolates used in the present study, locality, year of isolation, organ, DNA fingerprinting group affiliation, and GenBank accession number

Isolate code[a]	Year of isolation	Locality	Organ	DNA fingerprinting group			Accession number			
				BOX	ERIC	REP	*acnA*	*gapA*	*icdA*	*mdh*
Dd31[a,b]	2018	Obrovac	Stem	III	III	III	MK604559	MK604561	MK604563	MK604569
Dd32[a]	2018	Obrovac	Stem	III	III	III	–	–	–	–
Dd35	2018	Obrovac	Stem	III	III	III	–	–	–	–
Dd37	2018	Obrovac	Stem	III	III	III	–	–	–	–
Dd41	2018	Obrovac	Stem	III	III	III	–	–	–	–
Dd42[a]	2018	Obrovac	Stem	III	III	III	–	–	–	–
Dd44[a,b]	2018	Obrovac	Stem	III	III	III	MK604560	MK604562	MK604564	MK604570
Dd46[a]	2018	Obrovac	Stem	III	III	III	–	–	–	–
Dd47	2018	Obrovac	Stem	III	III	III	–	–	–	–
Pcb33[a,b]	2018	Obrovac	Stem	II	II	II	MK604547	MK604549	MK604551	MK604557
Pcb34[a]	2018	Obrovac	Stem	II	II	II	–	–	–	–
Pcb38	2018	Obrovac	Stem	II	II	II	–	–	–	–
Pcb39	2018	Obrovac	Stem	II	II	II	–	–	–	–
Pcb61	2018	Obrovac	Stem	II	II	II	–	–	–	–
Pcb62[a,b]	2018	Obrovac	Stem	II	II	II	MK604548	MK604550	MK604552	MK604558
Pcb64[a]	2018	Obrovac	Stem	II	II	II	–	–	–	–
Pcb67[a]	2018	Obrovac	Stem	II	II	II	–	–	–	–
Pcb2531	2019	Maglić	Stem	I	I	I	–	–	–	–
Pcb2538	2019	Maglić	Stem	I	I	I	–	–	–	–
Pcb2544[a,b]	2019	Maglić	Stem	I	I	I	MT134020	MT134026	MT134032	MT134038
Pcb2549[a]	2019	Maglić	Stem	I	I	I	–	–	–	–
Pcb2562[a,b]	2019	Maglić	Stem	I	I	I	MT134019	MT134025	MT134031	MT134037
Pcb2563[a]	2019	Maglić	Stem	I	I	I	–	–	–	–
Pcb2568[a]	2019	Maglić	Stem	I	I	I	–	–	–	–
Pcb2811[a,b]	2019	Kulpin	Tuber	IV	IV	IV	MT134018	MT134024	MT134030	MT134036
Pcb2812[a]	2019	Kulpin	Tuber	IV	IV	IV	–	–	–	–
Pcb2813[a]	2019	Kulpin	Tuber	IV	IV	IV	–	–	–	–
Pcb2815[a]	2019	Kulpin	Tuber	IV	IV	IV	–	–	–	–
Pcb2817[a,b]	2019	Kulpin	Tuber	IV	IV	IV	MT134017	MT134023	MT134029	MT134035
Pcb2819	2019	Kulpin	Tuber	IV	IV	IV	–	–	–	–
Pcb2833[a]	2019	Kulpin	Stem	V	V	V	–	–	–	–
Pcb2838[a]	2019	Kulpin	Stem	V	V	V	–	–	–	–
Pcb2839	2019	Kulpin	Stem	V	V	V	–	–	–	–
Pcb2841	2019	Kulpin	Stem	V	V	V	–	–	–	–
Pcb2842[a,b]	2019	Kulpin	Stem	V	V	V	MT134016	MT134022	MT134028	MT134034
Pcb2844[a]	2019	Kulpin	Stem	V	V	V	–	–	–	–
Pcb2847	2019	Kulpin	Stem	V	V	V	–	–	–	–
Pcb2861[a,b]	2019	Kulpin	Stem	V	V	V	MT134015	MT134021	MT134027	MT134033

[a] Isolates used in multilocus sequence analysis.
[b] Isolates deposited in National Center for Biotechnology Information.

that formed characteristic cavities on CVP were transferred onto nutrient agar (NA) and purified. A total of 38 isolates were selected for further study (Table 1). Pure cultures were stored in Luria–Bertani broth supplemented with 20% (vol/vol) of sterile glycerol at −20°C for short-term storage.

The bacterial isolates were checked for their pectolytic activity on surface-sterilized healthy potato tuber slices cultivar 'Lady Claire' (Schaad et al. 2001). Holes were bored in the center of slices and inoculated with a loopful of fresh bacterial culture (24 h). The tuber slices were incubated in Petri dishes at room temperature under high humidity and checked for the presence of macerated tissue over the next 48 h.

Preliminary identification. Polymerase chain reaction (PCR) was used for rapid identification of all isolates with the following specific primer sets for *Pectobacterium* spp.: for *P. c.* subsp. *carotovorum*, primer set F0145/E2477 (Kettani-Halabi et al. 2013); for *P. c.* subsp. *brasiliensis*, BR1f/L1r (Duarte et al. 2004); for *P. atrosepticum*, ECA1f/ECA2r (De Boer and Ward 1995); and for *Dickeya* spp., ADE1/ADE2 (Nassar et al. 1996) (Table 2).

As control strains, *P. c.* subsp. *carotovorum* strain Pcc10 (Institute for Plant Protection and Environment, Serbia) and *Dickeya solani* strain MK10 (SASA, Scotland; Toth et al. 2014) were used for all comparisons in the study.

DNA extraction was performed from a full loop (HiMedia, Nichrome Loop-D-2 diameter: 2 mm, double wound, calibrated to 0.005 ml) of bacterial isolates grown for 24 h on NA, suspended in test tubes containing 500 µl of sterile distilled water (SDW). Suspensions were heated for 10 min at 95°C in a water bath, cooled on ice, and centrifuged for 5 min at 7,600 *g*. Supernatants were used for PCR amplification.

A total of 25 µl of PCR mix contained 2.5 µl of 10 × KAPA Taq buffer with 1.5 mM of $MgCl_2$, 0.5 µl of deoxynucleoside triphosphate (10 mM), 1 µl of each of the primers (10 µM), 0.25 µl 5U/µl Taq DNA polymerase, 18.75 µl of UltraPure DNase/RNase-free water (Gibco, UK), and 1 µl DNA. PCR was carried out via the amplification programs listed in Table 2. All amplified PCR products were electrophoresed in a 1% agarose gel stained with ethidium bromide and checked for the presence of respective specific bands (666 bp for F0145/E2477, 322 bp for BR1f/L1r, 690 bp for ECA1f/ECA2r, and 420 bp for ADE1/ADE2 primer sets) under ultraviolet light.

Phenotypic features. Thirty-eight isolates were characterized via the following biochemical tests: gram reaction in 3% KOH; oxidative–fermentative metabolism of glucose; indole production; nitrate reduction; hydrogen sulfide (H_2S) production from peptone; the presence of arginine dihydrolase; gelatin liquefaction; aesculin, starch, and casein hydrolysis; tyrosinase activity; utilization of (D+) tartrate, lactic, tartaric, aspartic acids; L-leucine utilization; growth at 37°C and 4°C; salt tolerance (NaCl, 5%); and acid production from D-glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, D-sucrose, D-melibiose, amygdalin, and L-arabinose (Schaad et al. 2001).

