LAMP Detection of Four Plant-Pathogenic Oomycetes and Its Application in Lettuce Fields

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Abstract

In Kagawa Prefecture, Japan, the pathogens *Phytophthora pseudolactucae*, *Pythium irregulare*, *Pythium uncinulatum*, and *Pythium spinosum* have caused huge losses in lettuce production. We used loop-mediated isothermal amplification (LAMP) to analyze soil and plants in lettuce fields for the presence of these four pathogens. To develop an effective on-site detection method, we contrasted the Plant-LAMP and Plant Culture-LAMP procedures for plant samples, and five soil DNA extraction methods for soil samples. Plant-LAMP and a Soil DNA Isolation kit were selected to analyze three fields for the pathogen species present, infected sites, and level

of soil contamination. We found that the same wilting symptoms could be caused by *Phytophthora* or *Pythium*, or a mixture of species from both genera. *Ph. pseudolactucae* infects the pith of the lettuce in aboveground parts, whereas *Pythium* spp. mainly infect roots. *Ph. pseudolactucae* and *Py. uncinulatum* caused disease more frequently than the other two pathogens. Furthermore, not all of the pathogens existed in the soil near infected lettuce plants. Therefore, the LAMP method can be used to diagnose pathogenic oomycetes in the field, and will be useful in the development of control strategies in lettuce production.

Lettuce (*Lactuca sativa* L., Asteraceae), one of the most popular leafy vegetables, is susceptible to many diseases worldwide. These include several soil-borne diseases such as Verticillium wilt caused by *Verticillium dahliae*, Fusarium root rot caused by *Fusarium oxysporum* f. sp. *lactucae*, Phytophthora wilt caused by *Phytophthora pseudolactucae*, and Pythium damping-off caused by *Pythium ultimum* (Fradin and Thomma 2006; Fujinaga et al. 2001; Lynch et al. 1991; Rahman et al. 2015). The third largest cultivation area for winter lettuce in Japan is in Kagawa Prefecture, where huge losses have resulted from a damping-off caused by *Pythium* (since 2007) and a wilting disease caused by *Phytophthora* (since 2012). The oomycetes have quite different sensitivity to a range of fungicides compared with fungi. A series of systemic fungicides has been introduced for oomycete control. These generally have high activity, low toxicity, and low residual action, and include phenylamides (FRAC code 4), quinone outside inhibitors (FRAC code 11), and carboxylic acid amides (FRAC code 40) (Gisi and Sierotzki 2015). To use fungicides under appropriate and effective precautionary strategies, it is extremely important to develop rapid diagnostic methods to identify particular plant pathogens.

The visual symptoms of plant diseases are often very similar to those caused by other complex stresses. For example, visual symptoms can be caused by abiotic stresses such as drought, salinity, heat, or cold, or by biotic stresses such as plant pathogens or herbivores (Atkinson and Urwin 2012; Baligar and Fageria 2015; Suzuki et al. 2014; Wang et al. 2003). Vollenweider and Günthardt-Goerg (2005) proposed the use of visible symptoms for distinguishing between abiotic and biotic stresses; however, it remains difficult to identify plant diseases caused by pathogens using only visual symptoms. Furthermore, similar symptoms can be caused by different plant pathogens (Geraats et al. 2003;

Rojas et al. 2017; Samson et al. 2005). For instance, Fusarium root rot, Pythium damping-off, Verticillium wilt, and Phytophthora wilt all cause similar wilting symptoms in lettuce (Fradin and Thomma 2006; Koike 2016; Nordskog et al. 2008). Thus, there are many difficulties associated with identifying *Pythium* and *Phytophthora* pathogens based on visible symptoms alone.

Pythium and *Phytophthora* species can be isolated and detected using selective media containing antibacterial and antifungal agents (Gamo et al. 2004; Morita and Tojo 2007; Tsao 1970). However, this approach cannot distinguish between pathogenic and nonpathogenic *Pythium* and *Phytophthora* species that could be present in the same field. It is also difficult to isolate pathogens that grow more slowly than other species of the same genus using these media. Many studies have assessed the use of molecular methods for the initial and rapid detection of fungi, viruses, and other microorganisms, including *Pythium* and *Phytophthora* (Kageyama 2014; Reinhart et al. 2012). Two common approaches are conventional and real-time PCR. However, PCR-based techniques are time-consuming and require expensive specialized equipment that is usually only used in the laboratory. More recently, several isothermal DNA amplification methods have been developed for on-site diagnosis of plant pathogens. These include recombinase polymerase amplification (RPA) (Rojas et al. 2017), helicase-dependent amplification (HDA) (Schwenkbier et al. 2015), and loop-mediated isothermal amplification (LAMP) (Feng et al. 2018).

Since the LAMP method was first developed by Notomi et al. (2000), it has been widely used as an alternative to PCR due to its rapidity, simplicity, and practicality. The reaction has high specificity and, unlike PCR, it can be performed at a constant temperature. Amplification can be visually observed or measured using the turbidity of the reaction mixture (Mori et al. 2001, 2004), or by using color indicators (Goto et al. 2009; Iwamoto et al. 2003). Moreover, the LAMP reaction is effective even with crude template DNA extractions (Feng et al. 2015; Kitamura et al. 2016; Miyake et al. 2017; Shen et al. 2017). Therefore, LAMP is well suited for field diagnoses using only a simple water bath or heat block. The LAMP assay has been used to detect a wide range of plant pathogens including viruses, viroids, fungi, bacteria, and oomycetes (Boubourakas et al. 2009; Fukuta et al. 2012; Hodgetts et al. 2015; Miyake et al. 2017; Rigano et al. 2010; Tomlinson et al. 2010).

