Development of Three Loop-Mediated Isothermal Amplification (LAMP) Assays for the Rapid Detection of *Calonectria ilicicola*, *Dactylonectria macrodidyma*, and the *Dactylonectria* Genus in Avocado Roots

Louisamarie E. Parkinson,[†] Duy P. Le, and Elizabeth K. Dann

Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Dutton Park, Queensland 4102, Australia

Abstract

Black root rot of avocado is a severe disease of nursery trees and young orchard transplants, causing tree death within a year after planting. In Australia, key pathogens include species complexes *Calonectria ilicicola* and *Dactylonectria macrodidyma*; however, several other *Dactylonectria* species also cause the disease. Rapid detection of these pathogens in planta is important to speed up implementation of disease management and reduce loss. The purpose of this study was to develop three loop-mediated isothermal amplification (LAMP) diagnostic assays to rapidly identify species within the *C. ilicicola* and *D. macrodidyma* complexes and species in the *Dactylonectria* genus in avocado roots. Primers were designed from β -tubulin sequence data of *C. ilicicola* and from histone H3 of *D. macrodidyma* and the *Dactylonectria* genus. The LAMP primers were tested for specificity and sensitivity with 82 fungal isolates, which included the target species complexes *C. ilicicola* and *D. macrodidyma*; species within the target *Dactylonectria* genus viz. *D. macrodidyma*, *D. anthuriicola*, *D. novozelandica*, *D. pauciseptata*, and *D. vitis*; and isolates of nontarget species, including *Calonectria* sp., *Cylindrocladiella* sp., *Gliocladiopsis forbergii*, *G. peggii*, *G. whileyi*, *Ilyonectria* sp., *Mariannaea* sp., *Fusarium* sp., and *Phytophthora cinnamomi*. The species-specific LAMP assays were sensitive and specific at DNA concentrations of 1 pg/ μ l for *C. ilicicola* and 0.01 ng/ μ l for *D. macrodidyma*,

whereas the *Dactylonectria* genus-wide assay was sensitive to 0.1 ng/ μ l. Detection of *C. ilicicola* occurred within 10 to 15 or 15 to 30 min when the template was pure DNA or crude extracts obtained from suspending fungal cultures in sterile water, respectively. Detection of *D. macrodidyma* was between 12 to 29 min with pure DNA and 16 to 30 min with crude extracts. *Dactylonectria* spp. were detected within 6 to 25 min with pure DNA and 7 to 23 min with crude extracts. The specificity of the assays was found to be dependent on time and isothermal amplification temperature, with optimal specificity occurring in reactions of <30 min and at temperatures of 67°C for *C. ilicicola* and *D. macrodidyma* assays and 69°C for *Dactylonectria* genus-wide assays. The assays were modified to accommodate a DNA extraction step and use of avocado roots as DNA templates. Detection in avocado roots ranged between 12 to 25 min for *C. ilicicola*, 12 to 26 min for *D. macrodidyma*, and 14 to 30 min for species in the *Dactylonectria* genus. The LAMP assays are applicable across multiple agricultural industries, because *C. ilicicola*, *D. macrodidyma*, and *Dactylonectria* spp. are also important pathogens of various crops and ornamental plants.

Keywords: fungi, Nectriaceae, nectriaceous fungi, pathogen detection, *Persea americana*, techniques, tropical plants

Black root rot of avocado caused by soilborne nectriaceous pathogens is an important disease of nursery trees and young orchard transplants, causing tree stunting; wilt; black, rotten, and necrotic roots; and rapid tree decline and death within a year after planting (Parkinson et al. 2017). In Australia and globally, the important pathogens are *Calonectria ilicicola* (Dann et al. 2012) and *Dactylonectria macrodidyma* (*Ilyonectria macrodidyma* in Vitale et al. 2012). However, other studies have demonstrated that other species within these genera cause black root rot, including an undescribed *Calonectria* sp., *Dactylonectria anthuriicola*, *Dactylonectria novozelandica*,

and *Dactylonectria pauciseptata* (Parkinson et al. 2017). These pathogens are a threat to new plantings, and the disease can potentially be undetected in young nursery trees, because above ground symptoms of stunting and wilt may not develop until after outplanting (Parkinson et al. 2017). The Australian Avocado Nursery Voluntary Accreditation Scheme involves regular sampling of nursery material and testing by plant pathologists for a nursery to maintain disease-free accreditation. Standard diagnostic practices include diagnosing disease from symptomatic plant tissue, isolating and culturing the causal agents on selective media, and identifying microbial species by microscopy. In instances where a suspected pathogen is difficult to identify with microscopy alone (for example, nectriaceous genera, which have similar morphology), molecular techniques have been extremely useful to aid accurate identification. Molecular identification of suspect isolates to species level by isolation, establishment of pure cultures, microscopy, DNA extraction, polymerase chain reaction (PCR), and sequencing requires time and can take several days to weeks to achieve a diagnosis (Niessen 2015; Notomi et al. 2015). There is demand for rapid, sensitive, and specific molecular methods for early detection of plant diseases (Sankaran et al. 2010) to enable faster implementation of disease management strategies and reduce commercial loss (Fang and Ramasamy 2015).

Loop-mediated isothermal amplification (LAMP) is a rapid, highly specific, and sensitive nucleic acid amplification technique that uses autocycling strand displacement DNA synthesis and produces results from diseased plant tissue in <60 min (Fu et al. 2011; Notomi et al. 2000). LAMP is catalyzed by the *Bst* DNA polymerase and uses four primers that recognize six specific sequences on the target DNA (Niessen 2015). The reaction is initiated by an inner primer, which contains the sense and antisense strand sequence of target

Current address of D. P. Le: New South Wales Department of Primary Industries, Narrabri, NSW 2390, Australia.

[†]Corresponding author: L. E. Parkinson; l.parkinson@uq.edu.au

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DNA (Fu et al. 2011), called the F1 and F2 or B1 and B2 sequence (Notomi et al. 2000). These inner primers are called forward inner primer (FIP) and backward inner primer (BIP) (Notomi et al. 2000). A single-stranded synthesized DNA is released by an outer primer (Fu et al. 2011), called the F3 or B3 Primer, and this is used as a template for a second inner and outer primer (Notomi et al. 2000). On the other end of the target sequence, the second inner and outer primers hybridize to the target and produce a stem-loop DNA structure (Fu et al. 2011). In the cycles that follow, one inner primer hybridizes to the loop and initiates displacement DNA synthesis, which produces the original stem-loop structure and a new stem-loop DNA sequence with a stem twice as long (Notomi et al. 2000). The cycling of DNA displacement synthesis results in stem-loop DNA strands with several inverted repeats of the target DNA and loop structures (Notomi et al. 2000). Additional LAMP primers called loop primers (forward-loop [F Loop] and backward-loop [B Loop] primers) can be added to the reaction to dramatically reduce detection time (Niessen 2015) and increase the specificity and sensitivity of detection (Nagamine et al. 2002). Loop primers recognize and anneal to target DNA sequences between the F1 and F2 sequence and between the B1 and B2 sequence (Nagamine et al. 2002), providing broader coverage of a length of nucleotide sequences to be detected.

An advantage of LAMP is that the diagnostic is portable and simple to use (Notomi et al. 2015). Because it uses one continuous temperature, the reaction can also be carried out in a water bath or heating block (Notomi et al. 2000) using alternative reaction reagents, enabling cost-effective options (Fu et al. 2011) and accessibility in developing countries and resource-poor areas (Mori and Notomi 2009); detection is simplified by visualizing salt precipitation or fluorescence in the reaction tube (Tomita et al. 2008). LAMP offers the equivalent or better sensitivity and specificity of PCR (Fukuta et al. 2013, Vincelli and Tisserat 2008), and the LAMP procedure requires fewer preparation steps (Fu et al. 2011).

LAMP-based diagnostic methods have been developed for various plant pathogens, including some necrotrophic fungi, such as Boxwood blight fungi, *Calonectria henricotiae*, *Calonectria pseudonaviculata* (Malapi-Wight et al. 2016), and *Fusarium oxysporum* f. sp. *ciceris* of chickpea (Ghosh et al. 2015). So far, no LAMP assays exist for detecting *Dactylonectria* spp.

