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ORIGINAL ARTICLE

Detection of Esca-associated fungi in grapevine trunks using loop-mediated isothermal amplification (LAMP) assays

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Abstract

Esca is a grapevine trunk disease (GTD) that is caused by filamentous fungi. It is responsible for considerable economic losses in viniculture on a global scale. Despite many unknown factors contributing to the development of symptoms in affected plants, *Phaeoacremonium minimum* (PMI), *Phaeomoniella chlamydospora* (PCH) and *Fomitiporia mediterranea* (FMED) are generally considered as the main causative fungal species. Early detection and specific identification of these pathogens therefore play an important role in disease control and evaluation of suitable countermeasures. In this study, loop-mediated isothermal amplification (LAMP) assays were developed for each of the three pathogens. A genome-based approach was applied for detection and selection of unique target DNA sequences. The designed primer sets showed overall good specificities, with some observed cross-reactions towards closely related *Phaeoacremonium* species for the PMI primer set. The developed assays had detection limits of 100 pg (FMED, PMI) and 1 pg (PCH) per reaction (corresponding to 1460 [FMED]; 1950 [PMI]; 342 [PCH] genome copies per reaction). The application of the assays to field samples was demonstrated by testing individual infected grapevine trunks from two European viticultural regions using crude DNA obtained in a rapid sample preparation step. LAMP assay results matched those of PCR following a conventional DNA extraction protocol. The study showed that LAMP-based rapid molecular detection of major Esca agents can serve as a useful tool for further research and surveillance of a highly devastating grapevine disease. The application of computer-based whole genome comparison between target and non-target species for the identification of unique target sequences as the basis for LAMP (or PCR) primer design was demonstrated to be a useful approach in species for which scarce sequence information is available. Moreover, the developed method for rapid DNA preparation from grapevine trunks may potentially be adapted to the DNA-based detection also of other fungal species that cause grapevine trunk diseases.

KEYWORDS

Esca, fungi, grapevine trunk diseases, loop-mediated isothermal amplification, pathogen detection

1 | INTRODUCTION

Grapevine trunk diseases (GTDs) caused by fungal pathogens are responsible for significant harvest decrease and economic loss in viticultural areas around the globe (Cortesi et al., 2000; Fontaine et al., 2016; Kenfaoui et al., 2022; Ouadi et al., 2019) with Esca disease representing one of the most devastating, along with *Botryosphaeria* and *Eutypa* dieback. Esca can be considered a disease-complex involving fungal species of several genera, with ascomycetes like *Phaeomoniella chlamydospora* (PCH) (Crouse & Gams, 2000) and *Phaeoacremonium* minimum (PMI) (Crous et al., 1996), formerly *Phaeoacremonium aleophilum*, as well as the basidiomycete *Fomitiporia mediterranea* (FMED) (Fischer, 2002) being regarded some of the most important causal agents (Fischer & Kassemeyer, 2003). Further species associated with this disease complex include closely related members of the genera *Phaeoacremonium* and *Fomitiporia*, and occasionally other fungal pathogens with probably lower relevance (Bertsch et al., 2013; Larignon & Dubos, 1997; Mondello et al., 2019). So far, a variety of symptoms have been associated with the term Esca. The symptoms can occur independently from each other and at different developmental stages of the grapevine plant (Surico, 2001, 2009). Overall, internal symptoms with degradation of trunk and branch wood typically appear as darkened areas (brown wood streaking), or as white rot, mainly at later stages (Mugnai et al., 1999). External symptoms include decline in growth and vigour, wilting or leaf spotting and chlorosis developing into a characteristic 'tiger-stripe' pattern (Mostert et al., 2006; Mugnai et al., 1999). However, the mere presence of one or several of the mentioned fungi does not imply an immediate development of Esca symptoms. Reports have shown that fungal communities in healthy plant tissues did not differ significantly from those in infected ones (Hofstetter et al., 2012). On the other hand, grapevine plants inhabited by the pathogens can endure years without showing any symptoms (Bertsch et al., 2013; Whiting et al., 2001). Apparently, several biotic and abiotic factors play a significant role in pathogenesis (Fischer & Ashnaei, 2019; Mundy & Manning, 2011).

