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Special Report

A LAMP Assay for Rapid Detection of the Pitch Canker Pathogen *Fusarium circinatum*

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Abstract

The pine pitch canker pathogen *Fusarium circinatum* is endemic in the southeastern United States and Central America and represents an invasive threat globally. This ecologically adaptable fungus readily infects all parts of its pine hosts, leading to widespread mortality of nursery seedlings and decline in the health and productivity of forest stands. Because trees infected by *F. circinatum* can remain asymptomatic for long periods of time, accurate and rapid tools are needed for real-time diagnostics and surveillance at ports, in nurseries, and in plantations. To meet this need and to limit the spread and impact of the pathogen, we developed a molecular test using loop-mediated isothermal amplification (LAMP), a technology that allows for the rapid detection of pathogen DNA on portable, field-capable devices. LAMP primers were designed and validated to amplify a gene region unique to *F. circinatum*. Using a globally representative collection of *F. circinatum* isolates and other closely related species, we have demonstrated that the assay can be used to identify *F. circinatum* across its genetic diversity and that it is sensitive to as few as 10 cells from purified DNA extracts. The assay can also be used with a simple, pipette-free DNA extraction method and is compatible with testing symptomatic pine tissues in the field. This assay has the potential to facilitate diagnostic and surveillance efforts both in the laboratory and in the field and, thus, to reduce the spread and impact of pitch canker worldwide.

Keywords: crude DNA, forest health and protection, loop-mediated isothermal amplification, *Pinus*, rapid detection

Pitch canker, caused by the ascomycete fungus *Fusarium circinatum*, is one of the most important diseases of *Pinus* spp. globally. The pathogen is ecologically adaptable and able to cause a wide diversity of disease symptoms (Drenkhan et al. 2020; Wingfield et al. 2008), with severe losses seen in seedling nursery beds and managed forest stands (Blakeslee and Oak 1979; Mitchell et al. 2011; Viljoen et al. 1994; Wingfield et al. 2002). Infections result in considerable economic losses in nurseries due to recurring high mortality of seedling crops. In natural and planted forests, infections impair growth and vigor, reducing stand productivity, leaving trees susceptible to secondary pests and pathogens and, in some cases, resulting in tree death (Arvanitis et al. 1984; Blakeslee and Oak 1979; Wingfield et al. 2008).

The pitch canker pathogen is present throughout the southern United States and Central America (Britz et al. 2001; Hepting and Roth 1946; Wikler and Gordon 2000) where, despite being endemic, it is a constant threat to natural and plantation forests (Dwinell et al. 1985; Guerra-Santos 1998; Steenkamp et al. 2012). Through human activity and the commercial movement of contaminated materials, *F. circinatum* has also spread to nurseries and forests in many parts of the world (Drenkhan et al. 2020), including Japan (Kobayashi and Muramoto 1989), Korea (Lee et al. 2000), South Africa (Viljoen et al. 1994), Chile (Wingfield et al. 2002), Spain (Pérez-Sierra et al. 2007), Italy (Carlucci et al. 2007), Uruguay (Alonso and Bertucci 2009), and Portugal (Bragança et al. 2009). *F. circinatum* is a growing threat due to the susceptibility of pine trees in its native range and those naïve to the fungus (Davydenko et al. 2018; Martín-García et al. 2017). Moreover, the pathogen is expected to cause even greater damage under conditions of changing climate and in interactions with other native pathogens (Elvira-Recuenco et al. 2019; Quesada et al. 2019).

Trees infected by *F. circinatum* typically have flagging branches and conspicuous resin flow from stem and branch cankers (Dwinell et al. 1985). In seedlings, symptoms of infection are less obvious. The first symptoms of infected seedlings are wilting and stem discoloration, which only manifest when the belowground infections have progressed and the roots are already compromised (Viljoen et al. 1994). Closer inspection may reveal resinosis, and underdeveloped and discolored roots (Viljoen et al. 1994). The presence of the pathogen also may be cryptic because endophytic associations with seeds and cones have been reported and the hemibiotrophic relationship of *F. circinatum* with young seedlings is well documented (Storer et al. 1998; Swett et al. 2016). As such, infections and losses in nurseries may become visible only after significant resources have been expended to raise the crop, with seedlings dying in either the beds or after planting in the field (Starkey et al. 2007).

F. circinatum is able to persist in the environment in a diversity of reservoirs. Conidia may remain viable among pine slash and litter for up to 3 years (McNee et al. 2002). An interesting contemporary discovery has been that grasses (family Poaceae) in the proximity of infected trees also serve as a refuge for *F. circinatum* and apparently harbor the fungus asymptomatically as an endophyte (Carter and Gordon 2020; Herron et al. 2020; Swett et al. 2014). Several other natural reservoirs have been reported, including wild banana (*Musa acuminata*), boat orchids (*Cymbidium* sp.), elm-leaf blackberry (*Rubus ulmifolius*), meadow knapweed (*Centaurea debeauxii*), common

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sowthistle (*Sonchus oleraceus*), wood germander (*Teucrium scorodonia*), and maize (*Zea mays*) (Drenkhan et al. 2020).

There are no silvicultural interventions known to effectively suppress an established pitch canker outbreak in natural or planted forests (Wingfield et al. 2008). There are also no effective chemical fungicides for its treatment in forest stands or pine nurseries (Zamora-Ballesteros et al. 2019) and, consequently, other strategies for disease management must be considered. The most effective means of reducing risks due to tree diseases, including pitch canker, lie in preventing new introductions and infections through proactive management and early detection (Wingfield et al. 2015; Zamora-Ballesteros et al. 2019). For example, it is important in plantation settings to avoid the introduction of asymptomatic infected plants (Zamora-Ballesteros et al. 2019). Furthermore, operations that may injure trees should be limited, at least until periods when the ambient spore load is low. *F. circinatum* also responds to increases in nitrogen, potassium, and phosphorous, displaying greater incidence and severity in managed stands after the application of chemical fertilizers (Blakeslee et al. 1999; Fisher et al. 1981; Starkey et al. 2007).

