Development of the Automated Primer Design Workflow Uniqprimer and Diagnostic Primers for the Broad-Host-Range Plant Pathogen *Dickeya dianthicola*

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Abstract

Uniqprimer, a software pipeline developed in Python, was deployed as a user-friendly internet tool in Rice Galaxy for comparative genome analyses to design primer sets for PCR assays capable of detecting target bacterial taxa. The pipeline was trialed with *Dickeya dianthicola*, a destructive broad-host-range bacterial pathogen found in most potato-growing regions. *Dickeya* is a highly variable genus, and some primers available to detect this genus and species exhibit common diagnostic failures. Upon uploading a selection of target and nontarget genomes, six primer sets were rapidly identified with Uniqprimer, of which two were

specific and sensitive when tested with *D. dianthicola*. The remaining four amplified a minority of the nontarget strains tested. The two promising candidate primer sets were trialed with DNA isolated from 116 field samples from across the United States that were previously submitted for testing. *D. dianthicola* was detected in 41 samples, demonstrating the applicability of our detection primers and suggesting widespread occurrence of *D. dianthicola* in North America.

Keywords: blackleg, diagnostics, Galaxy, primer design

Bacterial plant diseases cause large global financial losses for numerous crop species. Pathogen detection is one of the most important management tools for both endemic and invasive bacterial diseases because it allows growers and regulators to exclude infected planting material and to avoid planting in contaminated soil or irrigating with contaminated water. At state and national borders, bacterial detection is crucial for identifying and quarantining seeds or other plant propagative materials infected with exotic bacterial pathogens. Cost-sensitive and time-sensitive decisions about crop destruction or quarantine may hinge on diagnostic test results; therefore, the tests used must be sensitive (i.e., able to detect all strains of the target group), specific (does not produce false-positive results for nontarget

groups), and rapid. Widely used molecular tools for bacterial phytopathogen detection include enzyme-linked immunosorbent assay (ELISA), PCR, and isothermal detection methods such as recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP), or a combination of these methods. Of these, PCR and other DNA-based methods are preferred for their sensitivity, specificity, and the ease with which the required reagents can be obtained. Thousands of available pathogen genomes have enabled comparative genomic analyses as a means to design PCR-based detection assays, and more than one dozen published pipelines or programs have been designed for genome-based differential primer designs. Alignment-based pipelines such as KPATH, Insignia, and TOPSI generate and parse alignments of whole genomes or coding sequences to identify signatures unique to the diagnostic targets (Phillippy et al. 2009; Slezak et al. 2003; Vijaya Satya et al. 2010). Alignment-free approaches, such as RUCS, PrimerHunter, PriMux, and the python package `find_differential_primers.py`, design primers or probes comprehensively or at intervals across a reference genome before screening them for target specificity across genomes (Duitama et al. 2009; Hysom et al. 2012; Pritchard et al. 2012; Thomsen et al. 2017). Although different existing programs are designed to suit a variety of different diagnostic needs, the adoption of comparative genomics methods for plant disease assay development has been limited in scope. Many published tools require comfort with a command-line environment, whereas web tools such as PrimerBLAST or MgenomSubtractor depend on genome annotation or are limited to the analysis of a few sequences (Shao et al. 2010; Ye et al. 2012). Some previously published web tools are no longer maintained (Frech et al. 2009; Phillippy et al. 2009; van Hijum et al. 2003). Together, these factors may have limited the widespread adoption of comparative genomics methods for primer design in bacterial phytopathology. Our goal was to develop a rapid, web-based platform to generate robust diagnostic primers from bacterial sequence data with a minimum of upstream preparation.

We developed an alignment-based primer design pipeline implemented in Python, named Uniqprimer, that combines whole-genome alignment and parsing with primer design and cross-validation. Similar

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Funding: We acknowledge the following agencies for funding support and sharing samples: CARE project 2017-68008-26731, Animal and Plant Health Inspection Service, United States Department of Agriculture Agricultural Research Service, USDA Specialty Crops Research Initiative project 2017-51181-26827, and the Faculty Development Program at the University of Agriculture, Faisalabad, Pakistan. The Rice Galaxy (and Uniqprimer in Galaxy) development and server are supported by the Taiwan Council of Agriculture Grant to IRRI, International Rice Informatics Consortium, IRD, the CGIAR Excellence in Breeding Platform, and the Genomic Open-source Breeding Informatics Initiative project. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the US Department of Agriculture.

*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary file is published online.

The author(s) declare no conflict of interest.

Accepted for publication 14 May 2019.

to some previous alignment-based programs (Vijaya Satya et al. 2010), Uniqprimer uses a series of pairwise genome alignments to identify sequences specific to diagnostic targets for primer design and screening steps. However, rather than identifying a large number of small primer “signatures,” Uniqprimer isolates and designs primers based on a small number of amplicon-length genomic regions that diverge between target and nontarget genomes. In-house versions of this pipeline have been used previously for the development of several validated conventional or LAMP primer sets (Ash et al. 2014; Lang et al. 2014; Langlois et al. 2017; Triplett et al. 2011, 2015). Recently, we released an online version of Uniqprimer as one of many tools on the Rice Galaxy resource (galaxy.iri.org; Juanillas et al. 2018) hosted by the International Rice Research Institute. Galaxy is a user-friendly internet platform that facilitates performing bioinformatics tasks in a reproducible way (Goedts et al. 2010), and workflows can be performed by anyone with a laptop and an internet connection. Although Uniqprimer on Rice Galaxy has been reported (Juanillas et al. 2018), its parameters and wet laboratory validation have not been previously reported.

