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Multilocus sequence and phenotypic analysis of *Pectobacterium* and *Dickeya* type strains for identification of soft rot *Pectobacteriaceae* from symptomatic potato stems and tubers in Pennsylvania

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ABSTRACT

Outbreaks of potato blackleg and soft rot caused by *Pectobacterium* species and more recently *Dickeya* species across the U.S. mid-Atlantic region have caused yield loss due to poor emergence as well as losses from stem and tuber rot. To develop management strategies for soft rot diseases, we must first identify which members of the soft rot *Pectobacteriaceae* are present in regional potato plantings. However, the rapidly expanding number of soft rot *Pectobacteriaceae* species and the lack of readily available comparative data for type strains of *Pectobacterium* and *Dickeya* hinder quick identification. This manuscript provides a comparative analysis of soft rot *Pectobacteriaceae* and a comprehensive comparison of type strains from this group using rep-PCR, MLSA and 16S sequence analysis, as well as phenotypic and physiological analyses using Biolog GEN III plates. These data were used to identify isolates cultured from symptomatic potato stems collected between 2016 and 2018. The isolates were characterized for phenotypic traits and by sequence analysis to identify the bacteria from potatoes with blackleg and soft rot symptoms in Pennsylvania potato fields. In this survey, *P. actinidiae, P. brasiliense, P. polonicum, P. polaris, P. punjabense, P. parmentieri,* and *P. versatile* were identified from Pennsylvania for the first time. Importantly, the presence of *P. actinidiae* in Pennsylvania represents the first report of this organism in the U.S. As expected, *P. carotorvorum* and *D. dianthicola* were also isolated. In addition to a resource for future work studying the *Dickeya* and *Pectobacterium* associated with potato blackleg and soft rot, we provide recommendations for future surveys to monitor for quarantine or emerging soft rot *Pectobacteriace* regionally.

Introduction

A variety of pectolytic organisms, referred to as soft rot *Pectobacteriaceae* (SRP), cause black leg and soft rot of potato (*Solanum tuberosum*) world-wide (van der Wolf et al., 2021). The diversity of pathogens causing outbreaks is generally regionally specific, although the spread on vegetative materials may mask some regional differences (Toth et al., 2011). The taxonomy of SRP has evolved and during the past four years, a significant number of new *Pectobacterium* Waldee, 1945 emend. Hauben et al., 1999 and *Dickeya* Samson et al., 2005 species have been described (Toth et al., 2021; Parte et al., 2020). Although descriptions of new species and taxonomic reviews of these genera provide sequences of individual or multiple genes or whole genomes for comparison, comprehensive comparisons of type strains for diagnostic characters are not available and the resultant identifications generally based solely on sequence analyses (Curland et al., 2021; Motyka-Pomagruk, 2021; Sarfraz et al., 2020; Theron et al., 2022).

The deposition of 16S rRNA sequences is required to describe new species according to various minimum standards to be followed according to the International Code of Nomenclature of Prokaryotes (Parker et al., 2019; Tindall et al., 2010) leading to their use as the most important barcode for species identification for bacteria including members of the SRP (Sławiak et al., 2009). The 16S rRNA locus has been extensively used to characterize the *Pectobacteriaceae* (Czajkowski et al., 2009; Raoul des Essarts, 2016; Ma et al., 2007) and to describe new species (van der Wolf, 2014; Gevers et al., 2005; Khayi et al., 2016; Nabhan et al., 2012). However, 16S rRNA sequence comparisons provide limited taxonomic discrimination at the species level (Ma et al., 2007; Ranjan et al., 2016). Chun et al. (2018) recommended a two-step process starting with 16S rRNA sequence analysis for the taxonomy of

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prokaryotes. Surveys of SRP have used other single genes as 'barcodes' (e.g., *gapA*, Sarfraz et al., 2020; *dnaX,* Sławiak et al., 2009), however, databases for these are not as widely available. Although whole genome sequence analysis is the new gold standard for differentiating species, whole genome sequences are not yet required to propose new species and may not be available for all type strains or isolates being evaluated. Additionally, plant disease clinics, the first line of defense in the detection of newly introduced phytopathogens, are not regularly using whole genome sequencing to identify bacterial pathogens (Bull and Koike, 2015). Nevertheless, some SRP surveys have used up to 13 housekeeping genes for MLSA (Sarfraz et al., 2020), but previous studies indicated that the concatenated housekeeping genes *gyrB-dnaJ-dnaX* are sufficient to distinguish species of *Dickeya* and *Pectobacterium* for plant health surveys (Brady et al., 2012; Curland et al., 2021; Marrero et al., 2013; van der Wolf et al., 2014; Khayi et al., 2016; Moretti et al., 2016; Oulghazi et al., 2019).

Likewise, REP-PCR has been used in a variety of studies to differentiate *Pectobacterium* and *Dickeya* strains (Czajkowski et al., 2009; Sławiak et al., 2009; Ngadze et al., 2012; Golanowska et al., 2017). However, it is not clear whether comparisons of field isolates to type strains is sufficient for isolate identification especially considering the recent taxonomic splintering of these genera.

Despite the tendency to rely on genetic data for identification, species proposals still require comparative biochemical and physiological data (Parker et al., 2019; Tindall et al., 2010). The methods used for acquiring phenotypic data vary for each nomenclatural proposal. The Biolog GEN III MicroPlate plates have been increasingly used to describe novel *Pectobacterium* and *Dickeya* species because of their ease of use and ability to consistently compare a wide array of substrates and traits to differentiate between closely related species (Portier et al., 2019; Waleron et al., 2018; Waleron et al., 2019a; Waleron et al., 2019b; Pédron et al., 2019; Tian et al., 2016). Alternatively, other commercial phenotyping tools such as bioMérieux Biotype assays have been used to define *Pectobacterium betavasculorum* (Thomson et al., 1981) Gardan et al., 2003, *P. wasabiae* (Goto and Matsumoto, 1987) Gardan et al., 2003, *D. dadantii* subsp. *dadantii* Samson et al., 2005, emend. Brady et al., 2012, *D. dianthicola* Samson et al., 2005, *D. dadantii* subsp. *dieffenbachiae* Samson et al., 2005, emend. Brady et al., 2012, and *D. zeae* Samson et al., 2005. Given the variations in the methodologies and the depth of phenotype testing carried out, there is no reference that directly compares the phenotypes of members of the SRP, particularly the type strains of *Pectobacterium* and *Dickeya* species. This manuscript aims to fill this gap by providing such a reference.

New species of *Pectobacterium* and *Dickeya* have become major concerns throughout the U.S. (Curland et al., 2021). Although the origin of these pathogens in the U.S. remains unknown (Johnson, 2015), *Dickeya* and *Pectobacterium* species have led to significant yield losses across the northeast since 2014, particularly in Maine (Johnson et al., 2017) and New York (Ma et al., 2018). The 2014 outbreaks sparked interest in monitoring not only endemic pathogens but also for the potential introduction of the highly virulent quarantine pathogen, *Dickeya solani* van der Wolf et al., 2014. *D. solani* was found to be associated with severe disease outbreaks in Europe in 2011 (Toth et al., 2011). To date, *D. solani* has not been identified in the U.S. (Charkowski, 2018), although two other *Dickeya* species, *D. dianthicola* Samson et al., 2005 and *D. chrysanthemi* (Burkholder et al., 1953) Samson et al., 2005, have been isolated from symptomatic potatoes in the U.S. Due to the threat posed by *D. solani* and other SRP, we sampled potatoes in Pennsylvania to determine the diversity of SRP present on symptomatic potatoes and to begin regional monitoring for introduced potato pathogens.

In this study, we have successfully identified SRP isolated from 2016 to 2018 from symptomatic potatoes in Pennsylvania, employing the aforementioned methodologies. Our study not only offers extensive comparative data for type strains of *Pectobacterium* and *Dickeya* species, which can be valuable for future identification efforts, but also marks the first reporting of various SRP species within Pennsylvania and the U. S. Furthermore, it introduces a strategic approach for regional monitoring of emerging SRP strains.

Methods

Sample collection

Symptomatic potato stems or tubers were collected or received at The Pennsylvania State University Plant Disease Clinic between April and October 2016, 2017 and 2018. Plants exhibited all or a combination of soft rot and black leg symptoms such as wilting, stunting, distinct black lesions, rotted petioles, a hollow pith on the above ground tissue, and/or rotting tubers.

In total, 456 isolates were cultured between 2016 and 2018 (Table S1, Table S2). After differentiating the isolates based on pectolytic activity, soft rot activity, rep-PCR banding pattern, and 16S rRNA sequence similarity, 99 isolates were used in this study. Of that 99, 44 were isolated in 2016, 28 in 2017, and 27 in 2018 (Table S1, Table S2). Isolates were cultured from symptomatic potato stems or tubers from 26 potato fields in Pennsylvania. Potato samples represented 11 cultivars (Chieftain, Fabula, Katahdin, Lehigh, MSW485-2, Norwis, NY-140, Reba, Red Norland, Snowden, and Superior) collected from eight Pennsylvania counties (Berks, Cambria, Centre, Franklin, Lancaster, Lehigh, Northampton, and Schuylkill) (Table S1, Table S3).