LAMP-based assays for detecting *C. ilicicola* and *Dactylonectria* spp. would be extremely useful for the timely identification of causal organisms and disease management for many horticultural, ornamental, and field crop industries. For example, *C. ilicicola* also causes crown and root rot of other Lauraceae trees, such as bay laurel (Polizzi et al. 2012); collar rot of papaya (Male et al. 2012); leaf spot in ornamental holly (Lechat et al. 2010); and diseases of various field crops, including *Cylindrocladium* black rot of peanut (as *Cylindrocladium parasiticum* in Wright et al. 2010) and crown rot of soybean (Ochi et al. 2011). *D. macrodidyma* causes black foot disease of grapevine (as *Cylindrocarpon macrodidymum* in Halleen et al. 2004), root rot of olive (as *I. macrodidyma* in Úrbez-Torres et al. 2012), and cherimoya (Auger et al. 2015), and it is associated with apple seedling replant disease (as *C. macrodidymum* in Tewoldemedhin et al. 2011) and dry root rot of citrus (as *Neonectria macrodidyma* in Adesemoye et al. 2016).

This investigation aimed to develop LAMP assays for the detection of two key necrotrophic pathogens, *C. ilicicola* and *D. macrodidyma*, and a genus-wide assay for detecting *Dactylonectria* spp. in avocado roots. The procedures involved (i) assessing multiple alignments of gene sequence data of a large collection of necrotrophic fungal isolates to identify genes with high nucleotide variation between species, (ii) selecting candidate genes and designing specific LAMP primers from species or genus-unique nucleotide sequences, and (iii) demonstrating the specificity, sensitivity, and rapid detection of the LAMP assay using DNA extracts, fungal cultures, and inoculated plant tissue. The *C. ilicicola* and *D. macrodidyma* isolates used in this study were part of species complexes, each containing potentially new and unresolved species within the phylogenetic clade of the target species (Parkinson 2017). The LAMP primers were designed to

detect all members of the *C. ilicicola* and *D. macrodidyma* species complexes, and these were treated as single species in the LAMP assay design.

Materials and Methods

Fungal isolates. Two species-specific LAMP assays for use with avocado roots were designed for detecting black root rot pathogens, *C. ilicicola* and *D. macrodidyma*, and a genus-wide assay was developed for detecting species in the genus *Dactylonectria*. Eighty-two fungal isolates from the Biosecurity Queensland Plant Pathology Herbarium (BRIP), Department of Agriculture and Fisheries (Brisbane, Queensland) were included in this study for demonstrating diagnostic specificity. The isolates tested included a representative number of target species, closely related species, and distantly related species (Supplementary Table S1).

Designing LAMP primers. Type DNA sequence data of the β -tubulin and histone H3 genes of species in the genera *Calonectria* and *Dactylonectria* were downloaded from GenBank (<https://www.ncbi.nlm.nih.gov/>). DNA was extracted from BRIP fungal isolates of the genera *Calonectria* and *Dactylonectria*, and the β -tubulin and histone H3 gene regions were sequenced following the methods described in Parkinson et al. (2017).

A multiple alignment of the β -tubulin and histone H3 individual gene regions was performed on each genus in Geneious v 7.1.9 (Biomatters Ltd) (Kearse et al. 2012) using the MAAFT Alignment (Katoh and Standley 2013) plugin and included sequence data of fungal species from related genera to identify gene regions unique to the target genus. The β -tubulin gene was selected for designing LAMP primers for *C. ilicicola*, and the histone H3 gene was selected for designing primers for *D. macrodidyma* and *Dactylonectria* spp., because these genes contained high sequence variation between species within the genus to enable target-specific primer annealing. β -tubulin was initially considered for *D. macrodidyma*; however, there was no sufficient sequence variation to design *D. macrodidyma*-specific LAMP primers.

Unique nucleotide sequences were identified for *C. ilicicola* and *D. macrodidyma* and used as the basis for designing specific LAMP primers and genus-specific *Dactylonectria* primers. For the *C. ilicicola* and *D. macrodidyma* assays, the outer F3 and B3 LAMP primers were designed from the unique sequences, and these were used to generate the FIP, BIP, F Loop, and B Loop primers in the LAMP primer designing software, Primer Explorer v 4 (<http://primerexplorer.jp>) (Table 1). These primer sets were modified manually.

For the genus-wide *Dactylonectria* assay, the consensus sequence of the genus-wide alignment was used as the backbone for designing LAMP primers. Histone H3 sequence data of the closely related genus *Ilyonectria* were included to ensure genus specificity. The F3, B3, FIP, BIP, F Loop, and B Loop primers (Table 1) were designed manually from the consensus sequence, and the target sequence of each primer was checked in the alignment for specificity to the target genus. Multiple combinations of the LAMP primers were tested in experiments for sensitivity, specificity, and optimal isothermal conditions. All LAMP optimization, sensitivity, and specificity experiments with pure fungal DNA and crude extracts obtained from suspending fungal cultures in sterile water were conducted once, whereas LAMP assays with avocado roots were performed twice.

Optimization of LAMP reactions. *Experiment 1: Initial sensitivity and specificity testing and optimizing isothermal conditions.* Experiment 1 tested the designed primers to confirm primer specificity, revealed an approximate level of sensitivity for detection, and determined the optimal isothermal temperature at which detection is the fastest. Unless otherwise stated, all isothermal reactions in this study were carried out using OptiGene Isothermal Master Mix and the isothermal block Genie II supplied by GeneWorks Pty Ltd.

The working primer mixture was made to 100 μ l containing 1.25 μ M F3 primer, 1.25 μ M B3 primer, 10 μ M FIP primer, 10 μ M BIP primer, and RNAase-free water. The reaction mixture for each test sample contained a total volume of 15 μ l composed of 2.6 μ l RNAase-free water, 9 μ l OptiGene Isothermal Master

Mix, 2.4 µl working primer mixture, and 1 µl DNA template of fungal DNA extracts at concentrations ranging from 0.01 pg/µl to 1 ng/µl. The template-free negative control in each LAMP diagnostic test run was RNase-free water.

For the species-specific assays, Genie II was set at 65°C for 60 min followed by 95 to 80°C annealing and termination rate of 0.5°C/s. If sensitivity was demonstrated at a DNA concentration <0.01 ng/µl, the experiment was repeated using 63 and 67°C isothermal amplification temperatures to determine the best isothermal temperature conditions for the LAMP diagnostic. The optimal isothermal temperature was used in subsequent experiments. The *Dactylonectria* genus-wide assay used an isothermal amplification temperature of 67°C and the same reaction parameters as the species-specific assays.

In reactions where target species were not detected when DNA concentrations of <0.01 ng/µl were tested, isothermal temperature comparisons and selection were made in experiment 2, where loop primers were introduced to the reaction and the optimal LAMP primer combination was determined.

Experiment 2: Testing loop primers for improved detection time, sensitivity, and specificity of species-specific LAMP assays. F Loop and B Loop primers were introduced individually or in combination with the LAMP reaction to test for increased reaction speed and improved sensitivity and specificity. The LAMP reagents and sample concentrations were as outlined in experiment 1; however, the working primer mixture contained 5 µM F Loop primer, 5 µM B Loop primer, or both primers each at 5 µM concentration.

Isothermal amplification for detecting *C. ilicicola* was at 67°C for 60 min followed by 95 to 80°C annealing and termination at 0.5°C/s. The test samples for the *D. macrodidyma* diagnostic underwent the same reaction conditions; however, the isothermal amplification temperature was set to 65°C. In both diagnostics, the best loop primer combination was selected from comparing the reaction times, sensitivity, and specificity, and this primer set was used in additional LAMP experiments.

For the *D. macrodidyma* diagnostic, additional experiments that compared isothermal amplification temperatures of 63, 65, and 67°C were carried out using the selected loop primer set to determine the optimal reaction conditions for this diagnostic. The selected temperature was used in the following experiments.

In the *Dactylonectria* genus-wide assay, the LAMP reaction parameters were set to 67°C for 45 min followed by 95 to 80°C annealing and termination at 0.5°C/s. The LAMP reaction components and master mix were as listed above and underwent additional testing for sensitivity using DNA between 1 ng/µl and 0.1 pg/µl following the

master mix requirements listed above. Isothermal amplification was at 67°C for 60 min followed by 95 to 80°C annealing and termination at 0.5°C/s, and then, it was repeated at 69°C for 30 min to test for an improvement in sensitivity.

Experiment 3: Screening DNA from multiple isolates to confirm LAMP specificity. The selected LAMP primer combination and isothermal amplification temperature with DNA templates of a representative number of isolates (target and nontarget species) were utilized to thoroughly confirm the specificity of all tests.