To identify the causal agents of lettuce damping-off in Kagawa Prefecture, oomycete species were collected from diseased roots and stem bases using a corn meal agar medium. Based on morphological characteristics and ribosomal DNA-ITS (rDNA-ITS) homologies, three *Pythium*

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species were isolated and identified as *Py. irregulare* Buisman, *Py. spinosum* Sawada, and *Py. uncinulatum* Plaats-Niterink & Blok (Kusunoki 2012). The causal agent of Phytophthora wilt in Kagawa Prefecture was isolated from diseased crowns and identified as a new taxon (Rahman et al. 2014). A phylogenetic tree based on rDNA-ITS sequences suggested that this isolate is closely related to *Phytophthora lactucae*; however, a tree based on mitochondrial *cox1* sequences and morphological characteristics demonstrated that the isolate is a new *Phytophthora* species, and it was named *Ph. pseudolactucae* M.Z. Rahman, S. Uematsu, Kanto, M. Kusunoki & Kageyama, sp. nov. (Rahman et al. 2015).

The objectives of this study were to develop simple LAMP detection methods for species-specific identification of *Py. irregulare*, *Py. spinosum*, *Py. uncinulatum*, and *Ph. pseudolactucae*. We used the LAMP assays to investigate the main pathogen species in plants and soils and the infectious sites of the pathogens in three lettuce cultivation fields.

Materials and Methods

Maintenance of isolates and mycelial DNA extraction. Isolates were collected from several scientific resource institutions, the CBS-KNAW Fungal Biodiversity Centre (CBS), Utrecht, the Netherlands, the NITE Biological Resource Center, Kisarazu, Chiba Prefecture, Japan (NBRC), the NIAS Genebank, Tsukuba, Ibaraki Prefecture, Japan (MAFF), the World *Phytophthora* Genetic Resource Collection, University of California, Riverside, CA, USA (P), and the Gifu University Cultures Collection, Gifu Prefecture, Japan (Tables 1 and 2, and Supplementary Table S1). All isolates were maintained on corn meal agar or potato-dextrose agar media at 20°C in the dark. Genomic DNA was extracted from the mycelia using the PrepMan Ultra Reagent (Applied Biosystems Inc., CA, USA) as described by Baten et al. (2014). DNA concentration was estimated using the Qubit 2.0 Fluorometer (Life Technologies, CA, USA). DNA was diluted to 100 pg/μl as the starting concentration for all assays.

LAMP and PCR primer design. We designed new LAMP and PCR primers targeted to the rDNA-ITS region for *Py. spinosum* and *Py. uncinulatum* and to the *cox1* region for *Ph. pseudolactucae*. Multiple alignments were constructed using these sequences and the same sequences from other species using the BioEdit Sequence Alignment Editor software. Based on the aligned sequences, the LAMP and PCR primers specific for each pathogen were designed and analyzed using the PrimerExplorer V5 software (<http://primerexplorer.jp>) and Primer3web (<http://bioinfo.ut.ee/primer3/>), respectively.

LAMP reaction. Each LAMP reaction was carried out in a total volume of 15 μl containing 1.6 μM FIP and BIP primers, 0.2 μM F3 and B3 primers, 0.8 μM B-loop primer (*Py. spinosum* and *Ph. pseudolactucae*), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 0.1% (vol/vol) Tween 20, 0.8 M betaine (Sigma-Aldrich) (or 1 M betaine for *Py. spinosum* only), 8 mM MgSO₄, 10 mM (NH₄)₂SO₄, 1.4 mM dNTPs (New England Biolabs Japan, Tokyo, Japan), 4.8 units of the *Bst* DNA polymerase large fragment (Nippon Gene Co. Ltd., Toyama, Japan), and template DNA (100 pg for specificity test). The mixtures were incubated for 60 min at different temperatures of 63, 64, 65, 66, and 67.5°C. The turbidity was recorded in real time using a loop amp real-time turbidimeter (LA-200, Teramecs, Kyoto, Japan). Each reaction was carried out in triplicate, and controls contained positive DNA or sterile distilled water (SDW) in place of template DNA.

LAMP specificity and sensitivity test. In total, 55 isolates of 45 *Pythium* species, 47 isolates of 44 *Phytophthora* species, 5 *Phytophythium* isolates, and 8 isolates of 7 other soil-borne oomycetes were used to test the specificity and sensitivity of each primer set (Tables 1 and 2). Series of 10-fold dilutions of *Py. spinosum* (NBRC100116), *Py. uncinulatum* (MAFF240295), and *Ph. pseudolactucae* (MAFF239556) genomic DNA were used to determine the sensitivity of the LAMP assay, with SDW as the negative control. The LAMP assay was carried out as previously described with the optimal LAMP primers and reaction parameters. The primers selected for use in field tests are listed in Table 3.

PCR reactions. In total, 36 isolates of 26 *Pythium* species, 31 isolates of 28 *Phytophthora* species, 3 *Phytophythium* isolates, and 8 isolates of 7 other soil-borne oomycetes were used to test the specificity and sensitivity of each primer set. Each PCR mixture (25 μl total volume) contained 1× PCR buffer (10 mM, pH 8.3 Tris-HCl, 50 mM KCl, and 1.5 mM MgCl₂), 0.2 mM dNTPs, 0.2 μM of each primer (0.5 μM for *Py. spinosum* only), 0.4 mg bovine serum albumin (Sigma-Aldrich, Tokyo, Japan), 2 units FastStart Taq DNA polymerase (Roche Applied Science, Tokyo, Japan), and template DNA (100 pg for specificity test). The amplification was performed using a Gene Amp PCR system 2700 thermal cycler (ABI, Tokyo, Japan) with the following conditions: 95°C for 5 min, 35 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 1 min, then 72°C for 10 min. The negative controls contained SDW instead of template. The amplification products were separated on a 3% agarose gel, stained with GelRed, and photographed under UV light. Each experiment was repeated three times.