In 2018, a more systematic and intensive sampling of symptomatic potato plants was conducted in three fields (designated 53, 54, and 55) in Cambria County, Pennsylvania each planted with a single cultivar (Red Norland, Superior, or Reba) (Table S1). In 2018, six symptomatic plants were randomly selected from two quadrats of 200–300 plants per field, for a total of twelve plants collected from each field. For all sampling times, plant samples were wrapped in paper towels and sealed in plastic bags for transport from the field to the laboratory. The samples were refrigerated at a constant temperature of 4 °C for up to nine days before processing.

Bacterial isolation

Stems and tubers were processed for microbial isolation using a slightly modified protocol described by Humphris et al. (2015). The plant samples were rinsed with tap water to remove attached soil and photographed for documentation. Instead, stems were surface sterilized by thoroughly spraying stem samples with 70 percent ethanol until run-off for two minutes followed by a sterilized MilliQ water rinse (Mendes et al., 2007).

For each symptomatic plant sample, one to three tissue pieces (Nikitin et al., 2018) of approximately 1 cm^3 were cut from the margins of symptomatic tissue from a single stem, macerated in ¼ strength Ringer's buffer (Humphris et al., 2015), and used to prepare a serial dilution from a composite sample. In 2016 and 2017, dilutions were plated on single-layer crystal violet pectate (SL-CVP) (Hélias et al., 2012) and incubated at 28 °C for 48 h in the dark in a standing low temperature incubator (Fisher Scientific). In 2018, to test the efficacy of different isolation parameters, dilutions were plated on SL-CVP and nutrient agar (NA). For each sample, one SL-CVP plate and one NA plate were incubated in the dark at 27 °C and 37 °C for 48 h.

In 2018, after 48 h, colonies with unique morphologies on NA were tested for their ability to pit on SL-CVP at 27 °C or 37 °C. Mixed cultures on NA were stored without further undergoing any purification. In all three years, bacterial colonies formed pits on SL-CVP plates (either from serial dilution on SL-CVP or from 2018NA morphology tests). Strains that consistently produced pits on SL-CVP in three replicates were considered pectolytic.

Potato slice inoculation

All pitting isolates were tested for their ability to cause soft rot on

organic Russet potato slices using methods modified from Ma et al. (2018). After surface sterilizing potato slices, sterile toothpicks were used to stab 5 mm deep holes into each potato slice. Individual holes were inoculated with the potato isolates and controls including a positive (*Pectobacterium carotovorum* (Jones, 1901) Hauben et al., 1998, LMG 02404 ^{T}), and negative control (sterile MilliQ water). Each potato slice tested three to six isolates plus one positive and one negative control depending on the slice size. Each isolate was replicated a total of three times. Potatoes were incubated in petri dishes for 24 h at 29 °C in a surface disinfested sealable plastic container with a sterile moistened paper towel. A slightly higher temperature was used for soft rot tests to enhance the development of rot symptoms. After 24 h, potato slices were evaluated for the presence or absence of soft rot symptoms (Ma et al., 2018). The identity of pectolytic soft rotting organisms was investigated in a stepwise manner. A total of 202 isolates from all years were pectolytic (pitting SL-CVP) of which 161 isolates were also capable of consistently rotting potatoes (Table S2).

Pathogen identification

Isolate diversity was characterized by rep-PCR, subsequently 16S rRNA, *gyrB, dnaJ*, and *dnaX* were sequenced for representative isolates having identical or near identical rep-PCR DNA fragment banding patterns and compared to the type strains (Table S4). Primer sequences and amplification conditions are presented (Table S5). Representative isolates were selected from different fields and/or plants when possible.

Rep-PCR

In 2017 and 2018, SL-CVP pitting isolates and *Dickeya* and *Pectobacterium* spp. type strains (Table S4) were grown on NA plates at 27 °C for 24–48 h prior to conducting a rep-PCR with the REP primers (REP1R and REP2I) according to the methods described by Rademaker et al. (1998; Table S5). PCR products were evaluated by gel electrophoresis (1 % TAE agarose gel) at 4 °C, and isolates were manually grouped into rep-PCR groups based on similar DNA fragment banding patterns.

16S rRNA PCR was conducted to amplify the 16S ribosomal gene region of one or two representatives from each 2018 REP-PCR group and all 2016 and 2017 isolates in preparation for sequencing and identification of pectolytic isolates. Representative isolates from unique clades were selected for further MLSA analysis.

Single and multilocus sequence analysis

For isolates collected in 2016 and 2017, amplicons for 16S rRNA, *gyrB, dnaJ*, and *dnaX* were generated from whole cells from a single colony lysed in NaOH (Rademaker et al. 1998) or genomic DNA extracted using the DNeasy Blood and Tissue Kit (QIAGEN) or the DNeasy Plant Mini Kit (QIAGEN) following manufacturer's protocol. Universal primers (27F and 1492R), and the methods described by Lane (1991) were used to amplify16S rRNA (Table S5). Amended methods from Marrero et al., 2013 were used to amplify *gyrB, dnaJ*, and *dnaX* (Table S5). The resulting PCR products were Sanger sequenced at The Pennsylvania State University Nucleic Acid Facility. Consensus sequences were generated from forward and reverse sequence aligned to *Pectobacterium carotovorum* (DSM 30168 ^{T}) using CLC Genomics Workbench 12.0.2 (QIAGEN Bioinformatics).

For isolates collected in 2018, 16S rRNA, *gyrB, dnaJ*, and *dnaX* sequences were extracted from whole genome sequences. Genomic DNA of 41 pitting isolates isolated in 2018 was extracted using the DNeasy Blood and Tissue Kit (QIAGEN) following manufacturer's protocol. Libraries were generated at The Pennsylvania State University Nucleic Acid Facility using a Nextera DNA Flex Kit (Illumina Incorporated 2018). Library preparation and sequencing were performed using 500-cycle Illumina MiSeq with 2 x 300 bp reads. Reads were processed and assembled *de novo* approximately following the pipelines describe in (Kovac et al., 2016). Available whole genome sequences of *Dickeya* and *Pectobacterium* type strains were downloaded from the NCBI database (Table S4). DNA sequences for *gyrB, dnaJ*, and *dnaX* were extracted from draft and downloaded genomes using BTyper v2.3.1 (Carroll et al., 2017). The genes extracted from each type strain of *Dickeya* and *Pectobacterium*, as well as pectolytic isolates, irrespective of their source, were trimmed to 1326 bp (16S rRNA), 714 bp (*gyrB*), 651 bp (*dnaJ*), 434 bp (*dnaX*). To test DNA sequences were in the correct reading frame, sequences were checked with Blastx (NCBI) to ensure they matched with the protein of interest. These genes were then concatenated in that order. All sequences were compared to those of *Dickeya* and *Pectobacterium* type strains using MLSA for species delineation.

Multilocus or single locus sequence analyses were performed using 16S rRNA, *gyrB, dnaJ*, and *dnaX* sequences to compare the SRP isolates to *Pectobacterium* and *Dickeya* type strains. Individual genes were concatenated. After model testing, either single genes or concatenated genes were aligned and the alignments were used to generate neighbor joining phylogenetic trees. All trees were constructed with Jukes-Cantor and 1000 bootstrap replicates using CLC Genomics Workbench 12.0.2 (QIAGEN bioinformatics). The single-gene and concatenated sequence phylogenies were manually compared for branching and clustering patterns for pectolytic isolates and *Dickeya* and *Pectobacterium* type strains.

The discriminatory power of 16S rRNA, *gyrB, dnaJ*, and *dnaX*, as well as the concatenated sequences of *gyrB-dnaJ-dnaX* were conducted (Table S6 and Table S7). A matrix of phylogenetic distances of the *Dickeya* and *Pectobacterium* type strains was constructed for each housekeeping gene and their concatenated sequences to evaluate the discriminatory power of each gene (Mulet et al., 2010). The modelled phylogenetic distance was calculated using the Jukes-Cantor model for each gene and concatenated sequence using CLC Genomics Workbench 12.0.2 (QIAGEN Bioinformatics). The gene with the largest average phylogenetic distance between strains was plotted against the phylogenetic distances between strains using each other gene or phylogenetic distance in a scatterplot in Microsoft Excel. The y-intercept was set to zero and a trend line was added for each set of points representing a gene. The trend line was used to calculate the correlation coefficient and slope for each set of points. The discriminatory power of each gene was calculated as the ratio between the slope of the gene with the largest average phylogenetic distance between strains, and the slopes of each other gene or concatenated sequence (Mulet et al., 2010).

Phenotype comparisons

The physiological and carbon utilization properties of the 99 pectolytic isolates representing all rep-PCR groups were assessed using GEN III MicroPlates (Biolog, Hayward, CA, U.S.), including 71 carbon source utilization assays and 23 chemical sensitivity assays following the manufacturer's protocol. The bacterial isolates were grown overnight on nutrient agar plates for 24 h. The bacteria were suspended in inoculation fluid A which was standardized to 95 % transmittance using a spectrophotometer. A 100 μl bacterial suspension was inoculated into each well of GEN III MicroPlates, which was then incubated for 24 h. The utilization pattern indicated by the color change was monitored using SpectraMAx I3X. Data were collected using SoftMax Pro software. For some type strains, especially those lacking published results, the assays were repeated by a commercial testing laboratory (Technology Access Center in Bio-innovation Center, Ottawa, ON) using manufacture's methods. In this case an Omnilog instrument was used to incubate the plate at 30 °C and read the color change within each reaction well at 24 h. All resulting values were normalized to the negative (A1) or positive (A10) control wells (Data Analysis Software v 1.7). The intensity of these changes was evaluated using an internal algorithm as a positive, moderate, or negative reaction (Data Analysis Software V 1.7). The results from all assays were compared to previously published data for *Dickeya* and *Pectobacterium* type strains.

