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Rapid Detection of Pine Pathogens *Lecanosticta acicola*, *Dothistroma pini* and *D. septosporum* on Needles by Probe-Based LAMP Assays

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Abstract: Needle blights are serious needle fungal diseases affecting pines both in natural and productive forests. Among needle blight agents, the ascomycetes *Lecanosticta acicola*, *Dothistroma pini* and *D. septosporum* are of particular concern. These pathogens need specific, fast and accurate diagnostics since they are regulated species in many countries and may require differential management measures. Due to the similarities in fungal morphology and the symptoms they elicit, these species are hard to distinguish using morphological characteristics. The symptoms can also be confused with those caused by insects or abiotic agents. DNA-based detection is therefore recommended. However, the specific PCR assays that have been produced to date for the differential diagnosis of these pathogens can be applied only in a well-furnished laboratory and the procedure takes a relatively long execution time. Surveillance and forest protection would benefit from a faster diagnostic method, such as a loop-mediated isothermal amplification (LAMP) assay, which requires less sophisticated equipment and can also be deployed directly on-site using portable devices. LAMP assays for the rapid and early detection of *L. acicola*, *D. pini* and *D. septosporum* were developed in this work. Species-specific LAMP primers and fluorescent assimilating probes were designed for each assay, targeting the beta tubulin (β-tub2) gene for the two *Dothistroma* species and the elongation factor (EF-1α) region for *L. acicola*. Each reaction detected its respective pathogen rapidly and with high specificity and sensitivity in DNA extracts from both pure fungal cultures and directly from infected pine needles. These qualities and the compatibility with inexpensive portable instrumentation position these LAMP assays as an effective method for routine phytosanitary control of plant material in real time, and they could profitably assist the management of *L. acicola*, *D. pini* and *D. septosporum*.

Keywords: loop-mediated isothermal amplification; brown spot needle blight; red band needle blight; *Pinus*; *Mycosphaerella dearnessii*; field-portable fungal diagnostics; forest health protection; phytosanitary surveillance

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1. Introduction

Needle blights are among the most serious fungal needle diseases affecting pine species worldwide both in plantations and naturally regenerating forest ecosystems. Among the different causal agents, the ascomycetous fungi *Lecanosticta acicola* (Thümen) H. Sydow, *Dothistroma pini* Hulbary and *D. septosporum* (G. Doroguine) M. Morelet are of particular concern [1], causing brown spot needle blight (BSNB) and Dothistroma needle blight (DNB), respectively. Needles infected by these fungi progressively die from the

tip and are prematurely shed. The proximal part of the branches and the lower crown are generally defoliated first. Eventually, almost all needles are lost, growth is severely impaired and the trees are weakened and may die after heavy and repeated attacks [2]. Because the photosynthetic activity of diseased needles is severely impaired, these diseases can cause major reductions in tree growth and limit wood production even when less than one-quarter of the canopy is affected [3]. These diseases can damage plants of a young age, thus inhibiting the growth in new plantations and locally impairing natural regeneration [4,5]. Currently, these three fungal pathogens are regulated in many countries of the world. According to the European and Mediterranean Plant Protection Organization (EPPO), *L. acicola* is categorized as a quarantine species in Morocco, Tunisia and Norway, and is included in the A1 list of quarantine species in Argentina, Brazil, Chile, Uruguay, Bahrain, Kazakhstan, Russia, Turkey and Ukraine and in the A2 list of quarantine species in Jordan [6]; *D. septosporum* is a quarantine species in Israel and Norway and it has the A2 quarantine status in Turkey and Jordan [7]; and *D. pini* is categorized as an A2 quarantine species in Turkey [8]. In the European Union, all three species are classified as Regulated Non Quarantine Pests (RNQPs), i.e., species regulated by implementing phytosanitary measures to reduce the economic impact in EU territories [9,10].

For most of the 20th century, DNB was known primarily as a destructive disease of pine plantations in the Southern Hemisphere (for a review of the historical records per country, the reader is referred to [11] and the supplementary data contained therein). Similarly, BSNB was initially confined to the southern part of the USA [12], but starting with a report in Spain in the 1940s [13], the pathogen was reported in several other countries in Europe, Asia and America (for a list of historical records see [14]). Nowadays, outbreaks of DNB and BSNB represent a global phenomenon [5,11,14–20], and the widespread mortality of natural pine forests and plantations is raising concern, given the commercial and environmental importance of the species. More than 95 pine species are currently confirmed as hosts of these pathogens, with varying degrees of susceptibility [11,14,21]. The infection of plants in other genera of Pinaceae has occasionally been reported for *D. septosporum*, possibly as a consequence of a high inoculum load from heavily infected neighboring pine plants [22–24], while for *L. acicola* there is a single and recent report of natural infection on a non-pine host, that is, on *Cedrus* Trew in Turkey [20]. Therefore, the exact number of host species is probably not yet well-defined. Moreover, an increase in DNB severity is expected, especially in the Northern Hemisphere, where natural woodlands and plantation forests are in close proximity, and where climate change could lead to increases in summer precipitation [25–27]. However, many other biotic, abiotic and anthropogenic factors could be important drivers for needle blights epidemics. Among these, the movement of infected planting material between regions and countries is thought to be the main anthropogenic pathway of *L. acicola, D. pini* and *D. septosporum* [28–31].