Sensitive diagnostic tools are required to detect *F. circinatum* in plants and substrates that could serve as a source of new infections. To serve this need, several molecular tools have been developed to enhance the detection of *F. circinatum* in various substrates (Vainio et al. 2019), including field-deployed spore traps (Schweigkofler et al. 2004), contaminated seed lots (Dreaden et al. 2012; Ioos et al. 2009), and infected plant tissue (Luchi et al. 2018). Molecular tools are now the standard (Luchi et al. 2020), providing much greater sensitivity to detection protocols relative to culture-based approaches. However, conventional and quantitative PCR (qPCR)-based procedures necessitate dedicated laboratory equipment such as PCR thermal cyclers that are costly and require specialized training (Li et al. 2017). Additionally, the *Taq* polymerase-based chemistry that underpins PCR is susceptible to inhibition by compounds present in plant tissues and requires thorough purification or heavy dilution of DNA before analysis (Capron et al. 2020; Notomi et al. 2015).

Loop-mediated isothermal amplification (LAMP) (Notomi et al. 2000) is a DNA amplification technique alternative to PCR that offers greater specificity and speed. Additionally, it is less sensitive to inhibitors, allowing the use of easily extracted crude DNA (Hamilton et al. 2020; Kogovšek et al. 2015; Mason and Botella 2020). LAMP reactions proceed under isothermal conditions through the activity of a recombinant *Bst* polymerase and can be prepared easily with commercially available all-in-one master mixes. The robust nature of LAMP reactions permits the use of inexpensive portable instruments in the field (Aglietti et al. 2019; Hamilton et al. 2020; Villari et al. 2017). For this reason, LAMP has become an increasingly popular tool for the diagnosis and surveillance of plant pathogens (Aglietti et al. 2021; Dai et al. 2019; Le and Vu 2017; Luchi et al. 2020; Sillo et al. 2018; Villari et al. 2013; Winkworth et al. 2020).

A LAMP assay targeting the pitch canker pathogen was recently developed (Stehlíková et al. 2020). Although that assay might be useful in some regions of the world, the primers are not sufficiently specific to differentiate *F. circinatum* from the agricultural pathogen *F. temperatum*. This is particularly relevant in areas such as North America, where *F. temperatum* occurs on maize (Lanza et al. 2016; Ridout et al. 2016) and where pine plantations and crop fields are often contiguous.

Thus, the aims of this study were to develop a new LAMP assay that is specific to *F. circinatum* and capable of detecting the fungus across its expanding range and global genetic diversity. Furthermore, we sought to validate the suitability of the assay for implementation as a diagnostic tool in the field via testing its compatibility with a rapid and simple, pipette-free DNA extraction method (Mason and Botella 2020) and to compare these results against a qPCR gold-standard method (Ioos et al. 2009).

Materials and Methods

Fungal isolates, plant material, and DNA extractions

A globally representative collection of 83 fungal isolates (Table 1) was used to develop and validate the LAMP assay. This collection consisted of 51 *F. circinatum* isolates and 32 nontarget fungi, including many closely related *Fusarium* spp. Each isolate was grown on sterile 2.0% potato dextrose agar (PDA; Becton Dickinson, Sparks, MD, U.S.A.) under ambient conditions for 7 to 14 days. Approximately 100 mg of mycelium was collected from each isolate, and total DNA was extracted using either the E.Z.N.A. Fungal DNA Mini Kit (Omega, Bio-tek, Norcross, GA, U.S.A.) or the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Waltham, MA, U.S.A.), according to manufacturer instructions. Concentrations of DNA extracts were quantified using a Qubit fluorometer (Invitrogen, Carlsbad, CA, U.S.A.) and normalized to a concentration of 1.0 ng/μl prior to use in LAMP reactions.

Crude DNA was extracted from wood tissue samples collected in Athens, GA, to determine the utility of the assay as a field-capable diagnostic tool. Samples were collected from *Pinus taeda* displaying visible symptoms of pitch canker, including stem cankers and conspicuous resin flow, as well as from asymptomatic trees. A pipette-free crude DNA extraction method that uses nucleic acid purifying filter paper dipsticks was adapted from the work of Mason and Botella (2020). Filter paper dipsticks with an absorptive area of 2 by 6 mm were prepared from Whatman Grade 1 filter paper (Cytiva, Marlborough, MA, U.S.A.) and Paraplast Plus (Leica Biosystems, Buffalo Grove, IL, U.S.A.) as described by Mason and Botella (2020). Tissues were excised from the samples using a sterilized knife to obtain 25 mg of wood tissue after removing the bark. The tissue was then placed into a 1.5-ml microcentrifuge tube containing two sterilized 4.5-mm steel bearings (Crosman Corporation, Bloomfield, NY, U.S.A.) and 800 μl of extraction buffer, as per Mason and Botella (2020). Tubes were then inverted five times to break open cells and release DNA. To purify DNA, a filter paper dipstick was immersed into the extraction buffer until saturated, dipped five times in 800 μl of wash buffer (Mason and Botella 2020), and eluted by dipping 15 times directly into a 25-μl LAMP or 20-μl PCR mixture.