Results & discussion

Pectolytic isolate collection

The pectolytic bacteria identified in this study were isolated from symptomatic potato stems and tubers collected between 2016 and 2018. In 2016 and 2017, when only the semi-selective SL-CVP media was used for isolation, only pectolytic *Pectobacterium* and *Dickeya* spp. were isolated. However, in 2018, because the general media, NA, was used in addition to SL-CVP for isolation, 353 pectolytic and non-pectolytic isolates were cultured (Table S1 and Table S2).

Regardless of the media on which they were originally cultured, the isolates were tested for pectolytic activity on SL-CVP and on potato slices for soft rot symptoms. Across the three years, 202 isolates were capable of pitting SL-CVP (71, 32, 99 from 2016, 2017, and 2018 respectively) (Table S2). Of the pectolytic isolates, only a few non-rotting (BP7000, BP7034, BP7035, BP7036, BP7048, BP7093, BP7101, and BP9130) or inconsistently rotting (BP9089 and BP9164) isolates were still included in the downstream analysis because of their 16S rRNA placement around *Pectobacterium* and *Dickeya* type strains. These isolates were further identified as *D. dianthicola, P. carotovorum,* and *P. versatile* Portier et al., 2019 (Table S1). The identification of BP7000 remains ambiguous.

As described below, 99 isolates were selected as representatives of rep-PCR groups (2017 and 2018) or 16S rRNA phylogenetic clades (2016) subjected to MLSA and phenotyped with Biolog GEN III MicroPlates™. These 99 isolates primarily came from *Solanum tuberosum* cultivars (cvs) Red Norland, Superior, and Lehigh (Table S3).

Rep-PCR to identify different genotypes

Rep-PCR using repetitive extragenic palindromic (REP) sequences was used to differentiate isolates and evaluate its effectiveness in identifying isolates for all years. For isolates from 2017 and 2018, we used rep-PCR to select representatives with similar DNA fragment banding patterns for further identification (Bull and Koike, 2015; Louws et al., 1999). Isolates from each year were evaluated separately, and their banding patterns were compared to those of the *Pectobacterium* and *Dickeya* type strains. In 2017, there were five unique rep-PCR groups, whereas in 2018, there were 11 unique rep-PCR groups and 15 singletons (data not shown). Select isolates collected in 2016 were analyzed by REP-PCR after 16S rRNA sequences were analyzed to evaluate the similarity of the REP-PCR banding patterns of isolates clustered in the

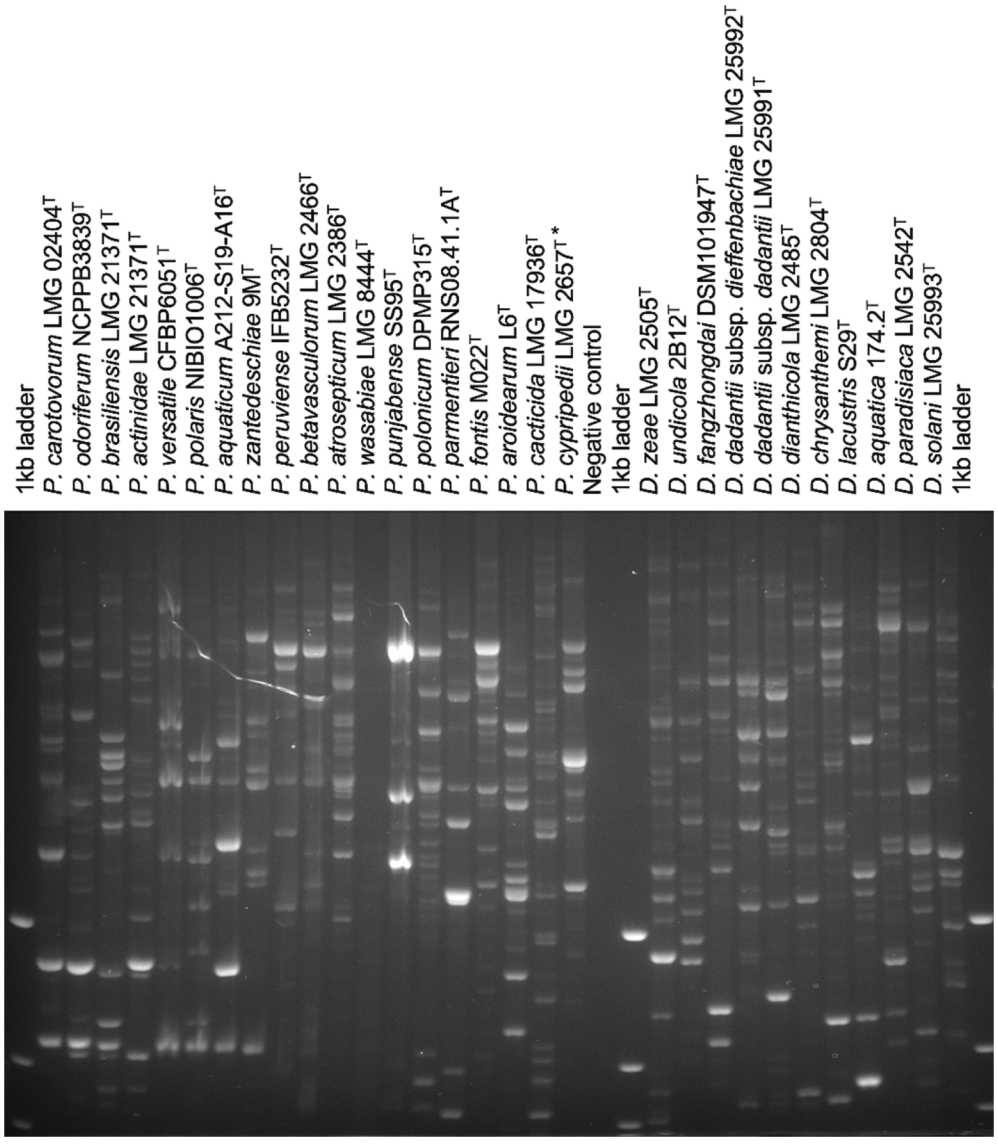

Fig. 1. Rep-PCR banding patterns of *Pectobacterium* and *Dickeya* species type strains. Astricks (*) indicates isolate is *Pantoea cypripedii* (formerly *Pectobacterium cypripedii*).

same clade. Among the chosen 2016 isolates, we identified four distinct groups (data not shown). Identities of isolates that were not further processed were extrapolated to be identical to isolates which were identified and had the same rep-PCR pattern.

DNA fragment banding patterns generated from rep-PCR have been shown to correspond to DNA-DNA homology for *Xanthomonas* species (Louws et al., 1999; Rademaker et al., 2000) and have been used in a variety of studies including strains of *Pectobacterium* and *Dickeya* species (Czajkowski et al., 2009; Slawiak et al., 2009; Ngadze et al., 2012; Golanowska et al., 2017). In this study, each species represented by the type strain exhibited a unique banding pattern (Fig. 1, Fig. S1). However, isolate banding patterns did not always match patterns of the type strains (Fig. S1). For example, isolates identified as *Pecobacterium carovotorum* had some band lengths that were similar to the band lengths produced by the type strain, but overall, isolates were more similar to one another in their banding patterns than to the type strain (Fig. S1). This made it difficult to tentatively identify the isolates using rep-PCR. However, rep-PCR banding patterns were very useful for this large-scale screening to identify similar isolates and to select reference isolates for further identification using other tools.

DNA sequencing and phylogenetic analyses

Initial identification based on 16S rRNA phylogeny and a 97 percent similarity cutoff value showed that, *P. carotovorum* was the most common species isolated from symptomatic potatoes in Pennsylvania (n = 38), followed by *P. brasiliense* (n = 20), *P. odoriferum* (Gallois et al., 1992) Portier et al., 2019 (n = 10), and *P. parmentieri* (n = 10). *Dickeya dianthicola* (n = 9) was the only *Dickeya* species identified in this survey and was isolated in 2016 and 2017 (Fig. 2, Table S1). In 2018, six species *P. aquaticum* (n = 2), *P. parvum* (n = 1), *P. polaris* (n = 1), *P. polonicum* Waleron et al., 2019 (n = 1), *P. punjabense* (n = 1), *P. versatile* (n = 5) not previously been reported from Pennsylvania were isolated from symptomatic potatoes (Fig. 2, Table S1, and Table S3). While the identities of