The ingress of plant pathogens from different origins could also lead to the mixing of different genetic populations, even when countries already harbor the same species. Since the three pathogens are known for having high intraspecific genetic variability carried out by sexual reproduction, the mixing of different populations may give rise to haplotypes containing new allele combinations [15,32–35], some of which might prevail under selective pressure if they were more capable of adapting to local environmental conditions, more virulent in attacking the host or able to better defeat existing resistance mechanisms [36,37]. Thus, it is crucial that efforts to prevent the spread of *L. acicola*, *D. pini* and *D. septosporum* across countries are strengthened. In this regard, there is a great need for user-friendly early detection methods that could be deployed at the point-of-care, that is, directly at the time and place of interest. Examples would be during phytosanitary inspections of commercial consignments at ports of entry or in plant nurseries, to rapidly screen for such pathogens in a highly specific way. These tools would help in the proper implementation of management and treatment measures [38] and in controlling and limiting the spread of *L. acicola*, *D. pini* and *D. septosporum* into pathogen-free areas. However, these three pathogens elicit very similar symptoms on their hosts, making it difficult to discriminate

one from the other based only on the morphological characteristics of the symptoms, even for an expert eye [14,39]. Moreover, there can be a lag of several months between infection and symptom expression for all of these species [40], making visual inspection an even less reliable approach for surveillance [29]. DNB symptoms may also be confused with abiotic damage and with damage caused by a number of needle sap-sucker and needle mining insects, such as the red-black pine bug *Haematoloma dorsatum* (Ahrens), widely distributed in Europe [41,42], *Ocoaxo* (Fennah) spittlebug species associated with pine forests in Mexico [43] and the Eurasian weevil beetle *Brachonyx pineti* (Paykull) [44]. DNA-based diagnostic methods are a more accurate alternative, especially because they allow for the species-specific detection of the pathogens even during the latent phase. However, the current available molecular diagnostic methods for the detection of *L. acicola, D. pini* and *D. septosporum* mostly rely on PCR [45–48] and qPCR [46], which are time consuming, and which require a well-equipped laboratory and molecular biology skills, thus being impractical for point-of-care implementation.

An alternative approach to PCR diagnostics would be the use of loop-mediated isothermal amplification (LAMP) [49]. This method allows one to amplify target DNA under constant temperatures, thus removing the need for expensive and bulky thermocyclers that can be replaced by user-friendly and field-suitable portable tools, without losing the benefits of molecular diagnostic methods. Moreover, LAMP is extremely rapid, being able to copy very large amounts of DNA in less than an hour [50], and it is more resistant to PCR inhibitors compared to standard PCR-based methods [51], thus allowing for the use of crude DNA extracts [52–54]. All these features make it an ideal solution for in-field point-of-care molecular diagnostics [55]. In addition, the technology has already proved successful for the detection of pathogens in forest systems [54,56–60]. The aim of this study was to develop three LAMP-based diagnostic assays for the rapid and early detection of the pine pathogens *L. acicola, D. pini* and *D. septosporum*.

2. Materials and Methods

2.1. Samples

Both axenic fungal cultures (Table 1) and naturally infected pine needle samples (Table 2) were used for optimizing each LAMP assay. The 58 fungal cultures used for this work included (i) 7 strains of *L. acicola* with different mating types, 17 strains of *Dothistroma pini*, and 8 strains of *D. septosporum*, (ii) species phylogenetically related to the former target species and iii) common colonizers of pine needles. Pine needle samples were collected from different symptomatic pine species (*Pinus mugo* Turra, *P. cembra* L., *P. halepensis* Mill., *P. brutia* Ten., *P. sylvestris* L., *P. nigra* J. F. Arnold, *P. nigra* subsp. *laricio* (Poir.) Maire, and *P. palustris* Mill.) in Italy [19], Slovenia [17] and Georgia (USA), and included both needles clearly displaying fungal fruiting bodies and needles showing only incipient symptoms of discolored banding (Table 2). Asymptomatic pine needles of *P. taeda* L. were collected in Athens (Georgia, USA), from disease-free areas and trees that had never before shown symptoms of BSNB or DNB, and used as a negative control.

Table 1. Fungal isolates used to test the specificity of the *Lecanosticta acicola, Dothistroma pini* and *D. septosporum* species-specific loop-mediated isothermal amplification (LAMP) assays.