Here, we describe the Rice Galaxy implementation of the Uniqprimer pipeline that we used to develop diagnostic tools for *Dickeya dianthicola*, a broad-host-range pathogen responsible for a recent outbreak of blackleg in potato in North America (Ma et al. 2018). *D. dianthicola* is a pectolytic Gram-negative bacterial pathogen that colonizes potato stems and tubers and causes stem blackening, plant wilt, and tuber decay in potato (Charkowski 2018). Dozens of *Dickeya* sequences are currently available, are numerous genome sequences from closely related species (Zhang et al. 2016). Primer development for *Dickeya* species, however, has been continually problematic because of its wide genomic diversity and frequent genetic exchanges. In 2013, Pritchard et al. used a comprehensive alignment-free pipeline to design >80 primers for *Dickeya* detection, of which DIA-A and DIA-C were successfully adopted for widespread diagnostic use. A primer set that was previously reliable, DIA-A, was found to yield false-negative results for a subset of recently described North American *D. dianthicola* strains that had lost the target operon for these primers (A.O. Charkowski and N.T. Perna, unpublished data). Recently

designed primers based on the arbitrarily chosen *dnaX* failed when tested with field samples (van der Wolf et al. 2014). Therefore, the North American potato industry is currently dependent on a single primer set, DIA-C, for the diagnosis and detection of this highly variable pathogen, which is likely to result in the selection for strains that lack this target sequence. This is a common problem for diagnostic assays of variable pathogens, and it highlights the need to have multiple distinct detection tools available. To provide additional assays for the potato industry, we used Uniqprimer to develop additional primer sets that can be used with specific and sensitive assays for *D. dianthicola* detection and disease management.

Materials and Methods

Uniqprimer design and implementation on Rice Galaxy. Uniqprimer is a Python script that can be run as a stand-alone script in a Linux command line environment. Uniqprimer incorporates whole-genome alignment and parsing with primer design tools and *in silico* cross-validation. The Uniqprimer Python code is hosted at the SouthGreen github repository (<https://github.com/SouthGreenPlatform/Uniqprimer>). A functional Uniqprimer tool is publicly available via Galaxy, which is an open-source bioinformatics internet portal (Afgan et al. 2018). The graphical interface of Uniqprimer in the Rice Galaxy accepts inputs of “include” (diagnostic target) genomes and “exclude” (diagnostic nontarget) genomes, which are uploaded by the user as a single or multiFASTA sequence format files (Fig. 1). The script uses NUCmer, a suffix tree-based nucleotide alignment program in the MUMmer package, to rapidly align one of the target genomes against the combined nontarget sequences (Delcher et al. 2002). Regions of the target genome identified as mismatches for all nontarget genomes are then aligned to the additional target genomes in an iterative fashion, and regions that are not identified as matches in all target genomes are eliminated. Regions that are both distinct to and conserved among the group of target genomes are parsed into a multiFASTA sequence file. This file is used as input for primer design using the Primer3 program (Koressaar and Remm 2007). Primer3 is set at the default stringency; users can modify the primer length and product size range as desired. Finally, primers are mapped to target and nontarget genomes using the Primersearch program from the package EMBOSS (Rice et al. 2000), thereby eliminating any primers that are predicted to amplify a nontarget genome or that do not perfectly match a target genome. The output is a file of primer pair sequences including predicted product length and melting temperature (T_m) and a logfile with details of the run. A step-by-step tutorial for using Uniqprimer is posted on the Rice Galaxy site (Supplemental Figure S1).

Primer design and *in silico* analyses. Uniqprimer was used for the design of primers specific to *D. dianthicola*. To identify relevant genomes for successful primer design, 39 complete or draft genomes representing *Dickeya*, *Pectobacterium*, and *Brenneria* isolates were collected from the ASAP online database (<https://asap.genetics.wisc.edu>) and subjected to whole-genome phylogenetic analysis using REALPHY 1.12 (Bertels et al. 2014) (Table 1 and Fig. 2). Six *D. dianthicola* genomes, including two draft contig assemblies, were uploaded as target genomes, and 33 other enterobacterial genomes were concatenated and uploaded as nontarget genomes (Table 1). All primer options were left on the default settings (product size range, 100 to 300 bp; optimal primer size, 20 nucleotides; minimum primer size, 18 nucleotides; and maximum primer size, 27 nucleotides). The option to cross-check primers for specificity was selected. Primer output was checked for the specificity for *D. dianthicola* among the Genbank nr and Refseq representative genomes databases using Primer-BLAST (Ye et al. 2012). *D. dianthicola* target genes identified through the Primer-BLAST were analyzed for conservation among *Dickeya* and *Pectobacterium* using BlastN (Altschul et al. 1990).

Source and identity of isolates and environmental samples. Bacterial strains used in this study are listed in Table 1. The collection included multiple *D. dianthicola* strains, *Dickeya* species, genera related to *Dickeya*, and other bacteria found on potato. The bacterial strains were identified through morphology and either multi-locus

Fig. 1. Flowchart of the Uniqprimer process for primer design. White boxes represent Uniqprimer stages. Gray boxes represent analytical processes.

sequence or genome sequence analyses. To confirm the specificity of our primers on diverse agricultural samples, DNA isolated from potato tissues samples and irrigation water was collected from multiple states, including California, Colorado, Florida, Michigan, Missouri, New Mexico, New York, North Carolina, Texas, and Wisconsin.

