Species Diversity of *Dickeya* and *Pectobacterium* Causing Potato Blackleg Disease in Pakistan

Sohaib Sarfraz,^{1,2} Shahbaz Talib Sahi,² Saïd Oulghazi,^{1,3} Kashif Riaz,² Nasir Ahmed Rajput,² Muhammad Atiq,² Muhammad Rizwan Tufail,² Akhtar Hameed,² and Denis Faure^{1,†}

¹ Université Paris-Saclay, CEA, CNRS, Institute for Integrative Biology of the Cell (I2BC), 91198, Gif-sur-Yvette, France

² Department of Plant Pathology, Faculty of Agriculture, University of Agriculture Faisalabad 38000, Pakistan

³ Department of Biology, Faculty of Sciences, Moulay Ismaïl University, Meknes, Morocco

Abstract

Potato blackleg is caused by a diverse species of pectinolytic bacteria. In Pakistan, approximately 90% of the pathogens involved belong to *Pectobacterium atrosepticum*. Survey (2014 to 2017), sampling, and isolation from different potato growing areas of Punjab, Pakistan depicted an overall disease incidence of approximately 15%. Thirty-six pectinolytic strains confirmed through biochemical and pathogenicity testing were characterized via *gapA* gene to identify them at the species level. To further validate the identification, one strain from each species SS26 (*P. atrosepticum*), SS28 (*Pectobacterium polaris*), SS70 (*Dickeya dianthicola*), SS90 (*Pectobacterium parmentieri*), SS95 (*Pectobacterium punjabense*), and SS96 (*Pectobacterium versatile*) were selected for draft genome sequencing and multilocus sequence analysis of 13 housekeeping genes (*fusA*, *rpoD*, *acnA*, *purA*, *gyrB*, *recA*, *mdh*,

mtlD, *groEL*, *secY*, *glyA*, *gapA*, and *rplB*). Phylogenetic analysis revealed considerable genetic diversity in the genus *Pectobacterium*. *In silico* DNA–DNA hybridization and average nucleotide identity values of the strains selected for genome sequencing were determined with other reference *Pectobacterium* and *Dickeya* strains. Moreover, all six representative strains were also phenotypically characterized on the basis of metabolism of different carbon sources. Overall, on the basis of genotypic and phenotypic characteristics, these 36 isolates were grouped into six species: *P. atrosepticum*, *P. versatile*, *P. parmentieri*, *P. polaris*, *P. punjabense*, and *D. dianthicola*.

Keywords: *Dickeya*, diversity, genotypic, pathogen diversity, *Pectobacterium*, pectinolytic, phenotypic, prokaryotes, vegetables

The causative agents of blackleg and soft rot diseases on potato plants and tubers belong to the *Pectobacterium* and *Dickeya* genera of the Pectobacteriaceae family (Adeolu et al. 2016). They are pectinolytic plant pathogens that represent a major threat to economically important crops of vegetables and ornamental plants (Czajkowski et al. 2011). *Pectobacterium* and *Dickeya* species have been included among the 10 most important bacterial plant pathogens based on their economic or scientific impact (Mansfield et al. 2012). Worldwide, they cause diseases on a broad spectrum of monocotyledonous and dicotyledonous plants such as potato, horseradish, yellow kiwifruit, tomato, cabbage, cucumber, artichoke, sunflower, bell pepper, cucurbit, and lettuce (Caruso et al. 2016). *Pectobacterium* and *Dickeya* species are responsible for causing potato blackleg in the field and tuber soft rot in the field and storage conditions, reducing the yield and quality of the tubers (Adeolu et al. 2016).

The *Pectobacterium* genus encompasses 18 species: *P. atrosepticum*, *P. wasabiae*, *P. betavascularum* (Gardan et al. 2003), *P. carotovorum* (Kado 2010), *P. cacticidum* (Alcorn and Oldfield 1991; Hauben et al. 1999), *P. parmentieri* (Khayati et al. 2016), *P. aroidearum* (Nabhan et al. 2013), *P. peruviense* (Waleron et al. 2017), *P. polaris* (Dees et al. 2017), *P. punjabense* (Sarfraz et al. 2018), *P. fontis* (Oulghazi et al. 2019a), *P. zantedeschiae* (Waleron et al. 2019a), *P. aquaticum* (Pédrón et al. 2019), *P. odoriferum*, *P. versatile*, *P. brasiliense*, *P. actinidiae* (Portier et al. 2019), and *P. polonicum*

(Waleron et al. 2019b). Along with *Pectobacterium*, *Dickeya* species including *Dickeya solani* (van der Wolf et al. 2014), *D. dadantii*, *D. zeae*, and *D. dianthicola* (Samson et al. 2005) were also found to be important plant pathogens (Potrykus et al. 2016). For several decades, *D. dianthicola* has been reported as a significant potato blackleg pathogen in the United States (Rosenzweig et al. 2016; Patel et al. 2019), Australia (Wright et al. 2018), and Europe (Toth et al. 2011).