Pathogenicity of 38 isolates was checked on young potato plants (cultivar 'Lady Claire'), three plants per isolate. Potato tubers were planted in the pots with sterile substrate and placed in a greenhouse at 22 to 25°C under natural light, with regular watering. After 5 weeks of growing, the plants were in the phase of the third and the fourth true leaf stage on the main stem defined by Biologische Bundesanstalt, Bundessortenamt, and Chemical Industry (BBCH 103–104). Isolates were grown on NA for 48 h and suspended in SDW. The pathogenicity test was performed by injection of bacterial suspension (adjusted to 10^7 to 10^8 CFU/ml) with a sterile hypodermic syringe and needle of 23G × 1 into the stem at the third node from the stem base, and soil inoculation with 20 ml of bacterial suspension given once (adjusted to 10^9 CFU/ml) to each pot in four holes (Tsror et al. 2009). Experiments were performed in three replicates. As a positive control treatment, *P. c.* subsp. *carotovorum* strain Pcc10 and *D. solani* strain MK10 were used. SDW served as a negative control treatment. Inoculated plants were kept in plastic boxes under controlled conditions at 25°C under high humidity (70 to 80%) and 16 h/8 h (day/night) photoperiod. Symptom development was observed visually on a daily basis.

Reisolations were performed on CVP, and reisolates were purified on NA. The identification of reisolates, to be the same as the original ones, was performed via PCR with primer pairs BR1f/L1r and ADE1/ADE2.

Table 2. Primers used in this study and their corresponding profiles

Primer name	Primer sequence	Fragment length (bp)	Annealing temperature (°C)	Reference
Specific primer sets for detection of *Pectobacterium* spp. *and Dickeya* spp.				
ADE1	5′-GATCAGAAAGCCCGCAGCCAGAT-3′	420	72	Nassar et al. (1996)
ADE2	5′-CTGTGGCCGATCAGGATGGTTTTGTCGTGC-3′			
F0145	5′-TACCCTGCAGATGAAATTATTGATTGTTGAAGAC-3′	666	55	Kettani-Halabi et al. (2013)
E2477	5′-TACCAAGCTTTGGTTGTTCCCCTTTGGTCA-3′			
ECA1f	5′-CGGCATCATAAAAACACG-3′	690	62	De Boer and Ward (1995)
ECA2r	5′-GCACACTTCATCCAGCGA-3′			
Br1f	5′-GCGTGCCGGGTTTATGACCT-3′	322	62	Duarte et al. (2004)
L1r	5′-CAAGGCATCCACCGT-3′			
Repetitive PCR fingerprinting primer sets				
BOXA1R	5′-CTACGGCAAGGCGACGCTGACG-3′	–	52	Versalovic et al. (1994)
ERIC1R	5′-ATGTAAGCTCCTGGGGATTCAC-3′	–	52	
ERIC2	5′-AAGTAAGTGACTGGGGTGAGCG-3′			
REP1R-I	5′-IIIICGICGICATCIGGC-3′	–	40	
REP2-I	5′-ICGICTTATCIGGCCTAC-3′			
Multilocus sequence analysis primer sets				
acnA3F	5′-CMAGRGTRTTRATGCARGAYTTTAC-3′	300	52	Ma et al. (2007)
acnA3R	5′-GATCATGGTGGTRTGSGARTCVGT-3′			
icdA400F	5′-GGTGGTATCCGTTCTCTGAACG-3′	520	52	
icdA977R	5′-TAGTCGCCGTTCAGGTTCATACA-3′			
gapA326F	5′-ATCTTCCTGACCGACGAAACTGC-3′	450	52	
gapA845R	5′-ACGTCATCTTCGGTGTAACCCAG-3′			
mdh2	5′-GCGCGTAAGCCGGGTATGGA-3′	500	52	Moleleki et al. (2013)
mdh4	5′-CGCGGCAGCCTGGCCCATAG-3′			

Genotypic features. *Total DNA extraction.* Total DNA from 38 isolates was extracted via a modified cetyl trimethylammonium bromide method described by Ausubel et al. (2003). Pure bacterial colonies (grown on NA for 24 h at 26°C) of the isolates were suspended in 500 µl (approximately 10^6 CFU/ml) of SDW and centrifuged at 10,000 *g* for 10 min. The obtained pellet was resuspended in TE buffer (50 mM of Tris, pH 8.0, 1 mM of EDTA) (567 µl), 10% (wt/vol) of sodium dodecyl sulfate (30 µl) with 20 mg/ml of proteinase K (3 µl). The mix was incubated for 30 min at 37°C, and after 100 µl of 5 M NaCl was added. The next step consisted of addition of 300 µl of 3% hexadecyltrimethylammonium bromide (cetyl trimethylammonium bromide, pH 8.0) and incubation for 20 min at 65°C. For additional DNA purification, 750 µl of chloroform was added and centrifuged at 10,000 *g* for 10 min. The upper phase was collected, transferred to new tubes, mixed with ice-cold isopropanol (750 µl), and centrifuged at 10,000 *g* for 15 min. The pellet was washed with 1 ml of 96% ice-cold ethanol, centrifuged at 10,000 *g* for 10 min, and dried at room temperature for 30 min. The obtained DNA was dissolved in 50 µl of TE buffer and stored at −20°C.

DNA fingerprinting. The rep-PCR fingerprinting was performed with 38 isolates and three oligonucleotide primers: BOX (BOXA1R), ERIC (ERIC1R/ERIC2), and REP (REP1R-I/REP2-I) (Versalovic et al. 1994). The concentrations of DNA and their purity were measured by fluorometric quantitation and equalized. PCR reactions were performed by Versalovic et al. (1994) (Table 2). A total volume of 25 µl of mix contained the following: 2.5 µl of 10 × KAPA Taq buffer B; 0.5 µl of deoxynucleoside triphosphate mixture (10 mM) (KAPA Biosystems, USA), 2 µl (10 µM) of each forward and reverse primer, 0.2 µl of (5 U/µl) KAPA Taq polymerase, 16.8 µl of UltraPure DNase/RNase-free water (Gibco, UK), and 1 µl of total sample DNA. After PCR amplification, DNA products were electrophoresed in a 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml). To calculate the differences in band positions and the level of genetic similarity between the obtained profiles, and unweighted pair group method with arithmetic mean (UPGMA) trees were constructed in PyElph version 1.4 software (Pavel and Vasile 2012).

Multilocus sequence analysis. Based on the results of rep-PCR performed with 38 isolates, a total of 25 isolates (five from each representative pattern group) were chosen for multilocus sequence analysis (MLSA) with four housekeeping genes: *acnA* (aconitate hydratase 1), *gapA* (glyceraldehyde-3-phosphate dehydrogenase A), *icdA* (isocitrate dehydrogenase, specific for NADP+), and *mdh* (malate dehydrogenase) (Ma et al. 2007; Moleleki et al. 2013; Table 2). The PCR amplifications were performed with 12.5 µl of DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), 1 µl of each of the used primers (10 µM), 9.5 µl of ultrapure DNase/RNase-free water (Gibco, UK), and 1 µl of sample total DNA, to obtain the final reaction volume of 25 µl. QIAquick/250 Gel Extraction and Purification Kits (QIAGEN GmbH, Hilden, Germany) were used for purification of the obtained PCR products. After purification, PCR products were sequenced by the Macrogen sequencing service (Amsterdam, the Netherlands). The obtained nucleotide sequences were checked for their quality in FinchTV version 1.4.0 (Geospiza, https://finchtv.software.informer.com/1.4/) and aligned by the ClustalW program implemented in BioEdit (version 7.0.5). The sequences were trimmed to 252 nt (*acnA*), 382 nt (*gapA*), 451 nt (*icdA*), and 283 nt (*mdh*) for phylogenetic analysis. They were simultaneously compared with the available *Pectobacterium* spp. and *Dickeya* spp. sequences retrieved from the National Center for Biotechnology Information (NCBI) database (Table 3) with the nucleotide BLAST*n* search tool. Representative isolates of all detected haplotypes were deposited into the NCBI GenBank (Table 1).