To date, the importance of PCH, PMI and FMED for Esca-related grapevine decay is widely recognized (Bruno & Sparapano, 2007; Surico, 2001). Identification of these pathogens therefore plays an important role in disease control and understanding of its dissemination mechanisms. Since grafting is a common and necessary practice in viticulture, transmission of the fungi via infected scions or rootstock material has been reported as a potential factor for propagation of GTDs (Aroca et al., 2010; Carbone et al., 2022; Gramaje et al., 2018; Gramaje & Armengol, 2011; Retief et al., 2006; Waite et al., 2015). Therefore, methods for the screening of the grafting material may contribute to avoid transmission of the main Esca-associated species. Moreover, such screening methods can help to gain further knowledge of the distribution of Esca-related fungi in viticultural systems. In the past, different advanced techniques have been described for the detection of fungi related to Esca or other GTDs, including immunochemical assays (Cardoso et al., 2014; Fleurat-Lessard et al., 2010, 2014) and molecular biological techniques that were based on the polymerase chain reaction (PCR) (Arzanlou & Narmani, 2014; Billones-Baaijens et al., 2018; Fischer, 2006; Groenewald et al., 2000).

As an alternative to PCR, loop-mediated isothermal amplification (LAMP) has increasingly gained importance in many fields, especially when it comes to direct testing of clinical or environmental samples for virus and microorganisms (Fu et al., 2011). LAMP is a sequence-specific molecular detection method developed by Notomi et al. (2000), which offers several advantages due to the high speed, robustness and specificity of the involved reaction mechanism. Moreover, low requirements regarding equipment make it a suitable technique for in-field applications. Especially in the field of phytopathology, a major advantage is that the LAMP reaction is less prone to inhibitors than PCR and does not depend on time-consuming extraction protocols to yield high quality DNA. Therefore, apart from clinical applications, LAMP assays have been developed for the detection of microorganisms that are relevant for food and agriculture, including a variety of filamentous fungi and yeasts (Niessen, 2015). In the context of grapevine diseases, published LAMP assays include virus (Almasi, 2018; Romero Romero et al., 2019), bacteria (Kogovšek et al., 2015, 2017) and oomycetes (Kong et al., 2016).

Due to the importance of Esca for viticulture, the aim of the current study was to develop three separate LAMP assays to detect each of the mainly associated species PCH, PMI and FMED with high specificity and to provide a proof-of-principle for their practical application in environmental samples. For this purpose, their performance was in a first step assessed using pure fungal DNA, and secondly the developed assays were validated to detect the presence of the respective fungi in a woody matrix from contaminated grapevine trunks using a rapid protocol for crude DNA preparation.

This work aims to spotlight the potential of LAMP technology as a useful tool for disease management in viticulture, with intended use on grafting material and to aid early detection of the fungi in nurseries.

2 | MATERIALS AND METHODS

2.1 | General procedure

For each of the three fungal species, a separate LAMP primer set was designed and tested for sensitivity and specificity with DNA that was extracted from pure cultures of the respective target species as well as from a variety of other fungal species. Performance of the optimized LAMP-assays in naturally contaminated sample materials was subsequently tested with crude DNA prepared from 10 grapevine trunks from plants with visible Esca-related symptoms. For validation, LAMP results obtained with crude DNA were compared to the results of a species-specific PCR that was run with highly purified DNA from corresponding grapevine trunk samples.

2.2 | Culture conditions and DNA preparation

All fungal strains that were used in this study are listed in Table 1 along with their source collections. Most GTD-associated fungal

TABLE 1 LAMP results for different fungal strains tested with the three primer sets.