The latest agricultural statistics (2017) show that potato is grown on 178,000 hectares with a total production of approximately 4.0 million tonnes. Pakistan ranks 6th and 19th in potato production in Asia and worldwide, respectively. The leading province in potato production is Punjab (95%), followed by Khyber Pakhtunkhwa, Balochistan, and Sindh (FAOSTAT 2017). In Pakistan, blackleg of potato and soft rot of tubers were first reported in 1984 from Swat (Khan et al. 1985). A pluriannual survey of 42 locations including the plains and hilly areas of Pakistan revealed that the disease severity of blackleg of potato ranged from 2 to 30% and disease incidence from 5 to 35% (Ali et al. 2010). Blackleg and soft rot diseases affect potato production by reducing yield when blackleg incidence is ≤10% and by rotting the tubers during storage, affecting tuber export and seed certification (Farran and Mingo-Castel 2006). Until now no studies have monitored the genetic diversity of *Pectobacterium* and *Dickeya* potato pathogens in Pakistan. In this study, we analyzed and deposited in an international database a set of molecular information on the *Pectobacterium* and *Dickeya* potato pathogens in Pakistan that could be used for epidemiologic and diagnostic purposes.

Materials and Methods

Survey and sample collection. Seventy samples of *Solanum tuberosum* presenting blackleg symptoms were collected from major potato growing regions, namely Okara, Pakpattan, Depalpur, Chiniot, and Faisalabad of province Punjab, Pakistan. Bacteria were isolated from 1-cm sections of symptomatic tissues suspended in 0.8% NaCl for 30 min. Serial dilutions were plated onto crystal violet pectate (CVP) agar medium, and plates were incubated for 48 h at 28°C (Hélias et al. 2012). Bacterial colonies producing pitting on CVP were purified on nutrient agar (beef extract 3, peptone 5, glucose 2.5, and agar 15 g/liter). All pectinolytic isolates were confirmed

†Corresponding author: D. Faure; denis.faure@i2bc.paris-saclay.fr

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as gram-negative through Gram staining and 3% KOH string test, and a 5% NaCl salt tolerance test was also done to determine whether the isolated strains were sensitive to salt (Schaad et al. 2001). All isolates were preserved at -80°C for storage in 25% glycerol.

gapA barcode analysis. Bacterial strains were subjected to PCR sequencing of the *gapA* barcode with the primers gapA-7-F (ATCAAAGTAGGTATCAACGG) and gapA-938-R (TCTACCA GAAACCAGTT). The *gapA* barcode was designed to identify *Pectobacterium* and *Dickeya* species (Cigna et al. 2017). Two microliters of a fresh liquid culture was diluted in 18 μl of Tris/EDTA buffer, followed by 10 min of heating at 99°C in a thermal cycler (Cigna et al. 2017). The reaction was completed at a final volume of 25 μl , including 1 μl of DNA, 2.5 μl of PCR buffer (10 $\times$), 2 μl of MgCl_2 (25 mM), 2.5 μl of 1 mM of each dNTP, 1 μl of each forward and reverse primer, and 0.1 μl of Taq polymerase. The amplification started by initial denaturation (95°C for 5 min), followed by 30 cycles of denaturation (95°C for 1 min), annealing (56°C for 30 s), elongation (72°C for 1 min), and a final extension step (72°C for 10 min). At the end of amplification, PCR products were examined through gel electrophoresis with 2% agarose gel dipped in a solution containing 0.5 $\mu\text{g}/\text{ml}$ of ethidium bromide for 30 min and visualized under the Gel doc system (Sambrook and Russell 2001). A standard 1-Kb DNA ladder was also run on each gel along with positive and negative controls. The obtained amplicons (932 bp) were sequenced via Sanger sequencing. Phylogenetic analyses were performed with Mega 7 software and the maximum likelihood method with 1,000 bootstrap replications.

