Development of a Loop-Mediated Isothermal Amplification Assay for the Detection of *Dickeya* spp.

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Accepted for publication 10 July 2017.

ABSTRACT

Dickeya and *Pectobacterium* spp. are responsible for soft-rotting diseases of several plant species, some with overlapping host range. On potato, symptoms caused by these pathogens cannot be clearly differentiated. Disease results in the downgrading and rejection of potato seed, thus requiring additional phytosanitary restrictions across Northern Europe and other parts of the world. In an effort to provide a more timely and accurate diagnostic to distinguish these two groups of pathogens, a method for detecting *Dickeya* spp. using loop-mediated isothermal amplification (LAMP) was developed.

The LAMP assay can be used to test crude extracts prepared directly from symptomatic lesions. The entire test can be completed in less than 30 min, making it faster than the current diagnostic standard, the *pelADE* conventional polymerase chain reaction. Additionally, the LAMP assay was able to detect *Dickeya* DNA in samples spiked with varying amounts of *Pectobacterium* DNA, thus demonstrating the highly specific and sensitive nature of the assay, which can be applied on survey samples with mixed soft-rotting bacterial populations.

World-wide, *Dickeya* and *Pectobacterium* are important genera containing several species of plant-pathogenic bacteria that are responsible for soft-rotting diseases on a variety of plant crops, including potato, maize, tomato, pineapple, sugar beet, rice, and banana, as well as many ornamental plants (Gardan et al. 2003; Ma et al. 2007; Samson et al. 2005). In global potato production, *Pectobacterium carotovorum* and *P. atrosepticum* have dominated the pathogenic populations observed on potato plants affected with blackleg and soft rot; other species of *Pectobacterium* have recently been reported in many of the potato-growing regions of the world (Pritchard et al. 2016). In temperate regions, *P. carotovorum*, *P. atrosepticum*, and *Dickeya dianthicola*, the latter of which has been detected in potato fields in Europe since the 1970s, pose significant production constraints to the potato industry. Furthermore, *D. solani*, a recently described pathogen (van der Wolf et al. 2014b), has emerged as an additional threat to potato production in Europe (Toth et al. 2011), whereas *D. dadantii* and *D. zeae* are known to cause diseases in potato stems and tubers in tropical and subtropical regions of the world (Ngadze et al. 2010; Samson et al. 2005).

Damage caused by pests and pathogens plays a significant role in crop losses throughout the world. New outbreaks of blackleg and soft-rot diseases are thought to be the result of movement of latently infected planting material, which has also been attributed to the worldwide distribution of *Pectobacterium* and *Dickeya* spp. Efforts to curtail the spread of these pathogens has prompted legislation such as the zero tolerance policy for all *Dickeya* spp. enacted by Scotland to protect its market on the export of “clean” seed for potato production (Pritchard et al. 2016). However, it should be noted that tolerance for blackleg in European seed-potato tubers differs from country to country (Toth et al. 2011). Therefore, diagnostic tests in support of legislation should be reliable and accurate, and able to discriminate between *Pectobacterium* and *Dickeya* spp.,

because efficient detection of these pathogens in plant material, or exclusion thereof, is necessary for timely implementation of eradication or containment measures to prevent or limit spread of these pathogens.

Currently, diagnostic testing for the detection and identification of *Pectobacterium* and *Dickeya* spp. has depended on the isolation of viable bacterial cells on semiselective crystal-violet pectate (CVP) culture medium, followed by biochemical analyses and the use of more sensitive conventional polymerase chain reaction (PCR) or species-specific real-time PCR. However, validated species-specific real-time PCR assays do not exist for all species of *Pectobacterium* or *Dickeya* (Czajkowski et al. 2015; Humphris et al. 2015). The drawback to current diagnostic testing methods is the need for high-tech facilities and equipment, as well as the associated costs of a skilled operator or diagnostician.