For both species-specific diagnostics, the working primer mixture was made to 100 µl containing 1.25 µM F3 primer, 1.25 µM B3 primer, 10 µM FIP primer, 10 µM BIP primer, 5 µM B Loop primer and RNAase-free water. The *Dactylonectria* genus-wide assay utilized these reaction components; however, 5 µM F Loop primer was also included. Each reaction was prepared as described in experiment 1, with the fungal DNA template at a concentration of 1 or 50 ng/µl and RNase-free water as the template-free negative control (Table 2). For all three assays, the reaction parameters were 67°C isothermal amplification for 30 min followed by 95 to 80°C annealing and termination at 0.5°C/s. The *Dactylonectria* genus-wide assay was repeated with the same parameters and an additional isothermal amplification temperature of 69°C to test for an improvement in specificity.

LAMP diagnostic detection with fungal cultures and inoculated necrotic avocado tissue. **Experiment 4: LAMP diagnostic for detection of target pathogens in fungal cultures.** The ability of the diagnostic to detect target DNA in fungal cultures suspended in sterile water was examined. Representative isolates of target and nontarget species were grown for 7 to 10 days on half-strength potato dextrose agar amended with 200 ppm streptomycin (sPDA) and incubated at room temperature under black light. Four 0.5-cm³ plugs of sPDA of each fungal isolate were added to microfuge tubes containing 1 ml sterile distilled water (sd water) and vigorously shaken to disperse the fungal conidia and mycelia into suspension; 1 µl of the latter suspension was used as the DNA template in each reaction sample, with one sample containing 1 µl of RNase-free water as the template-free negative control. Some microfuge tubes contained two isolates to represent mixed cultures, with three 0.5-cm³ plugs of sPDA of each fungal isolate in the tube (Table 3). The LAMP temperature conditions for species-specific diagnostics were set to 67°C isothermal amplification for 30 min followed by 95 to 80°C annealing and termination at 0.5°C/s. This was repeated for the *Dactylonectria* genus-wide assay with an additional isothermal amplification temperature of 69°C.

Table 1. List of primer sequences for each loop-mediated isothermal amplification (LAMP) assay^z

Target and LAMP primer	Primer sequence
<i>Calonectria ilicicola</i>	
F3	5'-TGTTGCTGCCCCCTGAGCG-3'
B3	5'-GTTACCCCTGATCGGAATGT-3'
FIP	5'-AGTCAGCAACCTTGTCTCCGACCGGTTCCGACCGCTTC-3'
BIP	5'-CTTTCTCAATTCTAGCTCCACCCAGGGCAGTTTGTGG-3'
F Loop	5'-TCGTCGAGCTTTGTTGTTGTC-3'
B Loop	5'-GTCAGTGCCTAAGTGATCATTCC-3'
<i>Dactylonectria macrodidyma</i>	
F3	5'-GTCCACTGGTGGCAAGG-3'
B3	5'-CACGGAGAGCGACGGTA-3'
FIP	5'-GTATGGCGATGCATTTTGTGATCTTCCAAGGCTGGTGAGT-3'
BIP	5'-ACCTTAACCATCAACAGCCCGCTAGCGGTGAGGCTTCTTG-3'
F Loop	5'-CGCGACGTGTCAAGTAAATGG-3'
B Loop	5'-GCCCCCTCTACCGGTGGTGT-3'
<i>Dactylonectria</i> spp.	
F3	5'-TCCAAGGCTGGTGAGTCTCG-3'
B3	5'-ACTCACGAGACGCTGGAA-3'
FIP	5'-GCTCTTGCGGGCTGTTGATATTTACTTGACGCGTCGC-3'
BIP	5'-TCAAGAAGCCTACCGCTACATGAGGAGCTCGGTGACT-3'
F Loop	5'-GGTTGAGGTTAGTATGGCGATG-3'
B Loop	5'-TACCGTCGCTCTCCGTGA-3'

^z BIP, backward inner primer; B Loop, backward loop; FIP, forward inner primer; F Loop, forward loop.

Table 2. Experiment 3—screening DNA from multiple isolates to confirm loop-mediated isothermal amplification (LAMP) specificity of optimized primer sets and isothermal conditions for detection of target species *Calonectria ilicicola*, *Dactylonectria macrodidyma*, and *Dactylonectria* spp. using pure fungal DNA extracts as templates^z

LAMP assay and isolate (BRIP) identification	Species	DNA concentration (ng/μl)	Detection time at 67°C (min:s)	Detection time at 69°C (min:s)
<i>C. ilicicola</i>				
54018 a	<i>C. ilicicola</i>	1	10:22	
60982	<i>C. ilicicola</i>	1	10:05	
60992	<i>C. ilicicola</i>	1	11:12	
61291	<i>C. ilicicola</i>	1	10:34	
60388	<i>C. ilicicola</i>	1	10:51	
60389	<i>C. ilicicola</i>	1	11:15	
60397	<i>C. ilicicola</i>	1	10:45	
53933 a	<i>C. ilicicola</i>	1	11:35	
55531 a	<i>C. ilicicola</i>	1	10:21	
53653 a	<i>C. ilicicola</i>	1	10:53	
61448	<i>Calonectria</i> sp.	1	ND	
60981	<i>Calonectria</i> sp.	1	ND	
63712	<i>Calonectria</i> sp.	1	ND	
15920 a	<i>Calonectria</i> sp.	1	ND	
16747 a	<i>Calonectria</i> sp.	1	ND	
60986	<i>Cylindrocladiella</i> sp.	1	ND	
61292	<i>Cylindrocladiella</i> sp.	1	ND	
62001 a	<i>D. macrodidyma</i>	1	ND	
62000 a	<i>D. novozelandica</i>	1	ND	
61428 b	<i>D. pauciseptata</i>	1	ND	
61429 b	<i>D. anthuriicola</i>	1	ND	
n/a	<i>Fusarium</i> sp.	1	ND	
60983	<i>Gliocladiopsis peggii</i>	1	ND	
61430	<i>G. whileyi</i>	1	ND	
61349 d	<i>Ilyonectria</i> sp.	1	ND	
53498 a	<i>Ilyonectria</i> sp.	1	ND	
63711 e	<i>Mariannaea</i> sp.	1	ND	
61192 c	<i>Mariannaea</i> sp.	1	ND	
n/a	RNase-free water	0	ND	
<i>D. macrodidyma</i>				
60979	<i>D. macrodidyma</i>	50	15:22	
61294 a	<i>D. macrodidyma</i>	50	14:17	
61431 c	<i>D. macrodidyma</i>	50	13:44	
61434 a	<i>D. macrodidyma</i>	50	18:54	
61436 a	<i>D. macrodidyma</i>	50	14:05	
60907 b	<i>D. macrodidyma</i>	50	14:31	
61090 c	<i>D. macrodidyma</i>	50	12:16	
61354 c	<i>D. macrodidyma</i>	50	14:25	
61195 d	<i>D. macrodidyma</i>	50	13:18	
61306 a	<i>D. macrodidyma</i>	50	13:48	
61442	<i>D. macrodidyma</i>	50	28:30	
61444 a	<i>D. macrodidyma</i>	50	13:31	
62000 b	<i>D. macrodidyma</i>	50	26:05	
62000 g	<i>D. macrodidyma</i>	50	25:02	
62001 b	<i>D. macrodidyma</i>	50	15:48	
62001 a	<i>D. macrodidyma</i>	1	15:29	
62001 a	<i>D. macrodidyma</i>	50	13:18	
62002 a	<i>D. macrodidyma</i>	1	15:34	
62002 a	<i>D. macrodidyma</i>	50	12:40	
61546 a	<i>D. macrodidyma</i>	1	17:15	
61546 a	<i>D. macrodidyma</i>	50	12:32	
62005 a	<i>D. macrodidyma</i>	1	26:40	
62005 c	<i>D. macrodidyma</i>	50	16:51	
61428 b	<i>D. pauciseptata</i>	1	ND	
61428 c	<i>D. pauciseptata</i>	1	ND	
62000 c	<i>D. novozelandica</i>	1	ND	
61433 a	<i>D. pauciseptata</i>	50	ND	
52550 a	<i>D. pauciseptata</i>	50	ND	
60991 a	<i>D. pauciseptata</i>	50	ND	
63707 a	<i>D. pauciseptata</i>	50	ND	
63713	<i>D. pauciseptata</i>	50	ND	

(Continued on next page)

^z BRIP, Biosecurity Queensland Plant Pathology Herbarium; FP, false positive as indicated by detection of a nontarget species in <30 min or an inconsistent or absent annealing curve; ND, not detected; n/a, not applicable.