Fungal Species	Isolate ID (Mating Type)	Host	Locality	Collector/ Collection	LAMP Detection Results		
					L. acicola Assay	*D. pini* Assay	*D. septosporum* Assay
D. septosporum [1]	DS 3212 (MAT2)	*P. sylvestris*	Võru County, Estonia	R. Drenkhan	-	-	+
D. septosporum [1]	Ds 57	*P. contorta*	Pärnu County, Estonia	R. Drenkhan	-	-	+
D. septosporum [2]	DSEP_KC_19_Ne1_TAIGA_504 (MAT2)	*P. contorta* var. *latifolia*	British Columbia, Canada	R. Hamelin	-	-	+
D. septosporum [2]	DSEP_CLG_22_TAIGA_601 (MAT1)	*P. contorta* var. *latifolia*	British Columbia, Canada	R. Hamelin	-	-	+

Table 1. *Cont.*

Fungal Species	Isolate ID (Mating Type)	Host	Locality	Collector/ Collection	LAMP Detection Results		
					L. acicola Assay	*D. pini* Assay	*D. septosporum* Assay
D. septosporum [2]	DSEP_PGTIS_P3_P16_Ne2_TAIGA_460 (MAT1)	*P. contorta* var. *latifolia*	British Columbia, Canada	R. Hamelin	-	-	+
D. septosporum [2]	DSEP_WC_27_Ne1_TAIGA_626 (MAT2)	*P. contorta* var. *latifolia*	British Columbia, Canada	R. Hamelin	-	-	+
D. septosporum [2]	DSEP_FLNRO2_19M_Ne1_TAIGA_486 (MAT1)	*P. contorta* var. *latifolia*	British Columbia, Canada	R. Hamelin	-	-	+
D. septosporum [2]	DSEP_SM_1_4_Ne1_TAIGA_484 (MAT2)	*P. contorta* var. *latifolia*	British Columbia, Canada	R. Hamelin	-	-	+
D. pini [2]	CMW 10951 CBS 116487	*P. radiata*	Michigan, USA	G. Adams	-	+	-
D. pini [2]	CMW 37634	*P. cembra*	North Dakota, USA	J. Walla	-	+	-
D. pini [2]	CMW 37786	*P. nigra*	Indiana, USA	J. Walla	-	+	-
D. pini [2]	CMW 38037	*P. ponderosa*	South Dakota, USA	J. Walla	-	+	-
D. pini [2]	CMW 42947	*P. nigra* subsp. *pallasiana*	Kherson, Ukraine	K. Davydenko	-	+	-
D. pini [2]	CMW 43903	*P. nigra* subsp. *laricio*	La Ferte Imbault, France	I. Barnes	-	+	-
D. pini [2]	CMW 29366	*P. pallasiana*	Tarasovsky, Russia	S.B. Timur	-	+	-
D. pini [2]	CMW 37633	*P. ponderosa*	North Dakota, USA	J. Walla	-	+	-
D. pini [2]	CMW 41496	*P. nigra*	France	I. Barnes	-	+	-
D. pini [2]	CMW 50237	*Pinus* sp.	Arkansas, USA	M.S. Mullett	-	+	-
D. pini [2]	A10	*P. nigra*	Ontario, Canada	S. McGowan	-	+	-
D. pini [2]	A11	*P. nigra*	Ontario, Canada	S. McGowan	-	+	-
D. pini [2]	A12	*P. nigra*	Ontario, Canada	S. McGowan	-	+	-
D. pini [2]	A13	*P. nigra*	Ontario, Canada	S. McGowan	-	+	-
D. pini [2]	A14	*P. nigra*	Ontario, Canada	S. McGowan	-	+	-
D. pini [2]	A20	*P. nigra*	Ontario, Canada	S. McGowan	-	+	-
D. pini [1]	E18/63-6	*Pinus* sp.	Slovenia	B. Piškur	-	+	-
L. acicola [2]	CV2019013	*P. palustris*	Georgia, USA	C. Villari	+	-	-
L. acicola [1]	8496 (MAT1)	*P. sylvestris*	Tartu County, Estonia	R. Drenkhan	+	-	-
L. acicola [1]	B1599 (MAT1)	*P. radiata*	France	R. Ioos	+	-	-
L. acicola [1]	B1569 (MAT11)	*P. radiata*	France	R. Ioos	+	-	-
L. acicola [3]	CMW 45427 CBS 133791	*P. strobus*	New Hampshire, USA	B. Ostrofsky	+	-	-
L. acicola [3]	CMW 45428 CBS 322.33	*P. palustris*	USA	P.V. Siggers	+	-	-
L. acicola [3]	MX7	*P. halepensis*	Nuevo León, Mexico	J.G. Marmolejo	+	-	-
L. brevispora [3]	CMW 45424 CBS 133601	*Pinus* sp.	Mexico	M. de Jesús Yáñez-Morales	-	-	-
L. brevispora [3]	CMW 46502	*P. pseudostrobus*	Chimaltenango, Guatemala	I. Barnes	-	-	-
L. gloeospora [3]	CMW 42645 IMI 283812	*P. pseudostrobus*	Nuevo León, Mexico	H.C. Evans	-	-	-
L. guatemalensis [3]	CMW 42206 IMI 281598	*P. oocarpa*	Guatemala	H.C. Evans	-	-	-
L. guatemalensis [3]	CMW 43892	*P. oocarpa*	Chiquimula, Guatemala	I. Barnes	-	-	-
L. jani [3]	CMW 38958 CBS 144456	*P. oocarpa*	Jalapa, Guatemala	I. Barnes	-	-	-
L. jani [3]	CMW 48831 CBS 144447	*P. oocarpa*	Alta Verapaz, Guatemala	I. Barnes	-	-	-