Molecular detection and differentiation techniques are more precise than classical and biochemical analyses and reduce the delay between sample collection and test results. In contrast to conventional PCR and real-time PCR, loop-mediated isothermal amplification (LAMP) is an amplification method that uses a *Bst* DNA polymerase with strand-displacement activity and two to three sets of primers to amplify DNA with high specificity under isothermal conditions in less than 1 h (Nagamine et al. 2001, 2002; Notomi et al. 2000). At present, LAMP assays have been developed for the detection of *P. atrosepticum* (Li et al. 2011) and *P. carotovorum* (Yasuhara-Bell et al. 2016) but none for the detection of *Dickeya* spp. In this study, a LAMP assay for the rapid detection of *Dickeya* spp. was developed. The specificity of the LAMP assay was tested against *Pectobacterium* spp. and other plant-pathogenic bacteria associated with potato or overlapping hosts of the genus *Dickeya*. Results of further validation tests involving environmental samples of field-grown pineapple infected with *Dickeya* spp. and laboratory-inoculated potato, as well as spiked competition assays, are presented.

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The GenBank accession number for the *dnaA* gene sequence used in this study was KY967613.

MATERIALS AND METHODS

Bacterial strains and DNA extraction. Strains of bacteria used in this study included the six plant-pathogenic species of *Dickeya* described currently in the literature, along with other plant-associated

pathogens with overlapping hosts or genera known to be closely related to *Dickeya* (Table 1). Bacterial strains were grown on TZC medium (agar at 17 g/liter, peptone at 10 g/liter, glucose at 5 g/liter, and 0.001% 2,3,5-triphenyl-tetrazolium chloride) or TZC-S (substitute sucrose at 5 g/liter in place of glucose for *Clavibacter* spp.) and allowed to incubate at 26°C (±2°C) until colony formation. Bacteria from single colonies were suspended in 0.75 to 1.0 ml of sterile distilled water and thoroughly mixed by vigorous vortexing. Suspensions were heated to 95°C for 10 min on a digital heat block, allowing cell lysis and DNA release into suspension, then allowed to cool to room temperature or held overnight at 4°C before use as a template for LAMP testing. All suspensions were stored at 4°C. The crude lysates were used in all LAMP assays, unless otherwise stated.

Primer design and LAMP protocol. The *mgIC* gene, a β -methylgalactoside transporter, was chosen as the candidate target for primer design. The sequences of the target region in *Dickeya* spp., including *D. dadantii* (PRJNA30, PRJNA172906, and PRJNA172953) and subsp. *dieffenbachiae* (PRJNA172911), *D. dianthicola* (PRJNA172901, PRJNA173463, PRJNA172903, and PRJNA173395), *D. chrysanthemi* (PRJNA172904, PRJNA172902, PRJNA172952, and PRJNA31295), *D. solani* (PRJNA173464, PRJNA172958,

PRJNA172957, and PRJNA173396), *D. zeae* (PRJNA33667, PRJNA229184, PRJNA172951, PRJNA172883, PRJNA172907, PRJNA172950, and PRJNA172959), *D. paradisiaca* (PRJNA172954 and PRJNA33069), and *Dickeya* spp. PRJNA173398, PRJNA172910, PRJNA172949, and PRJNA172956, were obtained from the National Center for Biotechnology Information (NCBI) GenBank nucleotide database. Molecular Evolutionary Genetics Analysis (MEGA) version 4.0 (Tamura et al. 2007) was used to perform a multiple sequence alignment of the sequences, followed by sequence comparison with the Species Demarcation Tool version 1.2 (Muhire et al. 2014) using default parameters.

LAMP primers (Table 2) were designed using the primer design program PrimerExplorer V4 (Eiken Chemical Co., Ltd, Tokyo), available at <http://primerexplorer.jp/e/>. The LAMP reactions were performed with 5 μ l of primer mix, consisting of MglC-F3 (0.2 μ M), MglC-B3 (0.2 μ M), MglC-FIP (1.6 μ M), MglC-BIP (1.6 μ M), MglC-Loop (0.8 μ M), MglC-Loop Probe (0.08 μ M), and Quencher Probe (0.16 μ M) (Kubota et al. 2011) per reaction; 15 μ l of Isothermal Mastermix (OptiGene, Horsham, West Sussex, UK); and 5 μ l of sample/template DNA. Positive and negative controls contained 5 μ l of *D. dadantii* (Collection Française de Bactéries Associées aux

TABLE 1. Bacterial strains and corresponding reaction results with loop-mediated isothermal amplification (LAMP) primers designed in this study