Species	Strain	Clone	LAMP result		
			FMED	PCH	PMI
Alternaria tenuissima	DLR 8785	TMW 4.3028	–	–	–
Aspergillus aculeatus	TMW 4.2390	TMW 4.2390	–	–	–
Aspergillus awamori	CBS 101704	TMW 4.1066	–	–	–
Aspergillus oryzae	IBT 28103	TMW 4.2208	–	–	–
Aureobasidium pullulans	TMW 4.2253	TMW 4.2253	–	–	–
Botryosphaeria obtusa	DLR Bot10	TMW 4.3018	–	–	–
Botryosphaeria obtusa	DLR Bot39	TMW 4.3020	–	–	–
Botrytis cinerea	DLR 10465	TMW 4.3026	–	–	–
Chaetomium aureum	DSM 63132	TMW 4.2981	–	–	–
Chaetomium globosum	SZMC 24508	TMW 4.2955	–	–	–
Cladosporium cladosporioides	DLR 10496	TMW 4.3029	–	–	n.t.
Colletotrichum acutatum	CBS 295.67	TMW 4.0652	–	–	–
Emericella astellata	IBT 21903	TMW 4.2202	–	–	–
Epicoccum nigrum	TMW 4.1407	TMW 4.1407	–	–	–
Fomitiporia mediterranea	F16/H1/M	TMW 4.3021	+	n.t.	–
Fomitiporia mediterranea	ITA 235.03	TMW 4.2933	+	–	–
Fomitiporia mediterranea	ITA 221.03	TMW 4.2934	+	n.t.	n.t.
Fomitiporia mediterranea	DLR F20 S2/11	TMW 4.3053	+	–	–
Fomitiporia mediterranea	DLR W1 S1/11	TMW 4.3054	+	–	–
Fomitiporia mediterranea	DLR R2.3 SL	TMW 4.3055	+	–	–
Fomitiporia mediterranea	DLR R1.1 VM	TMW 4.3056	+	–	–
Fomitiporia mediterranea	DLR R8.6 VM	TMW 4.3057	+	–	–
Fomitiporia mediterranea	DLR 49/S1/11	TMW 4.3058	+	–	–
Fomitiporia mediterranea	DLR R11.1 VB2	TMW 4.3059	+	–	–
Fomitiporia mediterranea	DLR F36 H14	TMW 4.3060	+	–	–
Fomitiporia mediterranea	DLR W45 S2/B	TMW 4.3061	+	–	–
Fomitiporia mediterranea	DLR 8195	TMW 4.3062	+	–	–
Fomitiporia punctata	CBS 100121	TMW 4.3039	(+)	–	–
Fomitiporia punctata	CBS 301.33	TMW 4.3040	(+)	–	–
Fusarium acuminatum	CBS 485.94	TMW 4.0701	–	–	–
Fusarium buharicum	CBS 769.70	TMW 4.0627	–	–	–
Inonotus hispidus	ITA 236.05	TMW 4.2935	–	n.t.	n.t.
Myrothecium roridum	CBS 212.92	TMW 4.0915	–	–	–
Paecilomyces dactylethromophus	TMW 4.2614	TMW 4.2614	–	–	–
Penicillium brevicompactum	MUM 14.27i	TMW 4.2545	–	–	–
Penicillium burgense	MUM 14.44	TMW 4.2541	–	–	–
Penicillium chrysogenum	TMW 4.2616	TMW 4.2616	–	–	–
Penicillium citrinum	MUM 14.29	TMW 4.2547	–	–	–
Penicillium digitatum	DSM 62840	TMW 4.1083	–	–	–
Phaeoacremonium alvesii	CBS 408.78	TMW 4.3079	–	–	–
Phaeoacremonium fraxinopennsylvanicum	CBS 211.97	TMW 4.3083	–	–	–
Phaeoacremonium inflatipes	CBS 391.71	TMW 4.3077	–	–	(+)
Phaeoacremonium iranianum	CBS 100400	TMW 4.3084	–	–	(+)
Phaeoacremonium krajdenii	CBS 423.73	TMW 4.3078	–	(+)	–
Phaeoacremonium minimum	CBS 110704	TMW 4.3043	–	–	+

TABLE 1 (Continued)