Table 1. *Cont.*

Fungal Species	Isolate ID (Mating Type)	Host	Locality	Collector/ Collection	LAMP Detection Results		
					L. acicola Assay	*D. pini* Assay	*D. septosporum* Assay
L. longispora [3]	CMW 45429 CBS 133602	*Pinus* sp.	Mexico	M. de Jesús Yáñez-Morales	-	-	-
L. longispora [3]	CMW 45430	*Pinus* sp.	Mexico	M. de Jesús Yáñez-Morales	-	-	-
L. pharomachri [3]	CMW 37134	*P. tecunumanii*	Baja Verapaz, Guatemala	I. Barnes	-	-	-
L. pharomachri [3]	CMW 37136 CBS 144448	*P. tecunumanii*	Baja Verapaz, Guatemala	I. Barnes	-	-	-
L. tecunumanii [3]	CMW 46805 CBS 144450	*P. tecunumanii*	Baja Verapaz, Guatemala	I. Barnes	-	-	-
L. tecunumanii [3]	CMW 49403 CBS 144451	*P. tecunumanii*	Baja Verapaz, Guatemala	I. Barnes	-	-	-
L. variabilis [3]	CMW 42205 CBS144453	*P. caribaea*	Santa Barbara, Honduras	H.C. Evans	-	-	-
L. variabilis [3]	MX1	*P. arizonica* var. *stormiae*	Nuevo León, Mexico	J.G. Marmolejo	-	-	-
Leptographium profanum [2]	CV20170072	*P. taeda*	Georgia, USA	C. Villari	-	-	-
Leptographium procerum [2]	CV2017311	*P. taeda*	Georgia, USA	C. Villari	-	-	-
Leptographium sp. [2]	CV20170049	*P. taeda*	Georgia, USA	C. Villari	-	-	-
Rhizosphaera sp. [2]	CV2018024	*P. taeda*	Georgia, USA	C. Villari	-	-	-
Cladosporium sp. [2]	CV2018023	*P. taeda*	Georgia, USA	C. Villari	-	-	-
Alternaria tenuissima [2]	CV2018022	*P. taeda*	Georgia, USA	C. Villari	-	-	-
Dothideomycetes sp. [2]	CV2018020	*P. taeda*	Georgia, USA	C. Villari	-	-	-
Leotiomycetes sp. [2]	CV2018019	*P. taeda*	Georgia, USA	C. Villari	-	-	-
Nigrospora oryzae [2]	CV2018018	*P. taeda*	Georgia, USA	C. Villari	-	-	-
Lophodermium conigeum [2]	CV2018002	*P. taeda*	Georgia, USA	C. Villari	-	-	-
Lophodermium australe [2]	CV2018001	*P. taeda*	Georgia, USA	C. Villari	-	-	-

[1] Reactions performed at the Department of Agricultural, Food, Environmental and Forest Sciences and Technologies (DAGRI), University of Florence (Italy). [2] Reactions performed at the University of Georgia, Athens (United States). [3] Reactions performed at the Forestry and Agricultural Biotechnology (FABI), University of Pretoria (South Africa). CBS = Culture collection of the Westerdijk Fungal Biodiversity Institute, Centraalbureau voor Schimmelcultures, Royal Netherlands Academy of Arts and Sciences (KNAW), Utrecht, The Netherlands. CMW = Culture collection of FABI (University of Pretoria, South Africa). CV = Culture collection of Villari Lab, Warnell School of Forestry & Natural Resources, University of Georgia, Athens, Georgia, United States. IMI = The UK National Fungus collection, CABI Bioscience, Egham, UK.

Table 2. Description of the pine needles samples tested with the three LAMP assays for the detection of *Lecanosticta acicola*, *Dothistroma pini* and *D. septosporum*, respectively, as well as with the qPCR assay described by [46]. LAMP results are shown as time of amplification (min) while qPCR results are shown as time of amplification (min) and cycle threshold (Ct). Negative result (-). Needles were classified as either fully symptomatic if they were discolored and bearing fruiting bodies (++), with incipient symptoms if they were only displaying discolored banding (+), or asymptomatic if they were displaying none of the above (N).