Number ^a	Strain ID ^b	Organism	LAMP
A2431	IRRI 1427-4	<i>Acidovorax avenae</i>	–
A2432	BCE-12	<i>A. avenae</i>	–
A2433	CHY9NF	<i>A. avenae</i>	–
A6168	EluW3L16	<i>Brenneria</i> spp.	–
A6169	Eni5D312	<i>Brenneria</i> spp.	–
A6170	Equ11D3	<i>Brenneria</i> spp.	–
A6171	ATCC 15712	<i>Brenneria salicis</i>	–
A1666	ATCC 23060	<i>Burkholderia andropogonis</i>	–
A2058	H160	<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	–
A6094	NCPPB 2579; LMG 3698	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	–
A6095	20037	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	–
A6096	200800460	<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	–
A2041	R8	<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	–
A5415	CFBP 2048 ^T	<i>Dickeya chrysanthemi</i> biovar <i>chrysanthemi</i>	+
A6062	CFBP 3701	<i>D. chrysanthemi</i> biovar <i>chrysanthemi</i>	+
A6063	CFBP 3262	<i>D. chrysanthemi</i> biovar <i>chrysanthemi</i>	+
A5574	IPO 2117	<i>D. chrysanthemi</i> biovar <i>parthenii</i>	+
A5641	CFBP 1270	<i>D. chrysanthemi</i> biovar <i>parthenii</i>	+
A5416	CFBP 1269 ^T	<i>D. dadantii</i> subsp. <i>dadantii</i>	+
A5642	CFBP 3855	<i>D. dadantii</i> subsp. <i>dadantii</i>	+
A5643	CFBP 6467	<i>D. dadantii</i> subsp. <i>dadantii</i>	+
A5419	CFBP 2051	<i>D. dadantii</i> subsp. <i>dieffenbachiae</i>	+
A5578	IPO 2124	<i>D. dadantii</i> subsp. <i>dieffenbachiae</i>	+
A5646	CFBP 3694	<i>D. dadantii</i> subsp. <i>dieffenbachiae</i>	+
A6060	CFBP 3698	<i>D. dadantii</i> subsp. <i>dieffenbachiae</i>	+
A6061	CFBP 1247	<i>D. dadantii</i> subsp. <i>dieffenbachiae</i>	+
A5418	CFBP 1200 ^T	<i>D. dianthicola</i>	+
A5571	IPO 1741	<i>D. dianthicola</i>	+
A5644	CFBP 2015	<i>D. dianthicola</i>	+
A5645	CFBP 4155	<i>D. dianthicola</i>	+
A6058	CFBP 1982	<i>D. dianthicola</i>	+
A6059	CFBP 3706	<i>D. dianthicola</i>	+
A5420	CFBP 4178 ^T	<i>Dickeya paradisiaca</i>	+
A5579	IPO 2127	<i>D. paradisiaca</i>	+
A5688	CFBP 3691	<i>D. paradisiaca</i>	+
A6064	CFBP 3696	<i>D. paradisiaca</i>	+
A6065	CFBP 1445	<i>D. paradisiaca</i>	+
A5581	IPO 2187	<i>Dickeya solani</i>	+
A5582	IPO 2188	<i>D. solani</i>	+
A5417	CFBP 1278	<i>Dickeya</i> sp.	+
A5421	CFBP 1272	<i>Dickeya</i> sp.	+
A6066	CFBP 1889	<i>Dickeya</i> sp.	+
A6067	CFBP 1890	<i>Dickeya</i> sp.	+

(continued on next page)

^a Acquisition number from the Alvarez laboratory at the University of Hawai'i at Mānoa.

^b ATCC, American Type Culture Collection, United States; CFBP, Collection Française de Bactéries Associées aux Plantes (French Collection for Plant-Associated Bacteria), France; IPO, Instituut voor Plantenziektenkundig onderzoek (Institute for Phytopathological Research), The Netherlands; LMG, Collection of the Laboratorium voor Microbiologie en Microbiële Genetica, Belgium; and NCPPB, National Collection of Plant Pathogenic Bacteria, United Kingdom.