Species	Strain	Clone	LAMP result		
			FMED	PCH	PMI
Phaeoacremonium minimum	CBS 631.94	TMW 4.3047	–	–	+
Phaeoacremonium minimum	DLR 9890	TMW 4.3036	–	–	+
Phaeoacremonium minimum	DLR 15709.11	TMW 43063	–	–	+
Phaeoacremonium minimum	CBS 631.94	TMW 43064	–	–	+
Phaeoacremonium minimum	DLR 11760	TMW 43065	–	–	+
Phaeoacremonium minimum	DLR 16	TMW 43066	–	–	+
Phaeoacremonium minimum	DLR 9890	TMW 43068	–	–	+
Phaeoacremonium minimum	DLR 9880	TMW 43069	–	–	+
Phaeoacremonium minimum	CBS 246.91	TMW 4.3046	–	–	+
Phaeoacremonium minimum	CBS 100398	TMW 4.3048	–	–	+
Phaeoacremonium minimum	CBS 101006	TMW 4.3049	–	–	+
Phaeoacremonium novae-zealandiae	CBS 110156	TMW 4.3085	–	–	–
Phaeoacremonium scolyti	CBS 112585	TMW 4.3086	–	–	–
Phaeoacremonium sphinctrophorum	CBS 337.90	TMW 4.3081	–	–	–
Phaeoacremonium sphinctrophorum	CBS 694.88	TMW 4.3080	–	–	–
Phaeoacremonium viticola	CBS 428.95		–	–	(+)
Phaeomoniella chlamydospora	DLR 9886	TMW 4.3031	–	+	–
Phaeomoniella chlamydospora	CBS 161.90	TMW 4.3045	–	+	–
Phaeomoniella chlamydospora	CBS 239.74	TMW 4.3044	–	+	–
Phaeomoniella chlamydospora	CBS 101571	TMW 4.3050	–	+	–
Phaeomoniella chlamydospora	CBS 116431	TMW 4.3051	–	+	–
Phaeomoniella chlamydospora	CBS 117179	TMW 4.3052	–	+	–
Phaeomoniella chlamydospora	DLR 8819	TMW 4.3033	n.t.	+	–
Phaeomoniella chlamydospora	DLR 6	TMW 43070	–	+	–
Phaeomoniella chlamydospora	DLR 9883	TMW 43071	–	+	–
Phaeomoniella chlamydospora	DLR 8830	TMW 43072	–	+	–
Phaeomoniella chlamydospora	DLR 8838	TMW 43073	–	+	–
Phaeomoniella chlamydospora	DLR 8831	TMW 43074	–	+	–
Phellinus igniarius	CBS 240.61	TMW 4.3041	–	–	–
Phellinus igniarius	CBS 349.74	TMW 4.3042	–	–	–
Trichoderma koningiopsis	DLR Tr30	TMW 4.3024	–	–	–
Trichothecium roseum	CBS 567.50	TMW 4.0691	–	–	–

Note: Target species are shown in bold. False positive results are shown in brackets.
Abbreviation: n.t, not tested.
DLR, State Education and Research Center of Viticulture, Horticulture and Rural Development (DLR Rheinpfalz), Institute for Plantprotection, Neustadt a. d. Weinstraße, DE.
TMW, Chair of Microbiology, Technical University of Munich, Freising, DE.
CBS, Westerdijk Fungal Biodiversity Institute Utrecht, NL.
IBT, Institut for Bioteknologi, Technical University of Denmark, Lyngby, DK.
DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Darmstadt, DE.
SZMC, Department of Microbiology, University of Szeged, Szeged, HU.
ITA, Strain collection of Laura Mugnai, University of Florence, Italy.
MUM, Micoteca da Universidade do Minho, PT.

strains derive from the Institute for Plant Protection, State Education and Research Center of Viticulture, Horticulture and Rural Development (DLR Rheinpfalz) and had previously been isolated from grapevine in Rhineland-Palatinate, Germany. Fungal genomic DNA from cultures was extracted according to Cenis (1992) with small modifications (all centrifugation steps were conducted at 13,000 x *g*

at ambient temperature): fungi were grown at ambient temperature (22°C ± 1°C) for 72 h in Erlenmeyer flasks containing 50 mL malt extract medium (20 g/L malt extract, 2 g/L peptone ex soya, pH 5.6) while horizontally shaking at 180 rpm. After growth was visible, fungal cultures were centrifuged for 2 min in sterile 50 mL Falcon tubes and the medium was decanted. Lumps of mycelium (approximately 200 mg) were then transferred into 1.5 mL tubes and washed by adding 500 µL of distilled water before brief vortexing and subsequent centrifugation for 2 min. The supernatant was discarded. In order to disintegrate mycelia, 300 µL extraction buffer (200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and one small sampling spoon (approximately 0.3 g) of sterile glass beads (1.25–1.65 mm diameter) and sea sand, respectively were added. Samples were processed for 45 s in a Fastprep® at a speed of 5 m/s (MP Biomedicals, Irvine, CA, USA). Subsequently, 150 µL of 3 M sodium acetate (pH 5.2) was added to the sample tubes before storage at −20°C for 10 min. Following an additional centrifugation step for 5 min, the supernatant was transferred to a fresh reaction tube, mixed with an equal volume of ice-cold isopropanol and stored overnight at −20°C. Finally, the tubes were centrifuged again for 5 min and the supernatant was discarded. DNA pellets were washed with 500 µL cold EtOH (70%) and centrifuged for 5 min. After thorough removal of EtOH and air drying, the DNA was suspended in 150 µL of distilled water by incubation for 10 min at 50°C with gentle shaking.