Table 2. (Continued from previous page)

LAMP assay and isolate (BRIP) identification	Species	DNA concentration (ng/μl)	Detection time at 67°C (min:s)	Detection time at 69°C (min:s)
62000 d	<i>D. novozelandica</i>	1	ND	
61429 b	<i>D. anthuriicola</i>	50	ND	
61195 b	<i>D. vitis</i>	50	ND	
63708 b	<i>D. vitis</i>	50	ND	
61263 g	<i>D. vitis</i>	50	ND	
54018 a	<i>C. ilicicola</i>	1	ND	
60986	<i>Cylindrocyladiella</i> sp.	1	ND	
n/a	<i>Fusarium</i> sp.	1	ND	
60983	<i>G. peggii</i>	1	ND	
61349 a	<i>G. forbergii</i>	50	ND	
53498 a	<i>Ilyonectria</i> sp.	1	ND	
61192 c	<i>Mariannaea</i> sp.	1	ND	
n/a	<i>Phytophthora cinnamomi</i>	>50	ND	
n/a	RNase-free water	0	ND	
<i>Dactylonectria</i> spp.				
60979	<i>D. macrodidyma</i>	50	10:15	9:00
61349 e	<i>D. macrodidyma</i>	50	7:30	7:45
61431 c	<i>D. macrodidyma</i>	50	7:30	7:15
61434 a	<i>D. macrodidyma</i>	50	11:45	11:30
61436 a	<i>D. macrodidyma</i>	50	7:30	7:15
60907 b	<i>D. macrodidyma</i>	50	8:45	8:15
61090 c	<i>D. macrodidyma</i>	50	7:00	7:00
61354 c	<i>D. macrodidyma</i>	50	8:00	8:00
61195 d	<i>D. macrodidyma</i>	50	7:30	7:15
61306 a	<i>D. macrodidyma</i>	50	8:00	8:00
61442	<i>D. macrodidyma</i>	50	9:45	9:00
61444 a	<i>D. macrodidyma</i>	50	8:00	7:45
62000 b	<i>D. macrodidyma</i>	50	12:45	11:30
62000 g	<i>D. macrodidyma</i>	50	13:15	12:30
n/a	RNase-free water	0	ND	ND
62001 b	<i>D. macrodidyma</i>	50	11:00	10:30
62001 a	<i>D. macrodidyma</i>	50	6:15	7:00
62002 a	<i>D. macrodidyma</i>	50	6:00	6:45
61546 a	<i>D. macrodidyma</i>	50	6:00	7:00
62005 c	<i>D. macrodidyma</i>	50	7:15	8:45
61295 d	<i>D. pauciseptata</i>	50	12:15	12:00
61428 b	<i>D. pauciseptata</i>	50	12:30	13:15
61428 c	<i>D. pauciseptata</i>	50	13:30	14:00
61428 d	<i>D. pauciseptata</i>	50	12:15	12:30
61433 a	<i>D. pauciseptata</i>	50	13:30	13:45
52550 a	<i>D. pauciseptata</i>	50	17:30	16:30
60991 a	<i>D. pauciseptata</i>	50	11:30	11:30
63707 a	<i>D. pauciseptata</i>	50	13:30	13:15
63713	<i>D. pauciseptata</i>	50	13:15	13:15
62000 a	<i>D. novozelandica</i>	50	11:00	12:30
n/a	RNase-free water	0	26:30 FP	ND
62000 c	<i>D. novozelandica</i>	50	12:00	13:00
62000 d	<i>D. novozelandica</i>	50	11:30	16:00
60985	<i>D. anthuriicola</i>	50	11:15	15:15
61429 b	<i>D. anthuriicola</i>	50	11:00	15:00
61306 b	<i>D. anthuriicola</i>	50	11:45	15:15
61437 b	<i>D. anthuriicola</i>	50	9:15	15:30
61195 b	<i>D. vitis</i>	50	10:00	12:00
63708 b	<i>D. vitis</i>	50	9:00	12:15
61263 f	<i>D. vitis</i>	50	10:30	11:45
61263 g	<i>D. vitis</i>	50	6:15	12:45
61352 c	<i>D. macrodidyma</i>	50	9:15	8:00
61354 c	<i>D. macrodidyma</i>	50	8:00	10:30
53498 a	<i>Ilyonectria</i> sp.	50	29:15 FP	ND
60980	<i>Ilyonectria</i> sp.	50	28:30 FP	ND
61349 d	<i>Ilyonectria</i> sp.	50	ND	ND
n/a	RNase-free water	0	ND	ND
61546 a	<i>D. macrodidyma</i>	50	6:15	7:15
61263 f	<i>D. vitis</i>	50	9:15	11:00
61294 a	<i>D. macrodidyma</i>	50	7:30	9:00
54018 a	<i>C. ilicicola</i>	50	21:00 FP	ND

(Continued on next page)

Experiment 5: Validation of the LAMP diagnostic for detection of target pathogens in inoculated necrotic avocado roots. Experiment 5 tested the LAMP diagnostic for its ability to detect the target species directly in avocado root tissue. Twenty-three 6-month-old avocado cultivar Reed seedlings were inoculated by amending the potting soil with ~250 ml sand:bran:water media mixed with vermiculite (grade 3), prepared as described in Parkinson et al. (2017), and colonized with *C. ilicicola*, *D. macrodidyma*, *D. anthuricola*, *D. novozelandica*, *D. pauciseptata*, *D. vitis*, or a mixture of equal parts (percentage volume:volume) *C. ilicicola* and one species of the listed *Dactylonectria* species. The roots of a healthy, uninoculated 6-month-old Reed seedling were included as a negative control. The plants were maintained in the glasshouse at 22 to 24°C during the day and 18°C at night. At 7 weeks post inoculation, the plants were uprooted and assessed for root disease, and necrotic or healthy avocado roots were collected for use in the LAMP assay.

The LAMP assays were modified to include a DNA extraction step using the OptiGene Plant DNA Extraction Kit (GeneWorks Pty Ltd) for detection with avocado root tissue. For each LAMP assay test sample, necrotic avocado root pieces were sliced into two 1.5-cm sections and added to a 10-ml tube containing a large steel ball bearing from the kit. Each test sample was diluted with three 1.5-cm sections of healthy, uninoculated avocado root pieces. Fungal DNA was extracted following the manufacturer's instructions; 1 ml Lysis Buffer was added to each tube containing root tissue, and the tubes were shaken vigorously by hand for 1 min to macerate the tissue. Approximately 10 µl of crude DNA extract was transferred by sterile loop into 1 ml dilution buffer, and the solution was mixed by inversion. Five µl of diluted crude extract was used as the DNA template in the LAMP reaction.

The LAMP reaction master mix was modified to suit detection with avocado root tissue. The reaction mixture for each test sample contained a total volume of 25 µl made up of 15 µl OptiGene Isothermal Master Mix (GeneWorks Pty Ltd), 5 µl working primer mixture as listed above, and 5 µl DNA template. The isothermal temperature conditions for detecting *C. ilicicola* and *D. macrodidyma* were set to 67°C isothermal amplification for 40 min (Table 4) followed by 95 to

80°C annealing and termination at 0.5°C/s. This was repeated for the *Dactylonectria* genus-wide assay with an additional isothermal temperature of 69°C for comparison (Table 4). Each LAMP assay was conducted twice, with freshly sampled roots for each diagnostic run.

Results

Sensitivity and specificity of LAMP diagnostic, chosen primer sets, and optimization of isothermal conditions. *Experiment 1: Initial sensitivity and specificity testing and optimizing isothermal conditions.* The results of experiment 1 are listed in Supplementary Table S2. In the initial sensitivity and specificity tests, the F3, B3, FIP, and BIP primers designed for detecting *C. ilicicola* were sensitive and specific to the target species at DNA concentrations as low as 1 pg/µl. Isothermal amplification temperatures were compared, and the optimal temperature was found to be 67°C, enabling detection of 1 ng/µl DNA at 17 min and 41 s, with the detection slowing down as the temperature decreased. Isothermal amplification temperature of 67°C was selected for use in all additional experiments for detecting *C. ilicicola*. In terms of sensitivity, *C. ilicicola* could be detected at DNA concentrations of 1 pg/µl; however, the speed of detection varied and was slower than at higher concentrations, with detection at 24 min and 56 s at 65°C and detection at 27 min and 24 s at 67°C. In all tests, detection was slowest at isothermal amplification temperature of 63°C.

False positive detection in any sample was determined by an annealing curve not consistent in peak height or position with the curve representing the positive control for detection, an absent annealing curve, or an absent annealing and terminating temperature record. Across all assays, reliable positive detection occurred in <30 min, and any detection after 30 min was considered nondetection.