The evaluation of the phylogenetic relations and genetic divergence between the bacterial isolates were assessed by treating analyzed strains as single multilocus genotypes. The concatenated sequences used in further MLSA analysis consisted of four housekeeping genes (*acnA*, *gapA*, *icdA*, and *mdh*) and were 1,368 bp long (Table 3). The best evolutionary model of nucleotide substitution was determined in jModelTest version 2.0.2 (Posada 2008) according to the Bayesian information criterion. The most appropriate substitution model suggested by the jModelTest was further selected in maximum likelihood phylogeny reconstructed in MEGA6 by applying 1,000 bootstrap replications (Tamura et al. 2013). The sequences of strain *Yersinia pestis* Yp91001 were used for tree rooting in all phylogenetic analyses, based on Ma et al. (2007). To determine evolutionary relatedness and genealogy of *P. c.* subsp. *brasiliensis* and *D. dianthicola*, two separate median-joining networks were calculated in Network version 5.0.1.1 (www.fluxus-engineering.com) (Bandelt et al. 1999), keeping parameter ε at its 0 value and applying maximum parsimony postprocessing in order to obtain a network containing all the shortest trees. One representative isolate of each of the detected genotypes of *P. c.* subsp. *brasiliensis* and *D. dianthicola* in Serbia, as well as the type strains from the other species of the genera *Pectobacterium* and *Dickeya*, were used in the analysis, all selected by the availability of the appropriate genes deposited in the NCBI database. The same *P. c.* subsp. *brasiliensis* and *D. dianthicola* strains were used in phylogenetic and network analysis in order to compare obtained results. Average genetic distances based on pairwise analysis (uncorrected p-distances) among *Pectobacterium* spp. and *Dickeya* spp. multilocus strains were estimated in MEGA6 software (Tamura et al. 2013).

Results

Potato field monitoring and assessment of yield loss. Blackleg symptoms occurring in potato crops in 2018 and 2019 (Fig. 1) had the same dynamics of disease progress. The first symptom appeared in the form of sporadic wilting of the youngest stem segment (top) observed in the middle of May 2018 or 2 weeks later in 2019. At the beginning of June, potato crops were in the phase of intensive growth (BBCH 209–301), and disease incidence was 5 to 10%, uniformly distributed in the fields. In the phase of tuber formation (BBCH 407–408), wilting was more widespread and recorded on one or two stems among an average of six stems per plant. The youngest leaves on plants rolled upwards, wilted, and necrosis of leaves started to progress from the top part and leaf edges. At the same time, a small number of plants developed typical symptoms of blackleg on stems: a light to dark brown discoloration of tissue at the stem base. In some cases, necrotic lesions were formed externally along the whole stem (2 to 3 cm in size), and internally necrosis of the vascular system was visible. When the diseased plants were removed from the soil, rotting of mother tubers was observed. At the end of June, wilting of lower leaves was noticed, followed by an expanded infection to previously healthy stems on the same plants. Stems with typical blackleg symptoms decayed completely. Foci of diseased plants were noticed in the fields, and disease incidence in all three fields was about 30%. In July (from the middle onwards), when the potato crops entered the end of the vegetative period (BBCH 905–907), the number and diameter of foci with completely dried and decayed plants increased. The disease incidence reached 45% in 2018 and 40 and 70% for T-25 and T-28, respectively, in 2019. After the potato harvest, rotten progeny tubers were found.

The achieved yield in the observed field was 29 ton/ha in 2018; in 2019 it was 34 ton/ha and 21 ton/ha for T-25 and T-28, respectively. In the Bačka region, the average 7-year yield data of cultivar 'Lady Claire' was 38 ton/ha. Yield was reduced by 23.7% in 2018 and in 2019 by 10.5% and 44.7% in the T-25 and T-28 fields, respectively.

During the vegetative period of potato in 2018 and 2019, rain occurred in short intervals and abundantly during the whole growing season (in 2018, locality Obrovac, 132 mm in May, 163 mm in June, and 71 mm in July; in 2019 for localities Kulpin and Maglić, 85 mm in May, 200 mm in June, and 77 mm in July). According to the data from Republic Hydrometeorological Service of Serbia, the average daily temperature was 2.6°C higher than the annual average, with 10% higher precipitation, and climate conditions in 2019 were within the annual average.

Bacterial isolation and potato rot test. After the isolation of bacteria from the diseased potato stems and tubers, most colonies formed

characteristic cavities on CVP through pectin degradation. Upon purification, two types of colonies were formed on NA after a 48 h incubation period: round, small colonies, 1 to 2 mm in diameter, smooth, creamy in color, obtained in both years; and irregular colonies, 2 to 3 mm in diameter, slimy, and with creamy white coloration, obtained only in 2018 (Supplementary Fig. S1A).

Tissue maceration of inoculated potato slices appeared 24 h after the inoculation. Two types of tissue decomposition were noticed: One group of isolates (nine isolates coded with Dd prefix) produced cream-colored rotting tissue with dark brown margins in the zone between the healthy and decomposed tissue, and another group (29 isolates coded with Pcb prefix) devastated the tissue, causing cream-colored rotting with no visible margins (Supplementary Fig. S1B).

Preliminary identification. Eight isolates from 2018 (coded as Pcb33, Pcb34, Pcb38, Pcb39, Pcb61, Pcb62, Pcb64, Pcb67) and 21 isolates from 2019 (coded as Pcb2531, Pcb2538, Pcb2544, Pcb2549, Pcb2562, Pcb2563, Pcb2568, Pcb2811, Pcb2812, Pcb2813, Pcb2815, Pcb2817, Pcb2819, Pcb2833, Pcb2838, Pcb2839, Pcb2841, Pcb2842, Pcb2844, Pcb2847, Pcb2861) amplified the products at 322 bp with the BR1f/L1r primer pair specific for *P. c.* subsp. *brasiliensis*. Nine isolates obtained in 2018 (Dd31, Dd32, Dd35, Dd37, Dd41, Dd42, Dd44, Dd46, Dd47) and control strain MK10 produced amplicons at 420 bp with the ADE1/ADE2 primer pair specific for *Dickeya* spp. (Supplementary Fig. S1C). Control strain Pcc10 amplified the product of 666 bp with the F0145/E2477 primer pair.

Phenotypic features. All 38 isolates were gram-negative, facultative anaerobic (O+/F+ test), positive for indole and hydrogen sulfide production, nitrate reduction, gelatin liquefaction, aesculin hydrolysis, and utilization of aspartic acid. They were also tolerant to 5% of NaCl and able to grow at 37°C. All tested isolates showed negative reactions to starch hydrolysis, tyrosinase activity, and L-leucine utilization. Additionally, all isolates produced acid from D-glucose, D-mannitol, L-rhamnose, D-sucrose, amygdalin, and L-arabinose but

Table 3. National Center for Biotechnology Information GenBank multilocus sequence analysis (MLSA) data of different *Pectobacterium* spp. and subspecies and *Dickeya* spp. used for comparison with our MLSA results for 25 Serbian potato blackleg isolates using the same set of genes (*acnA*, *gapA*, *icdA*, and *mdh*)