Design of LAMP primers

A set of LAMP primers consisting of external primers F3 and B3, internal primers FIP and BIP, and a single forward-loop primer was designed using the PrimerExplorer V5 software with default parameters (Eiken Chemical Co., Tokyo, Japan). Primers were designed to bind a target sequence of *F. circinatum* that was selected within a 12-kb quantitative trait locus (QTL) encoding five growth-related genes in the subtelomeric region of chromosome 3 (De Vos et al. 2011; Van Wyk et al. 2018). This region was reported as unique to *F. circinatum* and was found to be present in all available genome data at the time of its identification. Each of the five genes in this QTL was determined to have arrived in the *F. circinatum* genome via horizontal transfer due to the high similarity each bears to other species (Van Wyk et al. 2018). Thus, the target sequence for the LAMP assay was selected as a 210-bp section of the QTL spanning portions of the gene *FCIRG_04556* and the noncoding region between genes *FCIRG_04556* and *FCIRG_04555* (Fig. 1) to ensure a combination of primer binding sites that is specific to *F. circinatum*. Multiple primer sets were initially generated by the PrimerExplorer V5 software but only the primer set with the most negative ΔG value for target binding affinity was selected and reentered into the software to generate potential loop primer sequences. The program generated no reverse-loop primers and four forward-loop primers with equally negative ΔG values, of which the first option was selected. The forward-loop primer sequence was then used to design a 6-carboxy-fluorescein (FAM)-labeled primer-probe containing a sequence that binds to a black hole quencher (BHQ) strand as previously described (Kubota et al. 2011). This primer-probe acts as a loop primer, enhancing reaction speed and specificity, and enables real-time fluorescence monitoring as a proxy for amplification. The complete primer set is reported in Table 2.

The target-binding sequences of primers F3, B3, FIP, and BIP were compared with the complete genomes of 50 *Fusarium* spp. representing the American, African, and Asian clades of the *F. fujikuroi* species complex (FFSC) (Supplementary Material) (O'Donnell et al. 1998). The FFSC is a closely related group of

Table 1. Fungal isolates used to verify the specificity of the *Fusarium circinatum* loop-mediated isothermal amplification (LAMP) assay[a]

Fungal species	Collection number[b]	Host species	Country	LAMP result	LAMP reaction volume (µl)
Fusarium anthophilum	CBS 119858	Environmental	United States	0/3	10
F. awaxy	CN 058D3	*Zea mays*	South Africa	0/3	25
F. awaxy	CN 074B7	*Z. mays*	South Africa	0/3	25
F. awaxy	CN 165I5	*Z. mays*	South Africa	0/3	25
F. awaxy	CN 166B2	*Z. mays*	South Africa	0/3	25
F. awaxy	CN 129D2	*Melinis repens*	South Africa	0/3	25
F. awaxy	CN 130A3	*Chloris* sp.	South Africa	0/3	25
F. awaxy	CN 135D8	*Chloris* sp.	South Africa	0/3	25
F. bacteroides	CBS 100057[c]	*Cronartium conigenum* on *Pinus leiophylla*	United States	0/3	10
F. circinatum	CV_2020_001	Unknown	United States	**3/3**	25
F. circinatum	CV_2020_002	*P. elliottii*	Florida, United States	**3/3**	25
F. circinatum	CV_2020_003	Unknown	Florida, United States	**3/3**	25
F. circinatum	CV_2020_004	*P. elliottii*	Georgia, United States	**3/3**	25
F. circinatum	CV_2020_005	*P. palustris*	Alabama, United States	**3/3**	25
F. circinatum	CV_2020_006	*P. taeda*	Georgia, United States	**3/3**	25
F. circinatum	CV_2020_007	*P. palustris*	Florida, United States	**3/3**	25
F. circinatum	61F	*P. greggii*	KwaZulu Natal, South Africa	**3/3**	10
F. circinatum	CMWF 573	*P. radiata*	Western Cape, South Africa	**3/3**	10
F. circinatum	CMWF 1287	*P. oocarpa*	Nicaragua	**3/3**	10
F. circinatum	757F	*P. patula*	Limpopo, South Africa	**3/3**	10
F. circinatum	CMWF 1288	*P. oocarpa*	Nicaragua	**3/3**	10
F. circinatum	CMWF 1286	*P. rudis*	Guatemala	**3/3**	10
F. circinatum	CMWF 1635	*P. pseudostrobus* × *P. moniciminoii*	Colombia	**3/3**	10
F. circinatum	CMWF 532	*P. radiata*	California, United States	**3/3**	10
F. circinatum	CMWF 553	*P. radiata*	Florida, United States	**3/3**	10
F. circinatum	UG18	*P. greggii*	Eastern Cape, South Africa	**3/3**	10
F. circinatum	CMWF 24	*P. patula*	Mpumalanga, South Africa	**3/3**	10
F. circinatum	CMWF 1598	Unknown	Chile	**3/3**	10
F. circinatum	CMWF 1289	*P. oocarpa*	Nicaragua	**3/3**	10
F. circinatum	CMWF 660	*P. radiata*	Western Cape, South Africa	**3/3**	10
F. circinatum	CMWF 1807	*P. greggii*	KwaZulu Natal, South Africa	**3/3**	10
F. circinatum	CMWF 11	*P. patula*	Mpumalanga, South Africa	**3/3**	10
F. circinatum	CMWF 279	*P. patula*	Colombia	**3/3**	10
F. circinatum	CMWF 105	*P. radiata*	Spain	**3/3**	10
F. circinatum	CMWF 106	*P. radiata*	Spain	**3/3**	10
F. circinatum	CMWF 1599	Unknown	Chile	**3/3**	10
F. circinatum	CMWF 1608	Unknown	Chile	**3/3**	10
F. circinatum	CMWF 1284	*P. rudis*	Guatemala	**3/3**	10
F. circinatum	CMWF 1285	*P. hartwegii*	Guatemala	**3/3**	10
F. circinatum	CMWF 550	*P. radiata*	Mexico	**3/3**	10
F. circinatum	CMWF 1800	*P. radiata*	Mexico	**3/3**	10
F. circinatum	CMWF 107	*P. radiata*	Spain	**3/3**	10
F. circinatum	CMWF 540	*P. radiata*	California, United States	**3/3**	10
F. circinatum	FSP 34	Unknown	California, United States	**3/3**	10
F. circinatum	CBS 138822	Unknown	Unknown	**3/3**	10
F. circinatum	NRRL 25331 = MRC 7541 = CBS 405.97[c]	*P. radiata*	United States	**3/3**	10
F. circinatum	NRRL 25332 = CBS 100197	*P. taeda*	United States	**3/3**	10
F. circinatum	CBS 117843	*Pinus* sp.	Spain	**3/3**	10
F. circinatum	MRC 7488 = CBS 119864	*P. patula*	South Africa	**3/3**	10
F. circinatum	CBS 138821	*Pinus* sp.	United States	**3/3**	10
F. circinatum	CBS 141668	Unknown	Unknown	**3/3**	10
F. circinatum	MRC 6213 = CBS 119865	*P. patula*	South Africa	**3/3**	10
F. circinatum	CBS 122161	*P. radiata*	Spain	**3/3**	10
F. circinatum	CBS 122162	*P. radiata*	Spain	**3/3**	10
F. circinatum	CBS 122163	*P. radiata*	Spain	**3/3**	10
F. circinatum	CBS 122164	*P. radiata*	Spain	**3/3**	10
F. circinatum	MRC 6213 = CBS 122165	*P. radiata*	Spain	**3/3**	10