Field sample collection. In February 2016, a total of eight roots and 12 pith samples were collected from randomly selected diseased lettuce plants in two lettuce cultivation fields (A and B) in Kagawa Prefecture (Table 4 and Supplementary Figure S1). In December 2016, five diseased plants from each of three lettuce fields (1 to 3) in Kagawa Prefecture were each divided into pith, root base, and root tip samples (Table 5). Soil samples (c. 200 g) from around the same plants were also collected. In field 3, where plastic-film mulching was used, soil samples were collected from above and below the mulch.

Detection of pathogens in lettuce. We used the Plant-LAMP (P-LAMP) and Plant Culture-LAMP (PC-LAMP) detection methods developed by Feng et al. (2015) to analyze the pathogens in lettuce tissues. For P-LAMP, rotted or brown tissue samples were cut into 1 cm long (0.2 cm³) segments. The segments were placed into 1.5-ml tubes with 0.5 ml SDW, vortexed for 1 min using an MT-360 Multitube Fast Mixer (Tomy Seiko Co. Ltd., Japan), and then 4.5 μl of the supernatant was used directly for LAMP amplification. For PC-LAMP, each cut segment was placed on NARM (nystatin+ampicillin+rifampicin+miconazole) medium (Morita and Tojo 2007) and incubated at 25°C for 1–5 days. A sample of the medium (about 0.1 cm³) containing mycelia was excised and placed in a 1.5-ml tube with 100 μl SDW, vortexed as described above, and then 1 μl of the supernatant was used for LAMP amplification.

Soil DNA extraction methods. We compared five soil DNA extraction methods: (i) the Soil DNA Isolation kit (Norgen Biotek Corp., Thorold, ON, Canada), (ii) the Extrop soil kit plus ver. 2 kit (Nippon Steel & Sumikin Eco-Tech., Tsukuba, Ibaraki, Japan), (iii) the Mightyprep reagent for DNA kit (TaKaRa Bio., Kusatsu, Shiga, Japan), (iv) Kageyama + Mag method (Li et al. 2015), and (v) the Soil DNA Isolation mini kit (Favorgen Biotech Corp., Ping-Tung, Taiwan, China) (Table 6). A soil sample (200 g) collected from a lettuce cultivation field was autoclaved at 120°C for 60 min. *Ph. pseudolactucae* were inoculated on 10 ml liquid V8 medium (2 ml V8 juice, 0.025 g CaCO₃, and 8 ml distilled water) at 20°C for 1 to 2 weeks until mass formation of oospores. The oospores were separated from the mycelia using a homogenizer at 15,000 rpm on SDW for 5 min (Nissei AM-3, Nihonseiki Kaisha, Tokyo, Japan), collected using 50 μm and 27 μm stemless mesh sieve, and then counted under a microscope with a hemocytometer. The soil sample was inoculated with *Ph. pseudolactucae* oospores to a concentration of 33 oospores per gram of soil. After mixing thoroughly, 0.2-g samples were subjected to DNA extraction and then 4.5 μl of the extracted solution was used for LAMP assays. Each kit was tested twice (Extractions 1 and 2, Table 6). The Norgen Biotek Corp. kit was chosen for pathogen detection in the soil samples shown in Table 5.

Results

Specificity of the LAMP assay. A specific primer set targeted to the rDNA-ITS region was already available for *Py. irregulare* (Feng et al. 2015) (Table 3). We designed new primers targeted to the rDNA-ITS region for *Py. spinosum* and *Py. uncinulatum* and to

the *cox1* region for *Ph. pseudolactucaae*. Multiple alignments were constructed using these sequences and the same sequences from other species in the same phylogenetic clades, as proposed by Lévesque and De Cock (2004) for *Pythium* and Blair et al. (2008) for *Phytophthora*. We designed a total of 41 primer sets for *Py. spinosum*, 12 sets for *Py. uncinulatum*, and 13 sets for *Ph. pseudolactucaae*. These primers were tested using isolates from the same phylogenetic clades as the target species (see Tables 1 and 2). The primers that showed the greatest specificity (Psp, Pun, and Phps for *Py. spinosum*, *Py. uncinulatum*, and *Ph. pseudolactucaae*, respectively) were further tested using a broad range of oomycetes, as shown in Tables 1 and 2. Psp, Pun, and Phps (see Table 3) were highly specific for their target species and were selected for field testing. We further optimized the LAMP reactions for these primer sets using temperature gradients from 63 to 67.5°C (Supplementary Figure S2). Considering the high specificity, reactivity, and stability, optimal temperatures of 64, 66, and 66°C were selected for specific amplification of *Ph. pseudolactucaae*, *Py. spinosum*, and *Py. uncinulatum*, respectively.

Sensitivity of the LAMP assay. To assess the sensitivity of the LAMP assay, we designed specific PCR primers for each species (Table 3) and tested the specificity using a broad range of oomycetes. The PCR and LAMP primers were tested using serial dilutions of *Py. spinosum*, *Py. uncinulatum*, and *Ph. pseudolactucaae* DNA samples. As shown in Figure 1, the LAMP primer sets had high sensitivities, comparable with those of the PCR primers. In both the LAMP and PCR assays, the detection limits were 10, 100, and 100 fg for *Py. spinosum*, *Py. uncinulatum*, and *Ph. pseudolactucaae* respectively.