Conventional DNA extraction from wood followed the protocol by Inglis et al. (2018) with minor modifications: 150 mg of wood shavings were transferred into sterile 2 mL Eppendorf tubes and treated in 1 mL of sorbitol wash buffer (100 mM Tris–HCl pH 8.0, 0.35 M sorbitol, 5 mM EDTA pH 8.0, 1% [w/v] polyvinylpyrrolidone MW 40,000) in a Fastprep® homogenizer for 45 s at 5 m/s after adding 0.3 g of sterile glass beads (1.25–1.65 mm diameter) as well as one sterile 6.35 mm ceramic sphere (MP Biomedicals, Irvine, CA, USA) per tube. After three additional washing steps with sorbitol wash buffer, samples were further processed according to protocol: 600 µL of CTAB lysis buffer (100 mM Tris–HCl pH 8.0, 3 M NaCl, 3% [w/v] CTAB, 20 mM EDTA, 1% [w/v] PVP-40), preheated to 65°C was added to sample tubes. After incubation for 1 h at 65°C while shaking at 200 rpm, samples were cooled down for 5 min before adding 600 µL of chloroform: isoamyl alcohol (24:1 v/v) and vigorously vortexed for 20 s before centrifugation for 10 min at 5000 x *g* at ambient temperature. The upper aqueous phase was transferred to a new tube, mixed with approximately 1/10 volume of 3 M sodium acetate (pH 5.2) and 2/3 volume of ice-cold isopropanol and stored overnight at −20°C for DNA precipitation.

The supernatant was then discarded following centrifugation for 10 min at 13,000 x *g* and after a washing step with EtOh (70%), additional centrifugation and thorough removal of EtOH by pipetting and evaporation, the DNA pellet was suspended in 150 µL of distilled water while gently shaking at 50°C for 10 min.

Simplified sample preparation for LAMP was conducted similarly to the method described by Sillo et al. (2018): 500 mg of wood shavings were treated in a sterile 15 mL Falcon tube containing 10 mL alkaline PEG-buffer (Chomczynski & Rymaszewski, 2006) (50 g/L PEG 4000, 20 mM KOH, pH 13.5) and glass beads of two different sizes (1.25–1.65 and 2.85–3.45 mm diameter, approximately 2.5 g each) in a Fastprep® homogenizer for 60 s at a speed of 5 m/s. Wood debris was spun down at 2000 x *g* at ambient temperature and the supernatant was transferred to a new tube. Five microliters of a 1:20 dilution of the crude extract in distilled water was directly used as target in LAMP reactions.

2.3 | Copy number calculation

The genome copy numbers corresponding to the detectable DNA amounts were calculated based on the reference genome sizes of the respective species (accession numbers: GCA_001006345.1 [*P. chlamydospora*], GCA_000392275.1 [*P. minimum*]; GCA_000271605.1 [*F. mediterranea*]). An average weight of 650 Daltons per base pair was assumed for calculation (copy number = [amount DNA (g) × N_A]/[genome size (bp) × 650 g/mol]) with N_A being Avogadro's constant (6.022×10^{23}).

2.4 | Target sequence selection and LAMP primer design

For each of the three species, the first step was to find unique core sequences using the *fucs*-entry point of the *RUCS* program (Thomsen et al., 2017). In each of the separate runs, the reference genome of FMED, PMI and PCH, respectively, was used as 'positive' sequence that was queried against a multitude of 'negative' genomes of grapevine-associated fungi, including the respective two other Esca species, that were not used in this run as 'positive'. The corresponding GenBank Accession numbers are listed in Table S1. As there was only one genome available for FMED and PMI and two for PCH, the corresponding coding sequences files were added to the positive genome folder. By this means, non-coding regions with potentially high interspecies variation and multiple repetitive motifs were excluded from the output sequences. All runs were conducted using a k-mer size of 17, which was found to give good results regarding the number of obtained unique core sequences. In order to retrieve a suitable target sequence for subsequent primer design, sequences from the output file of each analysis were filtered using the *biostrings* package (Pagès et al., 2021) of the *R* statistics software according to properties such as length, GC-content, mean sequence complexity (*universalmotif* package [Tremblay, 2022]) and palindrome ratio. The finally selected sequences were analysed with BLAST (Altschul et al., 1990) to ensure a low degree of sequence homology with other fungal species and are shown in Table S2. Based on those core sequences, a set of LAMP primers was designed for each target species using the Primer Explorer V5 (http://primerexplorer.jp/lampv5e/index.htmL) software tool. Primers used during the current study are listed in Table 2.