Genome sequencing and analyses. One representative strain from each of the *gapA*-identified species (*P. atrosepticum* SS26, *P. polaris* SS28, *D. dianthicola* SS70, *P. parmentieri* SS90, *P. punjabense* SS95, and *P. versatile* SS96) was selected for genome sequencing. DNA extraction was done with a DNA and RNA purification kit. The extraction was carried out with 5 ml of the bacterial culture grown at 28°C overnight. Extracted DNA was mixed in 100 μl of DNase-free water, its quality was confirmed with a spectrophotometer, and it was preserved -20°C for additional studies. The genome sequence was determined with a genomic DNA sequencer at the Institute for Integrative Biology of the Cell. Genomic data were assembled via de novo assembly in CLC Genomics Workbench version 10.1.1 (<https://www.qiagenbioinformatics.com>). Multilocus sequence analysis (MLSA), which is considered a general method to obtain an exact phylogenetic relationship of species within a genus, especially in prokaryotes, was used (Glaeser and Kämpfer 2015). CLC Genomics Workbench version 10.1.1 and MEGA 7 were used for MLSA and phylogenetic analyses, respectively. A robust MLSA of the concatenated gene fragments of the 13 housekeeping genes (*fusA*, *rpoD*, *acnA*, *purA*, *gyrB*, *recA*, *mdh*, *mtlD*, *groEL*, *secY*, *glyA*, *gapA*, and *rplB*) of Pakistani isolates was performed in comparison with the reference strains of *Pectobacterium* and *Dickeya* spp. available in the NCBI database to authenticate their identification at the species level (Oulghazi et al. 2019a, b). Moreover, the Genome-to-Genome Distance Calculator (<http://ggdc.dsmz.de/>) was used to calculate the *in silico* DNA–DNA hybridization (isDDH) values that approached the wet-lab DNA–DNA hybridization (DDH) as closely as possible (Meier-Kolthoff et al. 2013), and pairwise average nucleotide identity (ANI) values were obtained with the dedicated pipelines (<http://enve-omics.ce.gatech.edu/ani/>) (Delcher et al. 2002). Therefore, we used genomic data for all six representative strains and the reference genomes from NCBI database to calculate the isDDH and ANI values by uploading the genomic DNA sequences on the respective web servers.

Pathogenicity test on potato tubers and plants. To confirm the pathogenicity of all six representative strains (SS26, SS28, SS70, SS90, SS95, and SS96), inoculations were done on potato tubers and plants (cultivar Sante) under controlled incubator and greenhouse conditions, respectively. Bacterial inoculum was prepared by inoculating a fresh, single bacterial colony in nutrient broth (NB) medium, and the bacterial culture was shaken at 200 rpm at 28°C for 16 to 18 h in 10 ml of NB media. The subsequent bacterial

culture was centrifuged at 4,500 rpm for 10 min. Bacterial pellet was washed twice with 0.8% NaCl and resuspended in sterile 0.8% NaCl, adjusted to a final concentration of 10^8 cfu/ml ($\text{OD}_{600} = 1$) with a spectrophotometer (Hoque and Mansfield 2005).

Healthy potato tubers were washed, surface sterilized with 2% sodium hypochlorite for 10 min, and rinsed with water, followed by air drying at room temperature. Ten microliters of bacterial cell suspension ($\text{OD}_{600} = 1$) of each strain was inoculated with five replications, and boxes were placed in an incubator at 24°C for 4 days. For negative control, tubers were inoculated with 0.8% NaCl solution.

Potato plants were grown in 20-cm diameter pots at 24 to 26°C with regular watering in a greenhouse. Three weeks after stem emergence, potato plants were injected at 5 cm above the stem base with 100 μl of bacterial cell suspension ($\text{OD}_{600} = 1$) in a sterile syringe. The development of disease symptoms was observed daily for 2 weeks. Five plants were infected by each of the selected strains. Negative controls were injected with 0.8% NaCl solution. For the tuber assay, we visually observed symptoms after 4 days of inoculation by cutting the tubers from the center, whereas in case of plants, inoculated stems were observed daily until clear blackleg symptoms appeared.

Phenotypic characterization. We phenotypically characterized all representative strains (SS26, SS28, SS70, SS90, SS95^T, and SS96) by inoculating the minimal medium (M9) supplemented with different carbon sources including 1-0 β methyl glucopyranoside, 1-0-methyl- α -D glucopyranoside, D-lactose, melibiose, gluconic acid, raffinose, galactose, cellobiose L-alanine, maltose, and D-mannose (Elbing and Brent 2002; Khayi et al. 2016). All six representative strains and the reference type strains including *P. parmentieri* RNS 08-42-1A^T, *P. wasabiae* CFBP 3304^T, *D. dianthicola* CFBP 2015, and *D. solani* 0423.1 were used to determine and compare the phenotypic characters. All tests were carried out at 28°C for 24 to 48 h under 200 rpm shaking (Terta et al. 2010).

Results

Isolation and identification of *Pectobacterium* and *Dickeya* pathogens. From 2014 to 2017, potato plants with blackleg symptoms were collected from five potato growing regions of Punjab, Pakistan. A total of 70 symptomatic plants were collected, from which 36 bacterial isolates showed pectinolytic activity on CVP medium (Table 1). All isolates were confirmed as gram-negative and sensitive to salt, because they did not grow in the presence of 5% NaCl. The *gapA* barcode was amplified with gapA-7-F and gapA-938-R primer and sequenced. BLAST searches of the NCBI database (<http://www.ncbi.nlm.nih.gov>) and phylogenetic analyses via maximum likelihood method (Mega7) (Fig. 1) revealed that 21 isolates belonged to *P. atrosepticum*, three isolates to *P. versatile*, three to *P. parmentieri*, three to *P. punjabense*, two to *P. polaris*, and four to *D. dianthicola*. GenBank accession numbers of the *gapA* sequences are listed in Table 1.