Optimization of the procedures for LAMP detection in field samples. To identify the most efficient plant LAMP procedure for use in the field, we collected 8 root and 12 pith samples from diseased lettuce plants in 2 fields (A and B) in Kagawa Prefecture, and analyzed them using both the P-LAMP and PC-LAMP methods (Table 4). Each of the four pathogens was detected in at least one sample. Pathogens were found in a total of 10 samples using P-LAMP and 11 samples using PC-LAMP. To confirm the detection, all isolates were recovered from the NARM media and identified by morphological characteristics. Therefore, similar numbers of

Table 1. Specificity tests of the LAMP primers for *Pythium spinosum* and *Py. uncinulatum*

Species	Clade ^a	Isolate ^b	Origin	Amplification ^c	
				Psp	Pun
<i>Pythium adhaerens</i>	A	CBS 520.74	Soil	-	-
<i>Py. aphanidermatum</i>	A	GUCC0354	Carrot field soil	-	-
<i>Py. monospermum</i>	A	N02E2 3-4	Flooded soil	-	-
<i>Py. arrhenomanes</i>	B1	NBRC100102	Zoysia grass	-	-
<i>Py. graminicola</i>	B1	MAFF425415	Corn	-	-
<i>Py. myriotylum</i>	B1	NBRC100113	Kidney bean	-	-
<i>Py. plurisporium</i>	B1	CBS 100530	Root	-	-
<i>Py. sulcatum</i>	B1	NBRC100117	Carrot	-	-
<i>Py. torulosum</i>	B1	GUCC0422	Carrot	-	-
<i>Py. aquatile</i>	B2	NBRC107450	Water	-	-
<i>Py. dissotocum</i>	B2	MAFF305576	Soil	-	-
<i>Py. pyrilobum</i>	B2	NBRC107365	Water	-	-
<i>Py. acanthicum</i>	D	MAFF241099	Soil	-	-
<i>Py. periplocum</i>	D	NBRC100114	Zoysia grass	-	-
<i>Py. oligandrum</i>	D	GUCC0830	Soil	-	-
<i>Py. hypogynum</i>	E1	CBS 234.94	Soil	-	-
<i>Py. rostratum</i>	E1	NBRC100115	Zoysia grass	-	-
<i>Py. middletonii</i>	E2	CBS 528.74	Soil	-	-
<i>Py. parvum</i>	E2	GUCC0832	Soil	-	-
<i>Py. takayamanum</i>	E2	NBRC104223	Soil	-	-
<i>Py. irregulare</i>	F	CBS 263.30	<i>Nicotiana tabacum</i>	-	-
		EP-2	Carrot	-	-
<i>Py. irregulare III</i>	F	PY26	Sugarbeet	-	N
<i>Py. irregulare IV</i>	F	PY61	Sugarbeet	-	N
<i>Py. mamillatum</i>	F	CBS 251.28	<i>Beta vulgaris</i>	-	N
<i>Py. paroecandrum</i>	F	CBS 157.64	Soil	-	N
<i>Py. spinosum</i>	F	NBRC100116	Carrot field soil	+	-
		OD231		+	-
		GUKan5		+	-
		Py73		+	-
		07KST-2		+	-
		1D1S032		+	-
<i>Py. sylvaticum</i>	F	NBRC100119	Carrot field soil	-	-
<i>Py. intermedium</i>	F	CBS 221.68	Soil	-	-
<i>Py. macrosporum (strain +)</i>	F	CBS 574.80	Root of flower bulb	-	N
<i>Py. macrosporum (strain -)</i>	F	CBS 575.80	Lilium	-	N
<i>Py. nagaii</i>	G	GUCC0828	Soil	-	-
<i>Py. paddicum</i>	G	MAFF241108	Water	-	-
<i>Py. anandrum</i>	H	CBS 285.31	<i>Rheum rhaponitium</i>	-	-
<i>Py. senticosum</i>	H	NBRC104222	Soil	-	-

(Continued on next page)

^a Molecular phylogenetic clades for *Pythium* and *Phytophthora* were obtained from Lévesque and De Cock (2004) and Blair et al. (2008), respectively.

^b Isolates were collected from the CBS-KNAW Fungal Biodiversity Centre (CBS), the NITE Biological Research Centre (NBRC), the NIAS Genebank (MAFF), the World Phytophthora Genetic Resource Collection (P), and the Gifu University Cultures Collection (GUCC).

^c Psp: *Py. spinosum* specific primer set; Pun: *Py. uncinulatum* specific primer set; symbols: + = amplified, - = no amplification, and N = no test.

positive results were obtained with each method. We concluded that P-LAMP is suitable for detection of pathogens in the field, due to its simplicity and rapidity.

To develop a simple method for the detection of pathogens in soil samples using the LAMP assay, we compared five soil DNA extraction methods using soil samples containing *Ph. pseudolactucae*. We obtained positive results from both extractions with the Norgen Biotek Corp. Soil DNA Extraction kit and from one extraction with the Toyobo Co. MagExtractor-Plant Genome kit (Table 6). The Norgen Biotek Corp. kit uses a rapid spin-column format to capture pure DNA and requires only 40 min for DNA extraction. Therefore, we selected this kit as a simple method for detection of the pathogens in field soil samples.

Infection sites in lettuce plants. To identify the sites where pathogens could be detected in diseased lettuce plants, we collected five diseased plants from each of three fields (fields 1–3). Each plant was separated into three tissue types: pith, root base, and root tip, and the samples were assayed for each pathogen species using P-LAMP (Table 5). *Py. spinosum* and *Py. irregulare* were each found in only

one root base sample in field 3. *Py. uncinulatum* was found in all three fields with totals of eight root base samples, five root tip samples, and five pith samples. *Ph. pseudolactucae* was found only in pith samples: four samples in field 1, and 5 samples in field 3. Overall, two or more pathogens were detected in one plant in field 1 and in four plants in field 3. Also, all isolates were recovered and identified by morphological characteristics.