(*Continued on next page*)

[a] Positive LAMP results, indicated in bold, were determined by amplification of at least two of three replicate reactions for each sample.
[b] Abbreviations for the culture collections: the culture collection of Dr. Villari at the D. B. Warnell School of Forestry (CV); the U.S. Agricultural Research Service culture collection (NRRL); the Westerdijk Fungal Biodiversity Institute (WI) collection (CBS); the working collection of *Fusarium* isolates at FABI (CMWF) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; the working collection of the Applied Mycology Group (CN) at FABI, University of Pretoria; and the Medical Research Center (MRC) Tygerberg, Cape Town, South Africa.
[c] Ex-type specimen.
[d] Ex-epitype specimen.

Fusarium spp. that includes *F. circinatum* and other species relevant to plant and human health (Yilmaz et al. 2021). The target-binding sequences were queried against each of the 50 *Fusarium* genomes using the BLAST algorithm (Altschul et al. 1990) in the software package CLC Main Workbench 20.0.2 (Qiagen, Aarhus, Denmark).

Reactions using purified DNA

All reactions to validate LAMP assay sensitivity were carried out on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.) at the Warnell School of Forestry and Natural Resources at the University of Georgia. Specificity testing was conducted using either the above-mentioned StepOnePlus system or a Bio-Rad iCycler real-time system (Bio-Rad, Hercules, CA, U.S.A.) at the Forestry and Agricultural Biotechnology Institute at the University of Pretoria.

Reactions were performed in volumes of either 10 or 25 µl. Reduced-volume (10 µl) reactions were primarily used during specificity testing due to the limited availability of the LAMP master mix at the time. For 25-µl volumes, the reaction mixture consisted of 4.0 µl of molecular-grade water, 15.0 µl of Isothermal Master Mix (ISO-001nd; OptiGene Limited, Horsham, U.K.), 2.8 µl of primer mixture, 0.7 µl of probe mixture, and 2.5 µl of template DNA. For 10-µl reactions, all volumes were reduced by a factor of 2.5, maintaining consistent reagent concentrations. The final concentrations of the primers and probes were 2.8 µM for each FIP and BIP, 0.28 µM for each F3 and B3, 0.096 µM for the loop primer with the attached probe, and 0.184 µM for the quencher strand. All primers, probes, and quencher strands were synthesized by Integrated DNA Technologies (Coralville, IA, U.S.A.). Reactions to determine the sensitivity of the

Table 1. (*Continued from previous page*)