Genome sequencing and *in silico* analyses. Genome sequencing was done to confirm the phylogenetic position of the different strains at species level and to provide reference genomes from Pakistan. The characteristics of the genome sequences are presented in Table 2. Genomic data were collected from each of the six strains and reference strains of *Pectobacterium* and *Dickeya* spp. available in the NCBI database to perform MLSA by concatenating 13 housekeeping genes (*fusA*, *rpoD*, *acnA*, *gyrB*, *recA*, *mdh*, *mtlD*, *groEL*, *secY*, *glyA*, *gapA*, and *rplB*) (Fig. 2). All six Pakistani strains (SS26, SS28, SS70, SS90, SS95, and SS96) first identified with the *gapA* gene were clustered with the same clades or groups as respective species (Figs. 1 and 2). Hence, the *gapA* barcode unambiguously assigned the appropriate taxon (genus, species, and subspecies).

Furthermore, genomic data were also used to calculate isDDH and ANI values by using bioinformatics websites (mentioned in Materials and Methods). IsDDH and ANI values were calculated for all six Pakistani strains (SS26, SS28, SS70, SS90, SS95, and SS96) and other *Pectobacterium* and *Dickeya* strains as queries to assess whether such *in silico* methods can be used as substitutes of wet-lab

DDH to conclude species allocation of these plant pathogenic strains. Therefore, the isDDH and ANI values were consistent with their phylogenetic relationships (Figs. 1 and 2) because strains grouped in the same phylogenetic clade also showed high isDDH and ANI values. The ANI and isDDH values calculated for different species of *Pectobacterium* and *Dickeya* were well below the suggested 96% cutoff for the ANI value and 70% cutoff for the isDDH value for delineating the species (Table 3).

Virulence on potato plant host. Results of the pathogenicity tests showed that all representative strains including SS26 (*P. atrosepticum*), SS28 (*P. polaris*), SS90 (*P. parmentieri*), SS95 (*P. punjabense*), SS96 (*P. versatile*), and *D. dianthicola* strain (SS70) produced typical blackleg symptoms on inoculated potato plants and induced maceration on potato tubers, with five replications for each of the six strains used. In case of potato plants, 2 weeks after inoculation, all inoculated stems showed black, rotten stems, including positive controls, but the negative control plants remained symptomless. Similarly, in the tuber assay, all infected tubers exhibited maceration 4 days after inoculation, but not those inoculated with 0.8% NaCl solution.

Phenotypic analyses. All six representative strains were phenotypically characterized on the basis of ability to use different carbon sources as compared with the closest type or reference strains. In case of *Pectobacterium* strains, positive results were obtained with 1-0 β methyl glucopyranoside, D-lactose, melibiose, gluconic acid, raffinose, galactose, cellobiose, and D-mannose, whereas negative results with 1-0-methyl- α -D glucopyranoside, L-alanine, and D-maltose. In case of *D. dianthicola* strains (SS70 and CFBP 1515), positive growth was

obtained with L-rhamnose but no growth with D-arabinose. In contrast, reference strain *D. solani* 0423.1 showed opposite results for both of the aforementioned carbon sources, thus differentiating the *D. dianthicola* strains on the basis of phenotypic characters (Table 3).

Discussion

Blackleg and soft rot diseases of potato are among the most important bacterial diseases because they adversely affect the potato (*S. tuberosum*) trade worldwide (McNally et al. 2017). Recent advancements in the classification of blackleg-causing bacteria and occurrence of newly emerging pathogens such as *Dickeya* species have encouraged many scientists to characterize the overall diversity of blackleg-related pathogens (Jiang et al. 2016). The results of this study highlight the diversity of blackleg pathogens that threaten potato production and can be used to reduce the risk of economic losses caused by this disease through better quarantine measures. Therefore, from 2014 to 2017, 70 potato plant samples exhibiting blackleg disease were collected from different farmers' fields in five potato growing regions of Punjab, Pakistan. These surveys found an overall disease incidence of approximately 15% in the country's most important potato producing regions.

A multiple approach of genomic pathogenicity tests and phenotypic (biochemical) characterization were done to explore the genetic diversity of pectinolytic bacteria isolated from typical blackleg symptoms of potato (Rosselló-Mora and Amann 2001). Homogeneity was almost constant in case of the phenotypic properties; in contrast, genomic analyses of the isolates showed the diversity among all