			GenBank accession number			
Strain name[a]	**Host**	**Origin**	***acnA***	***gapA***	***icdA***	***mdh***
Pectobacterium carotovorum subsp. *brasiliensis*						
BZA12	Cucumber	China	CP024780	CP024780	CP024780	CP024780
SX309	Cucumber	China	CP020350	CP020350	CP020350	CP020350
BC1	Chinese cabbage	China	CP009769	CP009769	CP009769	CP009769
HG1501090302	Cucumber	China	KX010008	KX010017	KX010026	KX010035
kbs-1	Potato	Japan	LC145701	LC145702	LC145703	LC145704
A45	Potato	Syria	HM156766	HM156826	HM156887	HM156948
C18	Potato	Syria	HM156768	HM156828	HM156889	HM156950
JKP4.3.22	Potato	Germany	HM156792	HM156851	HM156913	HM156974
88/157-2	Potato	Switzerland	KP404135	KP404136	KP404137	KP404138
1033	Potato	Canada	JF926764	JF926774	JF926784	JF926794
1073	Potato	Peru	HM156787	HM156848	HM156910	HM156969
213	Potato	Brazil	JF926771	JF926781	JF926791	JF926801
1692^T	Potato	Brazil	NZ_CP047495	NZ_CP047495	NZ_CP047495	NZ_CP047495
Pectobacterium carotovorum subsp. *carotovorum*						
ATCC 15713^T	Potato	Denmark	FJ895848	FJ895849	FJ895850	FJ895851
Pcc10	Cabbage	Bosnia and Herzegovina	MT452473	MT188696	MT452474	MT188698
Pectobacterium atrosepticum						
CFBP 1526^T	Potato	UK	JN600333	JN600336	JN600339	JN600342
Pectobacterium wasabiae						
CFBP 3304^T	*Eutrema wasabi*	Japan	CP015750	CP015750	CP015750	CP015750
Pectobacterium parmentieri						
SCC 3193^T	Potato	Finland	NC_017845	NC_017845	NC_017845	NC_017845
Pectobacterium carotovorum subsp. *odoriferum*						
CFBP 1878^T	*Witloof chicory*	France	JF926763	JF926773	JF926783	JF926793
Pectobacterium betavasculorum						
CFBP 2122^T	*Beta vulgaris*	USA	JN600334	JN600337	JN600340	JN600343
Pectobacterium actinidiae						
KKH3^T	Kiwi	South Korea	JRMH01000001	JRMH01000001	JRMH01000001	JRMH01000001
Dickeya dianthicola						
RNS04.9	Potato	France	CP017638	CP017638	CP017638	CP017638
IPO980	Potato	Netherlands	NZCM002023	NZCM002023	NZCM002023	NZCM002023
GBBC2039	Potato	Belgium	NZCM001838	NZCM001838	NZCM001838	NZCM001838
ME23	Potato	USA (Maine)	CP031560	CP031560	CP031560	CP031560
NCPPB 453^T	Dianthus	UK	NZ_CM001841	NZ_CM001841	NZ_CM001841	NZ_CM001841
Dickeya dadantii						
DSM 18020^T	Geranium	Comoros	CP023467	CP023467	CP023467	CP023467
Dickeya fangzhongdai						
DSM 101947^T	*Pyrus pyrifolia*	China	NZ_CP025003	NZ_CP025003	NZ_CP025003	NZ_CP025003
Dickeya solani						
IPO 2222^T	Potato	Netherlands	NZ_CP015137	NZ_CP015137	NZ_CP015137	NZ_CP015137
MK10	Potato	Israel	NZCM001839	NZCM001839	NZCM001839	NZCM001839
Dickeya zeae						
Ech586	Philodendron	USA (Florida)	CP001836	NC013592	NC013592	NC013592
Dickeya chrysanthemi						
NCPPB 516^T	*Parthenium argentatum*	Denmark	NZ_CM001904	NZ_CM001904	NZ_CM001904	NZ_CM001904

[a] Type strains are marked with superscript T.

not from D-sorbitol. The differences between the tested isolates were obtained in the tests for arginine dihydrolase and utilization of tartrate, lactic, and tartaric acid, where nine isolates coded with Dd prefix were positive, whereas 29 isolates coded with Pcb prefix were negative. Casein hydrolysis, acid production from inositol and D-melibiose, and growth at 4°C were positive for Pcb prefix isolates and negative for Dd prefix isolates. The results of biochemical tests indicated the presence of the genus *Pectobacterium* in 29 isolates with Pcb prefix and *Dickeya* in nine isolates with Dd prefix.

Pathogenicity was confirmed on young potato plants of cultivar 'Lady Claire'. When the injection method was used, the initial symptoms on stems appeared 2 days after inoculation (DAI) in the form of dark brown lesions at the sites of inoculation. Wilting symptoms occurred on the third DAI, and the lesions on the stems increased externally and were followed by internal necrosis of vascular tissue. Five DAI, necrosis extended, causing whole plant decay. When the soil inoculation method was used, the first symptoms were noticed 10 DAI in the form of wilting of the leaves and necrotic blackening of the stem bases. Later, 15 DAI, necrosis spread from the lower plant parts, leading to drying of the whole plant. Similar symptoms were observed for all 38 tested isolates and for the control strains Pcc10 and MK10. The negative control plants were symptomless.

Reisolations from symptomatic plants for all 38 isolates were successful on CVP media. The reisolated bacteria caused pitting on CVP and exhibited the same morphology as the original isolates on NA, and they were confirmed to be the same as the original via PCR with specific primer sets BR1f/L1r and ADE1/ADE2. Thus, Koch's postulates were fulfilled.

Genotypic features. The rep-PCR results from BOX, ERIC, and REP primer sets showed five different patterns among 38 tested isolates. The obtained UPGMA phylogenetic trees also distinguished five groups of isolates, placing them in the five separate clusters (Fig. 2).

Comparison of the isolates analyzed in this study with NCBI database strains via BLAST*n* revealed the presence of four multilocus haplotypes of *P. c.* subsp. *brasiliensis* among 20 analyzed isolates and a single haplotype of *D. dianthicola* present in all five genotyped isolates from Serbia. Isolates Pcb2833, Pcb2838, Pcb2842, Pcb2844, and Pcb2861 were attributed to the haplotype PCB-1 and showed 100% homology with *P. c.* subsp. *brasiliensis* strain kbs-1 (potato, Japan) for all four genes used in multilocus genotyping. Additionally,

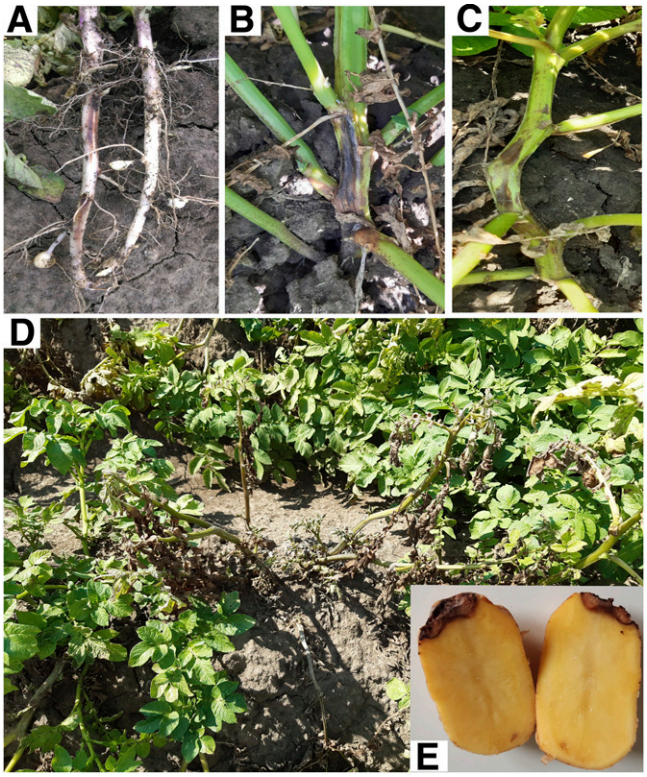

Fig. 1. Blackleg symptoms on potato cultivar 'Lady Claire' collected in the Bačka region, Serbia (2018, 2019). **A,** Lesions formed on the belowground part of the stem; **B,** brown discoloration of tissue at the stem base at soil–air level; **C,** lesions on the upper part of stems; **D,** decaying plants in the field; **E,** soft rot of progeny tuber.