Plantes [CFBP] 2051) or *D. chrysanthemi* (CFBP 2048) DNA, and molecular-grade water or *P. carotovorum* subsp. *carotovorum* DNA, respectively. All reactions were performed in triplicate and analyzed using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with the following parameters: 65°C for 30 min (could be extended to 40 min to an hour for low concentrations of DNA), with fluorescence readings being taken at 1-min intervals, and a final optional step of 2 min at 85°C.

LAMP specificity analysis. To determine whether the primers were specific to *Dickeya* spp., extracted DNA from *Pectobacterium* spp., *Clavibacter* spp., *Ralstonia solanacearum*, *Xanthomonas axonopodis* pv. *dieffenbachiae*, and other plant-associated pathogens (Table 1) were tested. Each sample was loaded in triplicate with positive and negative controls, as described above.

LAMP sensitivity assay for *Dickeya* spp. The sensitivity of the LAMP assay was assessed with 10-fold serial dilutions of *D. solani* (IPO 2187) DNA from 5 ng/μl to 5 fg/μl. Purified DNA was extracted using the Wizard Genomic DNA purification kit (Promega Corp., Madison, WI), followed by use of a NanoDrop Model ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE) to quantify and adjust the starting dilution of DNA. LAMP assays were performed as described above.

Additionally, to assess the sensitivity of the LAMP assay, reactions were performed with spiked amounts of *P. carotovorum* subsp. *carotovorum* WPP14 (A6155) DNA in the presence of *D. solani* (IPO 2187) DNA, which was held constant at the detection

limit of the LAMP assay (5 pg) or used at 1 ng/reaction. Reactions were spiked with increasing amounts of *P. carotovorum* subsp. *carotovorum* DNA at a final concentration of 5, 10, 20, or 100 ng/sample, along with *D. solani* DNA at both 5 pg and 1 ng/reaction, totaling 5 μl. Additionally, a 1:1 mixture of 100 ng of *D. solani* IPO 2187 DNA and *P. carotovorum* subsp. *carotovorum* WPP14 DNA was also assessed to check for inhibition of the LAMP reaction at high DNA concentrations. The following were used as negative controls: *P. carotovorum* subsp. *carotovorum* WPP14 DNA only at 1 ng, or molecular-grade water only; the positive control was 5 pg or 1 ng of *D. solani* IPO 2187 DNA only, corresponding to the amount included in the test sample. All tests were run in duplicate using the LAMP parameters described above.

Evaluation of LAMP for field application. LAMP was evaluated for field application via two separate in planta testing methods. First, samples of pineapple (*Ananas comosus*) showing symptoms of bacterial heart rot and a healthy-looking control were collected from the same field and row of a pineapple-growing field on Oahu, HI. Samples, one leaf each from an infected plant or healthy-looking control, were treated as described by Kaneshiro et al. (2008) for the isolation of *Dickeya* spp., except that the remaining volume of macerate was boiled at 95°C for 10 min in a digital heat block, allowed to cool and sediment overnight at 4°C, then used for testing with the LAMP assay. LAMP assays were performed in triplicate alongside a positive (*D. chrysanthemi* CFBP 2048) and nontemplate (sterile distilled water used in isolation

TABLE 1. (continued from preceding page)

