Meinecke, C. D., De Vos, L., Yilmaz, N., Steenkamp, E. T., Wingfield, M. J., Wingfield, B. D., Villari, C. 2022. A LAMP assay for rapid detection of the pitch canker pathogen *Fusarium circinatum*. Plant Dis.

Assessing *Fusarium circinatum* qPCR and LAMP assays for cross-reactivity with *F. pilosicola* DNA.

Materials and methods

Reactions using purified DNA

To assess whether other available *F. circinatum* molecular tests cross-react with the newly described *F. pilosicola*, we screened *F. pilosicola* DNA using the dual-labelled probe qPCR method developed by Ioos *et al.* (2009) and the probe-based LAMP method developed by Stehlíková *et al*. (2020).

The qPCR reaction mix (final volume of 20 µL) consisted of 5.0 µL of molecular grade water, 10 µL of GoTaq® Probe qPCR Master Mix (Promega, Madison, Wisconsin, USA), 1.0 µL of each FCIR-F and FCIR-R primers (10 µM) and 1.0 µL of the FCIR-P dual-labeled probe (10 µM). The LAMP reaction (final volume 25 µL) was prepared as described by Stehlíková *et al*. (2020), using identical reagents, primers, and reaction conditions. The DNA of *F. pilosicola* isolates CMWF 1183 and CMWF 1189 was tested by each assay. Each qPCR and LAMP run included the DNA of *F. circinatum* isolate CV-2020-006 (Table S1) as a positive control and two non-template negative controls. Each test of *F. pilosicola* or *F. circinatum* DNA was run in triplicate within the same run. All qPCR and LAMP reaction mixtures were assembled in eight-tube MicroAmp® Fast Reaction Tubes strips (Applied Biosystems TM).

All reactions were run on a StepOnePlus Real-Time PCR System (Applied Biosystems™, Foster City, California, USA). Reactions were run as described in Ioos *et al.* (2009) and Stehlíková *et al.* (2020) for the qPCR and LAMP tests, respectively.

Results

Reactions using purified DNA

Both isolates of the recently described *F. pilosicola* and the *F. circinatum* positive control tested positive by the previously published LAMP assay, whereas only *F. circinatum* DNA was amplified by the qPCR test (Table S1). The negative controls did not amplify.

Pathogenicity testing of *Fusarium pilosicola* on *Pinus* seedlings

Materials and methods

Inoculations

Nineteen-month-old *Pinus patula* x *Pinus tecunumanii* plants were housed at 25°C under the ambient day/night cycle. Plant stems were inoculated using a 10 mm cork borer to remove the bark, inserting an agar plug into the opening, and sealing the wound with Parafilm. Fungal isolates were grown on PDA at 25°C for 7 days. Trees were either inoculated with one of the only two known isolates of *Fusarium pilosicola* (isolates CMWF1183 and CMWF1189), *F. circinatum* (isolate FSP34) as a positive control, or sterile PDA as a negative control. Each treatment was applied to 26 trees. After 28 days, the bark surrounding the inoculation site was removed and the length of the lesion measured. Lesion lengths were compared between treatments using the Student's t-test.

Results

Inoculations

Neither isolate of *F. pilosicola* caused the formation of lesions on the pine seedlings ($P > 0.05$). *Fusarium circinatum* induced lesions that were significantly longer than those observed in the negative control and *F. pilosicola* inoculations ($P < 0.05$, Figure 1). Under the conditions tested, *F. pilosicola* is not virulent towards *Pinus patula* x *Pinus tecunumanii* seedlings.

Figure S1. Comparison of pathogenicity of *F. pilosicola* CMWF1183 and CMWF1189 and *F. circinatum* FSP34. Average lesion length was measured 28 days after inoculation on *P. patula* x *P. tecunumanii* seedlings. Error bars represent the standard deviation.