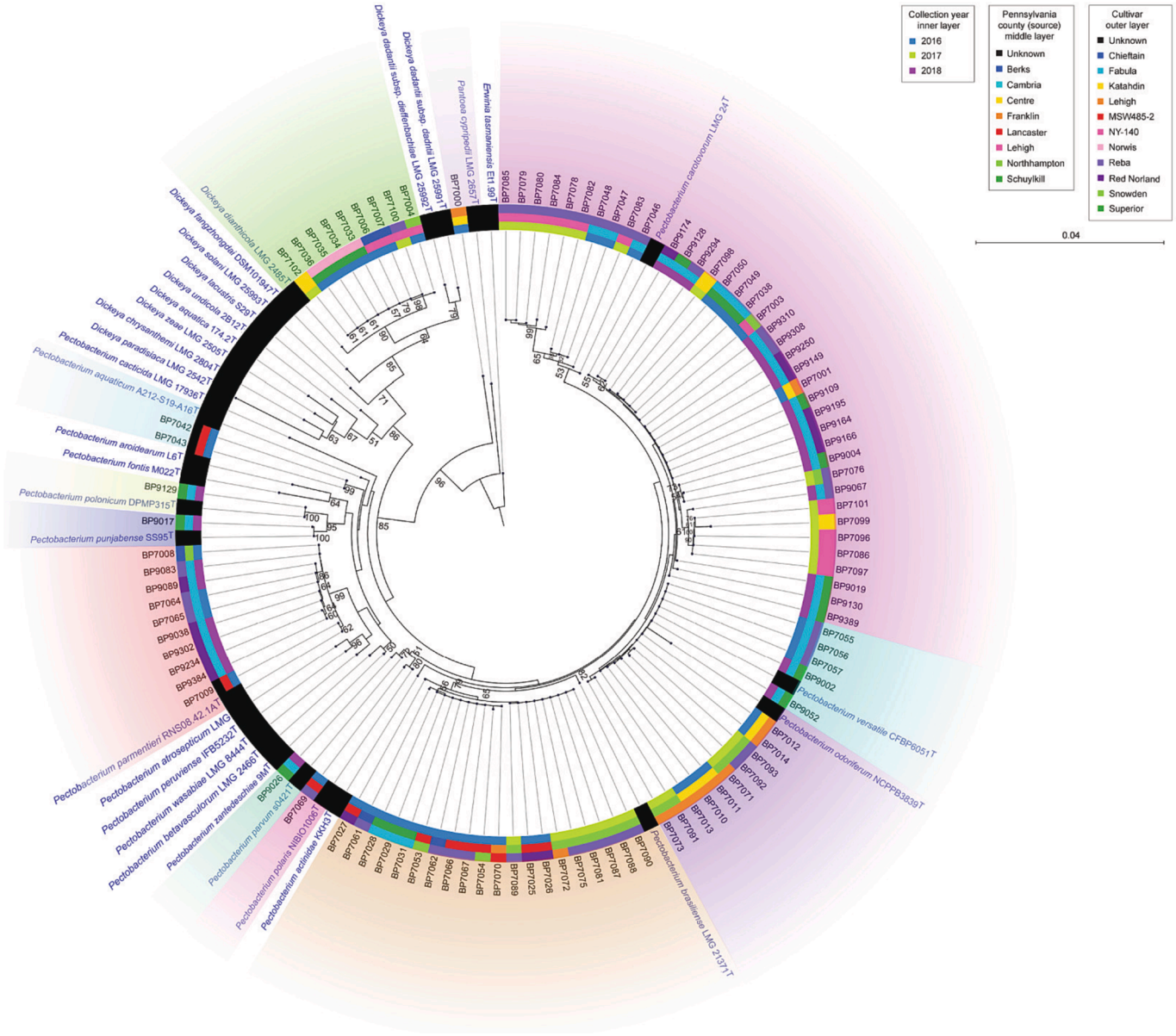

Fig. 2. Neighbor joining phylogenetic tree of concatenated gene sequences of 16S rRNA, of *Pectobacterium* spp. and *Dickeya* sp. isolated from Pennsylvania between 2016 and 2018 along with type strains of each species. The inner band represents the clades of isolates around each type strain with sequence similarity of 98 percent or greater. The outer band represents the cultivar from which each isolate was cultured. Node colors reflect year in which isolate was collected and whether or not the isolate is a type strain. Bootstraps (1,000 replicates) are shown when greater than 50 percent.

most of these isolates were further supported by additional analyses, for a few isolates (53) some we relied on MLSA (see below) and phenotypic results rather than 16S rRNA sequencing for the final determination of their identity species required further resolution to determine the identity of each isolate (Table S8).

The housekeeping gene sequences *gyrB, dnaJ* and *dnaX* were used to verify and refine the taxonomy of the representative pectolytic isolates within each 16S rRNA clade. Discriminatory power analysis and neighbor joining phylogenetic trees were constructed for each locus, as well as for concatenated sequences of *gyrB*, *dnaJ*, and *dnaX* to compare isolates to *Dickeya* and *Pectobacterium* type strains in a MLSA for species delineation. The locus *dnaJ* had the highest average phylogenetic distance between type strains of different species and was therefore used as a baseline to compare the phylogenetic distances between type strains for other genes and concatenated sequences. The *dnaJ* locus was shown to have the highest discriminatory power, and 5.81 times the discriminatory power of the 16S rRNA gene (Fig. 4, Table S6). The discriminatory power of the concatenated *gyrB-dnaJ-dnaX* sequences appeared to be reduced by the inclusion of *gyrB* which had the lowest discriminatory power of the three genes used in the concatenation. Despite the lower discriminatory power compared to *dnaJ* and *dnaX,* the concatenated *gyrB-dnaJ-dnaX* sequences were best for creating the neighbor-joining phylogenetic tree because it had the highest bootstrap support at each node (Fig. 3). The high bootstrap support and discriminatory power of the three gene MLSA ultimately support the use of these three housekeeping genes to represent the phylogenetic relationships between these isolates.

Because of their high discriminatory power, either *dnaJ* or *dnaX* are suitable genes for confirming species level identification for this group of isolates and type strains and are useful loci to include in MLSA for *Pectobacterium* and *Dickeya* species. Of the two, the topology the *dnaJ* phylogeny was most similar to the concatenated phylogeny, with only four isolate placements that did not match the concatenated placement (Fig. S2, S3, and S4). These placement discrepancies were mostly centered around *P. polaris, P. aquaticum, P. actinidiae, P. versatile,* and *P. odoriferum*, suggesting multiple loci are useful for correctly identifying isolates in these species. Other genes, such as *dnaX*, *leuS* and *recA,* may also be used to differentiate *P. polaris, P. aquaticum, P. actinidiae, P. versatile,* and *P. odoriferum* (Portier et al., 2019).

MLSA supported identification of several isolates as *D. dianthicola, P. versatile, P. brasiliense, P. carotovorum, P. polaris, P. punjabense,* and *P. parmentieri* (Figs. 2 and 3, Table S8) confirming tentative 16S rRNA identifications. However, there were some instances in which 16S rRNA identification was inconsistent when additional loci were evaluated. Isolates identified as *P. aquaticum* using only 16S rRNA clustered around *P. actinidiae* in MLSA. Likewise, BP9026 tentatively identified as *P. parvum* by 16S rRNA analysis in the MLSA, clustered closer to *P. polaris*. Finally, six isolates tentatively identified as *P. carotovorum* (BP7082, BP9004, BP9019, BP9109, BP9166, and BP9389) clustered around *P. versatile* when *gyrB-dnaJ-dnaX* were used for MLSA (Table S8). 16S rRNA sequences are often used for initial comparisons to evaluate whether unknown isolates form distinct clades (van der Wolf et al., 2014; Portier et al., 2019) but MLSA or whole genome sequence analysis may be better for surveys of diversity.