Number ^a	Strain ID ^b	Organism	LAMP
A6174	...	<i>Dickeya</i> sp.	+
A5422	CFBP 2052 ^T	<i>Dickeya zeae</i>	+
A5423	CFBP 6466	<i>D. zeae</i>	+
A5647	CFBP 1531	<i>D. zeae</i>	+
A6068	CFBP 1533	<i>D. zeae</i>	+
A6069	CFBP 1277	<i>D. zeae</i>	+
A6070	CFBP 1884	<i>D. zeae</i>	+
A6071	CFBP 1871	<i>D. zeae</i>	+
A6072	CFBP 3804	<i>D. zeae</i>	+
A6073	CFBP 1447	<i>D. zeae</i>	+
A6074	CFBP 3707	<i>D. zeae</i>	+
A6075	CFBP 1502	<i>D. zeae</i>	+
A3131	ATCC 13048	<i>Enterobacter aerogenes</i>	–
A5149	B193	<i>E. cloacae</i>	–
A6125	S-3	<i>Klebsiella oxytoca</i>	–
A6133	ATCC 13883	<i>K. pneumoniae</i>	–
A6126	S-4	<i>K. variicola</i>	–
A6140	CC27	<i>Pantoea agglomerans</i>	–
A6162	Eca6	<i>Pectobacterium atrosepticum</i>	–
A6163	Eca31	<i>P. atrosepticum</i>	–
A6164	SCRI1043; BAA-672	<i>P. atrosepticum</i>	–
A6165	Ecb1	<i>P. betavasculorum</i>	–
A6166	Ecb2	<i>P. betavasculorum</i>	–
A6167	Ecb6	<i>P. betavasculorum</i>	–
A6148	WPP1	<i>P. carotovorum</i> subsp. <i>brasiliensis</i>	–
A6149	WPP5	<i>P. carotovorum</i> subsp. <i>brasiliensis</i>	–
A6150	WPP17	<i>P. carotovorum</i> subsp. <i>brasiliensis</i>	–
A6151	WPP20	<i>P. carotovorum</i> subsp. <i>brasiliensis</i>	–
A6152	WPP165	<i>P. carotovorum</i> subsp. <i>brasiliensis</i>	–
A6153	WPP2	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	–
A6154	WPP12	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	–
A6155	WPP14	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	–
A6156	WPP16	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	–
A6157	WPP161	<i>Pectobacterium wasabiae</i>	–
A6158	WPP163	<i>P. wasabiae</i>	–
A6159	WPP168	<i>P. wasabiae</i>	–
A6160	WPP172	<i>P. wasabiae</i>	–
A6161	SCRI488	<i>P. wasabiae</i>	–
A3292	CC95; GMI1000	<i>Ralstonia solanacearum</i>	–
A1965	D6	<i>Xanthomonas axonopodis</i> pv. <i>dieffenbachiae</i>	–
A2046	1591	<i>X. axonopodis</i> pv. <i>dieffenbachiae</i>	–
A2108	D78-3	<i>X. axonopodis</i> pv. <i>dieffenbachiae</i>	–
A5603	V108LRUH1	<i>X. axonopodis</i> pv. <i>dieffenbachiae</i>	–

procedure) control, as described above. From one of the infected-plant tissues, a single colony (strain A6174) (Table 1) producing indigoidine on TZC medium was isolated and used in colony PCR with *pelADE* (Nassar et al. 1996) and *dnaA* (Marrero et al. 2013; Schneider et al. 2011) primers, followed by sequencing of the latter marker.

Second, store-purchased potato (*Solanum tuberosum*) tubers ($n = 2$) were stab inoculated in two distinct spots per sample using a sterile toothpick dipped in *D. solani* IPO 2187 suspended at an optical density at 600 nm (OD_{600}) of approximately 0.1. Each potato was also inoculated with sterile distilled water to use as a negative control on each potato. An additional potato was also inoculated at three distinct spots with sterile distilled water to use as a negative control in this assay. Briefly, inoculated potato tubers were incubated in a sealed plastic container at high humidity for 2 days at room temperature to allow symptom development. Macerated tissue (1 cm³) or mock-inoculated tissue (stabbed with sterile distilled water) was excised with a sterile scalpel and transferred to a 1.5-ml Eppendorf tube containing 500 μ l of sterile distilled water. Bacteria were allowed to diffuse for 20 min before removal of the sampled tissue. The remaining suspension was then boiled at 95°C for 10 min in a digital heat block and allowed to cool to room temperature or held at 4°C for LAMP testing. Plant-sample suspensions were tested with the LAMP assay as described above, alongside a suspension of *D. solani* IPO 2187 ($OD_{600} = 0.1$) prepared the same way as the plant samples as positive control and sterile distilled water as a negative control.

PCR. PCR were performed using the *pelADE* (Nassar et al. 1996) and *dnaA* (Marrero et al. 2013; Schneider et al. 2011) primers, with PCR conditions performed as described previously. The obtained *dnaA* sequence was deposited into the GenBank database (KY967613) and used for phylogenetic analysis.