Detection of pathogens in soil samples from the field. To assess the prevalence of detectable pathogens in soil samples from the field, we collected soil samples from around each diseased lettuce in fields 1–3. Plastic-film mulching had been used in field 3, and soil samples were also collected from above the mulch in this field. Each sample was extracted twice using the Norgen Biotek Corp. Soil DNA Isolation kit. All four pathogens were detected in multiple DNA extracts from fields 1 and 3, and the pathogens were detected in samples from above and below the mulch in field 3 (Table 5). In field 2, only one extract contained detectable amounts of *Py. spinosum* DNA, and five extracts contained detectable *Py. uncinulatum* DNA. Neither *Py. irregulare* nor *Ph. pseudolactucae* was detected in any of the

Table 1. (Continued from previous page)

Species	Clade ^a	Isolate ^b	Origin	Amplification ^c	
				Psp	Pun
<i>Py. undulatum</i>	H	NBRC107363	Water	-	-
<i>Py. heterothallicum</i>	I	GUCC0137	Soil	-	-
<i>Py. splendens</i>	I	C101	<i>Anigozanthus</i>	-	-
<i>Py. ultimum</i>	I	NBRC100122	Sugar beet	-	-
<i>Py. uncinulatum</i>	J	MAFF240295	Lettuce	-	+
		CH97LAC1	Lettuce	-	+
<i>Py. buismaniae</i>	J	CBS101356	Chrysanthemum	N	-
<i>Py. mastophorum</i>	J	CBS 375.72	<i>Apium graveolens</i>	N	-
<i>Py. sp. "jasmonium"</i>	J	C10-1		N	-
<i>Py. orthogonon</i>	J	CBS 376.72	Root	N	-
<i>Py. perplexum</i>	J	N02F2 1-1	Soil	N	-
<i>Py. nodosum</i>	J	02F5 12-2	Soil	N	-
<i>Py. acanthophoron</i>	J	MAFF425319	Soil	N	-
<i>Py. nunn</i>	J	CBS 808.96	Soil	-	-
<i>Py. polymastum</i>	J	CBS 811.70	Lettuce	-	-
<i>Phytophthora chamaehyphom</i>		CBS 259.30	Papaya	-	-
<i>Pp. helicoides</i>		NBRC100107	Rose	-	-
<i>Pp. oedochilum</i>		GUCC0829	Yacon	-	-
<i>Pp. ostracodes</i>		CBS 768.73	Soil	-	-
<i>Pp. vexans</i>		2D111	Soil	-	-
<i>Phytophthora nicotianae</i>	1	CBS 535.92	<i>Kalanchoe</i>	-	-
<i>Ph. citricola</i>	2	CH95PHE28	<i>Eustoma grandiflorum</i>	-	-
<i>Ph. nemorosa</i>	3	C71		-	-
<i>Ph. palmivora</i>	4	CH88-1	<i>Oncidium</i>	-	-
<i>Ph. heveae</i>	5	P1102		-	-
<i>Ph. humicola</i>	6a	P3826		-	-
<i>Ph. cambivora</i>	7a	MAFF305918	Apple	-	-
<i>Ph. cryptogea</i>	8a	CBS 113.19	<i>Anaphalis margaritacea</i>	-	-
<i>Ph. pseudolactucae</i>	8b	MAFF239556	Lettuce	-	-
		Kph2010-1-1	Lettuce	-	-
		Kph2010-3-2	Lettuce	-	-
		Kph2011-1-1	Lettuce	-	-
<i>Ph. insolita</i>	9	P6195	Soil	-	-
<i>Ph. boehmeriae</i>	10			-	-
<i>Aphanomyces</i> ssp.		GFHT2		-	-
<i>Fusarium oxysporum</i>		MAFF72510	Strawberry	-	-
<i>Plasmodiophora brassicae</i>		HY	Chinese cabbage	-	-
<i>Plasmodiophora brassicae</i>		AH		-	-
<i>Rhizoctonia solani</i>		SO1	Bacopa	-	-
<i>Saprolegnia</i> sp.		IFO32708	Brown trout	-	-
<i>Verticillium alboatrum</i>		Vaal	Potato	-	-
<i>Sclerotinia sclerotiorum</i>		AiTog	Wax gourd	-	-

plant or soil samples from field 2 (Table 5). Overall, the pathogens were detected much more frequently in soil samples than in plant samples.

Discussion

In this study, we developed a simple procedure for the detection of four plant-pathogenic oomycetes using the LAMP assay. We designed primers and optimized the assay for three of the species: *Py. spinosum*, *Py. uncinulatum*, and *Ph. pseudolactucae*. The detection limits of each LAMP primer set were at most 100 femptograms. This high level of sensitivity is similar to that obtained with PCR. Feng et al. (2018) developed a LAMP assay for detection of *Py. irregulare* in eustoma, and we confirmed the utility of the same primers and conditions for detection of *Py. irregulare* in lettuce. Thus, like PCR, LAMP has the advantages of high specificity and sensitivity. It has the additional advantages of simplicity, speed, and low cost, and skilled personnel are not required to perform the assay.