Table 1. Strains isolated and identified in this study

Strain code	Sample collection date	Location	Potato plant tissue	Pathogenicity on tubers ^a	Species assignment based on <i>gapA</i>	<i>gapA</i> accession number (GenBank)
SS12	07-01-2016	Depalpur1	Stem	2	<i>Pectobacterium atrosepticum</i>	MK392510
SS13	07-01-2016	Depalpur1	Stem	2	<i>P. atrosepticum</i>	MK392511
SS14	07-01-2016	Depalpur2	Stem	1	<i>P. atrosepticum</i>	MK392512
SS15	07-01-2016	Depalpur2	Stem	2	<i>P. atrosepticum</i>	MK392513
SS16	07-01-2016	Depalpur3	Stem	2	<i>P. atrosepticum</i>	MK392514
SS17	07-01-2016	Depalpur3	Stem	2	<i>P. atrosepticum</i>	MK392515
SS18	30-12-2015	Pakpattan1	Stem	2	<i>P. atrosepticum</i>	MK392516
SS22	30-12-2015	Pakpattan2	Stem	1	<i>P. atrosepticum</i>	MK392517
SS23	30-12-2015	Pakpattan2	Stem	1	<i>P. atrosepticum</i>	MK392518
SS24	30-12-2015	Pakpattan2	Stem	2	<i>P. atrosepticum</i>	MK392519
SS25	30-12-2015	Pakpattan2	Stem	1	<i>P. atrosepticum</i>	MK392520
SS26	16-01-2016	Okara 1	Stem	1	<i>P. atrosepticum</i>	MK392521
SS28	14-01-2017	Okara 2	Stem	1	<i>Pectobacterium polaris</i>	MH188893
SS32	24-12-2014	Okara 2	Stem	2	<i>P. atrosepticum</i>	MK392522
SS33	24-12-2014	Okara 2	Stem	1	<i>P. atrosepticum</i>	MK392523
SS54	16-01-2017	Chiniot 1	Stem	2	<i>Pectobacterium punjabense</i>	MH603864
SS56	16-01-2017	Chiniot 1	Stem	1	<i>P. punjabense</i>	MH603865
SS57	20-12-2015	Chiniot 2	Stem	1	<i>P. atrosepticum</i>	MK392524
SS64	15-01-2014	Okara 3	Stem	2	<i>P. atrosepticum</i>	MK392525
SS65	15-01-2014	Okara 3	Stem	2	<i>P. atrosepticum</i>	MK392526
SS70	14-01-2017	Okara 3	Stem	2	<i>Dickeya dianthicola</i>	MG845896
SS71	20-12-2015	Chiniot 4	Stem	2	<i>P. atrosepticum</i>	MK392527
SS72	20-12-2015	Chiniot 4	Stem	2	<i>P. atrosepticum</i>	MK392528
SS82	15-01-2014	Okara 3	Stem	2	<i>P. atrosepticum</i>	MK392529
SS84	14-01-2017	Okara 4	Stem	2	<i>D. dianthicola</i>	MG845897
SS85	14-01-2017	Okara 4	Stem	1	<i>D. dianthicola</i>	MG845898
SS86	14-01-2017	Okara 4	Stem	1	<i>D. dianthicola</i>	MG845899
SS90	20-12-2015	Chiniot 2	Stem	2	<i>Pectobacterium parmentieri</i>	MH188895
SS91	20-12-2015	Chiniot 2	Stem	2	<i>P. parmentieri</i>	MH188896
SS92	20-12-2015	Chiniot 2	Stem	2	<i>P. parmentieri</i>	MH188897
SS93	14-01-2017	Okara 3	Stem	2	<i>P. atrosepticum</i>	MK392530
SS94	14-01-2017	Okara 2	Stem	1	<i>P. polaris</i>	MH188894
SS95	16-01-2017	Chiniot 1	Stem	2	<i>P. punjabense</i>	MH603866
SS96	05-01-2016	Faisalabad	Stem	2	<i>Pectobacterium versatile</i>	MK029455
SS97	05-01-2016	Faisalabad	Stem	2	<i>P. versatile</i>	MK029456
SS98	05-01-2016	Faisalabad	Stem	1	<i>P. versatile</i>	MK029457

^a Numbers indicate aggressiveness levels in tuber maceration assays (1, virulent; 2, more virulent).

these pectinolytic strains (Gallelli et al. 2009; Yahiaoui-Zaidi et al. 2003). This study confirmed that the commonly described major potato pectinolytic pathogens including *P. atrosepticum*, *P. versatile*, *P. parmentieri*, *P. polaris*, *D. dianthicola*, and the recently identified *P. punjabense* species also exist in Pakistan (Sarfraz et al. 2018). These results are important because many *Pectobacterium* species have also been reported as the most important blackleg and soft rot pathogens in Morocco and

Turkey (Faquih et al. 2015; Ozturk et al. 2018; Terta et al. 2010). Therefore, to identify the bacteria belonging to the Pectobacteriaceae family and especially to characterize the pectinolytic bacteria (*Pectobacterium* and *Dickeya* genera), a PCR-sequencing tool designed by Cigna et al. (2017) was used. Hence, the analysis with *gapA* sequences enabled us to identify all pectinolytic bacteria at species level in six different clades. Clade I includes strains of *P.*