Phylogenetic analysis. Phylogenetic analysis of strain A6174 was performed using sequences obtained with the *dnaA* marker for species-level identification. Sequence alignment and phylogenetic analyses were performed using Geneious, version 10.0.5 (<http://www.geneious.com>) (Kearse et al. 2012). The *dnaA* sequences for known strains were obtained from NCBI GenBank (Table 3). Sequences were aligned using ClustalW, taking into account the corresponding amino acid alignments for protein-coding genes. Neighbor-joining trees (Saitou and Nei 1987) were constructed using the Jukes-Cantor method (Jukes and Cantor 1969) to compute evolutionary distances. Confidence intervals were assessed using the bootstrap method with 1,000 replications (Felsenstein 1985).

RESULTS

Specificity of the *Dickeya* LAMP assay. LAMP primers were designed to detect the *mgIC* gene, encoding a β -methylgalactoside transporter, after NCBI BLAST analysis revealed no homologous matches to closely related *Pectobacterium* spp. or low to no homology to other nontarget plant-associated pathogens. Sequence comparison of the full-length *mgIC* gene illustrated that this gene had a percent identity between 84 and 100% (Fig. 1) among the

Dickeya spp. tested, therefore making it a good candidate for primer design. Primers were tested initially with one strain each from all *Dickeya* spp., except for *D. solani* and *D. zeae*, for which two and three strains were tested, respectively. No amplification was observed in negative controls that lacked template DNA or contained *P. carotovorum* subsp. *carotovorum* DNA. An additional 30 *Dickeya* strains were tested; all strains tested had observable positive results (Table 1). Therefore, a threshold for reactions determined as positive was based on the amplification of 40 *Dickeya* strains and set at 30 min. Primer specificity was tested with an additional 44 strains, including *Pectobacterium* spp., *Clavibacter* spp., *Acidovorax avenae*, *Brenneria* spp., and other plant-associated or nontarget organisms. The LAMP assay did not react positively with any non*Dickeya* strains or with the negative controls.

Sensitivity of the LAMP assay. In a series of 10-fold dilutions starting with DNA at 1 ng/ μ l, the detection limit for an observable result was DNA at approximately 5 pg/reaction. At this reduced concentration of DNA, a shift in time was observed for a positive reaction; approximately 40 min/reaction was needed for an observable positive result. However, single colonies isolated from cultures grown on plates or crude extracts prepared from diseased tissue should contain an ample quantity of bacterial cells to provide enough DNA to fall within the threshold of 30 min for a positive reaction.

TABLE 3. GenBank accession numbers for strains used in phylogenetic analysis

Number ^a	Strain ID	Organism	GenBank ID
A5415	CFBP 2048 ^T	<i>Dickeya chrysanthemi</i> biovar <i>chrysanthemi</i>	JX434861.1
A5574	IPO 2117	<i>D. chrysanthemi</i> biovar <i>parthenii</i>	JX434870.1
A5641	CFBP 1270	<i>D. chrysanthemi</i> biovar <i>parthenii</i>	JX434879.1
A5416	CFBP 1269 ^T	<i>Dickeya dadantii</i> subsp. <i>dadantii</i>	JX434862.1
A5642	CFBP 3855	<i>D. dadantii</i> subsp. <i>dadantii</i>	JX434880.1
A5643	CFBP 6467	<i>D. dadantii</i> subsp. <i>dadantii</i>	JX434881.1
A5419	CFBP 2051	<i>Dickeya dadantii</i> subsp. <i>dieffenbachiae</i>	JX434865.1
A5578	IPO 2124	<i>D. dadantii</i> subsp. <i>dieffenbachiae</i>	JX434871.1
A5646	CFBP 3694	<i>D. dadantii</i> subsp. <i>dieffenbachiae</i>	JX434884.1
A5418	CFBP 1200 ^T	<i>Dickeya dianthicola</i>	JX434864.1
A5571	IPO 1741	<i>D. dianthicola</i>	JX434887.1
A5644	CFBP 2015	<i>D. dianthicola</i>	JX434882.1
A5645	CFBP 4155	<i>D. dianthicola</i>	JX434883.1
A5420	CFBP 4178 ^T	<i>Dickeya paradisiaca</i>	JX434866.1
A5579	IPO 2127	<i>D. paradisiaca</i>	JX434872.1
A5581	IPO 2187	<i>Dickeya solani</i>	JX434888.1
A5582	IPO 2188	<i>D. solani</i>	JX434889.1
A5422	CFBP 2052 ^T	<i>Dickeya zeae</i>	JX434868.1
A5423	CFBP 6466	<i>D. zeae</i>	JX434869.1
A5647	CFBP 1531	<i>D. zeae</i>	JX434885.1
A5421	CFBP 1272	<i>Dickeya</i> sp.	JX434867.1
A5417	CFBP 1278	<i>Dickeya</i> sp.	JX434863.1
	Et1/99	<i>Erwinia tasmaniensis</i>	CU468135.1