Multiplex PCR was developed by Chamberlain et al. (1988), and this method has been successfully applied in many areas of DNA

testing, including the multiple diagnosis of plant-pathogenic oomycetes (Ishiguro et al. 2014; Li et al. 2011; Rojas et al. 2017). In the LAMP assay, a lack of simple, sequence-dependent detection techniques makes it difficult to implement a multiplex approach. Currently, multiplex LAMP detection has been used successfully in the laboratory detection and clinical diagnosis of infectious diseases. These techniques involve one or more of the following: a fluorescence-based monitoring method, such as gel electrophoresis-based analysis with restriction enzyme digestion of the amplified DNA products (He and Xu 2011); measurement of amplicon annealing temperatures with a fluorometer (Mahony et al. 2013); fluorescence signal-based monitoring with assimilating probes (Kubota and Jenkins 2015); and the use of novel mediator displacement probes with universal reporters (Becherer et al. 2018). These high-cost and time-consuming methods are not suitable for routine pathogen detection in field samples. Thus, more research is needed to develop appropriate multiplex LAMP detection methods for plant pathogens in the field.

The LAMP detection process does not require the extraction and purification of DNA from plant materials. To identify the most

Table 2. Specificity test of the LAMP primer for *Phytophthora pseudolactucae*

Species	Clade ^a	Isolate ^b	Origin	Amplification ^c
<i>Pythium adhaerens</i>	A	CBS 520.74	Soil	-
<i>Py. arrhenomanes</i>	B1	NBRC100102	Zoysia grass	-
<i>Py. dissotocum</i>	B2	MAFF305576	Soil	-
<i>Py. acanthicum</i>	D	MAFF241099	Soil	-
<i>Py. hypogynum</i>	E1	CBS 234.94	Soil	-
<i>Py. middletonii</i>	E2	CBS 528.74	Soil	-
<i>Py. irregulare</i>	F	CBS 263.30	<i>Nicotiana tabacum</i>	-
		EP-2	Carrot	-
<i>Py. spinosum</i>	F	NBRC100116	Carrot field soil	-
		OD231		-
		GUKan5		-
		Py73		-
		07KST-2		-
		1D1S032		-
<i>Py. paddicum</i>	G	MAFF241108	Water	-
<i>Py. anandrum</i>	H	CBS 285.31	<i>Rheum rhaponitium</i>	-
<i>Py. ultimum</i>	I	NBRC100122	Sugar beet	-
<i>Py. uncinulatum</i>	J	MAFF240295	Lettuce	-
		CH97LAC1	Lettuce	-
<i>Py. polymastum</i>	J	CBS 811.70	Lettuce	-
<i>Phytophthora chamaehyphon</i>		CBS 259.30	Papaya	-
<i>Pp. helicoides</i>		NBRC100107	Rose	-
<i>Pp. oedoehilum</i>		GUCC0829	Yacon	-
<i>Pp. ostracodes</i>		CBS 768.73	Soil	-
<i>Pp. vexans</i>		2D111	Soil	-
<i>Phytophthora nicotianae</i>	1	CBS 535.92	<i>Kalanchoe</i>	-
<i>Ph. cactorum</i>	1a	MAFF731066	Strawberry	-
<i>Ph. tentaculata</i>	1b	C05	<i>Gazania</i> sp.	-
<i>Ph. infestans</i>	1c	MAFF236324		-
<i>Ph. citricola</i>	2	CH95PHE28	<i>Eustoma grandiflorum</i>	-
<i>Ph. citrophthora</i>	2a	CBS 950.87		-
<i>Ph. colocasiae</i>	2a	NBRC30695	<i>Colocasia antiquorum</i>	-
<i>Ph. meadii</i>	2a	CBS 219.88		-
<i>Ph. capsici</i>	2b	MAFF305920	Water melon	-
<i>Ph. nemorosa</i>	3	C71		-
<i>Ph. palmivora</i>	4	CH88-1	Oncidium	-
<i>Ph. heveae</i>	5	P1102		-
<i>Ph. humicola</i>	6a	P3826		-
<i>Ph. megasperma</i>	6b	NBRC32176	White trumpet lily	-

(Continued on next page)

^a Molecular phylogenetic clades for *Pythium* and *Phytophthora* were obtained from Lvesque and De Cock (2004) and Blair et al. (2008), respectively.

^b Isolates were collected from the CBS-KNAW Fungal Biodiversity Centre (CBS), the NITE Biological Research Centre (NBRC), the NIAS Genebank (MAFF), the World Phytophthora Genetic Resource Collection (P), and the Gifu University Cultures Collection (GUCC).

^c Symbols: + = amplified, - = no amplification

effective on-site detection method, we contrasted the procedures of P-LAMP and PC-LAMP. For P-LAMP, the plant sample is simply mixed with distilled water. In PC-LAMP, a selective culture medium is used, to allow the pathogen to grow, thus increasing the probability of detection. This procedure is also simple, but a few days are required to obtain the results. Feng et al. (2015) demonstrated that the detection sensitivity of PC-LAMP was higher than P-LAMP in the diagnosis of *Py. irregulare*. However, in PC-LAMP, the detection of a slow-growing pathogen such as *Py. uncinulatum* will be negatively impacted by fast-growing species that might be simultaneously present in the plant sample. In this study, we obtained positive results in similar numbers of samples with P-LAMP and PC-LAMP. Therefore, for the pathogens examined in this study, P-LAMP is the most simple and time-saving procedure for the sensitive detection of pathogens in lettuce samples.

Bait-based methods that use baiting materials to trap pathogens in the soil are not suitable for slow-growing species such as *Py. uncinulatum* and *Ph. pseudolactucae*, and in particular, they are not suitable for *Py. spinosum*, which does not form zoospores

(Van der Plaats-Niterink 1981). For this reason, we tested five soil DNA extraction kits and identified the one that showed the highest sensitivity. This kit also has the advantages of high extraction efficiency and simplicity.