Plant Species	Locality	Symptoms on Needles	LAMP Results (min) [Total Reaction Time 35 min]			qPCR Results (min/Ct) [Total Reaction Time 1 h 30 min]		
			L. acicola	*D. pini*	*D. septosporum*	*L. acicola*	*D. pini*	*D. septosporum*
Pinus cembra	Val Sarentino, Bolzano, Italy	+	-	-	15	-	-	64/26.95
P. cembra	Val Sarentino, Bolzano, Italy	+	-	-	20	-	-	67/28.53
P. cembra	Val Sarentino, Bolzano, Italy	+	-	-	-	-	-	-
P. mugo	Val Sarentino, Bolzano, Italy	+	-	-	-	-	-	-
P. mugo	Auronzo di Cadore, Belluno, Italy	++	-	-	20	-	-	75/32.73
P. mugo	Paluzza, Udine, Italy	++	20	-	-	71/30.58	-	-
P. mugo	Gardone, Brescia, Italy	+	-	-	-	-	-	-
P. nigra var. laricio	La Sila, Cosenza, Italy	+	-	-	15	-	-	73/31.66
P. nigra var. laricio	La Sila, Cosenza, Italy	++	-	-	14	-	-	63/26.74
P. palustris	Newton, Georgia, USA	+	20	-	-	69/29.61	-	-
P. palustris	Newton, Georgia, USA	++	20	-	-	72/30.88	-	-
P. palustris	Newton, Georgia, USA	+	20	-	-	75/32.76	-	-
P. radiata	La Sila, Cosenza, Italy	+	-	-	12	-	-	67/28.71
P. taeda	Athens, Georgia, USA	N	-	-	-	-	-	-
Pinus sp.	Slovenia	+	-	20	-	-	71/30.45	-
Pinus sp.	Slovenia	+	-	20	-	-	72/31.33	-
Pinus sp.	Slovenia	++	-	15	-	-	62/25.90	-
Pinus sp.	Slovenia	+	-	20	-	-	70/29.90	-
Pinus sp.	Slovenia	++	-	15	-	-	65/27.54	-

2.2. DNA Extraction

All fungal cultures (Table 1) were grown on sterile cellophane in 90 mm Petri dishes containing 1.5% MEA (malt extract agar) and maintained in the dark at 17–22 °C according to species requirements [61]. After 7–15 days, approximately 80 mg (fresh weight) of mycelium from each species was obtained by scraping off the mycelia from the cellophane surface. Total DNA was extracted using the E.Z.N.A.® Fungal DNA mini Kit (Omega, Bio-tek, Norcross, GA, USA), following the manufacturer's directions and concentrations measured using a Qubit™ Fluorometer (Invitrogen™, Carlsbad, CA, USA). For the extraction of DNA directly from pine needles, 2–3 needles per sample were cut in 5-mm-long pieces in which were included both symptomatic (yellow/red/brown bands and/or fruiting bodies depending on the sample) and asymptomatic (green) parts of the needle. These

were then ground into a fine powder using liquid nitrogen and a sterilized mortar and pestle, and 50 mg (fresh weight) was used for extraction using the DNAeasy® PowerPlant® Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.3. LAMP Primers and Probes Design

All available sequences of the elongation factor (EF1- α) and beta-tubulin (β-tub2) genes belonging to *L. acicola*, *D. pini* and *D. septosporum* described in [46,62,63] were retrieved from GenBank (NCBI) and compared with species reported as being phylogenetically closely related, using the multiple alignment server T-COFFEE (online access https://tcoffee.vital-it.ch/apps/tcoffee/index.html accessed on 8 March 2021) with default parameters. Sequence regions containing the highest genetic variability between target species and phylogenetically related fungi, but in which single nucleotide polymorphisms (SNPs) among individuals of the same target species were not present, were selected for primer design. Sets of six LAMP primers were designed for each pathogen using Primer Explorer (V.4, Eiken Chemicals, Tokyo, Japan, http://primerexplorer.jp/e/ accessed on 8 March 2021), following the specifications of [49,51]. The primer sets each consisted of the four primers necessary for amplification, and loop primers to enhance reaction rate and specificity. Primers were designed to target the beta-tubulin (β-tub2) gene of *D. septosporum* (GenBank Acc. No. FJ467298) and *D. pini* (GenBank Acc. No. FJ467304), and the elongation-factor (EF-1α) gene of *L. acicola* (GenBank Acc. No. KJ938441). Of the multiple primer sets generated by the software, those displaying strong mismatches at the 3′ end [64] between the targets and genetically related species were selected. The most specific of each loop primer was selected for each LAMP assay and used to design a sequence-specific assimilating probe for each target species following [65]. FAM (6-carboxyfluorescein) fluorescent strands of each assimilating probe were designed against the backward loop primer for *D. pini*, and against the forward loop primers for *D. septosporum* and *L. acicola*. Quencher strands were retrieved from [65]. All primers and probes were synthesized by either Eurofins Genomics (GmbH, Ebersberg, Germany) or Integrated DNA Technologies (IDT, Coralville, IA, USA) and are reported in Table 3.