Bacterial DNA extraction. Bacterial isolates were streaked onto nutrient agar and were grown at 25°C for 2 days (Schaad et al. 2001). Bacterial cells were harvested from the plate, and DNA was extracted using a FastPrep kit (MP Biomedicals). To extract bacterial DNA from plant stems and tubers, a tissue sample was excised from the border of the symptomatic regions. The sample was vortexed in 400 µl of sterile water, and DNA was extracted from the water with a FastDNA Spin Kit for Soil (MP Biomedicals). DNA quality and quantity were determined for DNA isolated from pure cultures and from diagnostic samples with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). DNA was stored at –20°C.

Specificity of conventional PCR and quantitative PCR assays. Purified bacterial genomic DNA was serially diluted in sterile water to concentrations ranging from 1 ng to 100 fg, and these standards were used to determine the sensitivity of conventional PCR (cPCR) and real-time (quantitative PCR [qPCR]) assays. For cPCR, assays

were considered positive if a band was visible by imaging on a 2.0% agarose gel after staining with SYBR safe DNA gel stain (Thermo Fisher Scientific); for qPCR, we used a C_T value of 28.22 ± 2 to detect *D. dianthicola*.

For primer set DDI-1, PCR was conducted in a 25-µl reaction that contained 10 µl of Q-solution (5×), 1 µl of dNTP mix (10 mM), 5 µl of reaction buffer (10×), 1 µl of DDI-1 primers (10 µM), 10 ng of DNA template, and 0.5 µl of HotstartTaq DNA polymerase (5 units/µl) (Table 2). Thermal cycling parameters consisted of 3 min at 95°C and 30 s at 95°C, followed by 25 cycles of 30 s at 57°C, extension at 72°C for 1 min, and incubation for 5 min at 72°C and 10°C for infinite hold.

The specificity of primer set DDI-2 was evaluated using the same bacterial isolate collection that was used for cPCR testing. For qPCR, the reactions were conducted in 20 µl, including 1 µl of primers (10 µM), 2 µl of the DNA template (50 ng), and 10 µl of Power SYBR Green PCR master mix (2×) (Applied Biosystems) (Table 2). The thermal cycling conditions were as follows: 2 min and 30 s at 98°C, followed by 30 cycles of 10 s for 98°C and 1 min at 60°C in an ABI-700 real-time PCR system (Applied Biosystems). The threshold cycles were analyzed and calculated based on absolute quantification, and values $< 24 \pm 3$ were considered a positive detection response.

Table 1. Strains and genomes used in this study

Species	Strain	Origin	Host	Uniqprimer ^a	ASAP Genome ID	DDI-1 ^b	DDI-2 ^b
<i>Brenneria</i>							
<i>B. goodwinii</i>	OBR1	nd ^c	nd	Excluded	WIS_BgoOBR1_v1.fas	–	–
<i>B. quercina</i>	Equ 11D3	California	Oak	nt ^d	–	–	–
<i>B. salicis</i>	ATCC 15712-T ^e	UK	Willow	Excluded	WIS_BsaATCC15712_v1.fas	–	–
<i>Brenneria</i> sp.	Eni D312	California	Walnut	Excluded	WIS_BspEniD312_DRAFTv2.fas	–	–
<i>Dickeya</i>							
<i>D. chrysanthemi</i>	ATCC 11663-T	US	Chrysanthemum	Excluded	WIS_Dch11663_DRAFTv1.fas	–	–
<i>D. chrysanthemi</i>	NCPBP 3533	nd	nd	Excluded	WIS_Dch3533_DRAFTv1.fas	–	–
<i>D. chrysanthemi</i>	M074	Malaya	nd	Excluded	WIS_DchM074_DRAFTv1.fas	–	–
<i>D. dadantii</i>	3937	France	African violet	Excluded	WIS_ECH3937_v6b.fas	–	–
<i>D. dadantii</i> subsp. <i>dieffenbachiae</i>	NCPBP 2976-T	US	Dieffenbachia	Excluded	WIS_Dda2976_DRAFTv1.fas	–	–
<i>D. dianthicola</i>	GBBC 2039	Belgium	Potato	Included	WIS_Ddi2039_DRAFTv1.fas	+	+
<i>D. dianthicola</i>	NCPBP 3534	Netherlands	Potato	Included	WIS_Ddi3534_DRAFTv1.fas	+	+
<i>D. dianthicola</i>	NCPBP 453-T	nd	Dianthus	Included	WIS_Ddi453_DRAFTv1.fas	+	+
<i>D. dianthicola</i>	IPO 980	Netherlands	Potato	Included	WIS_Ddi980_DRAFTv1.fas	+	+
<i>D. dianthicola</i>	RNS04.9	France	Potato	Included	WIS_DdiRNS049_DRAFTv1.fas	+	+
<i>D. dianthicola</i>	CSL RW240/1	England	nd	Included	WIS_DicCSLRW240_DRAFTv1.fas	+	+
<i>D. dianthicola</i>	IPO 1741	Netherlands	Potato	nt	–	+	+
<i>D. dianthicola</i>	IPO 3646	Netherlands	Potato	nt	–	+	+
<i>D. dianthicola</i>	IPO 502	Netherlands	Potato	nt	–	+	+
<i>D. dianthicola</i>	ME 23	US	Potato	nt	–	+	+
<i>D. dianthicola</i>	TXG 3	US	Potato	nt	–	+	+
<i>D. paradisiaca</i>	703	Australia	Potato	Excluded	WIS_Dic703_v3.fas	–	–
<i>D. paradisiaca</i>	ATCC 33242-T	nd	Banana	Excluded	WIS_Dpa2511_DRAFTv1.fas	–	–
<i>D. solani</i>	MK10	Israel	Potato	Excluded	WIS_DsoMK10_DRAFTv1.fas	–	–
<i>D. solani</i>	MK16	UK	nd	Excluded	WIS_DsoMK16_DRAFTv1.fas	–	–
<i>D. solani</i>	IPO 2222	Netherlands	Potato	nt	–	–	–
<i>D. solani</i>	IPO 2187	Israel	Potato	nt	–	–	–
<i>D. solani</i>	IPO 3648	Netherlands	Potato	nt	–	–	–
<i>Dickeya</i> sp.	NCPBP 3274	St. Lucia	Aglaonema	Excluded	WIS_Dic3274_DRAFTv1.fas	–	–
<i>Dickeya</i> sp.	NCPBP 569	Australia	Sugarcane	Excluded	WIS_Dic569_DRAFTv1.fas	–	–
<i>Dickeya</i> sp.	DW 0440	nd	nd	Excluded	WIS_DicDW0440_DRAFTv1.fas	–	–
<i>Dickeya fangzhongdai</i>	MK7	Scotland	nd	Excluded	WIS_DicMK7_DRAFTv1.fas	–	–
<i>D. zeae</i>	Ech1591	US	Corn	nt	–	–	–
<i>D. zeae</i>	Ech586	Florida	Philodendron	Excluded	WIS_Dic586_v1.fas	–	–
<i>D. zeae</i>	NCPBP 3531	Australia	nd	Excluded	WIS_Dze3531_DRAFTv1.fas	–	–
<i>D. zeae</i>	EC1	China	Rice	Excluded	WIS_DzeEC1_v1.fas	–	–
<i>D. zeae</i>	MK19	nd	nd	Excluded	WIS_DzeMK19_DRAFTv1.fas	–	–