We used the P-LAMP assay, with the species-specific primer sets for the four plant-pathogenic oomycetes, to analyze diseased lettuce plants collected from three cultivation fields. The wilting symptoms were visually similar in all of the plants. All four pathogens were detected in the fields with varying degrees, suggesting that even with the same wilting symptoms, the disease could be caused by species of *Phytophthora* and/or *Pythium*. We independently analyzed three different tissues from each plant: the root base, root tip, and the aboveground pith. Our results suggested that *Ph. pseudolactucae* infects the pith in the aboveground parts, whereas *Pythium* mainly infects roots. This is supported by previous studies. Kanto et al. (2005) found that the browning caused by *Ph. pseudolactucae* occurs mainly along the conduit from the pith. Kusunoki (2012) and Matsuura et al. (2010) surveyed the pathogenicity of the *Pythium* species in lettuce and found that all the pathogens caused browning and corruption of the roots.

Table 2. (Continued from previous page)

Species	Clade ^a	Isolate ^b	Origin	Amplification ^c
<i>Ph. asparagi</i>	6c	CBS 132095		-
<i>Ph. cambivora</i>	7a	MAFF305918	Apple	-
<i>Ph. cajani</i>	7b	P3105		-
<i>Ph. cinnamomi</i>	7b	MAFF238144	St. John's wort	-
<i>Ph. melonis</i>	7b	CH00ME1-1	Melo	-
<i>Ph. sojae</i>	7b	MAFF237500	White trumpet lily	-
<i>Ph. cryptogea</i>	8a	CBS 113.19	<i>Anaphalis margaritacea</i>	-
<i>Ph. drechsleri</i>	8a	P1087	Potato	-
<i>Ph. erythroseptica</i>	8a	CBS 129.23	Potato	-
<i>Ph. medicaginis</i>	8a	P7029	Medicago sativa	-
<i>Ph. richardiae</i>	8a	P7789	<i>Zantedeschia</i>	-
<i>aethiopica</i>	-			
<i>Ph. sansomeana</i>	8a	CBS 117692	<i>Silene latifolia</i>	-
<i>Ph. trifolii</i>	8a	CBS 117687	<i>Trifolium</i>	-
<i>Ph. sp. kelmania</i>	8a	P10613		-
<i>Ph. pseudolactucae</i>	8b	MAFF239556	Lettuce	+
		Kph2010-1-1	Lettuce	+
		Kph2010-3-2	Lettuce	+
		Kph2011-1-1	Lettuce	+
<i>Ph. brassicae</i>	8b	CBS 179.87	<i>Brassica oleracea</i>	-
<i>Ph. porri</i>	8b	CBS 688.79	<i>Brassica chinensis</i>	-
<i>Ph. primulae</i>	8b	CBS 620.97	<i>Primula acaulis</i>	-
<i>Ph. dauci</i>	8b	CBS 127102	<i>Carota</i>	-
<i>Ph. lactucae</i>	8b	BPIC1985	Lettuce	-
<i>Ph. foliorum</i>	8c	CBS 121655	<i>Azalea</i> sp.	-
<i>Ph. lateralis</i>	8c	CBS 168.42	<i>Lawson cypress</i>	-
<i>Ph. austrocedri</i>	8d	CBS 122911	<i>Austrocedrus chilensis</i>	-
<i>Ph. obscura</i>	8d	CBS 129273	Soil	-
<i>Ph. syringae</i>	8d	Fium1		-
<i>Ph. insolita</i>	9	P6195	Soil	-
<i>Ph. parsiana</i>	9	R6 (1)	<i>Medicago sativa</i>	-
<i>Ph. polonica</i>	9	P131445	<i>Alnus glutinosa</i>	-
<i>Ph. boehmeriae</i>	10			-
<i>Ph. kernoviae</i>	10	P1751		-
<i>Aphanomyces</i> sp.		GFHT2		-
<i>Fusarium oxysporum</i>		MAFF72510	Strawberry	-
<i>Plasmodiophora brassicae</i>		HY	Chinese cabbage	-
<i>Plasmodiophora brassicae</i>		AH		-
<i>Rhizoctonia solani</i>		SO1	Bacopa	-
<i>Saprolegnia</i> sp.		IFO32708	Brown trout	-
<i>Verticillium alboatrum</i>		Vaal	Potato	-
<i>Sclerotinia sclerotiorum</i>		AiTog	Wax gourd	-

The soil samples around the diseased lettuce were collected and analyzed using the LAMP assay. In many cases, the pathogens were detected more frequently in the soil samples than in the plant samples, suggesting that not all of the pathogens existing in the soil will

infect the lettuce plants. On the other hand, *Ph. pseudolactucae* and *Py. uncinulatum* were both detected in similar numbers of soil samples as lettuce samples, and in higher numbers of lettuce samples than the other two species. These results suggest that