Fig. 1. Phylogenetic analysis of all species identified in this study based on their *gapA* nucleotide sequences. Strains in bold letters are from this study, whereas all others are reference strains from the NCBI database. Phylogenetic analyses were conducted in MEGA7 software by maximum likelihood method. *Pseudomonas aeruginosa* strains were used as an outgroup to root the phylogeny tree.

atrosepticum, clustered with the reference strain *P. atrosepticum* 1526; clade II comprises strains of *P. polaris* clustered with the reference strain *P. polaris* NIBIO 1006; and clade III includes strains of *D. dianthicola* clustered with *D. dianthicola* GBBC2039. Similarly, clade IV includes strains of *P. parmentieri* clustered with its reference strain *P. parmentieri* RNS 08-42-1A, whereas clade V includes strains of *P. punjabense* clustered distinctly with its closest relatives, *P. wasabiae* CFBP 3304 and *P. parmentieri* RNS 08-42-1A. Meanwhile, clade VI consists of *P. versatile* strains, which also linked to its respective clade with *P. versatile* CFBP6051 (Figs. 1 and 2).

Overall, molecular characterization showed that 88% of isolates belonged to *Pectobacterium* and 12% to *Dickeya* species. Of the 36 isolates, Chiniot showed the presence of diverse species of *Pectobacterium*, including three strains of *P. atrosepticum*, three of *P. parmentieri*, and three of the newly identified *P. punjabense*. However, the representative isolates of the Okara region showed the presence of genus *Pectobacterium* and *Dickeya*, with two strains of *P. polaris*, eight strains of *P. atrosepticum*, and four strains of *D. dianthicola*. Moreover, the Faisalabad region showed 100% prevalence of *P. versatile* with three strains; similarly, the other two regions, Depalpur and Pakpattan, also showed 100% occurrence, but of *P. atrosepticum*, with five strains from each region. Therefore, our study clearly validates the predominance of *P. atrosepticum* in Pakistan. By and large, high variability between the *Pectobacterium* species isolated from five different regions of the Punjab province has been observed. Similarly, Ozturk et al. (2018) and Faquih et al. (2015) observed a good diversity among their isolates selected for molecular characterization, tested with multiplex PCR and with species-specific PCR, and identified different strains as *P. atrosepticum*, *P. carotovorum* subsp. *brasiliense*, *P. carotovorum*, and *P. parmentieri*.

Some recent studies have revealed changes in the positioning of a number of strains within *Pectobacterium* and *Dickeya* species and highlighted the need for species reclassification based on genomic data (Khayati et al. 2015; Nykyri et al. 2012; Pritchard et al. 2016; Zhang et al. 2016). Thus, on the basis of *gapA* sequence analyses, it was decided to genome sequence representative strains from each group to get more insight from genomic studies. Therefore, one strain from each of six species was selected for draft genome sequencing to confirm their taxonomic position in the tree via MLSA. Khayati et al. (2016) also reestablished the taxonomic position of *P. wasabiae* to *P. parmentieri* by using molecular methods such as MLSA (Glaeser and Kämpfer 2015) and genome-to-genome distance (Meier-Kolthoff et al. 2013). The new species-level clade, differentiated from the other group of *Pectobacterium* species based on the results of a phylogenetic tree constructed by concatenating seven housekeeping genes (*acnA*, *gapA*, *icdA*, *mdh*, *mtlD*, *pgi*, and *proA*), has also been reported by Ma et al. (2007) and Nabhan et al. (2012). Moreover, Oulghazi et al. (2019a, b) used 13 housekeeping genes (*fusA*, *rpoD*, *rpoS*, *glyA*, *purA*, *groEL*, *gapA*, *rplB*, *leuS*, *recA*, *gyrB*, *infB*, and *secY*) in MLSA to confirm the existence of new *Pectobacterium* and *Dickeya* species. Therefore, based on the earlier studies, a robust phylogenetic analysis of the six representative strains and reference strains from NCBI database was done via MLSA of 13 housekeeping genes (*fusA*, *rpoD*, *acnA*, *purA*, *gyrB*, *recA*, *mdh*, *mtlD*, *groEL*, *secY*, *glyA*, *gapA*, and *rplB*) that exist in nearly all *Pectobacteriaceae* belonging to the genera *Pectobacterium* and *Dickeya*

(Fig. 2). Therefore, all six representative strains were placed in six different groups or clades, showing the same results as with *gapA* sequence analysis (Fig. 2). In addition, at the genome scale, several *in silico* methods were also available to evaluate assignment to existing species and proposition of novel species. Khayati et al. (2016) used isDDH and ANI values to differentiate the pairings between *P. wasabiae* CFBP 3304T and two *P. parmentieri* strains (RNS 08-42-1AT and RNS14.18.2.1A), which showed 68.5 and 67.4% isDDH values, respectively, thus ensuring the transfer of the *P. wasabiae* to *P. parmentieri*. Similarly, Waleron et al. (2019a) identified and reported a new species, *Pectobacterium zantedeschiae*, by genome sequencing the strains and making their comparison with whole genome sequences of other *Pectobacterium* type strains by using isDDH and ANI values. Therefore, we also used genomic data from our six strains to calculate isDDH and ANI values. When phylogenetic relatives were compared, ANI and isDDH values were consistent with the existing identifications of the taxonomic position of these species, which had been generally depended on wet-lab DDH values, biochemical tests, and 16S rRNA sequencing (Brady et al. 2012; Gardan et al. 2003). However, the ANI and isDDH values found in this study were very much in line with the results of the phylogenetic studies of *gapA* sequences alone