(Continued on next page)

^a Status of genome in Uniqprimer analysis.

^b Combined results of conventional and quantitative PCR results; + = positive for *D. dianthicola* detection; – = no *D. dianthicola* detected.

^c Not determined.

^d Not tested.

^e T = type strain.

To validate the ability of qPCR to detect *D. dianthicola* in field samples, potato tubers and stems exhibiting blackleg and soft rot symptoms were collected from farmers throughout the United States. Tissue samples were suspended in 10 ml sterile water, and DNA was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals) and stored at -20°C .

Sensitivity of cPCR and qPCR assays. The sensitivity of primer sets DDI-1 and DDI-2 was evaluated with *D. dianthicola* DNA. Using the template set based on pure DNA, a 10-fold series of dilutions was made by diluting a purified genomic DNA solution to concentrations from 1 ng to 100 fg. The qPCR and cPCR were conducted with both template sets. All samples were run in three separate reactions.

Results

Description of the Uniqprimer Rice Galaxy Tool. Uniqprimer in Rice Galaxy is implemented using standard Galaxy tool development practices to wrap the Uniqprimer Python script that executes the primer design pipeline (uniqprimer.py, available at <https://github.com/SouthGreenPlatform/Uniqprimer/tree/master/uniqprimer-0.5.0>). The standalone python script collects the following as inputs (in the command-line parameters): FASTA files that should be included (the sequences for which you want to design a specific primer); one to several FASTA files that should be excluded (primers designed will not match these sequences); the product size range

(100 to 300 bases default), the optimal primer size (default is 20), the minimum primer size (default is 18), and the maximum primer size (default = 27); and a setting to cross-validate primers by Primersearch alignment to exclude files (default is YES). The Galaxy XML tool file implements the graphical interface to capture these parameters and passes these to the Uniqprimer python script (uniqprimer.xml, available at <https://github.com/SouthGreenPlatform/Uniqprimer>).

Design of *D. dianthicola* species-specific primers with Uniqprimer. Prior to the primer design, 39 genome sequences representing the Pectobacteriaceae were analyzed via whole-genome phylogenetic analysis (Fig. 2). Six genomes were confirmed or predicted to be *D. dianthicola* (Fig. 2). The six confirmed *D. dianthicola* genomes were used as inputs for the Uniqprimer analysis with 33 control genomes representing other *Dickeya*, *Pectobacterium*, and *Brenneria* species (Table 1 and Fig. 2). Uniqprimer analysis was executed in 9 minutes and 42 s in our experience; however, execution times may vary according to the number and size of input genomes, the number of initial primers screened, and the number of simultaneous users on Rice Galaxy. As part of its output, the Uniqprimer identified 24 genome regions as diagnostic candidate regions predicted to be distinct from and conserved among *D. dianthicola*. Of the initial primer pairs designed from those regions (two from each region), six primer pairs were confirmed via the Uniqprimer

Table 1. (Continued from previous page)