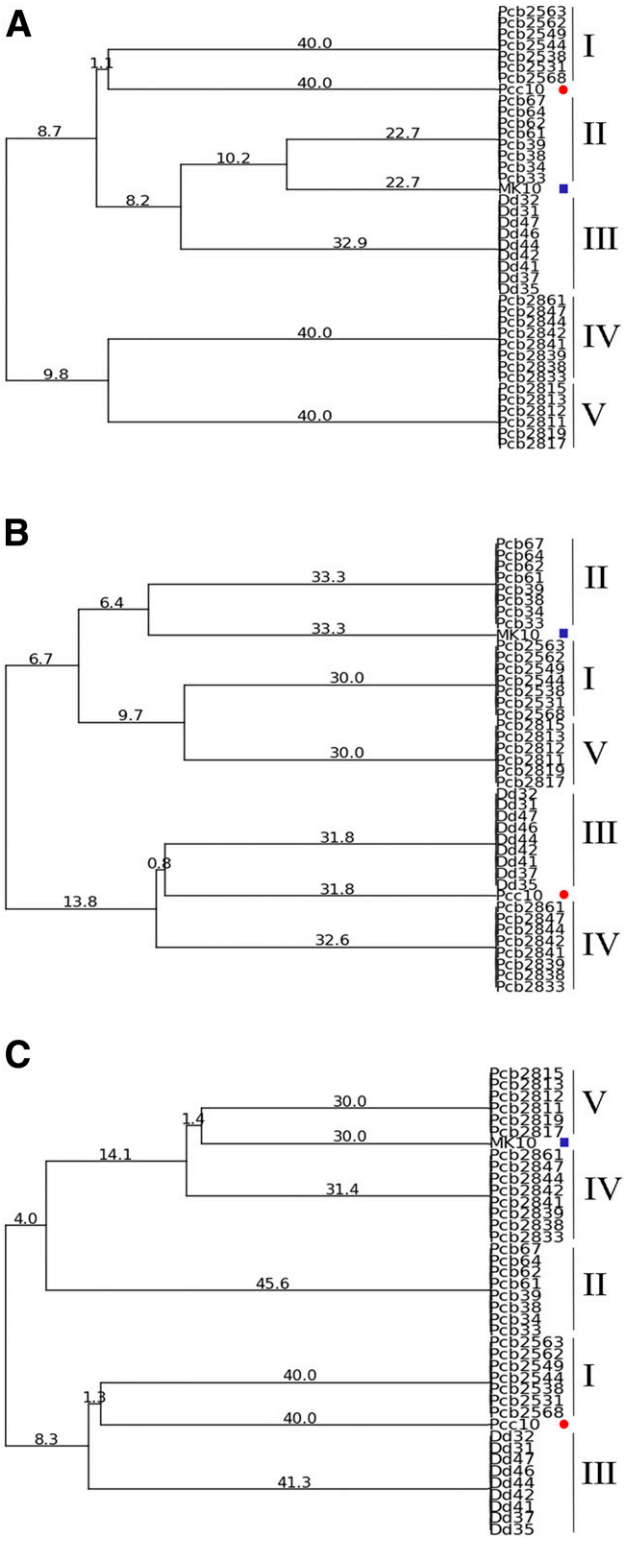

Fig. 2. Dendrogram generated via unweighted pair group method with arithmetic mean clustering method based on **A,** BOX-PCR, **B,** ERIC-PCR, and **C,** REP-PCR results for 38 *Pectobacterium carotovorum* subsp. *brasiliensis* and *Dickeya dianthicola* isolates from Serbia. Reference strains Pcc10 and MK10 are marked with a red dot and blue square, respectively. Genetic distances are presented with numbers placed on the branches.

three new multilocus haplotypes of *P. c.* subsp. *brasiliensis* were detected: PCB-2 (Pcb33, Pcb34, Pcb62, Pcb64, and Pcb67), PCB-3 (Pcb2811, Pcb2812, Pcb2813, Pcb21815, and Pcb2817), and PCB-4 (Pcb2544, Pcb2549, Pcb2562, Pcb2563, and Pcb2568). Haplotype PCB-2 showed 98.80 to 99.82% identity per locus with the *P. c.* subsp. *brasiliensis* strains: SX309 (cucumber, China) (99.42% *acnA*), BZA12 (cucumber, China) (99.79% *gapA* and 99.82% *icdA*), and BC1 (Chinese cabbage, China) (99.00% *mdh*). Isolates belonging to haplotype PCB-3 showed homology per locus of 99.48 to 100% with *P. c.* subsp. *brasiliensis* strains: SX309 (cucumber, China) (100% *acnA*), BZA12 (cucumber, China) (99.48% *gapA*), A45 (potato, Syria), (99.78% *icdA*), and kbs-1 (potato, Japan) (100% *mdh*). The fourth isolate group, PCB-4, was shown to be genetically closest to the *P. c.* subsp. *brasiliensis* strains JKP4.3.22 (potato, Germany) (99.6% *acnA*), A45 (potato, Syria) (99.74% *gapA* and 99.78% *icdA*), and kbs-1 (potato, Japan) (100% *mdh*).

Potato isolates Dd31, Dd32, Dd42, Dd44, and Dd46 were identified as the same multilocus haplotype of *D. dianthicola*, coded as DD-1, that shares 96.67 to 100% per locus identity with the *D. dianthicola* strains from NCBI: ME23 (potato, the USA [Maine]) and RNS04.9 (potato, France), with 96.67% homology for *acnA* and 100% for *gapA*, *icdA*, and *mdh*.