Fig. 2. Phylogenetic tree based on multilocus sequence analysis (MLSA). Six representative strains from each species (SS26, SS28, SS70, SS90, SS95, and SS96) were selected for MLSA based on concatenated genes *fusA*, *rpoD*, *rpoS*, *acnA*, *purA*, *recA*, *mdh*, *mtlD*, *groEL*, *secY*, *glyA*, *gapA*, and *rplB*. The evolutionary history was inferred via the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The evolutionary distances were computed via maximum composite likelihood method and are in the units of the number of base substitutions per site. The analysis involved 22 nucleotide sequences. There were a total of 17,940 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Table 2. Accession number and statistics for the draft genome sequences

Strain	GenBank accession number	Genome size (bp)	Contig N50 (bp)	No. of contigs	No. of coding DNA sequences	No. of transfer RNAs	Genome coverage
<i>Pectobacterium atrosepticum</i> SS26	QESY000000000	4,927,512	90,297	110	4,293	60	36
<i>P. polaris</i> SS28	QESX000000000	4,722,413	243,038	37	4,174	59	56
<i>Dickeya dianthicola</i> SS70	QESZ000000000	4,797,592	177,218	62	4,163	59	71
<i>P. parmentieri</i> SS90	QESW000000000	4,917,173	176,176	50	4,395	62	64
<i>P. punjabense</i> SS95	PYSO000000000	4,732,531	116,258	73	4,193	64	72
<i>P. versatile</i> SS96	QESV000000000	5,042,015	211,427	64	4,382	61	60

and by MLSA of 13 housekeeping genes. All genomes used in these *in silico* analyses found ANI and isDDH values well below the suggested 96% cutoff for the ANI value and 70% cutoff for the isDDH value when distinct species were compared (Table 3). Similarly, some previous studies reported that most intraspecies ANI values were $\geq 96\%$ and isDDH values were $\geq 70\%$ (Colston et al. 2014; Zhang and Qui 2015; Zhang et al. 2016).

Different *Pectobacterium* species can also be differentiated from all other strains solely on the basis of acid production from different carbon sources supplemented in minimal M9 medium (Elbing and Brent 2002). Positive results were obtained with 1-0 β methyl glucopyranoside, D-lactose, melibiose, gluconic acid, raffinose, galactose, cellobiose, and D-mannose, whereas negative results were obtained with 1-0-methyl- α -D glucopyranoside, L-alanine, and maltose (Table 3). These results were consistent with the results of Khayi et al. (2016), in which different *Pectobacterium* species were phenotypically distinguished from *P. wasabiae* strains by producing acids from (+)-raffinose, α -D(+)-a-lactose, D(+)-galactose, and (+)-melibiose but not from methyl α -D-glucopyranoside, (+)-maltose, or malonic acid. Moreover, recently proposed *P. punjabense* strain SS95^T showed completely

different results, when tested along with its nearby aligning strain *P. wasabiae* CFBP 3304T, by producing acids from melibiose, raffinose, and galactose (Table 4) (Sarfranz et al. 2018; Khayi et al. 2016).

Conclusion

Our work revealed the diversity of pectinolytic pathogens of potato plants in Pakistan. These characteristics are important for effective epidemiological observations and avoidance and management of these important plant pathogens. Thus, given the diversity of blackleg pathogens in different regions of Pakistan including Okara, Chiniot, Faisalabad, Depalpur, and Pakpattan, a high risk of potato blackleg outbreaks can be assumed in the near future as the numbers of fields are increased because of the demand for potato production. Because these pectinolytic bacteria are multihost pathogens, their occurrence in potato fields may put other crops at risk, such as chili, tomato, pepper, and cucumber. The presented study is the first step in characterizing the population of *Pectobacterium* and *Dickeya* spp. to evaluate the danger of pectinolytic bacteria in different regions of Pakistan.