2.5 | LAMP assay

LAMP reaction mixes contained for all three assays 1.4 mM of each dNTP, 8 mM $MgCl_2$, 8 U per 25 µL reaction *Bst* Polymerase (New

TABLE 2 LAMP primer sets designed in this study.

Primer name	Sequence	Target species
PCH_F3	CCTGCATTGTACCATTGGGA	*Phaeomoniella chlamydospora*
PCH_B3	TTACAACGACATCCACTGCC	
PCH_FIP	ACGGTGACAGGGACTACGTCTG-ATCGGATTGAATCGCTCCAG	
PCH_BIP	AGAGCCACTCTAATGCCTTGGG-GACGGGCGACTATGAACGA	
PCH_LF	GCTGTCTCTTTCGCCGTGG	
PCH_LB	GAGAAGCCAAGCCAAAGATTCGATG	
PMI_F3	ATCAGGTACGGCGGTTCC	*Phaeoacremonium minimum*
PMI_B3	GCGAAGCTTTACCGTCATCT	
PMI_FIP	CCGCCAGGAAGGCAATCGTTAT-TACTGAGCGGTCATCAGAGT	
PMI_BIP	TGTGTGCATGATCCCTACGACG-CGATGGTTTCCAGCCCAT	
PMI_LF	CGAGACGGACATGCTCTGG	
PMI_LB	CAGCGGGAGCCATGGCATT	
FMED_F3	CCAGAGACAATACGTGCTCC	*Fomitiporia mediterranea*
FMED_B3	TCACACGCATTTTGTCCGG	
FMED_FIP	GCGGGAAGGTCATCGTTGGA-AGAAAGCGACTGACCGAAC	
FMED_BIP	CGCGACGTCCCAGATCAAGAT-AAGTGGCTTTCTCGCCTG	
FMED_LF	CCTCTCAAAGGACATACTGACATT	
FMED_LB	GGTTTTGTCGTTAGAGCCAGAGAC	

England Biolabs, Ipswich, MA, USA), 2.5 µL 10× ammonium sulphate buffer (100 mM KCl, 100 mM ammonium sulphate, pH 8.7), primers at a concentration of 0.2 µM (F3 and B3), 1.6 µM (FIP and BIP) and 0.8 µM (LF and LB), 120 µM of pH indicator neutral red (SERVA Electrophoresis GmbH, Heidelberg, Germany) (neutral red reaction mix) and 5 µL per reaction template DNA. Sterile distilled water was added to a final volume of 25 µL per reaction.

Real-time LAMP reactions were run essentially under the same conditions replacing the respective components with 8 U per reaction *Bst* 2.0 Warm Start Polymerase, 0.2 µg/mL V13-01184 fluorescent intercalating dye (Dyomics, Jena, Germany) instead of neutral red and 2.5 µL 10× MOPS buffer (200 mM MOPS, 100 mM KCl, 100 mM ammonium sulphate, pH 8.8) (real-time reaction mix). Incubation temperature for all primer sets was 68°C and reactions were conducted in a conventional thermal cycler (Mastercycler Gradient, Eppendorf AG, Hamburg, Germany) for 60 min (neutral red mix) or in an ESEquant Tube Scanner TS95 (QIAGEN Lake Constance GmbH, Konstanz, Germany) for 45 min (real-time reaction mix).

2.6 | PCR reaction

The PCR reaction mix consisted of 0.5 µM of each respective forward and reverse primers, 200 µM of each dNTP, 5 µg/µL bovine serum albumin (BSA), 0.4 µL per reaction 5× Phire buffer and 0.4 µL per reaction Phire Hot Start II Polymerase (Thermo Fisher Scientific Inc., Waltham, MA, USA). The final $MgCl_2$ concentration was 1.5 mM. Sterile distilled water was added to a final volume of 20 µL per reaction. Conventionally extracted DNA from wood samples was diluted 1:10 in sterile distilled water and 1 µL of the dilution was added to the reaction mix as target. Dilution was necessary as previous tests had shown occasional inhibition of PCR reactions using undiluted DNA. For specific detection of the three fungal species PMI, PCH and FMED, primer pairs PmaleoF/PmaleoR (Arzanlou & Narmani, 2014), PCL1/PCL2 (Groenewald et al., 2000) and prFmed1/prFmed2 (Fischer, 2006), respectively, were used (see Table 3). Reactions were conducted with an initial denaturation of 1 min at 98°C, followed by 35 cycles with 10 s denaturation at 98°C, 10 s annealing at 47°C (FMED) or 68°C (PCH), 20 s elongation at 72°C and a final elongation step of 1 min at 72°C. The cycling protocol for PMI was 1 min at 98°C, 35 cycles of 5 s at 95°C, 5 s at 61°C and 15 s at 72°C, final elongation for 1 min at 72°C.