Phenotyping with Biolog GEN III plates

Here we provide the most extensive published comparison of

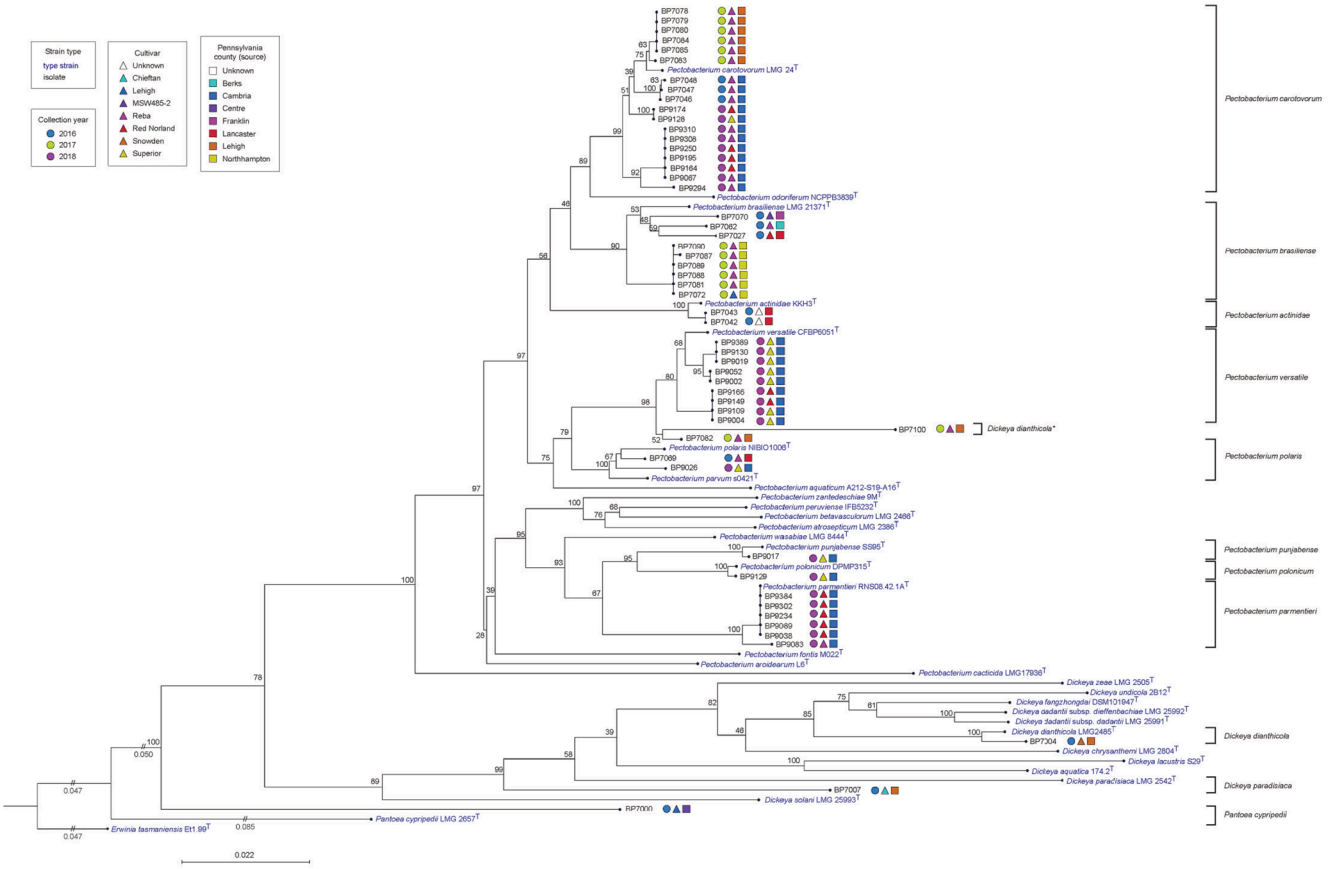

Fig. 3. Neighbor joining multi-locus (*gyrB-dnaJ-dnaX)* phylogeny of *Pectobacterium* and *Dickeya* spp. type strains compared to representative isolates from 2016 to 2018. Branch lengths are drawn to scale except when trimming was necessary (denoted by slashes). Brackets denote isolate identification based on rep-PCR, MLSA, and GENIII methods (Table S3). *BP7100 yields conflicting results depending on the classification method used but is identified as *Dickeya dianthicola* based on *dnaX* and 16S rDNA sequence similarities, and GENIII reaction results.

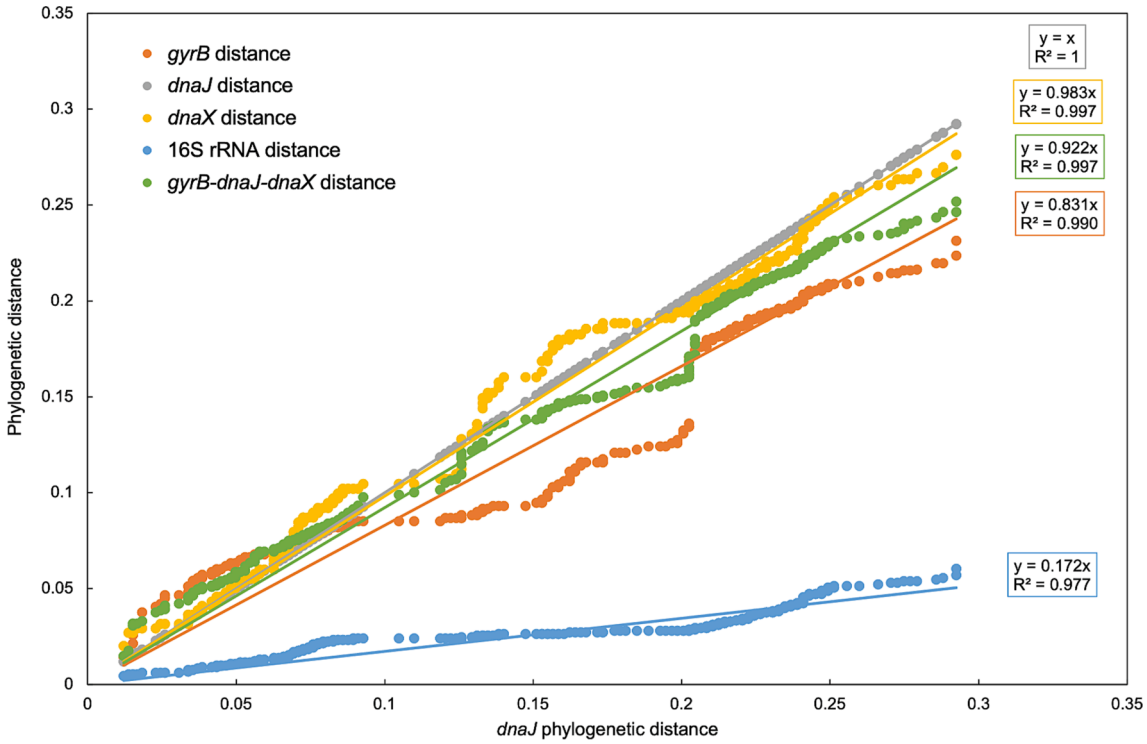

Fig. 4. Least square tendency lines generated from the comparisons of phylogenetic distances of *Dickeya* and *Pectobacterium* spp. type strains. Trend lines are set to zero for clarity. Correlation coefficients and slopes are indicated in the equations beside each line.

substrate utilization patterns based on Biolog GEN III MicroPlates™ (referred to here as GENIII) for *Dickeya* and *Pectobacterium* type strains available to date (Table S9). These comparisons should provide a taxonomic reference useful for future work differentiating between members of the SRP. Furthermore, we characterized representative SRP isolates from Pennsylvania with GENIII and compared them to the reactions of type strains to confirm sequence-based identification. Overall, *Dickeya* and *Pectobacterium* species used for GENIII comparisons reacted as expected with minimal contradictions with the published literature (Table 1 and Table 2. As is common among isolates within species, some phenotypes varied by isolates within species both in previously published literature and in these experiments. Distinguishing features for the species tested within each genus are provided (Table 1 and Table 2).

Based on the distinguishing characteristics reported in species description papers (Samson et al., 2005; van der Wolf et al., 2014; Hugouvieux-Cotte-Pattat et al., 2019; Oulghazi et al., 2019) and comparisons with our GENIII results of type strain reactions, *Dickeya* species can be differentiated from each other by D-Cellobiose, L-Alanine, L-Glutamic Acid, Guanidine Hydrochloride, Glucuronamide, Lithium Chloride, Formic Acid, and Sodium Butyrate (Table 1). Additionally, α-D-Lactose can be used to differentiate between *Dickeya* species, as *D. zeae* and *D. paradisiaca* are the only species that show a positive reaction, whereas others are negative or weak.

Dickeya species can be differentiated from *Pectobacterium* species using assays for Rifamycin SV, Niaproof 4, Pectin, D-Galacturonic Acid, L-Galactonic Acid Lactone, D-Saccharic Acid, Vancomycin, p-Hydroxyphenylacetic Acid, Citric Acid, γ-Amino-Butryric Acid, α-Hydroxy-Butyric Acid, β-Hydroxy-D,L-Butyric Acid, α-Keto-Butyric Acid, Propionic Acid, Sodium Bromate, and growth at pH 6 (Table 1 and Table 2). The type strains of all the species within each genus were similar in these tests.

Pectobacterium species can be differentiated using GEN III tests for Dextrin, D-Maltose, D-Trehalose, D-Cellobiose, D-Turanose, Stachyose, ph5, D-Raffinose, α-D-Lactose, D-Melibiose, β-Methyl-D-Glucoside, N-Acetyl-D- Glucosamine, 4 % NaCl, Inosine, Fusidic Acid, D-Serine, D-Sorbitol, D-Arabitol, myo-Inositol, D-Serine, Gelatin, L-Alanine, L-Glutamic Acid, L-Serine, Lincomycin, Guanidine Hydrochloride, D-Galacturonic Acid, L-Galactonic Acid Lactone, D-Glucuronic Acid, Glucuronamide, L-Lactic Acid, Citric Acid, α-keto-Glutaric Acid, Nalidixic Acid, and Lithium Chloride (Table 2). Furthermore, assays for D-Salicin, N-Acetyl-D-Galactosamine, α-D-Glucose, D-Mannose, D-Fructose, D-Mannitol, Glycerol, Rifamycin SV, L-Malic Acid, γ-Amino-Butryric Acid, α-Hydroxy-Butyric Acid, β-Hydroxy-D,L-Butyric Acid, and α-Keto-Butyric Acid are suitable for differentiating *Pectobacterium* species from other genera, as type strains for all species reacted similarly (Table 2). These conclusions are based on distinguishing characteristics reported in species description papers (Hauben et al., 1998; Gardan et al., 2003; Brady et al., 2010; Khayi et al., 2016; Sarfraz et al., 2018; Waleron et al., 2018; Oulghazi et al., 2019; Pédron et al., 2019; Portier et al., 2019; Waleron et al., 2019a; Waleron et al., 2019b; Pasanen et al., 2020) and comparisons to our results from type strains.