Estimation of the best substitution model in jModelTest for the *Pectobacterium* spp. phylogeny was done based on 15 *P. c.* subsp. *brasiliensis* ingroup sequences: three newly detected haplotypes (PCB-2, PCB-3, and PCB-4) and 13 *P. c.* subsp. *brasiliensis* NCBI strains (1692^T, kbs1 = PCB-1, BZA12, SX309, HG1501090302, A45, JKP4.3.22, BC1, 88/157-2, 1033, C18, 1073, and 213; Table 3). The proposed best model according to the Bayesian criterion was the K80 (Kimura 2 parameter) model with invariable sites (Kimura 1980), and it was further used for the maximum likelihood phylogenetic analysis. The reconstruction of *Pectobacterium* spp. phylogeny via maximum likelihood analysis and genealogical relations of *P. c.* subsp. *brasiliensis* isolates in a median joining network have shown congruent results (Fig. 3A, 3B). Two major *P. c.* subsp. *brasiliensis* genetic clusters are revealed in both the obtained phylogenetic tree and network, and they are marked according to the previous notation by Nabhan et al. (2012a) (Fig. 3A, 3B). Clade I of the obtained network, genetically distant from the rest of the analyzed haplotypes, includes three South American *P. c.* subsp. *brasiliensis* strains: the type strain 1692^T from Brazil, 213 also from Brazil, and strain 1073 from Peru (Fig. 3B). The second haplogroup is separated by 20 nucleotide differences and assembled of significantly more isolates (kbs1 = PCB-1, PCB-2, PCB-3, PCB-4, SX309, HG1501090302, A45, JKP4.3.22, BZA12, BC1, 88/157-2, C18, and 1033). This genetic cluster corresponds to *P. c.* subsp. *brasiliensis* Clade II according to the affiliation of strains 1033 and C18 described by Nabhan et al. (2012a). Closely positioned to these two strains (1033 and C18) are isolates BC1, 88/157-2, and JKP4.3.22. Two haplotypes detected in Serbia, PCB-1 = kbs-1 and PCB-2, are centrally positioned in the Clade II of the network and, jointly with strain BZA12, apparently interconnect five previous isolates (1033, C18, BC1, 88/157-2, and JKP4.3.22) with another haplogroup. This haplogroup consists of new haplotypes PCB-3 and PCB-4 from Serbia, along with strains SX309, HG1501090302, and A45 (Fig. 3B). Topology of the maximum likelihood tree shows highly supported (99%) basal positioning of the *P. c.* subsp. *brasiliensis* Clade I strains, from which diverse Cluster II may have evolved (Fig. 3A). Further branching of the Clade II mainly does not have good bootstrap supports but still reflects *P. c.* subsp. *brasiliensis* isolate clustering revealed in the median joining network. All three *P. c.* subsp. *brasiliensis* haplotypes from Serbia are clustered into a subclade with strains kbs-1 (potato, Japan), BZA12, SX309, and HG1501090302 (cucumber, China), A45 (potato, Syria), and JKP4.3.22 (potato, Germany). All *P. c.* subsp. *brasiliensis* isolates from Serbia and strains from the NCBI have formed a well-supported (100%) genetic group in the phylogenetic tree, showing inner genetic divergence between Clade I and Clade II (Fig. 3A). Strains of the *P. c.* subsp. *carotovorum* (ATCC 15713^T and Pcc10) and *Pectobacterium carotovorum* subsp. *odoriferum* (CFBP 1878^T) are shown as closely related and along with *Pectobacterium actinidiae* (KKH3^T) and all *P. c.* subsp. *brasiliensis* strains are segregated as a monophyletic lineage with high bootstrap support (97%). *P. atrosepticum* (CFBP 1526^T) and *Pectobacterium betavasculorum* (CFBP 2122^T) are grouped in a separate genetic lineage, similarly to the secluded *Pectobacterium wasabiae* (CFBP 3304^T) and *Pectobacterium parmentieri* (SCC 3193^T) as a more distant genetic branch. The determination of pairwise genetic distances showed a divergence between *P. c.* subsp. *brasiliensis* Clade I and Clade II of 2.3%, and their genetic distance in relation to the *P. c.* subsp. *carotovorum* is 3.9% and 3.4%, respectively (Table 4). Genetic distance between *P. c.* subsp. *brasiliensis* and other *Pectobacterium* species ranges from 4.4% (*P. actinidiae*) to 7% (*P. parmentieri*).

In case of the *Dickeya* spp. phylogenetic analysis, a suggested best fit model was Hasegawa–Kishino–Yano (Hasegawa et al. 1985), proposed for the 3 *D. dianthicola* in-group genotypes: DD-1 genotype detected in potato in Serbia, strain GBBC2039 from Belgium, and the type strain of *D. dianthicola* NCPPB 453^T from the United Kingdom that is genetically identical as the strains M23 (United States), RNS04.9 (France), and IPO980 (Netherlands). *D. dianthicola* multilocus genotype DD-1 from Serbia showed the same genetic divergence from the NCPPB 453^T, RNS04.9 IPO980, and M23 strains and strain GBBC2039 of 0.4% (Table 4). This equal distance and central position of the haplotype DD-1 (potato, Serbia) between the other two *D. dianthicola* genotypes as an interconnecting link is shown in the median joining network (Fig. 4B). The maximum likelihood phylogenetic tree has shown highly supported (100%) joint clustering of the *D. dianthicola* strains with well-supported inner divergence between the two branches, one consisting of Serbian DD-1 isolate and Belgium strain GBBC2039 and another branch with the type strain NCPPB 453^T, and another three identical strains: M23, RNS04.9, and IPO980 (Fig. 4A). Further genetic relations of other *Dickeya* species are unambiguous. Three species have shown genetic similarity to *D. dianthicola*: *Dickeya fangzhongdai* (DSM 101947^T), *D. solani* (IPO 2222^T and MK10), and *Dickeya dadantii* (DSM 18020^T) are jointly highly supported (100%) as a secluded genetic group with the *D. dianthicola* isolates. Strains of *Dickeya chrysanthemi* (NCPPB 516^T) and *Dickeya zeae* (Ech586) are basally positioned (Fig. 4A). The obtained p-distances between *D. dianthicola* isolates and *D. fangzhongdai*, *D. solani*, and *D. dadantii* range from 5 to 5.8%, whereas *D. chrysanthemi* and *D. zeae* express a divergence twice as big from *D. dianthicola*, ranging from 10.3 to 11.5% (Table 4).

Discussion

Blackleg disease in potato crops in Serbia, recorded for the first time in the 1990s, was at that time found to be caused by two bacterial species, *P. atrosepticum* and *P. c.* subsp. *carotovorum* (Arsenijević et al. 1994; Obradović 1996). Today, >20 years later, our results indicate *P. c.* subsp. *brasiliensis* as the causative agent of blackleg in Serbia, which appeared in 2 consecutive years (2018 and 2019). In 2018 this pathogen was found to a low extent in combined infection with *D. dianthicola.* To our knowledge, this is the first report on the presence of both pathogenic bacteria on potato in Serbia, and it indicates that the causal pathogen population has changed over the years. According to van der Wolf (2018), the population structure of blackleg-causing organisms can change rapidly. Recently, a shift in pathogen population structure has been noticed in the Netherlands, where *P. c.* subsp. *brasiliensis* has recently replaced *Dickeya* sp. as the main causal agent of blackleg (van der Wolf 2018; van der Wolf et al. 2007, 2017). Since the first isolation of *P. c.* subsp. *brasiliensis* from potato in Brazil (Duarte et al. 2004), an increasing number of outbreaks have been noticed worldwide in countries such as Peru, the United States, Canada, South Africa, Germany, Japan, Israel, Syria (Duarte et al. 2004; Ma et al. 2007; Nabhan et al. 2012a, b), and New Zealand (Panda et al. 2012). Portier et al. (2019) has proposed that *P. c.* subsp. *brasiliense* should be renamed or elevated to species level to *Pectobacterium brasiliense*. *D. dianthicola*, originally described as a pathogen of *Dianthus*, was later also found to cause blackleg in potato and has been reported for this crop by European countries as well as worldwide,

including the United States, Australia, Morocco, and Pakistan (Ma et al. 2018; Nasaruddin et al. 2019; Oulghazi et al. 2017; Sarfraz et al. 2018; Tsror et al. 2009; Wright et al. 2018). This bacterium is still not found on *Dianthus* in the European Union.