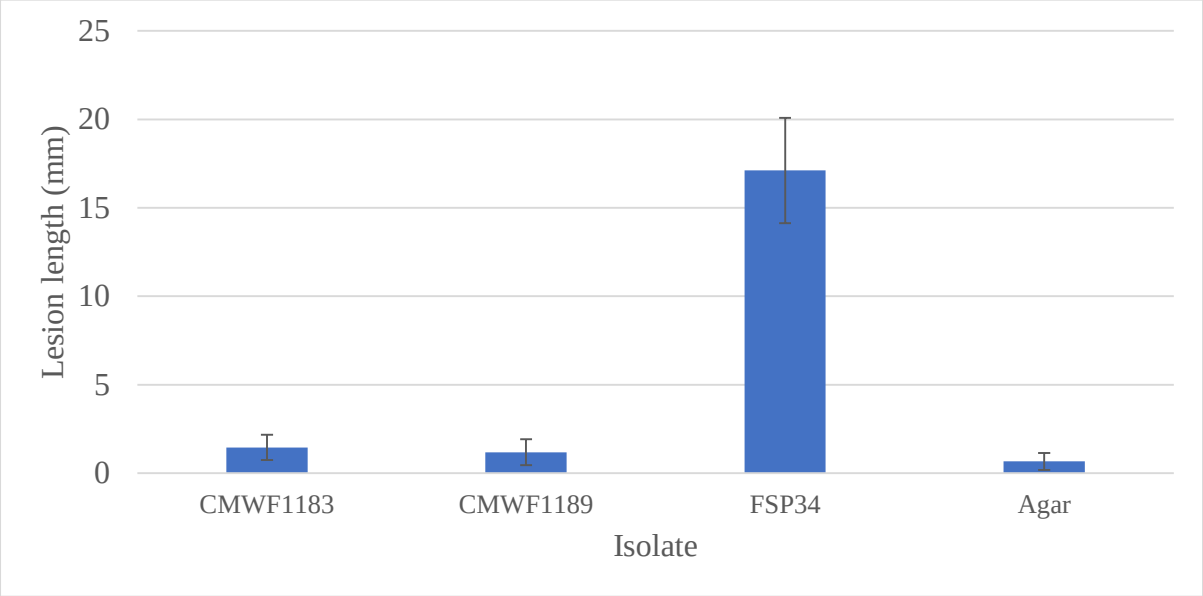

Table S1. Fungal isolates used to test available *Fusarium circinatum* qPCR and LAMP assays for cross-reactivity with the recently described *F. pilosicola*.

Fungal species	**Collection Number**[a]	**Host Species**	**Country**	**qPCR result**[b]	**LAMP result**
Fusarium circinatum	CV_2020_006	*Pinus taeda*	United States	**3/3** 40/26.41	**3/3** 13.6m
Fusarium pilosicola	CMWF 1183 = NRRL 29124[T]	*Bidens pilosa*	United States	0/3	**3/3** 10.0m
Fusarium pilosicola	CMWF 1189 = NRRL 29123	*Bidens pilosa*	United States	0/3	**3/3** 10.3m

[a] Abbreviations for the culture collections: the culture collection of Dr. Villari (CV) at the D.B. Warnell School of Forestry, University of Georgia, United States; the working collection of Fusarium isolates at FABI (CMWF) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

[b] Positive qPCR and LAMP results were determined by amplification of at least 2 of 3 replicate reactions for each sample. Average reaction time is given (min for LAMP and min/Ct for qPCR, total reaction time 60 minutes). Positive results are indicated in bold.

[T] Ex-type specimen

Table S2. Percent matches of each core LAMP primer binding site to the genomes of 50 *Fusarium* spp., representing the *Fusarium fujikuroi* species complex. Similarity of each primer binding sequence to genomic DNA was determined by BLAST comparison.

Species	Phylogeographic clade	Accession number	Isolate number	F3	B3	FIP	BIP
Fusarium acutatum	African A	GCA_012932015.1	NRRL 13308	0%	0%	0%	0%
Fusarium andiyazi	African A	ERX4077522	CBS 119857	0%	0%	0%	0%
Fusarium brevicatenulatum	African A	GCA_013363135.1	NRRL 25447	0%	0%	0%	0%
Fusarium denticulatum	African A	GCA_013396175.1	NRRL 25311	0%	0%	0%	0%
Fusarium ficicrescens	African A	ERX4078212	CBS 125178	0%	0%	0%	0%
Fusarium lactis	African A	ERX4077573	CBS 411.97	0%	0%	0%	0%
Fusarium mundagurra	African A	GCA_013396205.1	NRRL 66235	0%	0%	0%	0%
Fusarium musae	African A	GCA_019915245.1	F31	0%	0%	0%	0%
Fusarium napiforme	African A	GCA_013396005.1	NRRL 25196	0%	0%	0%	0%
Fusarium nygamai	African A	GCA_001262555.1	NRRL 66327	0%	0%	0%	0%
Fusarium phyllophilum	African A	GCA_013396025.1	NRRL 13617	0%	0%	0%	0%
Fusarium pseudoanthophilum	African A	GCA_013395995.1	NRRL 25211	0%	0%	0%	0%
Fusarium pseudocircinatum	African A	GCA_013396035.1	NRRL 36939	0%	0%	0%	0%
Fusarium pseudonygamai	African A	GCA_013186785.1	NRRL 13592	0%	0%	0%	0%
Fusarium ramigenum	African A	GCA_013186855.1	NRRL 25208	0%	0%	0%	0%
Fusarium secorum	African A	GCA_013363185.1	NRRL 62593	0%	0%	0%	0%
Fusarium thapsinum	African A	GCA_013186935.1	NRRL 22049	0%	0%	0%	0%
Fusarium tjaetaba	African A	GCA_013396195.1	NRRL 66243	0%	0%	0%	0%
Fusarium udum	African A	GCA_013186905.1	NRRL 25194	0%	0%	0%	0%
Fusarium verticillioides	African A	GCA_000149555.1	7600	0%	0%	0%	0%
Fusarium xylarioides	African A	GCA_013183765.1	KSU18978	0%	0%	0%	0%
Fusarium xyrophilum	African A	GCA_008711595.1	NRRL 62721	0%	0%	0%	0%
Fusarium dlamini	African B	GCA_013186775.1	NRRL 13164	0%	0%	0%	0%
Fusarium agapanthi	American	GCA_001654545.1	NRRL 54464	0%	0%	0%	0%
Fusarium ananatum	American	ERX4077703	CBS 118516	0%	0%	0%	0%
Fusarium anthophilum	American	GCA_013364935.1	NRRL 25214	0%	0%	0%	0%
Fusarium bactridioides	American	GCA_013623355.1	NRRL 66639	0%	0%	0%	0%