^a Acquisition number from the Alvarez laboratory at the University of Hawai'i at Mānoa.

TABLE 2. Loop-mediated isothermal amplification primers developed for the detection of *Dickeya* spp.

Oligonucleotide primer	Sequence (5'–3') ^a	Source, reference
MgIC-F3	TCGCTATCGGCGGTAACC	This study
MgIC-B3	ACCACCGGCAAAAGACAC	This study
MgIC-FIP	GCCGGACAGCATATACACCAAGGAAGCCGCCAAAGTGTC	This study
MgIC-BIP	CCTTCGGCGGTATGCTGGAAGCGATGGCGTCAAGTTTCGTA	This study
MgIC-Loop	CGGTAGTGCCACTAACAACCTGG	This study
MgIC-Loop probe	/56-FAM/ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGATTTTCGGTAGTGCCACTAACAACCTGG	This study
Quencher probe	TCGGCATCCGCATCCGCATTTCGCATCCGGGTCTCAGCGT/3BHQ_1/	Kubota et al. 2011

^a Abbreviations: /56-FAM/ = 5'-carboxyfluorescein, /3BHQ_1/ = 3' black hole quencher 1, and /5IABkFQ/ = 5' Iowa Black FQ.

In mixed cultures containing increasing amounts of DNA from the closely related pathogen *P. carotovorum* subsp. *carotovorum*, the detection limit of the LAMP reaction was not affected. All samples, including a combined mixture of 100 ng of *Dickeya* and *Pectobacterium* DNA, were positive, showing that LAMP could be reliably used without a loss in sensitivity or inhibition due to contaminant DNA, even at high concentrations.

Evaluation of the *Dickeya* LAMP assay from plant tissue samples. LAMP reliably amplified *Dickeya* DNA in the absence of time-consuming preparatory steps, such as pure culture isolation or purified DNA extraction, after infected tissue sampling. Direct addition of crude lysate from infected pineapple or potato tissue had no observable impact on amplification. Presence of the pathogen was verified from the infected pineapple tissue by direct plating, followed by confirmation via amplification with two separate conventional PCR and sequencing of the *dnaA* marker. Amplification by the *dnaA* primers and phylogenetic analyses with other isolates of *Dickeya* confirmed the identity of the pineapple isolate A6174 as a *Dickeya* sp. (Fig. 2). This strain clustered with two other strains (CFBP 1278 and CFBP 1282) that were originally isolated from plants showing pineapple heart rot in Malaysia (Samson et al. 2005). Amplification was not detected with this primer set when

Dickeya DNA was absent from the sample preparation (i.e., healthy-plant controls and sterile-water control).

Potato inoculated with *D. solani* strain IPO 2187 showed typical symptoms of tissue maceration 2 days postinoculation. No dilutions were necessary to detect the pathogen from inoculated plant extracts, whereas no amplification was observed from the water-inoculated control or negative controls. On average, amplification was observed within 15 to 25 min.

DISCUSSION

Detection of *Dickeya* spp. is challenging due to the genetic heterogeneity observed among strains, along with the complex nature of the pathogens; multiple species are known to cause similar symptoms on one host, and a single species can infect multiple hosts (Samson et al. 2005). Detection and identification of *Dickeya* spp. can be further complicated by its environmental proximity on overlapping hosts to closely related pathogens such as *Pectobacterium* spp. For example, under warm, wet conditions, symptoms of black-leg and soft rot in potato are markedly similar, whether caused by *Pectobacterium* or *Dickeya* spp., thus making identification of the causal agent impossible by visual inspection alone. Several molecular