Table 3. Primer sets used in this study

Target species	Primer sets ^a	Primers	Sequences (5' - 3')	Region amplified
<i>Py. spinosum</i>	L-Psp	F3	CTGCGTGGTGCTGTGTATG	rDNA-ITS
		FIP	ACTACCAGTACACCTCAAGGTCGGCTGGACAATGTTG	
		B3	ACGCAGGATTAACCCACATG	
		BIP	AGTGCGTTGTCGTGGATGCATCCCAAATTCAAACCTCCTCT	
		B-Loop	GTGCACCTTTGTGTGTGTCAGT	
<i>Py. uncinulatum</i>	P-Psp	For	TATCTGCGTGGTGCTGTGTA	rDNA-ITS
		Rev	ATCCACGACAACGCACTAC	
	L-Pun	F3	GAAGGTGAAGTCGTAACAAGG	rDNA-ITS
		FIP	GATGACGCTAAACTGGTTGGGTGAACCTGCGGAAGGATC	
		B3	CCAAGCCGTCTCACTACG	
<i>Py. irregulare</i> ^b	P-Pun	BIP	TACATTGCGAGAGAGGGGAAACAAAACAGCTCGACCTTCGT	rDNA-ITS
		For	ACCCAACCAGTTTAGCGTCATC	
		Rev	CTTGTTTCCCTCTCTCGCA	
	L-Pir	F3	CGTTTCTTCTTCCGTGTAGT	rDNA-ITS
		FIP	GCAATCATTGCAAACAATACTCCGGTGGAGGAGAGTTGCAGAT	
<i>Ph. pseudolactucae</i>	L-Phps	B3	ACCGCGAATCGAGGTCC	cox1
		BIP	TCTTTTTGTATGTGCGCGGTGCTCACCGAAGTCGCCGAC	
		For	GTTGTTAGTAGTGTGTGTRGCA	
		Rev	GATCAACCCGGAGTATACAAAAC	
		F3	ACCTGATATGGCATTTC	
	P-Phps	FIP	CGATTCAACAATAGCTGATGGGTTATTACCTCCAGCA	cox1
		B3	TGCTAAATCTACAGAAGG	
		BIP	GAGTACTGGTTGGACTGTTCTGAATGTGCCTGTAC	
		B-Loop	TCCACCATTATCAAG	
		For	GTGCTTTTGCGGGTATTATAG	
		Rev	AGAAGGTCCTGAATGTGCC	

^a L: LAMP primer set; P: PCR primer set.

^b The Primer sets of *P. irregulare* were designed by Feng et al. (2015).

Table 4. Comparison of two plant LAMP detection methods

Fields	Parts of lettuce	Number	<i>Py. spinosum</i>		<i>Py. uncinulatum</i>		<i>Py. irregulare</i>		<i>Ph. pseudolactucae</i>	
			P-LAMP ^a	PC-LAMP ^b	P-LAMP	PC-LAMP	P-LAMP	PC-LAMP	P-LAMP	PC-LAMP
A	Root	6	0	0	3	1	0	1	0	0
	Pith	10	0	0	0	1	0	0	4	5
B	Root	2	2	2	1	0	0	0	0	0
	Pith	2	0	0	0	1	0	0	0	0
Total		20	2	2	4	3	0	1	4	5

^a P-LAMP: diseased samples were mixed with distilled water and the supernatant liquid was used directly for amplification.

^b PC-LAMP: the diseased samples were placed in a culture medium and incubated for one day, then the medium was mixed with water and used for amplification.

Table 5. Investigation of infection sites by four pathogens in lettuce using P-LAMP with species-specific primer sets

Fields		Regions of samples	Number	<i>Py. spinosum</i>	<i>Py. uncinulatum</i>	<i>Py. irregulare</i>	<i>Ph. pseudolactucae</i>
1	Root	Base	5	0	0	0	0
		Tip	5	0	0	0	0
	Pith		5	0	1	0	4
		Soil	Around the plant	5	5	4	4
2	Root	Base	5	0	5	0	0
		Tip	5	0	3	0	0
	Pith		5	0	4	0	0
		Soil	Around the plant	5	1	5	0
3	Root	Base	5	1	3	1	0
		Tip	5	0	2	0	0
	Pith		5	0	0	0	5
		Soil	Below the mulch	5	4	2	5
		Above the mulch	5	1	2	5	2
Total			65	12	31	15	19

Table 6. Comparison of soil DNA extraction kits based on LAMP detection of *Phytophthora pseudolactucae*

Soil DNA extraction methods	Extraction time required for a sample (min)	Extraction	Detection of LAMP
Soil DNA Isolation kit	40	1	+
		2	+
Extrop soil kit plus ver.2 kit	50	1	-
		2	-
Mightyprep reagent for DNA kit	10	1	-
		2	-
Kageyama + Mag method	50	1	-
		2	+
Soil DNA Isolation mini kit	90	1	-
		2	-

Ph. pseudolactucae and *Py. uncinulatum* have higher disease incidence rates than *Py. irregulare* and *Py. spinosum*. Similarly, when Kusunoki (2012) compared the pathogenicity among *Pythium* species to lettuce, *Py. irregulare* and *Py. spinosum* were less pathogenic than *Py. uncinulatum*.

Based on the results of this study, we propose the following important strategies to prevent oomycete diseases in lettuce cultivation fields. (i) It is important to diagnose the soil prior to planting. If pathogens are found in the soil, it needs to be sterilized with fungicides effective against oomycetes. (ii) When plastic film mulch is used, the farmer should not put soil over the film, to reduce the risk of pith infection. (iii) Once disease has occurred, the soil surrounding the diseased plants should be excluded and sterilized to prevent spread of the disease. Certainly, if an oomycete-related disease occurs, soil sterilization is necessary after harvest.

Fig. 1. Comparison of the sensitivities of LAMP and PCR using species-specific primer sets for *Pythium spinosum* (A), *Py. uncinulatum* (B), and *Phytophthora pseudolactucae* (C). Genomic DNA was serially diluted from 1 ng to 1 fg. The LAMP reaction was monitored by measurement of turbidity. PCR products were detected by gel electrophoresis. M: 100 bp DNA ladder.

It is clear from this study that the LAMP method can be effectively used for the diagnosis of pathogenic oomycetes in plants and soil. This method has the potential to greatly enhance the management and prevention of diseases in the field.

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