Species	Strain	Origin	Host	Uniqprimer ^a	ASAP Genome ID	DDI-1 ^b	DDI-2 ^b
<i>Pectobacterium</i>							
<i>P. atrosepticum</i>	21A	Belarus	Potato	Excluded	WIS_Pat21A_v1.fas	—	—
<i>P. atrosepticum</i>	CFBP 6276	France	Potato	Excluded	WIS_Pat6276_DRAFTv1.fas	—	—
<i>P. atrosepticum</i>	SCRI1043	UK	Potato	Excluded	WIS_SCRI1043_v2.fas	—	—
<i>P. atrosepticum</i>	ATCC 33260	UK	Potato	nt	—	—	—
<i>P. betavascularum</i>	ATCC 43762-T	California	Sugar beet	Excluded	WIS_Pbe43762_DRAFTv1.fas	—	—
<i>P. betavascularum</i>	Ecb 2	nd	nd	nt	—	—	—
<i>P. betavascularum</i>	Ecb 6	nd	nd	nt	—	—	—
<i>P. betavascularum</i>	Ecb 1	nd	nd	nt	—	—	—
<i>P. carotovorum</i>	WPP14	Wisconsin	Potato	Excluded	WIS_EccWPP14_v2.fas	—	—
<i>P. carotovorum</i>	BC1	China	Chinese cabbage	Excluded	WIS_PcaBC1_v1.fas	—	—
<i>P. carotovorum</i>	ATTCC 15713	Denmark	Potato	nt	—	—	—
<i>P. carotovorum</i> subsp. <i>brasiliensis</i>	1692	Brazil	Potato	Excluded	WIS_WPP1692_v2.fas	—	—
<i>P. carotovorum</i> subsp. <i>brasiliensis</i>	WPP165	Wisconsin	Potato	nt	—	—	—
<i>P. carotovorum</i> subsp. <i>brasiliensis</i>	WPP5	Wisconsin	Potato	nt	—	—	—
<i>P. carotovorum</i> subsp. <i>brasiliensis</i>	WPP20	Wisconsin	Potato	nt	—	—	—
<i>P. carotovorum</i> subsp. <i>brasiliensis</i>	WPP1	Wisconsin	Potato	nt	—	—	—
<i>P. carotovorum</i> subsp. <i>carotovorum</i>	PCC21	Korea	Chinese cabbage	Excluded	WIS_PcaPCC21_v1.fas	—	—
<i>P. carotovorum</i> subsp. <i>carotovorum</i>	WPP127	Wisconsin	Potato	nt	—	—	—
<i>P. carotovorum</i> subsp. <i>carotovorum</i>	WPP156	Wisconsin	Potato	nt	—	—	—
<i>P. carotovorum</i> subsp. <i>carotovorum</i>	WPP2	Wisconsin	Potato	nt	—	—	—
<i>P. carotovorum</i> subsp. <i>carotovorum</i>	WPP12	Wisconsin	Potato	nt	—	—	—
<i>P. carotovorum</i> subsp. <i>carotovorum</i>	WPP16	Wisconsin	Potato	nt	—	—	—
<i>P. carotovorum</i> subsp. <i>carotovorum</i>	WPP17	Wisconsin	Potato	nt	—	—	—
<i>P. carotovorum</i> subsp. <i>odoriferum</i>	BC S7	nd	nd	Excluded	WIS_PcaBCS7_DRAFTv1.fas	—	—
<i>P. parmentieri</i>	RNS 08.42.1A-T	France	Potato	Excluded	WIS_PpaRNS08421A_v1.fas	—	—
<i>P. parmentieri</i>	WPP163	Wisconsin	Potato	Excluded	WIS_PwaWPP163_v1.fas	—	—
<i>Pectobacterium aroidearum</i>	PC1	Israel	Ornithogalum	Excluded	WIS_PcaPC1_v2.fas	—	—
<i>P. wasabiae</i>	ATCC 43316-T	Japan	Wasabi	Excluded	WIS_Pwa43316_v1.fas	—	—
<i>P. wasabiae</i>	WPP172	Denmark	Potato	nt	—	—	—
<i>P. wasabiae</i>	WPP161	Wisconsin	Potato	nt	—	—	—
Other							
<i>E. amylovora</i>	Ea 246	Illinois	Raspberry	nt	—	—	—
<i>E. amylovora</i>	Ea 321	France	Hawthorne	nt	—	—	—
<i>E. amylovora</i>	Ea 273	New York	Apple	nt	—	—	—

cross-check step to match all target genomes, but no excluded genomes were provided. Uniqprimer generated an output file of six primer pairs that were designated DDI-1 through DDI-6, with DDI-1 and DDI-4 originating from the same region and differing in only one nucleotide in the forward primer (Table 2). PrimerBLAST was used to confirm that all six pairs had strong predicted templates in *D. dianthicola* genomes, but not in any other bacterial genomes found in the RefSeq and nr databases. Mapping

primers to target regions in PrimerBLAST, followed by BLASTn searches of the target regions against Genbank, revealed that all of the target genes identified by Uniqprimer have homologs that are widely distributed among enterobacteria, with the exception of the *pehW* locus (Table 2). Primers DDI-1 and DDI-4 targeted the same region of heme-binding ABC transporter *ddpA* (WP_024104074). Primers DDI-3 and DDI-5 targeted different regions of the *coaABCD* locus involved in pantetheine 4'-phosphate synthesis. These results

Fig. 2. Whole-genome phylogeny of Pectobacteriaceae and control strains used for Uniqprimer *D. dianthicola*-specific detection primer design. Tree created with REALPHY 1.12.
^A[T] = type strain. ^BStatus in Uniqprimer analysis. ^CUndescribed (ASAP ID: WIS_DicCSLRW240_DRAFTv1.fas).