The F3, B3, FIP, and BIP primers designed for detecting *D. macrodidyma* were sensitive and specific to the target species at DNA concentrations of 1 ng/µl but detected after the 30-min threshold for reliability. The detection of the target species facilitated by these primers was slower and less sensitive than in the tests with *C. ilicicola* primers. Sensitivity was not demonstrated at DNA concentrations <0.1 ng/µl; therefore, no additional isothermal temperatures

Table 2. (Continued from previous page)

LAMP assay and isolate (BRIP) identification	Species	DNA concentration (ng/µl)	Detection time at 67°C (min:s)	Detection time at 69°C (min:s)
60982	<i>C. ilicicola</i>	50	29:15 FP	ND
61448	<i>Calonectria</i> sp.	50	24:00 FP	ND
60981	<i>Calonectria</i> sp.	50	29:15 FP	ND
60986	<i>Cylindrocladiella</i> sp.	50	ND	ND
61292	<i>Cylindrocladiella</i> sp.	50	ND	ND
63711 e	<i>Mariannaea</i> sp.	50	29:15 FP	ND
61192 c	<i>Mariannaea</i> sp.	50	ND	ND
60983	<i>G. peggii</i>	50	21:30 FP	ND
61430	<i>G. whileyi</i>	50	21:45 FP	ND
63711 f	<i>Ilyonectria</i> sp.	50	20:30 FP	17:00 FP
61090 a	<i>Ilyonectria</i> sp.	50	25:15 FP	ND
n/a	RNase-free water	0	29:15 FP	ND
61546 a	<i>D. macrodidyma</i>	50	9:15	7:30
60991 a	<i>D. pauciseptata</i>	50	12:00	12:30
61432 b	<i>Ilyonectria</i> sp.	50	27:45 FP	ND
61090 a	<i>Ilyonectria</i> sp.	50	25:15 FP	28:30 FP
63711 f	<i>Ilyonectria</i> sp.	50	9:45 FP	15:30 FP
63711 g	<i>Ilyonectria</i> sp.	50	18:00 FP	17:30 FP
61546 i	<i>Ilyonectria</i> sp.	50	ND	ND
61194 a	<i>Ilyonectria</i> sp.	50	25:30 FP	ND
61435 c	<i>Ilyonectria</i> sp.	50	29:00 FP	ND
62004 b	<i>Ilyonectria</i> sp.	50	ND	ND
60989	<i>Ilyonectria</i> sp.	50	ND	ND
61443	<i>Ilyonectria</i> sp.	50	29:15 FP	ND
61293	<i>Ilyonectria</i> sp.	50	ND	ND
53652 a	<i>Ilyonectria</i> sp.	50	29:15 FP	ND
61303 d	<i>Ilyonectria</i> sp.	50	29:15 FP	ND
n/a	RNase-free water	0	ND	ND

were tested for target species *D. macrodidyma*. Tests with loop primers in experiment 2 aimed to improve the sensitivity, specificity, and detection speed.

Initial specificity testing of F3, B3, FIP, and BIP primers for detecting *Dactylonectria* spp. showed detection between 16 min and 15 s and 25 min and nondetection from 30 min; 13 of 14 (~93%) *Dactylonectria* isolates were detected.

Experiment 2: Testing loop primers for improved detection time, sensitivity, and specificity. In experiment 2, combinations of loop primers were tested with the four F3, B3, FIP, and BIP primers for an improved speed of detection, sensitivity, and specificity for *C. ilicicola*, *D. macrodidyma*, or *Dactylonectria* spp. The results of experiment 2 are listed in Supplementary Table S3.

The B Loop primer in combination with the four F3, B3, FIP, and BIP primers performed the best, with the highest sensitivity and fastest detection of *C. ilicicola* at low DNA concentrations of 1 pg/μl

compared with the other loop primer combinations. The F Loop primer enabled faster detection at DNA concentrations of 1 ng/μl, detecting the DNA 1 min and 10 s faster than the B Loop primer. However, at DNA concentrations <0.01 ng/μl, detection with F Loop was slower than the detection with B Loop. Therefore, F Loop and B Loop individually improved detection time and had roughly equal sensitivity; however, B Loop was slightly faster at lower DNA concentrations than F Loop. When combined, the F Loop and B Loop primers enabled the fastest detection of DNA in 5 min and 51 s to 7 min and 5 s at concentrations of 1 to 0.1 ng/μl. However, sensitivity was reduced by using both loop primers, because DNA concentrations of 0.01 ng/μl were detected at a similar time to that with B Loop primers alone, and there was no detection at concentrations <0.01 ng/μl. Therefore, the B Loop primer was selected for use in the LAMP diagnostic for *C. ilicicola*, and it was tested in subsequent experiments.

Table 3. Experiment 4—testing optimized primer sets and isothermal conditions for specificity in detection of target species *Calonectria ilicicola*, *Dactylonectria macrodidyma*, and *Dactylonectria* spp. using crude extracts from a suspension of fungal cultures in sterile distilled water^z

LAMP assay and isolate (BRIP) identification	Species	DNA concentration (ng/μl)	Detection time at 67°C (min:s)	Detection time at 69°C (min:s)
<i>C. ilicicola</i>				
54018 a	<i>C. ilicicola</i>	Undetermined	15:15	
60982	<i>C. ilicicola</i>	Undetermined	15:00	
60992	<i>C. ilicicola</i>	Undetermined	15:48	
61291	<i>C. ilicicola</i>	Undetermined	16:10	
61291	<i>C. ilicicola</i>	Undetermined	17:28	
60389	<i>C. ilicicola</i>	Undetermined	29:15	
53933 a	<i>C. ilicicola</i>	Undetermined	23:16	
60981	<i>Calonectria</i> sp.	Undetermined	ND	
54018 a + 60981	<i>C. ilicicola</i> + <i>Calonectria</i> sp.	Undetermined	ND	
60982 + 60981	<i>C. ilicicola</i> + <i>Calonectria</i> sp.	Undetermined	24:05	
61291 + 60981	<i>C. ilicicola</i> + <i>Calonectria</i> sp.	Undetermined	17:49	
n/a	<i>Ilyonectria</i> sp. fungal culture	Undetermined	ND	
n/a	RNase-free water	0	ND	
<i>D. macrodidyma</i>				
61294 a	<i>D. macrodidyma</i>	Undetermined	18:16	
61294 a	<i>D. macrodidyma</i>	Undetermined	17:27	
61349 e	<i>D. macrodidyma</i>	Undetermined	16:35	
61349 e	<i>D. macrodidyma</i>	Undetermined	22:38	
62001 b	<i>D. macrodidyma</i>	Undetermined	16:28	
62001 b	<i>D. macrodidyma</i>	Undetermined	25:14	
61428 d	<i>D. pauciseptata</i>	Undetermined	ND	
62000 d	<i>D. novozelandica</i>	Undetermined	ND	
60985	<i>D. anthuriicola</i>	Undetermined	ND	
61294 a + 62000 d	<i>D. macrodidyma</i> + <i>D. novozelandica</i>	Undetermined	16:13	
61349 e + 62000 d	<i>D. macrodidyma</i> + <i>D. novozelandica</i>	Undetermined	29:21	
62001 b + 62000 d	<i>D. macrodidyma</i> + <i>D. novozelandica</i>	Undetermined	20:41	
61428 d + 62000 d	<i>D. pauciseptata</i> + <i>D. novozelandica</i>	Undetermined	ND	
n/a	<i>Ilyonectria</i> sp. fungal culture	Undetermined	ND	
n/a	RNase-free water	0	ND	
<i>Dactylonectria</i> spp.				
61546 a	<i>D. macrodidyma</i>	Undetermined	6:30	7:15
61349 e	<i>D. macrodidyma</i>	Undetermined	17:00	12:15
61429 b	<i>D. macrodidyma</i>	Undetermined	20:15	19:00
60985	<i>D. anthuriicola</i>	Undetermined	17:00	16:00
62000 d	<i>D. novozelandica</i>	Undetermined	13:15	14:15
61428 d	<i>D. pauciseptata</i>	Undetermined	20:30	17:15
63708 b	<i>D. vitis</i>	Undetermined	13:15	14:45
61349 e + 54018 a	<i>D. macrodidyma</i> + <i>C. ilicicola</i>	Undetermined	14:15	10:30
61429 b + 54018 a	<i>D. macrodidyma</i> + <i>C. ilicicola</i>	Undetermined	19:45	23:00
60985 + 54018 a	<i>D. anthuriicola</i> + <i>C. ilicicola</i>	Undetermined	16:45	18:15
62000 d + 54018 a	<i>D. novozelandica</i> + <i>C. ilicicola</i>	Undetermined	25:15	18:30
61428 d + 54018 a	<i>D. pauciseptata</i> + <i>C. ilicicola</i>	Undetermined	16:30	21:45
63708 b + 54018 a	<i>D. vitis</i> + <i>C. ilicicola</i>	Undetermined	20:15	20:00
54018 a	<i>C. ilicicola</i>	Undetermined	25:15 FP	ND
n/a	RNase-free water	0	ND	ND

^z BRIP, Biosecurity Queensland Plant Pathology Herbarium; FP, false positive as indicated by detection of a nontarget species in <30 min or an inconsistent or absent annealing curve; LAMP, loop-mediated isothermal amplification; ND, not detected; n/a, not applicable.