Fungal species	Collection number[b]	Host species	Country	LAMP result	LAMP reaction volume (µl)
F. circinatum	CBS 122448	*P. radiata*	Spain	**3/3**	10
F. circinatum	CBS 141670	Unknown	Unknown	**3/3**	10
F. circinatum	CBS 141671	Unknown	Unknown	**3/3**	10
F. guttiforme	NRRL 25295 = CBS 409.97[c]	*Ananas comosus*	Brazil	0/3	10
F. fujikuroi	MRC 1836 = CBS 119854	Unknown	Unknown	0/3	10
F. konzum	MRC 8427 = CBS 119849[c]	*Sorghastrum nuttans*	United States	0/3	10
F. lactis	NRRL 25338 = CBS 420.97	*Ficus carica*	United States	0/3	10
F. oxysporum	CV_2019_014	*P. palustris*	Florida, United States	0/3	10
F. pilosicola	CMWF 1183 = NRRL 29124[c]	*Bidens pilosa*	United States	**3/3**	10
F. pilosicola	CMWF 1189 = NRRL 29123	*B. pilosa*	United States	**3/3**	10
F. proliferatum	CBS 125014	*Homo sapiens*	United States	0/3	10
F. subglutinans	CMWF 530	*P. radiata*	Mexico	0/3	10
F. subglutinans	NRRL 20844 = CBS 215.76	*Z. mays*	Germany	0/3	10
F. fracticaudum	CMWF 280	*P. tecunamanii*	Colombia	0/3	10
F. succisae	NRRL 13613 = CBS 219.76[d]	*Succisa pratensis*	Germany	0/3	10
F. temperatum	CN 057I5	*Z. mays*	South Africa	0/3	25
F. temperatum	CN 061E4	*Z. mays*	South Africa	0/3	25
F. temperatum	CN 061G2	*Z. mays*	South Africa	0/3	25
F. temperatum	CN 061H5	*Z. mays*	South Africa	0/3	25
F. temperatum	CN 074B4	*Z. mays*	South Africa	0/3	25
F. temperatum	CN 074B6	*Z. mays*	South Africa	0/3	25
F. temperatum	CN 119D1	*Z. mays*	South Africa	0/3	25
F. temperatum	CN 122H1	*Z. mays*	South Africa	0/3	25
F. temperatum	CN 129E5	*Melinis repens*	South Africa	0/3	25
F. udum	NRRL 22949 = CBS 178.32	Unknown	The Netherlands	0/3	10
F. werrikimbe	CBS 125535[c]	*Sorghum leiocladum*	Australia	0/3	10

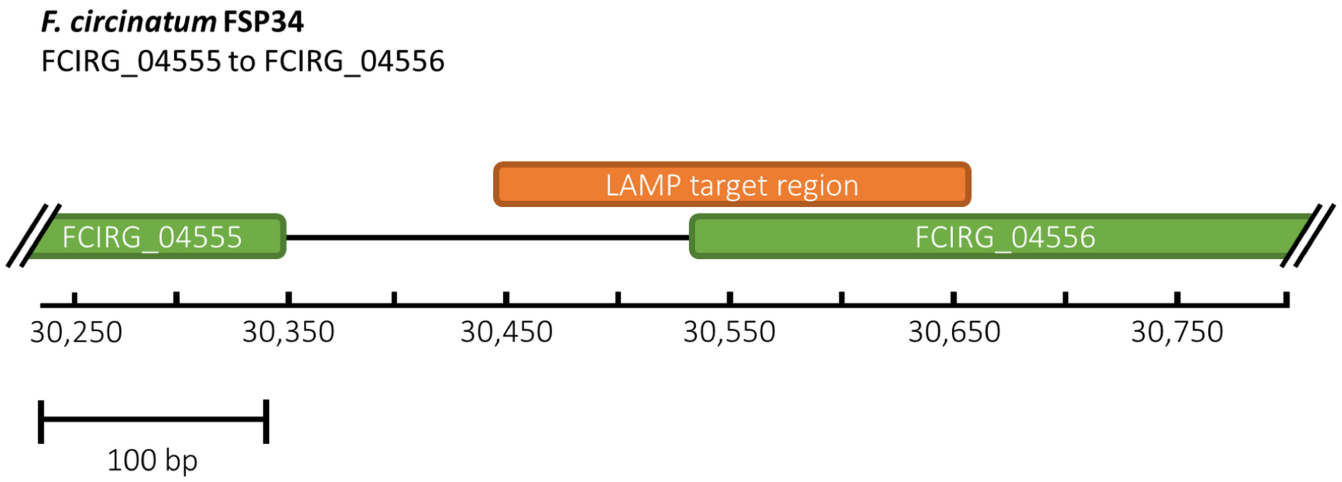

Fig. 1. Location of the *Fusarium circinatum* loop-mediated isothermal amplification (LAMP) target region in relation to the positions of genes *FCIRG_04555* and *FCIRG_04556*. The target region is located within a 12-kb quantitative trait locus that was identified by Van Wyk et al. (2018). Left and right blocks represent gene position while the center block indicates the 210-bp target of the LAMP assay. Numbers represent the base pair position in chromosome 3 of *F. circinatum* isolate FSP34 (accession number CM004513).

assay were conducted at a final volume of 25 µl, while reactions to validate assay specificity were run in either 10- or 25-µl volumes, as indicated in Table 1. All LAMP reaction mixtures were assembled in eight-tube MicroAmp Fast Reaction Tubes strips (Applied Biosystems), amplified for 60 min at 65°C, and finally incubated at 85°C for 5 min to inactivate the enzyme.

Assay sensitivity was determined by testing a 1:10 serial dilution of DNA from *F. circinatum* isolate CV-2020-006. The amounts of the DNA template added ranged from 1.0 ng to 0.1 pg (Table 3). Serially diluted spore suspensions were also tested as an additional sensitivity metric and one more relevant to spore-trapping applications. Isolate CV-2020-006 was grown on 2% water agar for 10 days to promote sporulation. The plate was then flooded with 5.0 ml of sterile water and the conidia loosened by gently agitating the surface of the plate with a sterile glass spreader. The suspension was collected from the plate and filtered through sterile cheesecloth (Hamilton et al. 2020). The concentration of cells was determined using a hemacytometer (Thermo Fisher Scientific), and dilutions were prepared at 1,000, 500, 100, 50, 10, 5, and 1 cells μl^{-1} (Table 3). Spore dilutions were boiled for 5 min and agitated for 15 s in a vortex mixer to release DNA for testing (Hamilton et al. 2020).