Table 2. Uniqprimer output of candidate *Dickeya dianthicola* diagnostic primers

Primer	Sequence (5'-3')	Length (bp)	Location ^a	Amplified (##)		T _m (°C) ^c	Source
				Target	Nontarget ^b		
DDI-F1 ^d	CTGACTATGCCTGCGTGAAA	206	4601359-564	5/5	0/36	55–65	This publication
DDI-R1	CGGAATCAGGCAGAACAGAT		(<i>dppA</i>)				
DDI-F2 ^d	GTATTTCAGCTCCGCCACTTC	201	4654393-593	5/5	0/36	50–65	This publication
DDI-R2	TTTAACCTGACCAGCGGAGT		(<i>ubiE</i>)				
DDI-F3	GAACCTGCAACTGGCCAAAAT	201	4618221-421	5/5	2/36	nt ^e	This publication
DDI-R3	AACGACAGGTTCGCTTTCAGT		(<i>coaD</i>)				
DDI-F4	TGACTATGCCTGCGTGAAAC	205	4601359-564	5/5	1/36	nt	This publication
DDI-R4	CGGAATCAGGCAGAACAGAT		(<i>dppA</i>)				
DDI-F5	CTGCATCAGGAAATGCGATA	194	4615678-871	5/5	5/36	nt	This publication
DDI-R5	GTGTTTCCCTGCAAGGTGTT		(<i>coaBC</i>)				
DDI-F6	CCGCCATACCACAGGTTATC	200	DDI_4012	5/5	3/36	55–65	This publication
DDI-R6	CAGAGTCGCACCTTTTGACA		(<i>pehW</i>)				
pelADE1	GATCAGAAAGCCCGCAGCCAGAT	420	3120359-765	nt	nt	–	Nassar et al. 1996
pelADE2	CTGTGGCCGATCAGGATGGTTTGTCTGTC		(<i>pel</i> cluster)				
Df	AGAGTCAAAAGCGTCTTG	130	4674870-5002	nt	nt	60	Laurila et al. 2010
Dr	TTTCAACCACCGTCAGTC		(tRNA-Glu)				
DIA-A_F	GGCCGCTGAATACTACATT	100	1154606-708	nt	nt	59	Pritchard et al. 2013
DIA-A_R	TGGTATCTCTACGCCCATCA		(oxidoreductase)				
DIA-C_F	CCAACGATTAGTCGGATCT	100	1115639-733	nt	nt	59	Pritchard et al. 2013
DIA-C_R	TAGTTGGTGCCAGGTTGGTA		(reductase)				

^a Location in chromosome of *D. dianthicola* RNS04.9 (GCA_000975305).

^b Nontarget amplification appeared 30 cycles after target amplification.

^c T_m = melting temperature range at which primers amplified target DNA.

^d Primers presented in this study.

^e nt = not tested.

Fig. 3. Sensitivity and specificity of primers detecting *D. dianthicola* determined through conventional polymerase chain reaction (PCR). **A**, Amplification using DDI-1 detection primers of the targeted gene presented by 200-bp to 250-bp bands. **B**, Sensitivity of conventional polymerase chain reaction of pure DNA from *D. dianthicola* ME23.

suggested that *ddpA* and *coaABCD* could represent regions of particular diagnostic value in Pectobacteriaceae. No regions present only in *Dickeya* or present only in Pectobacteriaceae were identified as primer targets by Uniqprimer.

Sensitivity and specificity of cPCR and qPCR assays. DDI primer sets identified by Uniqprimer were tested with purified DNA from *D. dianthicola* and related species. DDI-3, DDI-4, and DDI-6 primer sets amplified DNA from nontarget species in addition to *D. dianthicola*; therefore, they were excluded from subsequent analysis (Table 2). Primer sets DDI-1 and DDI-2 were sensitive to all strains of *D. dianthicola*, and no amplification was detected in other non-*D. dianthicola* species (Figs. 3 and 4 and Table 2). This represents a laboratory validation rate comparable with that of Pritchard et al. (2013). Using primers DDI-1 and DDI-2, neither cPCR nor qPCR amplified nontarget DNA from any of the other plant- and soil-associated bacteria that were tested, including the soft rot bacterial pathogen *Pectobacterium* and the closely related plant pathogenic genera *Brenneria* and *Erwinia* (Fig. 3).

Detection limit of cPCR and qPCR assays. DDI-1 and DDI-2 were tested to confirm detection limits with known *D. dianthicola* genomic DNA and field samples. To prepare for sensitivity testing, *D. dianthicola* primers were tested to confirm efficiency. Both DDI-1 and DDI-2 exhibited efficiency values within accepted limits ($100 \pm 10\%$, Fig. 5). To determine detection limits for our assays, 100 ng of template DNA was serially diluted to 1 pg and tested via cPCR and qPCR. The cPCR assays with DDI-1 detected *D. dianthicola* DNA at 10 pg. The qPCR assays with DDI-2 exhibited detection limits down to 1 pg of DNA template. DDI-1, DDI-2, and DIA-C consistently identified *D. dianthicola* in field

samples, whereas DIA-A, pelADE, and Df/Dr inconsistently identified it in field samples, suggesting poor specificity (Table 3; Figs. 4 and 6).