Several differences were observed between the strains of *P. brasiliense* isolated in Pennsylvania and the type strain. The type strain for *P. brasiliense* reacted as reported in the literature for Dextrin (+), D-maltose (+), D-turanose (+), and L-Lactic Acid (+) on GENIII plates, however all SRP isolates identified by sequencing as *P. brasiliense* did not react positively to these substrates. Nabhan et al. (2012) evaluated phenotypic diversity for *P. brasiliense* strains from Brazil, Syria, Peru, and Canada using Biolog GN2 microplates and found two unique clades of *P. brasiliense.* The two clades separate by MLSA (using *mtlD, acnA, icdA, mdh, pgi, gabA, proA* and *rpoS*) and 16S rRNA, and show slight differences in phenotypes. Clade I more closely match the phenotypic profile of the type strain (212 ^{T}) because it utilizes lactic acid, however Clade II does not. Our Pennsylvania strains results more closely matched the phenotypes of Clade II *P. brasiliense* as the 20*P. brasiliense* isolates did not utilize dextrin, maltose, or lactic acid (Table S9). The reaction of *Pectobacterium brasiliense* Clade II to D-turanose (+) is not reported (Nabhan et al., 2012). This is an example of the variability within a species that is iteratively defined. It would be beneficial to include the type strain and references for *P. brasiliense* clades for the identification of this organism.

Table 1
Phenotypic differentiation of between species with within the genus *Dickeya*.

Biolog GENIII assay		*Dickeya* species														
		Dickeya aquatica LMG 27354[T]	*Dickeya chrysanthemi* LMG 02804[T]	*Dickeya dadantii* subsp. *dadantii* LMG 25991[T]	*Dickeya dadantii* subsp. *dieffenbachiae* LMG 25992[T]	*Dickeya dadantii* subsp. *dieffenbachiae* LMG 25992 [T§]	*Dickeya dianthicola* LMG 02485[T]	*Dickeya fangzhongdai* DSM 101947[T]	*Dickeya lacustris* S29[T§]	*Dickeya lacustris* S29[T]	*Dickeya paradisiaca* LMG 02542 [T§]	*Dickeya paradisiaca* LMG 02542[T]	*Dickeya solani* LMG 25993 [T§]	*Dickeya solani* LMG 25993[T]	*Dickeya undicola* LMG 30903[T]	*Dickeya zeae* LMG 02505[T]
A05	D-Cellobiose	+/-	+/-	+	+	-	-	+	-	+	-	-	-	-	+/-	+
A11	ph6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B02	α-D-Lactose	+/-	+/-	–	+/-	–	–	–	–	–	–	+	–	–	+/-	+
D06	D-Glucose-6-PO_4†	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+
D11	Rifamycin SV†	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E03	L-Alanine	+/-	+/-	+/-	+	–	–	+/-	–	+	–	–	–	–	+/-	+
E06	L-Glutamic Acid	+	+	+	+	–	+/-	+/-	–	+	–	–	–	–	+	+
E11	Guanidine Hydrochloride	+	+	+	+	–	+/-	+	–	–	–	–	–	–	+	–
E12	Niaproof 4†	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F01	Pectin†	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F02	D-Galacturonic Acid†	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F03	L-Galactonic Acid Lactone†	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F06	Glucuronamide	+/-	–	+/-	+/-	–	–	+/-	–	+	–	+	–	+	+/-	+
F09	D-Saccharic Acid†	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F10	Vancomycin†	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
G01	p-Hydroxy-Phenylacetic Acid†	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
G05	Citric Acid†	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
G11	Lithium Chloride	+/-	+	+	+	–	–	+	–	–	–	–	–	–	+	–
H02	γ-Amino-Butryric Acid†	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
H03	α-Hydroxy-Butyric Acid†	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
H04	β-Hydroxy-D,L-Butyric Acid†	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
H05	α-Keto-Butyric Acid†	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
H07	Propionic Acid†	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
H09	Formic Acid	+/-	+	+/-	+	–	+/-	+	–	–	–	–	–	–	+/-	+
*H*11	Sodium Butyrate	+	+/-	+	+/-	–	+/-	+/-	–	–	–	–	–	–	+	+
H12	Sodium Bromate†	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

+ refers to positive response on Biolog GEN III plates.
- refers to negative response on Biolog GEN III plates.
w refers to weak response on Biolog GEN III plates.
† refers to substrates to be used for differentiating *Dickeya* spp. from other genera.
§ refers to Biolog GEN III plate results from type strains generated by a commercial laboratory that conflict with results generated by the Bull lab and additional isolates were not tested to further support results.
[T] type strain of species.

Table 2
Phenotypic differentiation between species within the genus *Pectobacterium*.

		Pectobacterium species																												
	Biolog GENIII assay	*Pectobacterium actinidiae LMG 26003T* §	*Pectobacterium actinidiae LMG 26003T*	*Pectobacterium aquaticum* CFBP 8637^{T}	*Pectobacterium aroidearum* LMG 02417^{T}	*Pectobacterium atrosepticum* LMG 02386^{T}	*Pectobacterium betavasculorum* LMG 02466^{T§}	*Pectobacterium betavasculorum* LMG 02466^{T}	*Pectobacterium brasiliensis* LMG 21371^{T}	*Pectobacterium cacticida* LMG 17936^{T§}	*Pectobacterium cacticida* LMG 17936^{T}	*Pectobacterium carotovorum* LMG 02404^{T}	*Pantoea cypripedii* LMG 02657^{T}*§	*Pantoea cypripedii* LMG 02657^{T}*§	*Pectobacterium fontis* LMG 30744^{T§}	*Pectobacterium fontis* LMG 30744^{T}	*Pectobacterium odoriferum* LMG 06688^{T}	*Pectobacterium parmentieri* LMG 29774^{T§}	*Pectobacterium parmentieri* LMG 29774^{T}	*Pectobacterium peruviense* LMG 30269^{T}	*Pectobacterium polaris* NCPPB 4611^{T§}	*Pectobacterium polaris* NCPPB 4611^{T}	*Pectobacterium polonicum* LMG 31077^{T§}	*Pectobacterium polonicum* LMG 31077^{T}	*Pectobacterium punjabense* LMG 30622^{T§}	*Pectobacterium punjabense* LMG 30622^{T}	*Pectobacterium versatile* CFBP 6051^{T}	*Pectobacterium wasabiae* LMG 08444^{T}	*Pectobacterium zantedeschiae* PCM 2893^{T}
A02	Dextrin	-	+	-	+	+/-	-	+	+	-	-	+/-	-	-	+/-	-	+/-	-	+	+/-	-	+/-	-	-	+/-	-	+/-	+/-	+
A03	D-Maltose	-	+	-	-	-	-	+	+	-	-	+/-	+/-	-	-	-	+/-	-	-	-	-	-	-	-	-	-	+/-	-	-
A04	D-Trehalose	-	+	-	+	+/-	-	+	+	+/-	+	+	+/-	+	+	+	+	+	+	+/-	-	+	+	-	+	+	+/-	+	-
A05	D-Cellobiose	+	+	-	+	+	-	+	+	-	-	+	+/-	+	+/-	-	+	+	+	+	+	+	+	+	+	+	+/-	+	+
A08	D-Turanose	-	-	-	-	+/-	-	+	+	-	-	+/-	-	-	+/-	-	+/-	-	-	+/-	-	+/-	-	-	+/-	-	+/-	+/-	-
A09	Stachyose	+	+	-	+	+	-	-	+	-	-	+	-	-	-	-	+	+	+	+	+	+	+/-	-	+	+	+/-	-	-
A12	ph5	-	-	+	-	+/-	-	+	+	-	-	+/-	+/-	-	+/-	-	+/-	-	+	+/-	-	+/-	+/-	-	+/-	-	+/-	+/-	-
B01	D-Raffinose	+	+	+	+	+	-	+	+	-	-	+	-	-	-	-	+	+	+	+/-	+	+	+	+	+	+	+	-	+
B02	α-D-Lactose	-	+	+	+	+	-	+	+	+/-	-	+	-	-	+/-	-	+	+	+	+	+	+	+	+	+	+	+/-	-	+
B03	D-Melibiose	+	+	+	+	+	-	+	+	-	-	+	+/-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+
B04	β-Methyl-D-Glucoside	+	+	+	+	+	+	+	+	+/-	+	+	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B05	D-Salicin†	+	+	+	+	+/-	+	+	+	+/-	+	+	+	+/-	+	+	+	+	+	+	+	+/-	+	+	+	+	+	+	+/-	+
B06	*N*-Acetyl-D-Galactosamine†	+	+	+	+	+	+	+	+	+/-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B08	N-Acetyl-D- Glucosamine	-	-	-	-	-	-	+	-	-	-	+/-	+/-	-	+/-	-	+/-	+	+	-	-	-	-	-	-	-	+	-	-
B11	4% NaCl	-	-	+	-	+/-	-	+	+	+/-	-	+	+/-	-	+/-	-	+/-	-	-	+/-	-	+/-	+	-	+/-	-	-	+/-	-
C01	α-D-Glucose†	+	+	+	+	+/-	+	+	+	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+/-	-
C02	D-Mannose†	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C03	D-Fructose†	+	+	+	+	+	+	+	+	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C09	Inosine	-	+	-	+	+/-	-	+	+	-	-	+/-	+/-	-	+/-	-	+/-	-	-	+/-	-	+/-	+/-	-	+/-	-	+/-	+/-	-
C11	Fusidic Acid	-	+	+	+	+/-	-	+	-	-	-	+/-	+/-	-	+/-	-	+/-	-	+	+	-	+	+/-	-	+	-	+/-	+/-	-
C12	D-Serine	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D01	D-Sorbitol	-	+	-	+	-	-	+	-	-	-	+/-	-	-	+/-	-	+	-	-	+/-	-	+/-	-	-	-	-	+/-	-	+
D02	D-Mannitol†	+	+	+	+	+	+	+	+	+/-	+	+	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+/-	+	+
D03	D-Arabitol	-	-	-	+	-	-	+	-	-	-	+/-	+/-	-	+/-	-	+	-	+	+/-	-	-	-	-	-	-	+/-	-	-
D04	*myo*-Inositol	-	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+/-	+	+	+	+	+