The sensitivity of the developed LAMP assay was compared with that of a a dual-labeled probe qPCR method developed by Ioos et al. (2009). The qPCR mix (final volume of 20 µl) consisted of 5.0 µl of molecular-grade water, 10 µl of GoTaq Probe qPCR Master Mix (Promega Corp., Madison, WI, U.S.A.), 1.0 µl of each FCIR-F and FCIR-R primer (10 µM) and 1.0 µl of the FCIR-P dual-labeled probe (10 µM). For all LAMP and qPCR sensitivity testing, the volume of template DNA added was reduced to 1.0 µl for ease of dilution. Each run contained two nontemplate controls, in which the volume of the DNA template was replaced with an equivalent amount of water.

The specificity of the LAMP assay was validated by testing genomic DNA obtained from the fungi listed in Table 1. Every run contained two negative water controls, and the DNA of one known *F. circinatum* isolate was included as a positive control.

Reactions using crude DNA

To determine whether the LAMP assay could reliably detect *F. circinatum* from infected host tissue, we prepared crude extracts from five asymptomatic and five symptomatic plants (Table 4). Three sample types were tested: (i) symptomatic, discolored, wood samples from symptomatic trees; (ii) asymptomatic wood samples from the same symptomatic hosts; and (iii) asymptomatic samples from different, asymptomatic hosts. Crude extracts were amplified using the LAMP assay and compared against the qPCR assay developed by Ioos et al. (2009). For all reactions, template DNA was added directly to the reaction mixture by filter paper dipstick (see details above, under the DNA extraction section). The amount of water in each reaction was increased by 2.5 µl for LAMP and by 2.0 µl for qPCR to account for the difference when adding template by filter paper dipstick. Each run contained a single nontemplate control and a positive control using purified DNA of *F. circinatum* isolate CV-2020-006 and was run following the conditions described above or in Ioos et al. (2009) for the LAMP and qPCR assays, respectively.

To estimate the number of cells needed to detect *F. circinatum* DNA from crude DNA extracts, a series of known spore quantities was prepared using the extraction and dipstick purification method described by Mason and Botella (2020). Spore suspensions, obtained using the same procedure described above, were aliquoted onto cut Parafilm squares of 2 by 2 cm (Bemis, Neenah, WI, U.S.A.) and allowed to air dry under sterile conditions. Dried spore spots were prepared in a series to range from a maximum of 1,000,000 to as low as 500 cells/square (Table 5). Each Parafilm square was then placed into the extraction buffer, and the DNA was purified by the filter paper dipstick described above. All spore spots were tested using the LAMP method and by qPCR, following the conditions described above. The number of cells in a reaction was estimated by dividing the number of cells extracted by the volume of the extraction buffer (800 µl) and then multiplying by the lower absorptive capacity of the filter paper dipstick, approximately 2 µl (Mason and Botella 2020).

Results

Design of LAMP primers

The set of LAMP primers designed to amplify a gene region specific to *F. circinatum* consisted of outer primers F3 and B3, inner primers FIP and BIP, and a FAM-labeled forward-loop primer-probe (Table 2). The fluorescent loop primer probe was designed to be compatible with a BHQ-1 labeled quencher strand described by Kubota et al. (2011) and enabled monitoring of amplification in real time.

In the in silico assessment of specificity, the BLAST searches of the target-binding sequences of primers F3, B3, FIP, and BIP returned 100% matches against all available *F. circinatum* genomes (Supplementary Material). No other genomes available at the time of analysis contained matches to all four primers when compared against other closely related members of the FFSC.

Table 2. Loop-mediated isothermal amplification primer and probe sequences for the detection of *Fusarium circinatum*[a]

Primers and probes	Sequence 5′–3′
Primers	
Fc_F3	ATTTCGACGCATTCCCATCT
Fc_B3	ACTCGAAGTCGTCCCAAAGT
Fc_FIP	GGCCGGCTTCAGTTGAGTGTA CGTTGACTTGGGGACTATGG
Fc_BIP	TCAACAGCGAACTTGTCGCTCCAG GTCATCCCCAACTGCC
Assimilating sequence-specific probes	
Fc_LB	FAM-ACGCTGAGGACCCGGATGCGAA TGCGGATGCGGATGCCGAGTTGAGC CAGCGTGGGACAT[b]
Quencher strand[c]	TCGGCATCCGCATCCGCATTCGCAT CCGGGTCCTCAGCGT-BHQ[d]

[a] Primers are designed against a portion of the 12,000-bp subtelomeric locus predicted to be species-specific to *F. circinatum* (Van Wyk et al. 2018).
[b] FAM = 6-carboxyfluorescein. The underlined fragment acts as a loop primer.
[c] Quencher strand was designed as described by Kubota et al. (2011).
[d] BHQ = black hole quencher-1 (Biosearch Technologies, Novato, CA, U.S.A.).

Table 3. Results of sensitivity testing using purified *Fusarium circinatum* DNA and lysed spore suspension dilutions[a]

	LAMP results		qPCR results	
DNA and spores	*N*	Time (min)	*N*	Time (min)/Ct
DNA added				
1 ng μl^{-1}	**3/3**	12	**3/3**	29/15.92
100 pg μl^{-1}	**3/3**	14	**3/3**	39/25.00
10 pg μl^{-1}	**2/3**	16	**2/3**	43/28.30
1 pg μl^{-1}	0/3	–	1/3	56/39.25
100 fg μl^{-1}	0/3	–	0/3	–
Spores added				
1,000 μl^{-1}	**3/3**	12	**3/3**	40/25.94
500 μl^{-1}	**3/3**	12	**3/3**	41/26.43
100 μl^{-1}	**3/3**	12	**3/3**	43/28.24
50 μl^{-1}	**3/3**	14	**3/3**	44/28.73
10 μl^{-1}	**2/3**	16	**3/3**	46/30.75
5 μl^{-1}	1/3	47	**3/3**	49/33.51
1 μl^{-1}	0/3	–	0/3	–

[a] Positive loop-mediated isothermal amplification (LAMP) and quantitative PCR (qPCR) results, indicated in bold, were determined by amplification of at least two of three replicate reactions for each dilution and are shown as the average amplification time (Time) or the average amplification time and cycle threshold (Time/Ct) for LAMP and qPCR, respectively (total reaction time = 60 min). *N* = number of positive replicates.