Table 3. Primers and probes used for the detection of *Lecanosticta acicola*, *Dothistroma pini* and *D. septosporum* using LAMP assays.

Primers	Sequence 5′->3′
LAMP primers—*Lecanosticta acicola*	
La_F3	GTACGCATGGGTCCTCGA
La_B3	GAAATCACGGTGACCAGGAG
LA_FIP	CGTACAGTTACGTAATATGAGCGTGAGCGTGGTATC
LA_BIP	GGACTCTTCGCTGCCGCCCGATGACCTTTCACGGGTTA
LA_LoopB	TCGCTGTCGCAACACCC
LAMP primers—*Dothistroma pini*	
Dp_F3	GTTGGGATGTATGTGGTGTTA
Dp_B3	CTCCATCGACATCTCCAAGA
Dp_FIP	GAAGTAAACATTCAACCGCTCGCACTCGTGAAGAAAGCTTGTG
Dp_BIP	CGAGGTACGGACTTCACTTCACAGTAAAGTGATGCTGTGCTG
Dp_LoopF	CCTCGTATCTGCGAGTCTTC
LAMP primers—*Dothistroma septosporum*	

Table 3. *Cont.*

Primers	Sequence 5′->3′
Ds_F3	TTTCTGGCAGACCATTTCTG
Ds_B3	ACGGCTCTTTCAAATGACTT
Ds_FIP	GTGCCTTCGTATCTGCATTTCATCCAGGACAGTATGTGGAATCC
Ds_BIP	CGAGAGCGACTGAGTGTCTATTTCGCATAGTGTTGAAGCACTGG
Ds_LoopB	GATGAGGTAGGTGCTCCTCT
Assimilating sequence-specific probes	
LA_LFPr [1]—*Lecanosticta acicola*	FAM [2]-ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGAGGCGTTTCAAACTTCCACAGAG
DP_LBPr [1]—*Dothistroma pini*	FAM [2]-ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGATTCCAGTGTGCTATGGCAAT
DS_LFPr [1]—*Dothistroma septosporum*	FAM [2]-ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGAAGTACGAATCTGCATGACGC
Quencher strand [3]	TCGGCATCCGCATCCGCATTCGCATCCGGGTCCTCAGCGT-BHQ [4]

[1] The underlined fragment acts as a loop primer; [2] FAM = 6-carboxyfluorescein; [3] Quencher strand was designed as reported in [65]; [4] BHQ = Black HoleQuencher-1 (Biosearch Technologies, Novato, CA, USA).

2.4. LAMP Reactions

LAMP reactions were performed and optimized in three different laboratories at the University of Florence (Italy), the University of Georgia (United States) and FABI at the University of Pretoria (South Africa) on a Bio-Rad® iCycler Real-time system (BioRad, Hercules, CA, USA), a StepOnePlus™ Real-Time PCR System (Applied Biosystems™, Foster City, CA, USA) and a Bio-Rad® CFX 96 Real-Time System (BioRad), respectively. DNA samples were amplified for 35 min in MicroAmp® Fast Reaction Tubes (Applied Biosystems™) strips at 65 °C, measuring fluorescence values in real-time every 30 s. Each reaction was terminated with a denaturing step at 85 °C for 5 min. Except where otherwise stated, each isothermal amplification was performed in duplicate in a final volume of 25 µL. The reaction mixture contained 15 µL Isothermal Master Mix (ISO-001nd) (OptiGene Limited, Horsham, UK), 3.05 µL LAMP primer mixture (at final concentrations of 0.28 µM of each F3 and B3, 0.8 µM of Loop primer without probe and 2.8 µM of each FIP and BIP), 0.6 µL of probe mixture (at final concentrations of 0.08 µM for each fluorescent strand and 0.12 µM for the quencher strand), 1.35 µL water (molecular biology grade, Fisher BioReagents™, Pittsburgh, PA, USA) and 5 µL of template DNA. For each run, two no-template controls (NTC), in which 5 µL of water were used instead of DNA, were included. The limit of detection of each LAMP assay was determined by testing in triplicate an 11-fold 1:5 serial dilution of target DNA template (ranging from 10 ng μL^{-1} to 0.001 pg μL^{-1}) for each target species (isolates CV2019013-*L. acicola*, CMW 29366-*D. pini*, WC27 Needle 1 Taiga 626-*D. septosporum*). The same points of DNA dilution retrieved from the same tubes used for LAMP sensitivity tests were also processed for comparison with a qPCR protocol specific for the same three target species [46] as a gold standard. qPCR reactions were performed at a final volume of 20 µL. The reaction mixture contained 1x DreamTaq Green Buffer (Thermo Scientific, Waltham, MA, USA), 2 × 0.2 mM each dNTP (Thermo Scientific), 2.5 µM each of the two respective forward and reverse primers, 0.2 µM of the respective dual-labeled probe, 1 U DreamTaq polymerase (Thermo Scientific), 1 µL of template DNA and water to reach the final volume. Each run included two NTCs and a positive control using the DNA of the target species. The real-time PCR cycling conditions included an initial denaturation step at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and elongation at 60 °C for 55 s. The specificity of each LAMP assay was tested against the genomic DNA extracted from all the fungal strains reported in Table 1, each at a final concentration of 2 ng μL^{-1}.