Latently infected potato seed imported from different parts of the world could explain the outbreaks caused by both pathogens in many countries (Czajkowski et al. 2011; Tsror et al. 2009). During both years of our potato monitoring, climatic conditions were

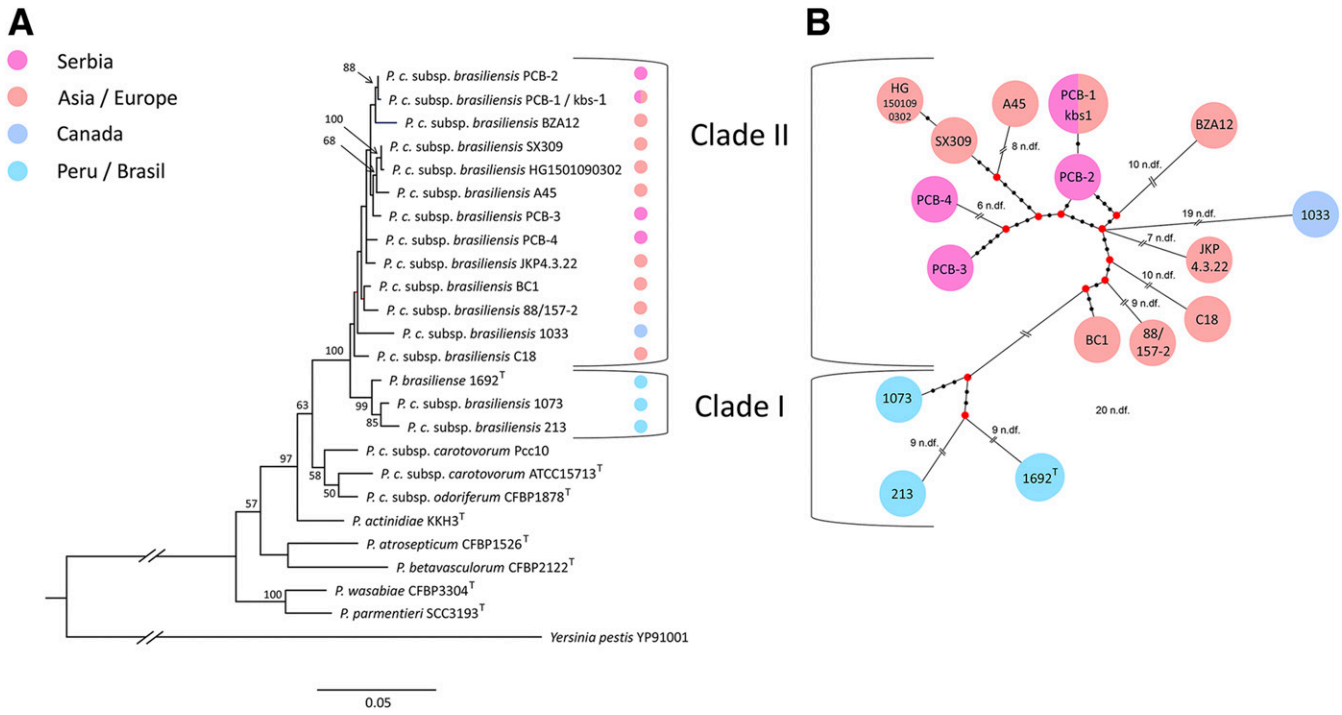

Fig. 3. Reconstruction of the phylogenetic relations evaluated between four gene–multilocus genotypes (*acnA*, *gapA*, *icdA*, and *mdh*) of four *Pectobacterium carotovorum* subsp. *brasiliensis* haplotypes detected in Serbia and sequence data of a selection of 21 *Pectobacterium* spp. strains from the National Center for Biotechnology Information database (12 from potato). Geographic origin of the haplotypes is color-marked as given in the legend. **A,** Maximum likelihood phylogenetic tree reconstructed via the K2P+I model with bootstrap support values >50 given at the nodes (bar: the estimated nucleotide substitutions per site are 0.02). **B,** Median joining network obtained for the *P. c.* subsp. *brasiliensis* haplotypes. Circle sizes are proportional to the number of strains belonging to a specific haplotype. Each black dot on the lines connecting the haplotypes marks one mutation; >5-nucleotide differences are shown with the corresponding number and abbreviation "n.df." (nucleotide differences). Red interconnecting dots are median vectors that represent missing or unsampled intermediate haplotypes. Matching of the genetic Clades I and II on the phylogenetic tree and network is noted in between.

Table 4. Average genetic divergence according to pairwise analysis (p-distance method) based on concatenated partial sequences of genes *acnA*, *gapA*, *icdA*, and *mdh* between *Pectobacterium carotovorum* subsp. *brasiliensis* and *Dickeya dianthicola* and selected strains belonging to other subspecies and species

	P/SE[a]									
Species and subspecies	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1. *P. c.* subsp. *brasiliensis* Clade II		0.003	0.004	0.004	0.005	0.007	0.006	0.007	0.007	0.010
2. *P. c.* subsp. *brasiliensis* Clade I	0.023		0.004	0.005	0.005	0.007	0.006	0.007	0.007	0.010
3. *P. c.* subsp. *carotovorum*	0.034	0.039		0.003	0.004	0.006	0.006	0.006	0.007	0.010
4. *Pectobacterium carotovorum* subsp. *odoriferum*	0.036	0.040	0.022		0.005	0.006	0.006	0.006	0.007	0.010
5. *Pectobacterium actinidiae*	0.044	0.044	0.040	0.035		0.006	0.006	0.006	0.007	0.010
6. *Pectobacterium atrosepticum*	0.060	0.065	0.058	0.057	0.058		0.006	0.007	0.007	0.010
7. *Pectobacterium betavasculorum*	0.061	0.067	0.063	0.062	0.070	0.060		0.006	0.007	0.010
8. *Pectobacterium wasabiae*	0.067	0.070	0.066	0.064	0.061	0.066	0.067		0.005	0.010
9. *Pectobacterium parmentieri*	0.070	0.069	0.069	0.064	0.064	0.066	0.074	0.032		0.010
10. *Yersinia pestis*	0.179	0.180	0.177	0.178	0.176	0.179	0.177	0.176	0.179	

	***P*/SE**								
Species and subspecies	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1. *D. dianthicola* Serbia		0.002	0.002	0.006	0.006	0.006	0.008	0.008	0.011
2. *D. dianthicola* Belgium	0.004		0.002	0.006	0.006	0.006	0.008	0.008	0.011
3. *D. dianthicola* USA, UK[T], France, Netherlands	0.004	0.007		0.006	0.006	0.006	0.008	0.008	0.011
4. *Dickeya fangzhongdai*	0.050	0.051	0.050		0.005	0.005	0.008	0.008	0.011
5. *Dickeya dadantii*	0.055	0.056	0.054	0.053		0.006	0.007	0.008	0.010
6. *Dickeya solani*	0.056	0.058	0.054	0.045	0.056		0.008	0.008	0.010
7. *Dickeya chrysanthemi*	0.104	0.105	0.103	0.104	0.105	0.104		0.008	0.010
8. *Dickeya zeae*	0.114	0.115	0.113	0.110	0.110	0.107	0.098		0.010
9. *Y. pestis*	0.200	0.199	0.200	0.195	0.195	0.194	0.196	0.190	

[a] *P*, p-distance over sequence pairs between groups; *SE*, standard error. *SE*s are shown above the diagonal and were obtained by a bootstrap procedure (1.000 replicates).

favorable for blackleg development. In 2018 daily temperatures were higher than in 2019, with abundant rainfall, providing a more favorable environment for *D. dianthicola* development, as it was also observed during warm summers in north Finland (Degefu et al. 2013).

Blackleg disease in the three infested fields reached a high disease incidence (40 to 70%). The symptoms in the observed potato fields, in a single or combined infection, were identical and corresponded to those described previously (De Haan et al. 2008; Pérombelon 2002; van der Merwe et al. 2010). Yield loss of 23.7% was established in case of the combined infection (*P. c.* subsp. *brasiliensis* and *D. dianthicola*) in 2018, whereas in 2019 losses were 10.5 and 44.7%, respectively, when *P. c.* subsp. *brasiliensis* was present as the only causal agent. No observed correlation was found between yield and detected bacteria in the field. According to Tsror et al. (2009), *Dickeya* spp. can cause, under favorable climatic conditions in Israel, a potato yield decrease of 20 to 25%. However, it is not possible to differentiate losses caused by *Pectobacterium* and *Dickeya* (Toth et al. 2011).