Reactions using purified DNA

All *F. circinatum* isolates across all geographic origins tested positive using the LAMP assay (Table 1). The assay cross-reacted with two isolates of a single nontarget species, the newly described *F. pilosicola* (Yilmaz et al. 2021). No other nontarget species tested positive.

When testing dilutions of purified *F. circinatum* DNA, all replicates down to as low as 100 pg and two of three replicates of the 10-pg dilution tested positive within 16 min (Table 3). None of the more dilute solutions tested positive. Identical results were obtained when testing DNA dilutions as low as 10 pg by qPCR, albeit with amplification times as late as 43 min. A single replicate of the 1.0-pg dilution amplified late, at 56 min. Of the serially diluted boiled spore suspensions, all replicates to as low as the suspension at 50 cells μl^{-1} and two of three replicates of the suspension at 10 cells μl^{-1} amplified by LAMP within 16 min. One of three replicates of the suspensions of 5 cells μl^{-1} amplified considerably later, at 47 min. When testing equivalent serially diluted boiled spore suspensions by qPCR, all replicates to as low as the suspension at 5 cells μl^{-1} amplified within 49 min. Amplification times by LAMP ranged from 12 to 16 min, with a single late reaction at 47 min. Amplification by qPCR could be detected starting at 29 min and as late as 56 min. None of the negative controls amplified at any time.

Reactions using crude DNA

Using easily extracted crude DNA, both the LAMP and qPCR assays detected *F. circinatum* in all five symptomatic wood tissue samples (Table 4). When testing healthy tissue from symptomatic plants, one of five samples tested positive by both LAMP and qPCR (Table 4). All positive LAMP and qPCR assays occurred within 50 min. All five samples from asymptomatic hosts tested negative by both LAMP and qPCR.

Crudely extracted DNA from *F. circinatum* spores dried on Parafilm was amplified by both LAMP and qPCR (Table 5). Two of three replicates of the 1,000,000-cell crude extract, corresponding to approximately 2,500 cells/reaction, tested positive with LAMP within 17 min, and the third replicate amplified at 38 min. Only one replicate of each of the 500,000- and 100,000-cell crude extracts, corresponding to approximately 1,250 and 250 cells/reaction, respectively, amplified within 50 min. All replicates of each crude extract to as low as 50,000 cells, corresponding to approximately 125 cells/reaction, amplified by qPCR with times ranging from 40 to 50 min. Two replicates of the 10,000-cell crude extract, corresponding to approximately 25 cells/reaction, amplified by qPCR later, at 56 min. None of the crude extracts containing fewer cells or the negative controls amplified with either LAMP or qPCR.

Discussion

A new, portable LAMP assay was designed to detect the important pitch canker pathogen *F. circinatum.* This assay consistently detected the pathogen and, differently from the other available species-specific LAMP assay, did not cross-react with the closely related and common agricultural pathogen *F. temperatum* (Stehlíková et al. 2020). The only nontarget cross-reaction was with the newly described and apparently uncommon *F. pilosicola* (Yilmaz et al. 2021). When testing purified DNA, the LAMP assay was sensitive to as little as 10 pg of DNA, or as few as 10 cleanly lysed conidia, in a 25-µl reaction. The results showed that the assay was suitable for use in the field as a rapid diagnostic tool because it is compatible with simple, field-ready DNA extraction methods and equipment.

The LAMP assay developed in this study was validated both in silico, using a comprehensive selection of *F. circinatum* genomes available at the time of development, and in vitro, using representative isolates from nearly all major infection centers in North and South America, Africa, and Europe. In all of these assessments, the assay was shown to detect the pathogen across its genetic diversity. Even though a small number of false-negative reactions would not be unusual when developing a DNA amplification assay (Ioos et al. 2019), all *F. circinatum* isolates included in the validation of the LAMP assay were detected.

Inspection of its genome sequence explained why *F. pilosicola* was recorded to cross-react with the new LAMP assay. A large proportion of the 12-kb QTL (98%) chosen as the target of the test is also present in *F. pilosicola*, and the primer binding sites show high similarity to sequences found in *F. pilosicola* (Duong et al. 2021). *F. circinatum* and *F. pilosicola* are, indeed, sister taxa and display high similarity in the sequences of some other phylogenetically informative loci as well (Yilmaz et al. 2021). In fact, the LAMP assay designed by Stehlíková et al. (2020) to target the *F. circinatum* translation elongation factor also cross-reacts with *F. pilosicola*, whereas the internal transcribed spacer-specific qPCR assay designed by Ioos et al. (2009) does not (Supplementary Material). Although closely related to *F. circinatum*, *F. pilosicola* has been recorded only among isolates from the herbaceous annual *Bidens pilosa* (Yilmaz et al. 2021), and it does not elicit symptoms of disease when inoculated into pine stem tissues, indicating that it is not a pine

Table 4. Results of testing crude DNA extractions from healthy and symptomatic loblolly pine (*Pinus taeda*) wood tissue collected in Athens, GA with the new *Fusarium circinatum* loop-mediated isothermal amplification (LAMP) assay and the quantitative PCR (qPCR) assay described by Ioos et al. (2009)[a]