The initial tests with the loop primers and four LAMP primers F3, B3, FIP, and BIP for the *D. macrodidyma* diagnostic were performed at an isothermal amplification temperature of 65°C, and loop primer combinations were compared to find the optimal primer combination. Including loop primers with the four standard LAMP primers improved sensitivity and detection speed compared with in experiment 1, which had no loop primers. Furthermore, LAMP primers with the B Loop primer were the most sensitive, detecting target DNA at 0.01 ng/μl within 17 min and 7 s. The F Loop primer enabled the fastest detection at 14 min and 51 s for concentrations of 1 ng/μl compared with 16 min and 4 s for B Loop and 18 min and 2 s for F Loop and B Loop combined. However, despite faster detection, use of the F Loop primer alone resulted in reduced sensitivity, with DNA concentrations detected at concentrations >0.01 ng/μl compared with successful detection at <0.01 ng/μl with use of the B Loop primer. Use of both loop primers combined resulted in the slowest detection speed and the lowest specificity, with detection occurring between 18 and 19 min and sensitivity to 0.1 ng/μl.

Although sensitivity and detection time were improved with introduction of B Loop primers, time seems to be an inhibiting factor in maintaining specificity to the target species. Across all loop primer combinations tested at 65°C, the nontarget species *D. novozelandica*, a close relative of *D. macrodidyma*, was detected within 29 to

55 min, with the F Loop primer detecting nontarget species the fastest and both loop primers combined detecting nontargets the slowest. It was, therefore, concluded that the success of target species specificity is dependent on time and the loop primers used. The optimal isothermal amplification time for this diagnostic design was 30 min to maintain target species specificity. However, the B Loop primer was found to be the most appropriate primer to satisfy the full criteria to a time cutoff for specificity, high sensitivity, and adequate detection speed. Therefore, the B Loop primer was chosen for subsequent LAMP diagnostic tests.

Additional tests in experiment 2 used the B Loop primer and compared the efficacy of the diagnostic between isothermal amplification temperatures of 63, 65, and 67°C. At DNA concentrations of 1 ng/μl, detection was fastest at 14 min and 37 s, with an isothermal temperature of 67°C, and it slowed to just >16 min at 63 and 65°C. Although the greatest sensitivity of detection (at 1 pg/μl DNA) was observed with isothermal temperature of 65°C, the speed of detection was 53 min and 20 s, which was beyond the previously determined optimal amplification time of 30 min. At DNA concentrations of 0.01 ng/μl, an isothermal temperature of 67°C performed detection faster than at 65°C. Therefore, the optimal isothermal temperature of 67°C for *D. macrodidyma* detection was used in all additional experiments.

Table 4. Experiment 5—validation of the loop-mediated isothermal amplification (LAMP) diagnostic for detection of target pathogens *Calonectria ilicicola*, *Dactylonectria macrodidyma*, and *Dactylonectria* spp. in inoculated necrotic avocado roots^z

Plant number	Isolate (BRIP) identification	Inoculum	Detection of <i>C. ilicicola</i>		Detection of <i>D. macrodidyma</i>		Detection of <i>Dactylonectria</i> spp.			
			Trial 1 at 67°C (min:s)	Trial 2 at 67°C (min:s)	Trial 1 at 67°C (min:s)	Trial 2 at 67°C (min:s)	Trial 1 at 67°C (min:s)	Trial 2 at 67°C (min:s)	Trial 1 at 69°C (min:s)	Trial 2 at 69°C (min:s)
n/a	62001 a	<i>D. macrodidyma</i> (50 ng/μl DNA)	ND	ND	7:45	8:00	5:30	5:15	5:45	7:30
n/a	54018 a	<i>C. ilicicola</i> (50 ng/μl DNA)	7:45	7:45	ND	ND	ND	28:00 FP	36:15 ND	34:45 ND
1	60985	<i>D. anthuricola</i>	ND	ND	ND	ND	16:00	15:30	18:15	18:45
2	60985	<i>D. anthuricola</i>	ND	ND	ND	ND	15:00	13:45	22:45	29:45
3	62000 d	<i>D. novozelandica</i>	ND	ND	39:15 ND	ND	15:30	39:15 ND	ND	22:30
4	62000 d	<i>D. novozelandica</i>	ND	ND	ND	ND	17:30	19:45	39:15 ND	28:45
5	61428 d	<i>D. pauciseptata</i>	34:15 ND	ND	ND	39:15 ND	20:00	28:45	ND	29:15
6	61428 d	<i>D. pauciseptata</i>	ND	ND	ND	ND	18:15	20:00	18:45	39:15 ND
7	63708 b	<i>D. vitis</i>	ND	ND	ND	ND	20:00	15:00	20:45	16:30
8	63708 b	<i>D. vitis</i>	ND	ND	ND	ND	16:00	15:15	17:30	17:30
9	60985 + 54018 a	<i>D. anthuricola</i> + <i>C. ilicicola</i>	13:00	15:45	ND	ND	17:15	15:15	18:45	30:45 ND
10	62000 d + 54018 a	<i>D. novozelandica</i> + <i>C. ilicicola</i>	14:45	15:15	ND	ND	ND	20:30	ND	ND
11	61428 d + 54018 a	<i>D. pauciseptata</i> + <i>C. ilicicola</i>	35:15 ND	36:00 ND	ND	ND	20:15	16:15	27:00 FP	ND
12	63708 b + 54018 a	<i>D. vitis</i> + <i>C. ilicicola</i>	13:45	ND	ND	39:15 ND	23:30	20:15	31:45 ND	ND
13	n/a	Uninoculated	ND	ND	ND	ND	30:30 ND	36:30 ND	33:15 ND	39:15 ND
n/a	n/a	Template free	ND	ND	ND	ND	30:30 ND	32:45 ND	39:15 ND	39:15 ND
n/a	62001 a	<i>D. macrodidyma</i> (50 ng/μl DNA)	ND	36:45 ND	8:00	8:00	15:15	5:45	7:45	8:00
n/a	54018 a	<i>C. ilicicola</i> (50 ng/μl DNA)	6:15	6:30	ND	ND	20:30 FP	24:00 FP	17:45 FP	39:15 ND
14	61349 e	<i>D. macrodidyma</i>	ND	ND	ND	21:00	ND	13:45	22:00	ND
15	61349 e	<i>D. macrodidyma</i>	ND	ND	12:00	13:45	10:45	29:00 FP	14:00	9:30
16	61349 e + 54018 a	<i>D. macrodidyma</i> + <i>C. ilicicola</i>	22:00	12:30	24:45	23:00	29:15 FP	20:00	15:15	28:00
17	61349 e + 54018 a	<i>D. macrodidyma</i> + <i>C. ilicicola</i>	13:15	11:45	13:30	18:45	15:15	16:45	16:30	18:00
18	61349 e + 54018 a	<i>D. macrodidyma</i> + <i>C. ilicicola</i>	17:30	15:15	15:15	19:00	ND	15:30	18:45	21:15
19	61349 e + 54018 a	<i>D. macrodidyma</i> + <i>C. ilicicola</i>	16:15	12:45	20:45	16:15	14:15	13:45	18:00	14:00
20	61349 e + 54018 a	<i>D. macrodidyma</i> + <i>C. ilicicola</i>	27:15 FP	15:00	25:30	18:15	ND	14:15	14:00	18:00
21	54018 a	<i>C. ilicicola</i>	17:00	14:30	ND	ND	28:45 FP	27:45 FP	ND	ND
22	54018 a	<i>C. ilicicola</i>	21:00	24:45	ND	ND	ND	27:30 FP	39:15 ND	ND
23	54018 a	<i>C. ilicicola</i>	ND	16:15	ND	ND	ND	37:00 ND	16:45 FP	39:00 ND
24	54018 a	<i>C. ilicicola</i>	12:30	13:45	ND	ND	ND	35:45 ND	ND	ND
n/a	n/a	Template free	ND	ND	ND	ND	27:15 FP	38:15 ND	39:15 ND	39:15 ND

^z BRIP, Biosecurity Queensland Plant Pathology Herbarium; FP, false positive as indicated by detection of a nontarget species in <30 min or an inconsistent or absent annealing curve; ND, not detected or nondetection as indicated by detection after 30 min; n/a, not applicable.