2.5. Detection on Naturally Infected Pine Needle Samples

In order to assess the performance of each LAMP assay on naturally infected plant tissue, DNA samples extracted from symptomatic needles (Table 2) were tested with all three primer sets (Table 3). The DNA samples were also processed for comparison with the gold-standard qPCR assay [46] described above, following the same protocol and conditions.

3. Results

3.1. LAMP Specificity and Sensitivity

Each LAMP assay demonstrated consistent amplification for all fungal isolates of its corresponding target species, regardless of their geographic origin (Table 1). No amplifications were observed for the non-target species tested by any of the three LAMP primer sets (Table 1). Positive reactions were visible starting at approximately 10 min for each target species (Figure 1; Table 4). Testing serially diluted DNA extracts, the detection limits of each LAMP assay were determined to be 0.128 pg μL^{-1} for *L. acicola*, 3.2 pg μL^{-1} for *D. septosporum*, and 0.64 pg μL^{-1} for *D. pini* (Table 4). The detection limit of the qPCR using the protocol of [46] was 0.128 pg μL^{-1} for all of the three species, taking an hour and 30 min to complete the analysis (Table 4).

Table 4. Comparison of the sensitivity of the LAMP assays developed for the detection of *Lecanosticta acicola*, *Dothistroma pini* and *D. septosporum*, respectively, and the qPCR assay described by [46]. Tested DNA was obtained from axenic cultures of each target species. LAMP results are shown as time of amplification (min) while qPCR results are shown as time of amplification (min) and cycle threshold (Ct).

Target DNA Concentration (pg μL^{-1})	LAMP Results (min) [Total Reaction Time 35 min]			qPCR Results (min/Ct) [Total Reaction Time 1 h 30 min]		
	L. acicola	*D. pini*	*D. septosporum*	*L. acicola*	*D. pini*	*D. septosporum*
10,000	10	10	10	40/15.10	37/13.51	42/16.23
2000	11	12	11	45/17.48	40/15.15	47/18.54
400	12	14	13	54/22.23	47/18.50	53/21.36
80	13	16	14	59/24.30	51/20.55	56/23.22
16	15	18	18	62/25.94	56/23.20	61/25.61
3.2	16	20	21	68/28.97	61/25.37	71/30.47
0.64	18	22	-	72/31.21	68/29.22	74/32.15
0.128	22	-	-	77/33.78	74/32.16	80/35.00
0.02	-	-	-	-	-	-

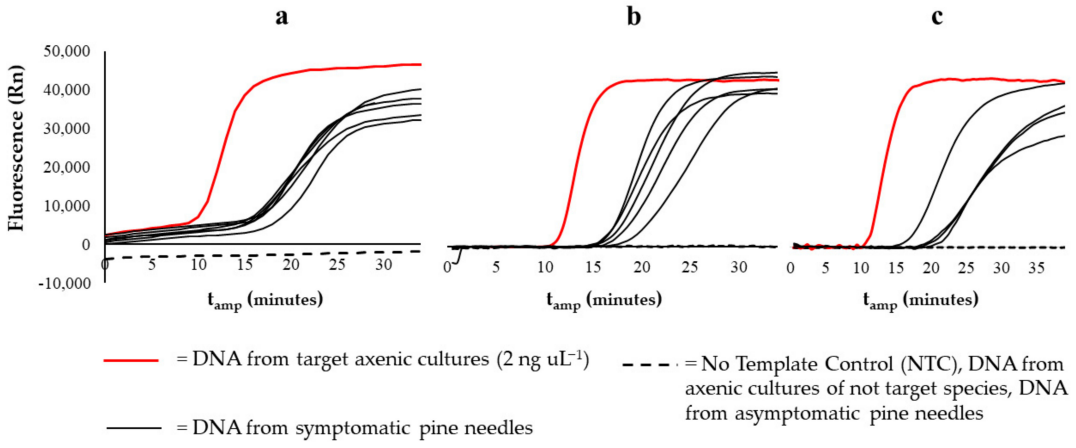

Figure 1. Selection of kinetics showing amplification results of the LAMP assays targeting *Lecanosticta acicola* (**a**), *Dothistroma pini* (**b**) and *D. septosporum* (**c**), respectively. Red solid lines represent DNA (2 ng uL^{-1}) extracted from axenic cultures of the corresponding target species. Black solid lines represent DNA extracted from pine needles showing symptoms of the corresponding target species. Black dotted lines represent both DNA extracted from non-symptomatic pine needles and no-template controls.