Sample	Plant symptoms	Tissue symptoms	LAMP result	qPCR result
1	Symptomatic	Asymptomatic	**3/3**	**3/3**
		Symptomatic	**3/3**	**3/3**
2	Symptomatic	Asymptomatic	0/3	1/3
		Symptomatic	**3/3**	**3/3**
3	Symptomatic	Asymptomatic	1/3	1/3
		Symptomatic	**3/3**	**3/3**
4	Symptomatic	Asymptomatic	0/3	0/3
		Symptomatic	**3/3**	**3/3**
5	Symptomatic	Asymptomatic	1/3	0/3
		Symptomatic	3/3	3/3
6	Asymptomatic	Asymptomatic	0/3	0/3
7	Asymptomatic	Asymptomatic	0/3	0/3
8	Asymptomatic	Asymptomatic	0/3	0/3
9	Asymptomatic	Asymptomatic	1/3	1/3
10	Asymptomatic	Asymptomatic	1/3	0/3

[a] Positive LAMP and qPCR results (indicated in bold) were determined by amplification of at least two of three replicate reactions for each sample.

Table 5. Results of testing crude DNA extractions from known spore quantities, with the newly developed *Fusarium circinatum* loop-mediated isothermal amplification (LAMP) assay and the quantitative PCR (qPCR) assay described by Ioos et al. (2009)

		LAMP results[b]		qPCR results[b]	
Cells in extraction buffer	Cells[a]	*N*	Time (min)	*N*	Time (min)/Ct
1,000,000	2,500	**3/3**	23	**3/3**	40/25.89
500,000	1,250	1/3	47	**3/3**	42/27.60
100,000	250	1/3	50	**3/3**	44/29.32
50,000	125	0/3	–	**3/3**	50/34.66
10,000	25	0/3	–	**2/3**	56/39.39
5,000	12.5	0/3	–	0/3	–
1,000	2.5	0/3	–	0/3	–
500	1.25	0/3	–	0/3	–

[a] Approximate cells per reaction were calculated by dividing the number of cells by the volume of the extraction buffer (800 µl) and multiplying by the lower absorptive capacity of the filter paper dipstick (approximately 2 µl) (Mason and Botella (2020).

[b] Positive LAMP and qPCR results (indicated in bold) were determined by amplification of at least two of three replicate reactions for each sample and are shown as the average amplification time (Time) or the average amplification time and cycle threshold (Time/Ct) for LAMP and qPCR, respectively (total reaction time = 60 min). *N* = number of positive replicates.

pathogen (Supplementary Material). Hence, *F. pilosicola* is unlikely to cause false-positive results when testing the wood tissue of diseased pine trees. Additionally, *F. pilosicola* is unlikely to appear among spore trap catches in pine stands or as a contaminant of pine tissues, because this fungus has not been recorded in these settings (Yilmaz et al. 2021).

The LAMP assay developed in this study is comparable with the commonly used qPCR method (Ioos et al. 2009), offering slightly less sensitivity but providing much shorter times to reach a result. The two assays conducted in this study achieved equivalent levels of sensitivity when testing kit-extracted pure DNA from mycelia (as low as 10 pg). The qPCR test demonstrated slightly greater sensitivity when testing pure DNA from spore suspensions (as low as 5 cells μl^{-1} as compared with 10 cells μl^{-1}) and far greater sensitivity when testing crudely extracted spores (as low as 10,000 cells/extraction by qPCR and 1,000,000 cells/extraction by LAMP). We speculate that this discrepancy in sensitivity may be the result of the qPCR assay's initial denaturation step, a 10-min incubation at 95°C. The heating step may have caused additional spore cell lysis and increased the availability of template DNA in the qPCR assays relative to the LAMP reactions, which lacked an initial heating step. However, the LAMP assay provided substantially more rapid amplification and a shorter time to detection, and it has the advantage of being suitable for direct application in the field using portable devices (Hamilton et al. 2020).

The LAMP assay consistently detected *F. circinatum* in the symptomatic tissues of infected trees using a rapid, pipette-free sample preparation method. It also detected *F. circinatum* in asymptomatic tissue from one infected tree, possibly indicating that the assay would be useful in identifying presymptomatic infections, as has been shown in LAMP detection of other forest disease systems (Hamilton et al. 2020). A time course study using artificially inoculated seedlings could be used to help test this utility. In this way, it would be possible to determine the stage at which the pathogen might be detected during the infection process.

Sensitive and rapid detection methods are important in order to prevent the spread of *F. circinatum* by cryptically infected plant materials, which is a major avenue for the pathogen's global spread (Zamora-Ballesteros et al. 2019). We believe that the *F. circinatum* LAMP assay developed in this study is well suited for such applications, considering its sensitivity, short time to result, and simple implementation. This assay is the first shown to be compatible with a simple, high-throughput crude DNA extraction method. In addition, the results of this study demonstrate that the qPCR method developed by Ioos et al. (2009) can be used with crude DNA extracts.

The simplicity of the LAMP assay developed in this study offers technicians the potential to diagnose pitch canker infections with relatively little training and inexpensive portable instrumentation. This flexibility establishes an opportunity to bring *F. circinatum* LAMP testing and the confidence of molecular confirmation into the field or to other settings where rapid diagnosis is needed, such as customs inspection facilities. Portable LAMP-based testing has been demonstrated in other forest disease systems (Hamilton et al. 2020), although never with a pipette-free sample-processing method. Granting forest and nurseries managers access to laboratory-grade diagnostics with minimal equipment will greatly facilitate the confirmation of pitch canker and enable better-informed management decisions in real-time.

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