2.7 | Preparation of wood samples

Ten different samples (see Table 4) of grapevine trunks were analysed with each of the developed LAMP assays. The sample material consisted of sections that had been cut from individual grapevine trunks with visible symptoms of Esca disease. Each sample was taken from a

TABLE 3 PCR primer sequences.

Primer name	Sequence	Detected organism	Target region	Approximate product length (bp)	Reference
PmaleoF	CTCTGCGACGCGTCCCAGATTG	*Phaeoacremonium minimum*	β-tubulin gene	500	(Arzanlou & Narmani, 2014)
PmaleoR	TCGCGATGGCCCACTGCCTAC				
PCL1	TACATGTGACGTCTGAACGG	*Phaeomoniella chlamydospora*	Internal transcribed spacer (ITS 1&2) region	325	(Groenewald et al., 2000)
PCL2	AGGACCACCTCAGTGTATGC				
prFmed1	GCAGTAGTAATAATAACAATC	*Fomitiporia mediterranea*	Internal transcribed spacer (ITS 1&2) region	550	(Fischer, 2006)
prFmed2	GGTCAAAGGAGTCAAATGGT				

TABLE 4 Grapevine wood samples used in this study.

Sample ID	Grapevine cultivar	Location	Region
S1	Gewürztraminer	Tramin	South Tyrol, Italy
S2	Sauvignon	Piglon	
S3	Pinot noir	Pfatten	
S4	Sauvignon	Pfatten	
S5	Gewürztraminer	Pfatten	
R1	Riesling	Gimmeldingen	Rhineland-Palatinate, Germany
R2	Dornfelder	Ruppertsberg	
R3	Riesling	Mußbach	
R4	Dornfelder	Mußbach	
R5	Riesling	Mußbach	

different vineyard and the most important grape varieties (red and white) grown in Europe were represented. Sample vineyards were located in Rhineland-Palatinate (Germany) and South Tyrol (Italy), representing two major central-European wine-growing areas.

Wood shavings were obtained from affected parts of each of the samples using a rasp. After each sample, the rasp was thoroughly cleaned and sterilized by immersion in pure ethanol and flaming. The obtained material was subsequently used for conventional DNA extraction as well as for the rapid preparation of crude DNA for LAMP-reactions as previously described.

3 | RESULTS

3.1 | Specificity testing

Specificity of each primer set was tested using purified gDNA. A total number of 80 strains representing 39 different species was tested by adding 25 ng of DNA to the respective LAMP master mix using neutral red for visual signal detection. Results obtained after 60 min of incubation at optimum temperature are summarized in Table 1. Each strain of *P. minimum*, *P. chlamydospora* and *F. mediterranea* resulted in a positive LAMP reaction with the respective primer set. For all three primer sets, negative reactions occurred with gDNA from most of the non-target species tested, which demonstrates the applicability of the assays for the intended purpose. However, some false positive results were observed for closely-related species, mostly belonging to the *Phaeoacremonium* species complex: Out of nine additionally tested *Phaeoacremonium* species, three resulted positive using the PMI primer set (*Phaeoacremonium inflatipes*, *Phaeoacremonium iranianum* and *Phaeoacremonium viticola*), as well as one in case of the PCH primer set (positive for *Phaeoacremonium krajdenii*). Similarly, the FMED primer set showed a cross-reaction for *Fomitiporia punctata*. As further discussed below, practical relevance of these false positive results is probably small, because the respective pathogens are also associated with the disease of the corresponding target species, but occur less frequently.

3.1.1 | Sensitivity testing

The sensitivity of all three primer sets was tested by using a ten-fold serial dilution of purified gDNA of the respective species. Neutral red was used for visual signal detection. Results are shown in Figure 1. The observed detection limits after 60 min of isothermal incubation were 100 pg (FMED, PMI) and 1 pg (PCH) per reaction, respectively, which corresponded to a theoretical genome copy number of 1460 (FMED), 1950 (PMI), and 342 (PCH).