The results of conventional bacteriological tests of Serbian potato isolates matched mainly the characteristics of *Pectobacterium* sp. and *Dickeya* sp. as described previously (Baghaee-Ravari et al. 2011; Czajkowski et al. 2009; Duarte et al. 2004; Nabhan et al. 2012a; Tsror et al. 2009; van der Merwe et al. 2010). Deviations were obtained for *D. dianthicola* isolates in tests such as negative reaction in casein hydrolysis and acid production from D-meliobiose, and positive reactions for arginine dihydrolase and tartrate utilization (Baghaee-Ravari et al. 2011; Czajkowski et al. 2009; Tsror et al. 2009). Pathogenicity, confirmed on young potato plants, manifested identical symptoms of wilting, blackleg, and decaying of plants, as was observed in fields and cited by other authors (Tsror et al. 2009; van der Merwe et al. 2010).

In preliminary identification of Serbian potato isolates, we obtained positive results with species-specific primers ADE1/ADE2 (Nassar et al. 1996) and Br1f/L1r (Duarte et al. 2004). In work with these primer sets, Czajkowski et al. (2015) recommended the use of pure bacterial colonies or purified genomic DNA, considering that sometimes false-positive reactions could be yielded. The authors suggested that several PCRs developed in the early 1990s still remain widely used and recognized as the gold standard in molecular detection of *Pectobacterium* and *Dickeya* spp. bacteria.

DNA fingerprinting via rep-PCR provided five different groups among 38 potato isolates, proving to be a useful tool for distinguishing blackleg-causing bacteria. The MLSA approach used to identify and characterize Serbian *P. c.* subsp. *brasiliensis* and *D. dianthicola* isolates yielded insight into the genetic relatedness of the detected isolates and previously described strains belonging to the same and other related taxa in phylogenetic analyses. Four housekeeping genes (*acnA*, *gapA*, *icdA*, and *mdh*) used in this study have been shown to be equally informative for revealing the phylogenetic and genealogic relations of *P. c.* subsp. *brasiliensis* and *D. dianthicola*, by confirming overall genetic clustering as previously done by using ≤8 housekeeping genes (Ma et al. 2007; Nabhan et al. 2012a, 2012b). Almeida et al. (2010) stated that MLST consists of sequencing of multiple loci, typically four to eight housekeeping genes, and usually allows strains to be distinguished below the species level. Phylogenetic analysis based on *gapA* and *mdh* housekeeping genes is an accurate method to characterize and differentiate *Pectobacterium* isolates (Baghaee-Ravari et al. 2011) or to distinguish *Dickeya* species (Palacio-Bielsa et al. 2010). Sławiak et al. (2009) developed a method for rapid characterization of *Dickeya* species based on *dnaX* sequence, which has proven to yield accurate clustering and identification. Ma et al. (2018) stated that the phylogenetic relationships reconstructed from *dnaX* data alone are congruent with the results of using MLSA data with housekeeping genes when characterizing North American blackleg-associated bacteria.

Analysis of the genetic structure of Serbian potato *P. c.* subsp. *brasiliensis* isolates and strains from NCBI based on *acnA*, *gapA*, *icdA*, and *mdh* housekeeping genes confirmed overall isolates clustering into two main genetic groups previously proposed by Nabhan et al. (2012a). Three new genotypes from Serbia (PCB-2, PCB-3, and PCB-4) showed their affiliation to Clade II and suggest possible further subclustering in this haplogroup. Based on the presented MLSA results, any assumption about infection origin would be speculative. Geography should not be taken as a decisive variable when interpreting phylogeny and network results because of the intensive global trade of the planting material, but still it should be taken into account, as shown by the fact that North American *P. c.* subsp. *brasiliensis* strain 1033 was detached by a 19-nucleotide difference from the heterogeneous Clade II, as well as the other two strains from South America (1073 and 213) that form separate Clade I. In case of *D. dianthicola*, high bootstrap values support the phylogenetic relatedness of the *D. fangzhongdai*, *D. solani*, and

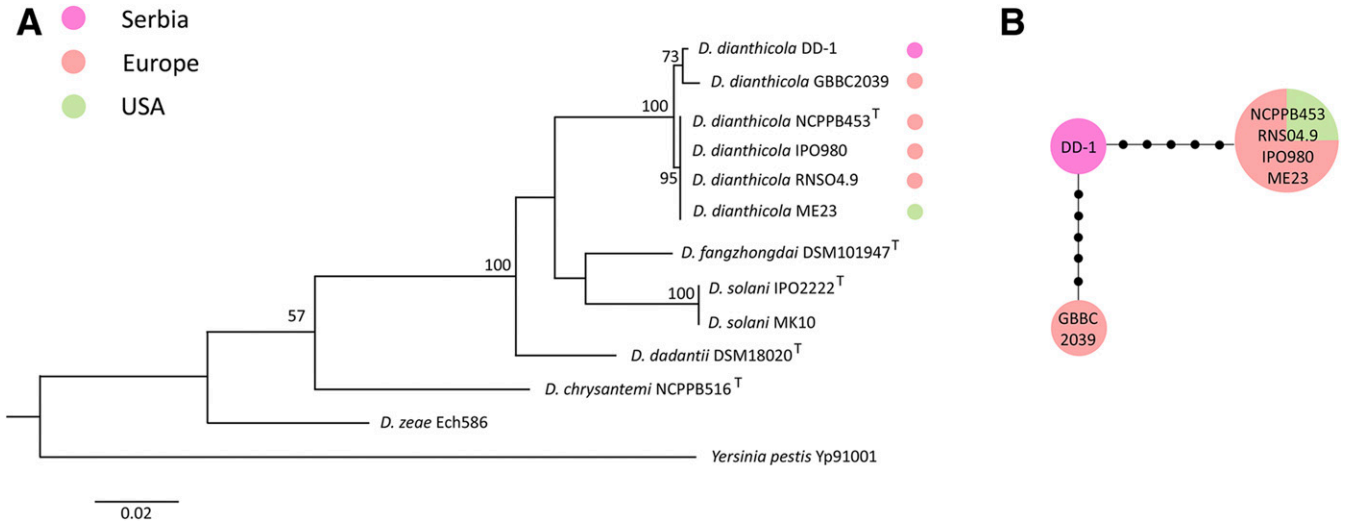

Fig. 4. Reconstruction of the phylogenetic relations based on four gene–multilocus genotypes (*acnA*, *gapA*, *icdA*, and *mdh*) evaluated between six *Dickeya dianthicola* isolates (one Serbian haplotype and five *D. dianthicola* NCBI database strains) and five strains of other *Dickeya* spp. selected from the National Center for Biotechnology Information database. Geographic origins of the isolates are marked in specific colors, as given in the legend. **A,** Maximum likelihood phylogenetic tree reconstructed via the Hasegawa–Kishino–Yano model with bootstrap support values >50 given at the nodes (bar: the estimated nucleotide substitutions per site are 0.02). **B,** Median joining network obtained for the *D. dianthicola* haplotypes. Circle sizes are proportional to the number of isolates belonging to a specific haplotype. Each black dot on the lines connecting the haplotypes marks one mutation.

D. dadantii to the *D. dianthicola*, forming a genetic clade of which the in-group relations are insufficiently understood.

In conclusion, our study identified *P. c.* subsp. *brasiliensis* as the main causal pathogen of blackleg disease of potato in some fields in northern Serbia, as well as *D. dianthicola*, found in the combined infection in 2018. These results represent the first detailed study of the genetic structure of the detected isolates of both bacteria. This new group of plant pathogens will be further studied for epidemiological features, including survival and dissemination.

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