Field sample analysis. To confirm the utility of our *D. dianthicola* detection primers, and to further demonstrate their specificity, we validated our Uniqprimer assays with DNA from field samples collected across the United States. In total, 52 potato stem samples, 14 potato tuber samples, 9 potato tissue culture samples, and 41 irrigation water samples from California, Colorado, Florida, Michigan, Missouri, New Mexico, New York, North Carolina, Texas, and Wisconsin were tested (Table 3). *D. dianthicola* was detected in 30 stem and 11 tuber samples from California, Florida, Michigan, Missouri, New Mexico, New York, North Carolina, Texas, and Wisconsin. We compared the results with those of previously published assays (Fig. 6), and our results were consistent with those of DIA-C *D. dianthicola* detection primers (Pritchard et al. 2013).

Comparison of DDI-1 and DDI-2 with extant tools for *D. dianthicola* detection. Currently, DNA-based *D. dianthicola* detection relies on the use of species-specific primers DIA-A and DIA-C combined with *Dickeya* general primers pelADE and Df/Dr (Nassar et al. 1996; Potrykus et al. 2014; Pritchard et al. 2013). We compared our Uniqprimer assays to current diagnostic standards, DIA-A and DIA-C, *in silico* using a Primer-BLAST analysis of the NCBI nonredundant whole-genome database (Fig. 7). Primer-BLAST detects potential amplification products in the NCBI genomic databases (Ye et al. 2012). As expected, DDI-1, DDI-2, DIA-A, and DIA-C shared a high level of nucleotide sequence identity with their cognate targets in all available complete *D. dianthicola* genomes (Fig. 7). DDI-1 and DDI-2 also shared a lower level of identity with amplicon-size

Fig. 4. Detection of *Dickeya dianthicola* (Ddi) in potato field samples from across the United States with genomic bacterial DNA (gDNA) control using DDI-1. + = *D. dianthicola* detected; - = *D. dianthicola* not detected.

Fig. 5. Primer efficiencies calculated for quantitative polymerase chain reaction *Dickeya dianthicola* detection primers DDI-1 (diamond), DDI-2 (square), DIA-A (triangle), and DIA-C (cross) (Pritchard et al. 2013). Results repeated in triplicate using *D. dianthicola* ME23 genomic DNA template. Efficiency calculated $[10^{-1/m} - 1] \times 100$.

Fig. 6. Detection of *Dickeya dianthicola* isolates ME23 and TXG3 using a quantitative PCR (qPCR). **A**, qPCR detection of *D. dianthicola* isolates using diagnostic primer DIA-A (Pritchard et al. 2013). **B**, qPCR detection of *D. dianthicola* isolates using diagnostic primer DDI-2. C_T = cycle threshold as calculated by the ABI-700 real-time PCR system.

regions in a greater number of off-target genomes than DIA-A or DIA-C (Fig. 7, blue shaded regions). Although this level of identity was not high enough to result in any false-positive results for DDI-1 or DDI-2 on strains tested in the laboratory (Table 1), they illustrated that the stringency settings of Uniqprimer may result in diagnostic targets that share a low level of identity with those in closely related genomes rather than targets completely missing in those genomes.

Discussion

We used the Rice Galaxy implementation of the Uniqprimer diagnostic primer design pipeline to efficiently develop primers for the sensitive and specific detection of *D. dianthicola*, a difficult-to-detect pathogen that causes tuber soft rot and potato blackleg. Given a set of target and nontarget genome files, Uniqprimer generated six primer pairs with sensitivity and specificity *in silico*, of which two also demonstrated absolute sensitivity and specificity against a library of strains in the laboratory. Uniqprimer can be used by anyone with an internet connection and access to genome sequences of diagnostic target bacteria, although the speed of the process may vary according to the size of the dataset input and the number of simultaneous users on RiceGalaxy. Novice users can follow the supplemental tutorial to apply Uniqprimer for their own diagnostic uses. Although the in-house Python code of Uniqprimer has previously been used to develop primers for the specific detection of pathogens and geographic pathogen clades, this work demonstrated the utility of the user-friendly internet-accessible implementation of the pipeline for solving difficult diagnostic problems.

The Uniqprimer pipeline shares similarities with several previous alignment-based primer design approaches because the pipeline uses a series of rapid whole-genome alignments to isolate sequences that are distinct to and conserved among diagnostic target strains (Marinier et al. 2017; Phillippy et al. 2009; Slezak et al. 2003; Vijaya Satya et al. 2010). This is in contrast to the computational approach of alignment-free methods, which comprehensively design primer pairs at regular intervals or for all coding sequences prior to filtering. Similar to other pipelines, Uniqprimer uses the popular tools Primer3 (used in many published tools), Primersearch (used by Pritchard et al. 2012), and BLAST (Pritchard et al. 2012; Thomsen et al. 2017; Vijaya Satya et al. 2010) for primer design and screening steps. Other alignment-based programs generate a large number of primer-length signatures that are paired to primer sets after screening, but Uniqprimer generates a smaller number of amplicon-length primer design templates that share increased conservation among targets

rather than between targets and nontargets. Our goal was not to compare the performance of Uniqprimer to other pipelines, but the approach of limiting the primer design to a small number of highly prefiltered regions was intended to limit the computationally intensive final screening step of aligning candidate primers to the input genomes. One benefit of isolating amplicon-length regions is that Uniqprimer output includes multiFASTA files of the candidate diagnostic target regions. This output file can be useful for nonconventional primer design, such as primers for LAMP (Ash et al. 2014).