Fig. 1. Color-coded matrix displaying pairwise identity scores between the *mgIC* gene sequences of *Dickeya* spp.

amplification methods and procedures have been developed in order to detect *Dickeya* spp. or distinguish them from *Pectobacterium* spp. (Diallo et al. 2009; Laurila et al. 2010; Pritchard et al. 2013; van der Wolf et al. 2014a; Van Vaerenbergh et al. 2012). Nucleic acid-based assays are available for the detection of *Dickeya* spp., including a multiplex PCR (Diallo et al. 2009) and real-time quantitative (q)PCR (Laurila et al. 2010) assay; however, these methods were not tested against *D. zeae* or *D. paradisiaca* and *D. paradisiaca* (Laurila et al. 2010), respectively. Species-level TaqMan qPCR assays have been developed to detect individual *Dickeya* spp. (van der Wolf et al. 2014a; Van Vaerenbergh et al. 2012) but cross-reactivity was observed, demonstrating reduced specificity.

In this study, LAMP primers designed to target a region of the *mgIC* gene had broader detection ability for *Dickeya* spp. than nucleic-acid methods described previously, which may be more advantageous in testing regimes where more than one *Dickeya* sp. may be present on a host such as potato. This broader detection ability also presents no disadvantages for detection where only a given species (i.e., *D. solani* or *D. dianthicola*) may be the target pathogen of interest for seed certification programs.

Relative to conventional PCR and other PCR-based methods, the LAMP assay is rapid, requires lower equipment costs, obviates the need for highly skilled personnel, and can be used without the need for time-consuming DNA extraction procedures. In samples prepared from crude extracts of infected pineapple suspected of harboring *Dickeya* spp. and *Dickeya*-inoculated potato, positive results were observed upon simple bacterial diffusion from the infected tissue, followed by a boil step. This is in contrast to some assays that require the use of an enrichment step prior to analysis for the detection of *Dickeya* (de Haan and van den Bovenkamp 2009).

Further optimization of the DNA extraction method may be required for different sample types; however, LAMP has been shown to be less sensitive overall to plant inhibitors (phenolic compounds) (M. Arif, unpublished information).

The *mgIC* LAMP primers designed in this study led to specific amplification of the 40 strains of *Dickeya* spp. tested. Additionally, the presence of a high amount of competitor or contaminant DNA in the reaction tube (*P. carotovorum* subsp. *carotovorum*) had no effect on the specific amplification of *Dickeya* DNA and resulted in no concomitant loss in sensitivity. The presence of competitor DNA did not reduce the detection limit of the LAMP assay, therefore showing the good overall specificity and high sensitivity of the assay. The LAMP assay developed in this study was assessed on two different plant samples: a naturally infected pineapple from the field and a lab-inoculated potato sample. The LAMP assay was able to detect *Dickeya* spp. in the infected pineapple tissue, while showing negative results in healthy-looking pineapple tissue. Bacteria were isolated from the diseased tissue and confirmed to be *Dickeya* spp. by *pelADE* (Nassar et al. 1996) and *dnaA* (Marrero et al. 2013; Schneider et al. 2011) PCR analysis. The genus *Dickeya* was also detected from diseased potato tissue while showing negative results in control potato tissue. The LAMP assay was able to detect *Dickeya* spp. in these plant tissues with minimal sample preparation. Plant samples were simply macerated or soaked in water to allow bacteria to diffuse into suspension and then boiled at 95°C for 10 min.

The specificity and sensitivity of this assay demonstrates its utility as a specific test for detecting *Dickeya* sp. Moreover, this assay is suitable for field tests due to the simplicity of sample preparation and the portability of the LAMP assay using hand-held real-time amplification technologies. Additionally, the gene target used for detection has application across several molecular detection platforms

Fig. 2. Phylogenetic analysis of the pineapple field-sample strain A6174 *dnaA* gene.

such as PCR, nicking-enzyme amplification reaction (Maples et al. 2009a,b; Van Ness et al. 2003), and recombinase polymerase amplification (Piepenburg et al. 2006).

ACKNOWLEDGMENTS

This work was supported by the United States Department of Agriculture National Institute of Food and Agriculture Hatch project HAW00987-H and the University of Hawaii Foundation-Plant Disease Control fund 120-50604.

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