(*continued on next page*)

Table 2 (*continued*)

		Pectobacterium species																											
Biolog GENIII assay		*Pectobacterium actinidiae LMG 26003T §*	*Pectobacterium actinidiae LMG 26003T*	*Pectobacterium aquaticum* CFBP 8637^{T}	*Pectobacterium aroidearum* LMG 02417^{T}	*Pectobacterium atrosepticum* LMG 02386^{T}	*Pectobacterium betavasculorum* LMG 02466^{T§}	*Pectobacterium betavasculorum* LMG 02466^{T}	*Pectobacterium brasiliensis* LMG 21371^{T}	*Pectobacterium cacticida* LMG 17936^{T§}	*Pectobacterium cacticida* LMG 17936^{T}	*Pectobacterium carotovorum* LMG 02404^{T}	*Pantoea cypripedii* LMG 02657^{T}*§	*Pantoea cypripedii* LMG 02657^{T}*§	*Pectobacterium fontis* LMG 30744^{T§}	*Pectobacterium fontis* LMG 30744^{T}	*Pectobacterium odoriferum* LMG 06688^{T}	*Pectobacterium parmentieri* LMG 29774^{T§}	*Pectobacterium parmentieri* LMG 29774^{T}	*Pectobacterium peruviense* LMG 30269^{T}	*Pectobacterium polaris* NCPPB 4611^{T§}	*Pectobacterium polaris* NCPPB 4611^{T}	*Pectobacterium polonicum* LMG 31077^{T§}	*Pectobacterium polonicum* LMG 31077^{T}	*Pectobacterium punjabense* LMG 30622^{T§}	*Pectobacterium punjabense* LMG 30622^{T}	*Pectobacterium versatile* CFBP 6051^{T}	*Pectobacterium wasabiae* LMG 08444^{T}	*Pectobacterium zantedeschiae* PCM 2893^{T}
D05	Glycerol†	+/-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D09	D-Serine	-	-	-	+	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
D11	Rifamycin SV†	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E01	Gelatin	-	-	-	-	-	-	+	-	-	-	+/-	-	-	+/-	-	+/-	-	-	-	-	-	-	-	-	-	+/-	-	-
E03	L-Alanine	-	+	-	+	-	-	+	+	-	-	+/-	+/-	-	+/-	-	+/-	-	-	-	-	-	+/-	-	-	+/-	+/-	-	-
E06	L-Glutamic Acid	-	+	-	+	+/-	-	+	+	-	-	+	+/-	-	+/-	-	+	+	+	+/-	-	+	+/-	+/-	-	+	-	+	+/-	-
E09	L-Serine	-	+	+	+	-	-	+	+	-	-	+	+/-	-	+/-	-	+	-	+	+	+/-	+	+	+	+	+/-	+	+	+
E10	Lincomycin	-	+	+	+	+/-	-	+	+	+/-	+/-	+/-	+	-	+/-	-	-	-	+	+/-	-	+/-	+	-	+	-	+	+/-	-
E11	Guanidine Hydrochloride	-	+	+	-	+/-	-	+	+	+/-	+	+	+	-	+	+	+	+	+	+	-	+	+	-	+	+/-	+	+	+
F02	D-Galacturonic Acid	-	+	+	+	+/-	-	+	+	-	-	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+	+	+
F03	L-Galactonic Acid Lactone	-	+	+	+	+/-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F05	D-Glucuronic Acid	-	-	-	+	+/-	-	-	-	-	-	+	+/-	+/-	-	-	+/-	-	+	+/-	-	+/-	-	-	-	-	+/-	+/-	-
F06	Glucuronamide	-	-	-	+	-	-	-	-	-	-	+/-	+/-	-	-	-	+/-	-	-	+/-	-	+/-	-	-	+/-	-	+	+/-	-
G04	L-Lactic Acid	-	+	-	+	+/-	-	+	+	-	-	-	+/-	-	-	-	-	+	+	+/-	-	-	+/-	-	+	-	-	+	-
G05	Citric Acid	+	+	+	+	+	-	-	+	+/-	+	+	+/-	+	+	+	+	-	+	+	+	+	+	+	+	-	+	-	+
G06	α-keto-Glutaric Acid	-	-	-	+	+/-	-	-	-	-	-	-	-	-	+/-	-	-	-	-	-	-	+/-	-	-	-	-	-	-	+
G08	L-Malic Acid†	+	+	+	+	+	+	+	+	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
G10	Nalidixic Acid	-	-	-	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	+	+/-	-	-	-	-	-	-	-	-	-
G11	Lithium Chloride	-	+	+	+	+/-	-	+	+	-	-	+	+/-	-	-	-	+/-	-	+	+/-	-	+	+	-	+	-	+	-	-
H02	γ-Amino-Butryric Acid†	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H03	α-Hydroxy-Butyric Acid†	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H04	β-Hydroxy-D,L-Butyric Acid†	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H05	α-Keto-Butyric Acid†	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

^{T}type strain of species.

\+ refers to positive response on Biolog GEN III plates.

\- refers to negative response on Biolog GEN III plates.

+/- refers to weak response on Biolog GEN III plates.

*Pantoea cypripedii (formerly Pectobacterium).

†refers to substrates to be used for differentiating *Pectobacterium* species from other genera.

§refers to Biolog GEN III plate results from type strains generated by La Cite that conflict with results generated by the Bull lab and additional isolates were not tested to further support results.

The GEN III plates were useful for supporting or clarifying the tentative identifications of SRP isolates previously based on single and multi-locus phylogenies. In most cases, the identifications made from MLSA and GEN III phenotyping were identical and most SRP isolates reacted as type strains with which they clustered and other strains described for those species in the literature (Waleron et al., 2019a, b; Tian et al., 2016; Portier et al., 2019; Table S6). The Pennsylvania isolates identified as *P. carotovorum*, *P. polaris, P. versatile, P. punjabense*, and *P. parmentieri* Khayi et al., 2016 by 16S and MLSA phylogenies (Figs. 2 and 3) matched the GEN III phenotypes of the respective type strains, further supporting their identification as these species.

However, discrepancies were observed for a few isolates and the justification for the identification given for these isolates is explained in Table S8. For example, several isolates (BP7010, BP7011, BP7012, BP7055, BP7056, BP7057, BP7092, BP7093, BP9002, BP9052, and BP9130) that clustered around *P. versatile* and *P. odoriferum* (Fig. 2) using 16S rRNA but were not included in the MLSA (Fig. 3). The inability to react with D-arabitol is a key distinguishing phenotype between *P. versatile* and *P. odoriferum* (Portier et al., 2019) and none of the Pennsylvania isolates reacted with D-arabitol, thus, the were identified as *P. versatile*. Another isolate, BP7100, clustered around the *D. dianthicola* type strain on both the 16S rRNA (Fig. 2) and *dnaX* single locus phylogenies (Fig. S4), but around the *P. versatile* type strain on the *dnaJ* (Fig. S3), *gyrB* (Fig. S2), and *gyrB-dnaJ-dnaX* (Fig. 3) phylogenies. However, the GENIII phenotypic profile of BP7100 is much more similar to that of *D. dianthicola* than to that of *P. versatile*. For BP7042 and BP7043, both isolates clustered in a clade of their own around *P. aquaticum* Pédron et al., 2019 using only 16S rRNA (Fig. 2); however, in the *gyrB-dnaJ-dnaX* MLSA, they clustered around *P. actinidiae* (Fig. 3). The GENIII results further supported the identification of these two isolates as *P. actinidiae,* particularly for D-trehalose, D-cellobiose, stachyose, and L-lactic Acid (Pédron et al., 2019; Portier et al., 2019). Lastly, BP9026 clustered around *P. parvum* with 16S rRNA but around *P. polaris* corrig. Dees et al., 2017 in MLSA (Fig. 3). The GENIII plate reactions further support the identification of *P. polaris* particularly because of its positive reaction to citric acid (Pasanen et al., 2020; Table S9).

Identification of soft rot Pectobacteriaceae from Pennsylvania

Our research identified *Dickeya dianthicola*, and eight *Pectobacterium* species (*P. actinidiae, P. brasiliense* Portier et al., 2019*, P. carotovorum, P. parmentieri, P. polaris, P. polonicum, P. punjabense* Sarfraz et al., 2018, and *P. versatile*) based on their isolation at least once from symptomatic potatoes in Pennsylvania within the three-year study period. Previously, only *D. chrysanthemi*, *P. atrosepticum* (van Hall, 1902) Gardan et al., 2003, and *P. carotovorum* were reported in Pennsylvania (https://www.prevalentbacteria.org). The only species of *Dickeya* identified*, Dickeya dianthicola,* was isolated from symptomatic potatoes in 2016 (7 isolates) and 2017 (2 isolates). Our previous study demonstrated that the pathogen was present in Pennsylvania since 2015 (Curland et al., 2021).