Dickeya is a diverse genus with at least seven known species and additional subspecies that show significant genetic overlap and exchange; developing primers for species in this genus has been difficult. Various immunodetection methods and DNA-based assays have been published that describe *D. dianthicola* detection, but many lack the specificity and the sensitivity necessary to produce pathogen-free stock. We confirmed that the Df/Dr primer set resulted in false-negative results, the pelADE primer set amplified spurious DNA fragments, and the target locus of the DIA-A primer set is missing from some North American strains of *D. dianthicola*. This was unavoidable because of the unavailability of representative genomes from North America during the time of the DIA-A primer design (Nassar et al. 1996; Potrykus et al. 2014; Pritchard et al. 2013). Primers designed in this study performed comparably with the industry standard, DIA-C, with DNA from pure bacterial isolates and field samples (Pritchard et al. 2013). The remaining four resulted in false-positive results on nontarget strains in our collection, which is always a possible consequence when the sequence is unavailable for some relevant organisms. The primer pairs targeted loci that have predicted homologs in multiple genera of enterobacteria, demonstrating the utility of the pipeline for identifying distinct regions of conserved diagnostic targets that may be missed in alignment strategies targeting the presence/absence of entire loci.

To date, no primer sets are available that amplify DNA from all strains within the *Dickeya* genus. We also attempted to use Uniqprimer to design primers for the detection of all *Dickeya* species, but the pipeline failed to identify any primer pairs that did not also amplify other pectolytic or enteric bacteria. This suggested that Uniqprimer may require a certain degree of genetic closeness within the diagnostic target group, as well as a threshold of genetic distance between target and nontarget groups. Therefore, the application of Uniqprimer may be limited for groups genetically broader than the highly specific groups currently validated, including species, pathovar, and subpathovar geographic groups (Lang et al. 2014; Langlois

Table 3. Comparison of PCR detection results using *Dickeya dianthicola*-specific and *Dickeya*-general diagnostic primers

Sample type (n)	Location (n)	Pel ADE			Df/Dr			DIA-A			DIA-C			DDI-1			DDI-2		
		+	–	*	+	–	*	+	–	*	+	–	*	+	–	*	+	–	*
Stems (52)	California (2)	0	2	0	0	2	0	0	2	0	0	2	0	0	2	0	0	2	0
	Colorado (17)	0	17	2	nt ^d	nt	nt	0	17	0	0	17	0	0	17	0	0	17	0
	Florida (6)	2	0	4	2	0	4	6	0	0	6	0	0	6	0	0	6	0	0
	Missouri (4)	3	0	1	4	0	1	4	0	0	4	0	0	4	0	0	4	0	0
	New Mexico (1)	1	0	0	0	0	2	0	1	0	1	0	0	1	0	0	1	0	0
	New York (5)	0	1	4	1	0	3	4	1	0	4	1	0	4	1	0	4	1	0
	North Carolina (3)	3	0	0	3	nt	nt	3	0	0	3	0	0	3	0	0	3	0	0
	Texas (2)	2	0	0	2	0	0	0	2	0	2	0	0	2	0	0	2	0	0
	Wisconsin (12)	3	5	4	nt	nt	nt	2	10	0	8	4	0	8	4	0	8	4	0
	Florida (6)	1	0	5	3	2	1	6	0	0	6	0	0	6	0	0	6	0	0
Tubers (14)	Texas (2)	0	0	2	2	0	0	0	2	0	2	0	0	2	0	0	2	0	0
	Wisconsin (6)	2	2	6	nt	nt	nt	2	4	0	3	3	0	3	3	0	3	3	0
	Colorado (9)	0	9	0	0	9	0	0	9	0	0	9	0	0	9	0	0	9	0
Tissue culture (9)	Colorado (23)	0	23	0	0	20	3	0	23	0	0	23	0	0	23	0	0	23	0
	Michigan (10)	0	10	0	0	10	0	0	10	0	0	10	0	0	10	0	0	10	0
Water (41)	Wisconsin (8)	0	8	0	nt	nt	nt	0	8	0	0	8	0	0	8	0	0	8	0

^a + = positive results.

^b – = negative results.

^c * = Ambiguous results displaying faint or nonspecific banding.

^d nt = not tested.

Fig. 7. *In silico* analysis of Pectobacteriaceae diagnostic primers and predicted targets. **A**, Nucleotide conservation was determined via a Primer3-BLAST analysis of the NCBI nonredundant complete genome database. Percent nucleotide identity is presented for true-positive results (red, black border) and false-positive results (blue, gray). Results were compared with primers from **B**, Pritchard et al. (2013), **C**, Nassar et al. (1996), and **D**, Laurila et al. (2010). Primer–genome interactions predicted to produce multiple amplicons are noted (dotted border). ^T = Type strain.

et al. 2017). Uniqprimer prioritizes rapid and user-friendly use over exhaustive primer design; however, previously published strategies using more comprehensive alignment-free or unique signature approaches may be better suited to develop diagnostics for genus-level detection or high-resolution differentiation of closely related strains, or to address other difficult diagnostic situations. The use of this pipeline to develop primers both distinct and comparable in performance to the *D. dianthicola* primers currently used in many testing programs that were previously generated through an alignment-free pipeline (Pritchard et al. 2013) illustrated the value of utilizing multiple complementary primer design strategies to maximize diagnostic resources. Sampling for *Dickeya* species has been limited to only a few crops and a few regions in the world; therefore, the discovery of new diversity and the ensuing search for new diagnostics tests will undoubtedly continue well into the future.

Acknowledgments

We thank John Herndon for the initial development work on the Uniqprimer pipeline, with funding from the Colorado State University Infectious Diseases Supercluster.

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