An initial specificity comparison between loop primer combinations was conducted on the *Dactylonectria* genus-wide assay before testing the assay for sensitivity. Including any combination of the loop primers in the assay improved the success rate of detection, with 100% of *Dactylonectria* isolates detected with 21 min compared with 93% in experiment 1. Including both loop primers resulted in faster detection of targets by ~2 min on average compared with using either loop primer alone. All primer combinations resulted in false positive detection of nontarget species and negative controls. Specificity was improved by including both loop primers, with detection of nontarget *Ilyonectria* sp. (BRIP 53498a) occurring at 44 min compared with 32 min and 15 s and 37 min and 45 s with F and B Loop primers, respectively. However, because these were detected after 30 min, it was nevertheless considered nondetection rather than true false positive detection.

The full primer set for detecting *Dactylonectria* spp. underwent sensitivity testing at isothermal amplification temperatures of 67°C for 60 min and subsequently, 69°C for 30 min with representative species from this genus. Two temperatures were tested for improvement in sensitivity. The isothermal amplification time was reduced to 30 min in the second assay, because nonspecific amplification of template-free samples was found in the first assay at 67°C after 30 min. Detection of target samples after this time was, therefore, considered a false positive result. The LAMP assay at 67°C for 60 min demonstrated sensitivity averaging 0.1 ng/μl, with *D. macrodidyma* (BRIP 61546a) detection as low as 1 pg/μl. The LAMP assay at 69°C for 30 min demonstrated sensitivity averaging 0.1 ng/μl, although one isolate (*D. anthuriicola* BRIP 60985) could not be detected at <1 ng/μl and another isolate (*D. macrodidyma* BRIP 61546a) was detected at 1 pg/μl but not at 0.01 ng/μl. The LAMP assay at 69°C for 30 min was then tested with nontarget *Ilyonectria* isolates in the specificity screening experiment (experiment 3) (Table 2).

LAMP diagnostic for detection of target pathogens in DNA samples, fungal cultures, and inoculated necrotic avocado tissue.
Experiment 3: Screening DNA samples in isolate collection to confirm LAMP specificity. Experiment 3 confirmed the specificity of the LAMP diagnostic design for detecting *C. ilicicola*, *D. macrodidyma*, and *Dactylonectria* spp. using 1 and 50 ng/μl fungal DNA extracts and fungal mycelia from a representative number of fungal target species, closely related nontarget species, and other fungal genera associated with root rot disease.

The *C. ilicicola* LAMP diagnostic design was confirmed to be specific, detecting the target species within 10 to 11 min and 30 s at DNA concentrations of 1 ng/μl (Table 2). None of the other isolates in the genus *Calonectria* or the tested genera were detected (Table 2).

The *D. macrodidyma* diagnostic was confirmed to be specific, detecting target DNA within 12 min and 15 s to 28 min and 30 s (averaging 15 to 16 min) at concentrations of 50 ng/μl. None of the nontarget *Dactylonectria* spp. (Table 2) or species in the other genera were detected. Some *D. macrodidyma* isolates were tested twice at concentrations of 1 and 50 ng/μl to observe any differences in detection speed (Table 2). The 50-ng/μl DNA templates were detected considerably faster (by ~2 to 9 min) than the 1-ng/μl DNA templates.

The *Dactylonectria* genus-wide assay at an isothermal amplification temperature of 67°C was demonstrated to amplify all *Dactylonectria* spp. isolates within 6 to 17 min and 30 s (Table 2). However, this assay was also found to amplify nontarget genera between 9 min and 45 s and 25 min and 15 s, including one isolate of *C. ilicicola*, *Calonectria* sp., *Gliocladiopsis peggii*, *G. whileyi*, and five isolates of *Ilyonectria* sp. (Table 2). A number of false positives were also found in nontarget genera detected within ~2 min and 15 s of the 30-min amplification time (Table 2) and some template-free controls. The alignment of the annealing curve for each sample to the positive control was used to visually assess true positive detection, with false positives denoted by no annealing curve or a short peak compared with the positive control and amplification of nontarget DNA often denoted by annealing curves not aligned to the positive control (with the curve positioned to the left or right of the positive control curve). However, considerable care was taken when using the curve

alignment to decide false or true positives, because large differences in amplification time between *Dactylonectria* species also had an effect on the annealing curve alignment.

The assay at an isothermal amplification temperature of 69°C was demonstrated to have improved specificity, with no detection of isolates in the *Calonectria*, *Cylindrocladiella*, *Gliocladiopsis*, or *Mariannaea* genera or the template-free control (Table 2). Of the 16 representative isolates of *Ilyonectria*, which were selected from five different phylogenetic clades within this genus (Parkinson 2017), only 2 *Ilyonectria* sp. isolates, BRIP 61090 and BRIP 63711 (BRIP 63711f and BRIP 63711g are duplicate cultures of the same isolate), were detected in this assay (Table 2). These isolates are likely to represent two separate new species, and they were recorded only from one sample each in an Australia-wide fungal black root rot disease survey between 2013 and 2016 of healthy and symptomatic avocado trees and thus, are considered to have a very limited distribution (Parkinson 2017).

Experiment 4: LAMP diagnostic detection of target pathogens in fungal cultures. Experiment 4 tested the LAMP diagnostic design for detecting *C. ilicicola*, *D. macrodidyma*, and *Dactylonectria* spp. using aliquots of sd water containing fungal mycelia (Table 3).

In the *C. ilicicola* diagnostic (Table 3), all of the target species as pure cultures were detected between 15 and 29 min and 15 s, averaging ~16 min for detection. None of the nontarget species as pure cultures were detected (Table 3). *C. ilicicola* was detected in two of three mixed cultures, with detection occurring between 17 min and 49 s and 24 min and 5 s (Table 3); the unknown *Calonectria* sp. was not detected.

In the *D. macrodidyma* diagnostic (Table 3), all of the target species as pure cultures were detected within 16 min and 30 s and 25 min and 15 s. None of the nontarget species as pure cultures were detected (Table 3); 100% of mixed cultures containing the target species resulted in positive detection within 16 min and 15 s to 29 min and 20 s (Table 3). None of the mixed cultures containing only nontarget species showed detection (Table 3).

In the *Dactylonectria* genus-wide assay, 100% of *Dactylonectria* isolates had positive detection for both isothermal amplification temperatures (Table 3), with detection speeds ranging from 6 min and 30 s to 25 min and 15 s. The detection time varied between the temperatures, suggesting that there was no link between detection speed and temperatures from 67 to 69°C. Specificity was demonstrated at 69°C, with no detection of *C. ilicicola* at this temperature compared with nonspecific detection at 25 min and 15 s at 67°C (Table 3).

Experiment 5: Validation of the LAMP diagnostic detection of target pathogens in inoculated avocado roots. This experiment tested the efficacy of the LAMP diagnostic on target species detection in symptomatic roots of inoculated avocado Reed seedlings. Each LAMP sample tested corresponded to a single plant inoculated with a pathogenic isolate or mixture of isolates (Table 4). Two diagnostic trials were conducted with freshly collected root tissue. Any detection after 30 min was considered to be nondetection, and this was confirmed by visual assessment of the annealing curve and temperature record. Any detection from 27 min and 30 s was subject to confirmation by visual assessment of annealing curves.

In the *C. ilicicola* assay, across two diagnostic trials, *C. ilicicola* was detected in 100% of plants inoculated with *C. ilicicola* alone, with detection time ranging from 12 min and 30 s to 24 min and 45 s (Table 4). However, *C. ilicicola* in one plant (plant #23) was not detected in the first trial but was subsequently detected in the second trial. Across two diagnostic trials, eight of nine plants coinoculated with *C. ilicicola* had positive detection from 12 min and 45 s to 22 min; *C. ilicicola* in plant #11 was not detected in both trials, and two coinoculated plants of nine had positive detection in only one trial (Table 4). Nondetection occurred at 34 min and 15 s in one plant (plant #5) of 24 and at 36 min and 45 s in the *D. macrodidyma* 50 ng/μl DNA sample (Table 4). There was no detection in healthy roots or template-free controls.

In the *D. macrodidyma* assay, across two diagnostic trials, *D. macrodidyma* was detected in 100% of plants inoculated with

D. macrodidyma, with detection time ranging from 12 to 21 min (Table 4). However, one plant (plant #14) was not detected in the first trial but was subsequently detected in the second trial. Across two diagnostic trials, 100% of plants coinoculated with *D. macrodidyma* were detected from 13 min and 30 s to 25 min and 30 s. Nondetection of *Dactylonectria* spp. occurred at 39 min and 15 s in 3 of 24 plants (Table 4) across two diagnostic trials. There was no detection in healthy roots or template-free controls.