3.2. Detection on Naturally Infected Pine Needles Samples

Positive amplifications were obtained from both pine needles samples bearing fungal conidiomata and from the ones showing only incipient symptoms (Table 2). All results obtained with the LAMP assays were consistent with those of the qPCR assays developed by [46]. No amplification was observed when testing the DNA of asymptomatic pine needles (Figure 1; Table 2).

4. Discussion

For notifiable pathogens, molecular diagnostic methods that enhance the rapid, accurate identification and interception of infected specimens are crucial to preventing their introduction and spread, especially in the case of morphologically similar species such as *L. acicola, D. pini* and *D. septosporum*. The LAMP-based assays developed in this work were capable of rapidly identifying these three pathogens in less than 30 min (Figure 1); a substantial improvement compared to the currently available DNA-based diagnostics for these species [45–48]. These assays also have the potential to be deployable in-field, directly at point-of-care with the use of portable devices developed for supporting LAMP reactions (e.g., Genie® II and III by OptiGene Limited, Horsham, UK) [55,56,59]. To the best of our knowledge, there are no published LAMP-based assays targeting *L. acicola* and *D. pini*, while for *D. septosporum* a LAMP-based assay has been recently published [60].

The LAMP assays demonstrated high specificity, with each test amplifying only the DNA of its respective target species (Table 1). This result was in part aided by the use of loop primers to enable probe-based detection [65], which further reduces the possibility of nonspecific binding. Positive amplification was observed for all tested target strains of each target species, belonging to different mating types and from different geographic origins, showing that the protocol is robust and that geographic variability in target fungi does not affect LAMP primer binding amplification. This indicates that each assay can be implemented across the world without the risk of losing specificity. However, it is noteworthy that the *Dothistroma* and *Lecanosticta* species are known to reproduce both asexually and sexually [32,66], reflecting the possibility of genetic recombination and thus the emergence of new haplotypes among their populations [33,34,36]. The emergence of new haplotypes could, in future, interfere with the function of the LAMP assay. We hence recommend that the specificity of the assays is re-confirmed intermittently, as it is good practice for every molecular diagnostic assay targeting sexually reproducing organisms. In addition, LAMP specificity should also be tested when new strains on new hosts and in new areas are discovered.

The detection limit of the assays was found to be 0.128 pg μL^{-1} for *L. acicola*, 0.64 pg μL^{-1} for *D. pini* and 3.2 pg μL^{-1} for *D. septosporum*. The detection limit for *L. acicola* is comparable to that of the qPCR assay developed by [46], which is adopted by the EPPO as the official tool for the diagnosis for these fungal species [1]. However, for both *Dothistroma* species, the qPCR method showed higher sensitivity than the LAMP assays, which nevertheless were sensitive enough to detect the target fungal species directly from host tissues (Table 2; Figure 1), including those from samples showing only incipient symptoms. With regard to the tests using needle samples, all results of the LAMP assays were consistent with those obtained with the qPCR method [46], further demonstrating the high specificity and efficiency of the developed assays. It is also worth noting that the LAMP assays developed in this study have been validated on different equipment in three different laboratories across three different continents, ensuring repeatability of the assays, which is a coveted attribute for molecular diagnostic approaches.

Future work should focus on the possibility for the LAMP assays to confirm the presence of *L. acicola*, *D. pini* and *D. septosporum* even before the development of any symptoms, hence testing the efficacy of the assays to detect latent infections. Efforts should also be directed toward validating the developed assays for use at point-of-care on portable devices and using crude DNA extracts, as this has already been successfully performed in numerous pathosystems, including forest pests and pathogen species such as *Heterobasidion*

irregulare Garbel and Otrosina, *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz and Hosoya, *Phytophthora* spp, *Raffaelea lauricola* Harrington, Fraedrich and Aghayeva, *Xylella fastidiosa* Wells, Raju, Hung, Weisburg, Parl and Beemer, *Ceratocystis* spp., and *Fusarium* spp. [54,56–59].

5. Conclusions

In many parts of the world, *Dothistroma* and *Lecanosticta* needle blights are spreading in pine plantations and natural forests over larger areas, showing a general increase in the severity of symptoms and causing increasing damage to local economies, ecosystem functionality and landscapes. In order to avoid further spread of the pathogen to disease-free areas, or the introduction of a second species or new, more virulent genotypes in areas already infected by one of these pathogens, it is critical to implement strict and efficient surveillance measures. It is well-established that the main and riskiest route of medium- to long-distance spread of these fungi is commercial trade and the movement of infected plant material [67], a pathway that is crucial to inspect with maximum efficiency [29,67]. Easy-to-use specific and sensitive diagnostic methods that provide rapid results and that can be used on small portable instruments directly at the points of entry and in the field, such as the LAMP assays developed in this work, which also require minimal training [53,68], would make phytosanitary controls of live plants or plant parts easier and more effective. This would allow for immediate management decisions to be made, and the necessary measures to contain risk and prevent further damage to be applied more quickly and efficiently.

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