Identification of all strains of *P.carotovorum, P. brasiliense, P. polonicum, P. punjabense, P. parmentieri* were straight forward because the *gyrB-dnaJ-dnaX* MLSA validating the identifications made by the 16S rRNA phylogeny and these results were consistent with the GENIII and rep-PCR results (Table S8). For some isolates of *P. polaris, P. actinidiae, P. versatile,* and *D. dianthicola* GENIII results were used to clarify which isolates were associated with disease each year (Table S8).

For exceptionally few isolates, identification of a previously reported species remains tentative, as the identifications generated from different methods conflicted. For example, isolate, BP9026, was initially identified as *P. parvum* by16S rRNA sequence analysis but MLSA identified it as *P. parmentieri*. Results from the GENIII plates indicate BP9026 reacted exactly like the *P. parmentieri* type strain when tested in our laboratory, further supporting this isolate as *P. parmentieri.* Therefore, we have included this strain with the nine other strains identified as *P. parmentieri* here in our first report. An additional isolate, BP7000 clustered near the *Pantoea. cypripedii* (Brady et al., 2010; formerly *Pectobacterium cypripedii*) type strain in both MLSA and 16S phylogenies, however, there was considerable distance between the two isolates. Additionally, when the 16S rRNA sequence was compared to other type strain sequences in Blast (NCBI), the sequence was more similar to Lelliottia species. The GENIII reactions of BP7000 were similar, but not identical, to those of *Pantoea cypripedii*. This isolate was initially tentatively identified as *Pantoea cypripedii* but further analysis within *Pantoea* is required. Likewise, isolate BP7100, was identified as a *P. versatile* in the *gyrB-dnaJ-dnaX* MLSA, however, it clustered with *D. dianthicola* in the *dnaX* and 16S rRNA phylogenies. We report BP7100 as *D. dianthicola* because although multilocus identification is more robust than 16S rRNA alone, the GENIII results for BP7100 are better matched to those of *D. dianthicola* than to *P. versatile*.

This survey represents the first report of *Pectobacterium actinidiae* in the U.S. as well as in Pennsylvania. *P. actinidiae* was identified twice from among the 99 isolates from potato characterized in this work, both in 2016 from a single field, but different plants of an unknown potato cultivar. *Pectobacterium actinidiae* was originally isolated in New Zealand in 2012 from kiwifruit showing canker symptoms and identified as *P. carotovorum* subsp. *actinidiae* (Koh et al., 2012). Later work elevated *P. actinidiae* to species level (Portier et al., 2019). Internationally, *P. actinidiae* has been reported to cause cankers on pear trees in South Korea (Choi et al., 2023) and kiwifruits in China (Wu et al., 2017; Lu et al., 2020). Although it was able to rot potatoes, further tests including the completion of Koch's postulates will be needed with these isolates and the type strain to confirm that this is a potato pathogen.

Additional first reports for Pennsylvania include isolation and identification of *P. brasiliense, P. polonicum, P. polaris, P. punjabense, P. parmentieri,* and *P. versatile* in this screening. *Pectobacterium brasiliense* was identified from other outbreaks in North America including Florida, Hawaii, Minnesota and North Dakota, as early as 2015 (Table S10). Isolates from 2017, along with a 2016 isolate of *P. brasiliense* for inclusion in a North American survey (Curland et al., 2021) represent the first reports of the species in Pennsylvania. *Pectobacterium polonicum* has only been reported in groundwater samples in Poland (Waleron et al., 2019b) and in blackleg symptomatic potatoes in China (Han et al., 2023). In this Pennsylvania survey, *Pectobacterium polaris* was isolated in 2016 and 2018, and *P. punjabense* in 2018. Although, both species were previously isolated from symptomatic potatoes in the northern Midwest region of the U.S. in 2015 and (Curland et al., 2021) this is the first report in Pennsylvania.

This is the first report of *P. parmentieri* in Pennsylvania, however, within the last decade, *P. parmentieri* has become the most commonly reported causal agent of blackleg and soft rot in the U. S. (Charkowski et al. 2018). In 2012, *P. parmentieri* (at the time described as *P. wasabaie*) was associated with potato blackleg in Washington, U.S. (Dung et al., 2012) and later was reported in Hawaii (Arizala et al., 2019), Maine (Ge et al., 2017), New York (Ma et al., 2018), and Minnesota and North Dakota (McNally et al., 2017b). Along with *D. dianthicola*, *P. parmentieri* has been a key species associated with the 2014 blackleg outbreak in the northeastern U.S. (Ge et al., 2017; Charkowski, 2018; Ma et al., 2018).

Pectobacterium carotovorum and *P. versatile* were the only species identified in all three years. This species is globally distributed (de Haan et al., 2008; Hu et al., 2008; Maisuria and Nerurkar, 2013), and has long been associated with potato blackleg and soft rot in the U.S. Historically, *P. carotovorum* was not distinguished from more recently named species, however, it is likely that *P. carotovorum* was historically present in Pennsylvania because it was the most widely isolated species in this study.

In contrast to *P. carotovorum, P. versatile* has not been previously reported in Pennsylvania. This may be partly because it was not described as a novel species until 2019 (Portier et al., 2019). Our findings suggest that *P. versatile* has been associated with diseases in Pennsylvania since at least 2016. This was further supported by Curland et al.

(2021) who found *P. versatile* in Minnesota in 2015 and Maine in 2016. Since its description, *P. versatile* has been reported on soft rotting potatoes in Oregon and Washington (Ma et al., 2022), New York (Ma et al., 2018), Minnesota, Maine, and North Dakota (Curland et al., 2021) and internationally in Serbia (Marković et al., 2022) and Pakistan (Sarfraz et al., 2020).

Based on these identifications, we demonstrate that in most instances, several species were isolated from each cultivar sampled. Between 2016 and 2018, *D. dianthicola, P. brasiliense, P. odoriferum, P. parmentieri, P. polaris, P. polonicum, P. punjabense, P. versatile,* and *P. carotovorum* were isolated from *S. tuberosum* cv. Reba stems and tubers (Table S1 and Table S3). *Dickeya dianthicola* was isolated from the cvs. Snowden, Atlantic, Reba, MSW485-2, and Lehigh (Table S1 and Table S3). *Pectobacterium brasiliense* was isolated from the cvs. Lehigh, Reba, Fabula, Norwis, Fabula, Snowden, and Keuka Gold. *Pectobacterium odoriferum* was isolated from cvs. Chieftain, Lehigh, Fabula, and Reba. *Pectobacterium parmentieri* was isolated from cvs. Chieftain, Fabula, Reba, and Katahdin. *Pectobacterium punjabense* and *P. polonicum* were both only isolated from cv. Reba. The most abundant species, *P. carotovorum,* was isolated from the cvs. Fabula, Keuka Gold, Lehigh, NY-140, Reba, Red Norland, and Snowden. To our knowledge, this is the first report of these species causing disease in these cultivars. Further research may elaborate on how the diversity of *Pectobacterium* and *Dickeya* species varies among cultivars.

Although not the primary goal of this study, pectolytic organisms from other pectolytic organisms have been isolated. One *Chryseobacterium* sp., two *Pseudomonas* spp., and six *Stenotrophomonas* spp. were isoled from the symptomatic potatoes. All of these isolates resulted in pits on SL-CVP and all but four *Stenotrophomonas* isolates were capable of macerating potato slices, if inconsistently, in addition to pitting SL-CVP. Further sequencing and phenotypic characterization may further clarify the identity of these isolates or explore their roles in this pathosystem.

Conclusions

We relied on a combination of isolation methods, pitting on SL-CVP, potato soft rot assays, rep-PCR, phylogenetic analyses, and phenotypic testing with Biolog GENIII plates for the species level identification of members of the soft rot *Pectobacteriaceae* from symptomatic soft rot and black leg of potato in Pennsylvania. To complete these analyses, we generated sequences for a three gene MLSA and GENIII dataset for the type strain of all *Dickeya* and *Pectobacterium* species that were described in this study. These resources are available to aid in the identification of these species. Sequences are available through NCBI and FASTA files of the gene alignments and concatenates can be downloaded (Table S7, https://bullpennblog.wordpress.com/).

In this survey, *P. actinidiae, P. polonicum, P. polaris, P. punjabense, P. parmentieri,* and *P. versatile* were identified from Pennsylvania for the first time. Importantly, the presence of *P. actinidiae* in Pennsylvania represents the first report of this organism in the U.S. As expected, *P. carotorvorum, D. dianthicola*, and *P. brasiliense* were also isolated. Surprisingly, *P. atroseptica* and *D. chrysanthemi* were not isolated in these surveys despite being previously reported in Pennsylvania (https://www.prevalentbacteria.org, Table S10).

Because of the threat posed by the potential introduction of *Dickeya solani* (Toth et al., 2011; van der Wolf et al., 2014), we will continue to survey potatoes in Pennsylvania. Based on our experience with this project, after isolation from SL-CVP our approach will be to compare 16S rRNA sequences from isolates to previous pathogens isolated in this study. The 16S rRNA sequence analysis will serve as a screening tool for novel sequence types not yet described in Pennsylvania. Any novel sequence types will be further analyzed by *gyrB-dnaJ-dnaX* MLSA or whole genome sequence analysis, and potentially by Biolog GENIII based on sequencing results. For general surveys of diversity of SRPs, use of a more general medium and MLSA and/or whole genome sequence analysis may yield a broader diversity of organisms involved in these diseases.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data has been uploaded to the NCBI. Fasta files are provided as supplemental data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2023.126476.

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