Fusarium begoniae	American	GCA_013186755.1	NRRL 25300	0%	0%	0%	0%
Fusarium bulbicola	American	GCA_013186765.1	NRRL 22947	0%	0%	0%	0%
Fusarium circinatum	American	GCA_000497325.3	FSP34	100%	100%	100%	100%
Fusarium fracticaudum	American	GCA_003353625.1	CBS 137234	0%	0%	0%	0%
Fusarium guttiforme	American	GCA_013186795.1	NRRL 22945	0%	0%	0%	0%
Fusarium marasasianum	American	GCA_022833035.1	CMW 25512	0%	0%	0%	0%
Fusarium mexicanum	American	GCA_013396015.1	NRRL 53147	0%	0%	0%	0%
Fusarium pilosicola	American	GCA_020615335.1	CMWF1183	100%	100%	95%	98%
Fusarium pininemorale	American	GCA_002165215.1	CMW 25243	0%	0%	0%	0%
Fusarium sororula	American	GCA_017579625.1	FCC 5425	0%	0%	0%	0%
Fusarium sterilihyphosum	American	GCA_013186845.1	NRRL 25623	0%	0%	0%	0%
Fusarium subglutinans	American	GCA_013396075.1	NRRL 66333	0%	0%	0%	0%
Fusarium succisae	American	GCA_013186925.1	NRRL 13298	0%	0%	0%	0%
Fusarium temperatum	American	GCA_001513835.1	CMWF389	0%	0%	0%	0%
Fusarium tupiense	American	GCA_013364945.1	NRRL 53984	0%	0%	0%	0%
Fusarium annulatum	Asian	GCA_019189775.1	F8_4S_2B	0%	0%	0%	0%
Fusarium coicis	Asian	GCA_013781345.1	NRRL 66233	0%	0%	0%	0%
Fusarium concentricum	Asian	GCA_014824425.1	NRRL 25181	0%	0%	0%	0%
Fusarium fujikuroi	Asian	GCA_900079805.1	IMI 58289	0%	0%	0%	0%
Fusarium globosum	Asian	GCA_013396165.1	NRRL 26131	0%	0%	0%	0%
Fusarium mangiferae	Asian	GCA_900044065.1	MRC7560	0%	0%	0%	0%
Fusarium proliferatum	Asian	GCA_900067095.1	ET1	0%	0%	0%	0%
Fusarium sacchari	Asian	GCA_017165645.1	FS66	0%	0%	0%	0%

Table S3. Results of sensitivity testing using purified *Fusarium circinatum* DNA and lysed spore suspension dilutions, and crude DNA extractions from known spore quantities.

DNA added	LAMP Results[a]		qPCR Results	
	Number of positive replicates	Reaction times (min) [total reaction time 60 minutes]	Number of positive replicates	Reaction times (Ct) [total reaction time 60 minutes]
1 ng μL^{-1}	**3/3**	12, 12, 12	**3/3**	15.85, 15.98, 15.93
100 pg μL^{-1}	**3/3**	14, 14, 13	**3/3**	25.13, 24.89, 24.99
10 pg μL^{-1}	**2/3**	16, 16	**2/3**	28.35, 28.26
1 pg μL^{-1}	0/3	-	1/3	39.25
100 fg μL^{-1}	0/3	-	0/3	-
Spores added				
1000 μL^{-1}	**3/3**	11, 12, 12	**3/3**	25.83, 25.93, 26.07
500 μL^{-1}	**3/3**	12, 12, 12	**3/3**	26.87, 25.98, 26.43
100 μL^{-1}	**3/3**	13, 12, 12	**3/3**	28.15, 28.19, 28.37
50 μL^{-1}	**3/3**	14, 14, 14	**3/3**	28.88, 28.77, 28.55
10 μL^{-1}	**2/3**	16, 16	**3/3**	31.12, 30.63, 30.50
5 μL^{-1}	1/3	47	**3/3**	33.19, 33.94, 33.39
1 μL^{-1}	0/3	-	0/3	-
Spores (crude)				
1000000	3/3	17, 18, 35	**3/3**	25.09, 25.74, 26.84
500000	1/3	47	**3/3**	27.19, 27.71, 27.91
100000	1/3	50	**3/3**	29.83, 29.05, 29.09
50000	0/3	-	**3/3**	35.42, 35.32, 33.25
10000	0/3	-	**2/3**	39.8, 39.0
5000	0/3	-	0/3	-

1000	0/3	-	0/3	-
500	0/3	-	0/3	-

[a]Positive LAMP and qPCR results, indicated in bold, were determined by amplification of at least 2 of 3 replicate reactions for each dilution and are shown as the average amplification time (min) or the average amplification time and cycle threshold (Ct) for LAMP and qPCR, respectively.