In the *Dactylonectria* genus-wide assay, across two diagnostic trials for each isothermal amplification temperature tested, the genus *Dactylonectria* was detected in 100% of plants inoculated or coinoculated with any species of the genus *Dactylonectria*, with detection time ranging 13 min and 45 s to 28 min and 45 s (Table 4) at 67°C, and 17 of 19 plants (~89.5%) ranging from 9 min and 30 s to 29 min and 15 s at 69°C. Within this group, 12 of 19 plants were detected in both of the diagnostic trials at 67°C, and 10 of 19 plants were detected in both of the diagnostic trials at 69°C. False negative detection occurred at 69°C in 3 of 19 plants in trial 1 and 4 of 19 plants in trial 2; at 67°C, false negative detection occurred in 4 of 19 plants in trial 1 and 1 plant in trial 2. False positive detection of nontarget *C. ilicicola* occurred in trials of both isothermal temperatures tested, with detection times ranging from 17 min and 45 s to 28 min in 50 ng/μl DNA samples. In trial 1 at 67°C, one of four plants inoculated with *C. ilicicola* alone resulted in false positive detection at 28 min and 45 s; in trial 2 at 67°C, 50% of plants inoculated with *C. ilicicola* alone resulted in false positive detection from 27 min and 30 s to 27 min and 45 s, whereas the other 50% resulted in non-detection after 30 min. In trial 1 at 69°C, only one plant inoculated with *C. ilicicola* alone resulted in false positive detection at 16 min and 45 s. Template-free controls and healthy plants resulted in non-detection as indicated by detection after 30 min in all trials at both temperatures; however, false positive detection occurred at 27 min and 15 s in trial 1 at 67°C.

Discussion

In this study, three LAMP diagnostic assays for detecting *C. ilicicola*, *D. macrodidyma*, and species in the *Dactylonectria* genus in avocado roots were developed from β-tubulin (*C. ilicicola*) and histone H3 (*D. macrodidyma* and *Dactylonectria* spp.) fungal DNA sequence data. Five primers were designed for the species-specific assays, which included one loop primer to increase the specificity, sensitivity, and speed of the reaction, allowing reliable detection in avocado roots and fungal DNA of both target species within 30 min. Six primers, including two loop primers, were designed for the *Dactylonectria* genus-wide assay, with detection in avocado roots occurring within 30 min. The speed of detection across all assays was fastest and most reliable for DNA extracts, with the detection speed decreasing as fungal cultures and plant tissue were introduced to the diagnostic.

The specificity of the diagnostic for detecting the target species or genus was found to be subject to time and isothermal amplification temperature, with specificity being most reliable in amplifications of <30 min and temperatures of 67°C for detecting *C. ilicicola* and *D. macrodidyma* and 69°C for *Dactylonectria* spp. Nondetection was assumed at time points after 30 min, and any detection after 27 min and 30 s was subject to the judgement of the user in checking for anneal curve consistency with the positive control to rule out false positive or nondetection. Nevertheless, nondetection after 30 min was less frequent in the species-specific assays compared with the genus-wide assay. False positives also did not occur at <30 min for the species-specific assays compared with the genus-wide assay, which suggests that the species-specific assays are reliable for in planta detection of the target pathogens.

A limitation of the assay is the likelihood of false negatives, with some inoculated root samples in experiment 5 failing to amplify in one trial but amplifying in the second trial with a fresh batch of roots (Table 4). Although the majority of inoculated roots resulted in positive detection, the detection in avocado roots was found to be variable and possibly subject to the amount and quality of necrotic root tissue used in each sample. In preliminary work (data not shown)

using too much necrotic root tissue (e.g., several necrotic roots >1.5 cm) often resulted in false negative detection; however, the detection rates improved when fewer roots (up to two necrotic roots <1.5 cm) were used, and these were also diluted by including a proportion of healthy roots (two to four healthy roots <1.5 cm) in the sample tube for extraction. Perhaps too much necrotic material may have an inhibitory effect on detection, and adjusting the concentration of fungal DNA using proportions helped to alleviate these effects. However, an accurate concentration of the target fungal DNA in the root tissue could not be determined, because the crude extracts contained target fungal DNA, avocado DNA, and DNA of soil microbial contaminants. Additional testing of the assays in field samples naturally infected with the pathogens should be done to fully validate the diagnostic, because artificial inoculation in the glasshouse may not represent varying pathogen titer in nursery or orchard environments. Nevertheless, the LAMP assays have been used for diagnostic testing of a symptomatic nursery tree submitted through the Australian nursery accreditation scheme, and *C. ilicicola* presence was confirmed and supported with identification by fungal isolation. LAMP detection speeds could provide an estimate of the fungal concentration, with faster speeds indicating higher target DNA; however, inhibitory compounds in the crude extract may also have an effect on the detection speed or success. Testing multiple samples of a single plant within the assay may also reduce the likelihood of false negative detection in a single diagnostic run.

The genus-wide *Dactylonectria* assay was demonstrated to have efficacy in detecting all *Dactylonectria* species available for testing, with no detection of representative isolates in the *Cylindrocladiella*, *Gliocladiopsis*, or *Mariannaea* genera; 2 of 16 representative isolates of the genus *Ilyonectria* selected were also detected in this assay (Table 2). The genus *Ilyonectria* is a close relative of the genus *Dactylonectria*, in which the latter genus was separated from the former in a multigenic phylogeny study (Lombard et al. 2014). In the LAMP primer design phase, the genera *Dactylonectria*, *Ilyonectria*, and *Calonectria* shared multiple conserved regions within each gene examined, rendering it challenging to maintain 100% specificity for all species within the genus *Dactylonectria* while also excluding all species in the sister genera. A limitation found in primer design software for designing genus-wide LAMP primers was that the primer combinations produced did not satisfy full exclusion of species in closely related genera. Manual design of *Dactylonectria* genus-wide primers improved the specificity. However, the false positive detection of nontarget *C. ilicicola* in <30 min in the *Dactylonectria* genus-wide assay demonstrated a shortfall in the primer design. For the purpose of the diagnostic assay in detecting multiple species in a known pathogenic genus, the LAMP primers could still be sufficient despite the chance of also detecting nontargets.

Some studies have demonstrated reliable detection without a DNA extraction step, with macerated plant tissue in water used directly as the DNA template (Fukuta et al. 2013). In LAMP studies by Fukuta et al. (2013), tomato roots were cut and placed into 1 ml sd water. The solution was shaken for 1 min. Then, 5 μl solution was used as the LAMP DNA template for each reaction sample, and the LAMP assay was repeated three times (Fukuta et al. 2013). Other alternative extraction methods using NaOH and Tris-HCl buffers were outlined by Fukuta et al. (2003) for extraction of DNA from young tomato leaf tissue, which was then used as the DNA template in 25-μl LAMP reactions. These methods were tested in preliminary avocado root tissue assays; however, there was inconsistent positive detection in inoculated avocado roots (data not shown), and DNA extraction with a commercial kit had improved the efficacy of the assays. DNA extraction from plant material using a lateral flow device (Tomlinson et al. 2010a, b) or with simple buffer solutions (Fukuta et al. 2003) could provide an alternative or potentially reduced cost DNA extraction before the LAMP reaction.

The findings of this study demonstrate the application and adoption of LAMP-based diagnostics in the Australian avocado industry. The simplicity of the testing method enables plant pathology service providers to use the tool as an initial test for confirming the presence of important pathogens in nurseries or orchards. Black root rot

caused by nectriaceous pathogens poses a significant risk to new plantings in the first year after planting in the field, causing rapid decline and death (Parkinson et al. 2017). Although *C. ilicicola* is infrequently found in Australian avocado nurseries, *Dactylonectria* spp. have a higher prevalence (Parkinson 2017), and if either are present in the nursery, the potential loss in new orchard plantings could be devastating. Use of the LAMP assays for testing nursery trees routinely and before dispatching to orchards could potentially prevent industry-wide loss of new plantings in Australia. The LAMP primer sequences are available via publication, and the flexibility of the diagnostic procedure to be used with a range of reagents and equipment enables global accessibility. This diagnostic technology is not limited to testing in avocados, because the nectriaceous pathogens of interest are also important pathogens of various crops and ornamental plants, revealing an opportunity for use of this diagnostic tool across multiple agricultural industries around the world.

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