Table 3. Average nucleotide identity (ANI) and *in silico* DNA–DNA hybridization (isDDH) values^a

Strains	ANI values															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 <i>Pectobacterium atrosepticum</i> SS26	–	99.5	88.8	88.9	88.8	88.8	89.1	89.1	88.7	89.3	92.8	88.7	91.1	79.2	79.2	79.3
2 <i>P. atrosepticum</i> ICMP1526T	94.6	–	88.7	89.0	89.0	89.0	89.1	89.1	88.0	89.5	93.4	88.7	92.1	79.2	79.4	79.5
3 <i>P. versatile</i> SS96	38.5	38.2	–	95.1	93.5	93.4	88.0	88.0	88.0	88.2	87.9	92.2	88.0	79.6	79.4	79.4
4 <i>P. carotovorum</i> subsp. <i>carotovorum</i> ICMP5702T	38.6	39.1	63.3	–	92.9	92.2	88.0	88.0	88.2	88.2	88.0	92.9	88.1	79.0	78.8	79.0
5 <i>P. polaris</i> SS28	38.2	38.2	54.1	52.2	–	96.8	88.1	88.0	88.1	88.1	88.1	93.7	88.1	79.2	79.0	79.4
6 <i>P. polaris</i> NIBIO 1006T	38.4	38.8	54.3	52.3	73.9	–	88.0	88.0	88.2	88.2	87.8	93.6	88.0	79.5	79.3	79.5
7 <i>P. parmentieri</i> SS90	39.2	39.3	36.5	36.3	36.4	36.4	–	99.0	90.7	93.6	88.1	88.2	87.8	79.2	79.0	79.4
8 <i>P. parmentieri</i> RNS 08-42-1AT	39.2	39.3	36.3	36.5	36.4	36.4	91.0	–	90.7	93.6	88.2	88.3	87.7	79.1	79.0	79.7
9 <i>P. punjabense</i> SS95	38.4	38.0	36.4	36.8	36.7	36.9	44.1	44.1	–	90.4	87.8	88.7	87.6	79.2	79.1	78.9
10 <i>P. wasabiae</i> CFBP 3304T	39.9	40.0	36.8	36.8	36.5	36.8	54.8	54.7	43.3	–	88.3	88.2	88.0	79.3	79.2	79.8
11 <i>P. peruvienne</i> IFB5232T	51.4	49.2	36.4	36.1	36.4	35.6	36.2	36.7	36.2	36.7	–	90.1	90.5	77.5	77.2	77.6
12 <i>P. carotovorum</i> subsp. <i>brasiliense</i> LMG21371T	38.7	38.8	49.2	51.7	54.8	55.7	36.9	37.4	38.1	37.1	49.4	–	88.8	79.4	79.2	79.3
13 <i>P. betavascularum</i> NCPPB2795T	45.3	49.0	36.0	36.6	36.6	36.5	36.0	35.8	35.5	36.6	44.2	39.8	–	77.6	77.0	77.2
14 <i>Dickeya dianthicola</i> SS70	20.7	20.7	21.0	20.4	20.9	21.1	20.7	20.9	20.5	20.8	20	20.9	19.6	–	99.6	91.7
15 <i>D. dianthicola</i> NCPPB453T	20.8	20.7	21.0	20.5	20.9	21.1	20.7	20.9	20.4	20.8	19.9	20.9	19.4	96.4	–	91.8
16 <i>D. solani</i> IPO 2222T	21.2	21.2	20.8	21.0	21.1	21.6	21.2	21.5	20.8	21.5	19.8	21.6	19.6	46.3	46.4	–

^a Strains in bold are from this study. Dashes (–) differentiate the ANI and DDH value columns.

Table 4. Phenotypic characterization^a

Carbon source (20% conc.)	Bacterial strain									
	<i>Pectobacterium atrosepticum</i> SS26	<i>P. polaris</i> SS28	<i>P. parmentieri</i> SS90	<i>P. punjabense</i> SS95	<i>P. parmentieri</i> RNS 08-42-1A ^T	<i>P. wasabiae</i> CFBP 3304 ^T	<i>P. versatile</i> SS96	<i>P. dianthicola</i> SS70	<i>Dickeya dianthicola</i> CFBP 2015	<i>D. solani</i> 0432.1
l-o-Methyl-β-D glucopyranoside	+	+	+	+	+	+	+	NT	NT	NT
l-o-Methyl-α-D glucopyranoside	–	–	–	–	–	–	–	NT	NT	NT
D-lactose	+	+	+	+	+	–	+	NT	NT	NT
Melibiose	+	+	+	+	+	–	+	+	+	+
Glyconic acid	+	+	+	+	+	+	+	NT	NT	NT
L-alanine	–	–	–	–	–	–	–	NT	NT	NT
D-raffinose	+	+	+	+	+	–	+	+	+	+
D-maltose	–	–	–	–	–	–	–	NT	NT	NT
D-galactose	+	+	+	+	+	–	+	NT	NT	NT
D-mannose	+	+	+	+	+	+	+	NT	NT	NT
D-cellobiose	+	+	+	+	+	+	+	NT	NT	NT
L-rhamnose	NT	NT	NT	NT	NT	NT	NT	+	+	–
D-arabinose	NT	NT	NT	NT	NT	NT	NT	–	–	+

^a (+) indicates optical density (OD₆₀₀) >1.5; (–) indicates OD₆₀₀ <0.04; NT, not tested.

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