3.2 | Testing of field samples

Field samples were tested for the presence of *P. chlamydospora*, *P. minimum* and *F. mediterranea*, respectively, using the designed

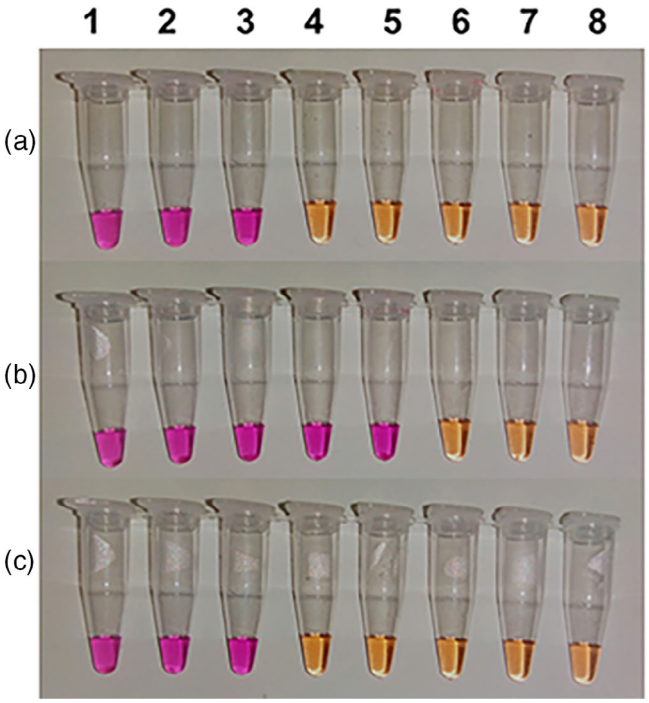

FIGURE 1 Sensitivity tests for all three primer sets. Purified gDNA of the respective species was added to the LAMP master mix from a 10-fold serial dilution. (a) *Fomitiporia mediterranea*; (b) *Phaeomoniella chlamydospora*; (c) *Phaeoacremonium minimum*; lanes 1–8, respectively: 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg (DNA per reaction). A pink colouring indicates a positive reaction.

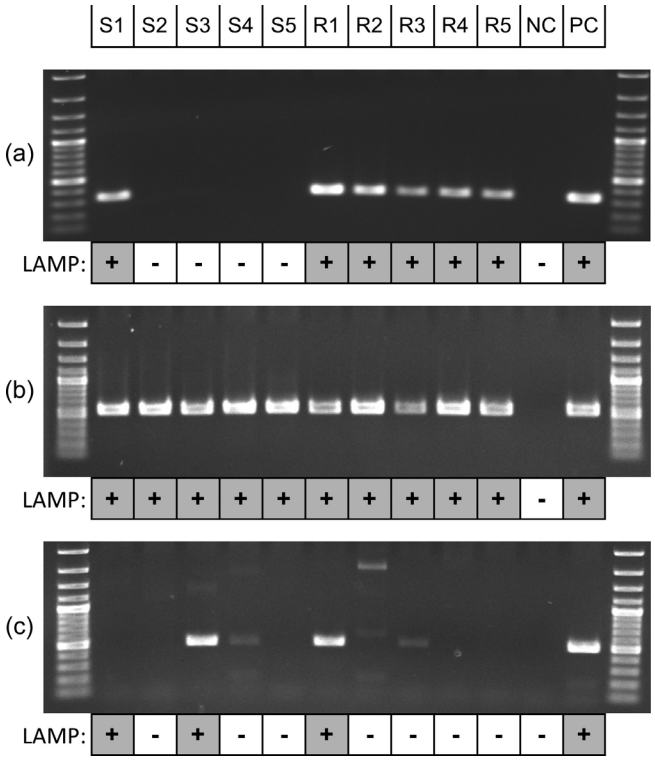

FIGURE 2 PCR- and real-time LAMP results of field samples using species-specific primers. LAMP reactions followed crude DNA extraction from wood shavings, whereas for PCR DNA was extracted and purified conventionally prior to assays. (a) *Phaeomoniella chlamydospora*, (b) *Fomitiporia mediterranea*, (c) *Phaeoacremonium minimum*. S1-R5: Sample ID according to Table 4). NC, negative control with water instead of DNA; PC, positive control, with 1 ng per reaction of purified gDNA of the respective species.

LAMP primer sets with crude DNA preparations as amplification target. Control PCR reactions were conducted with purified DNA extracted from wood shavings using PCR primers PCL1/PCL2 (PCH), prFmed1/prFmed3 (FMED) and PmaleoF/PmaleoR (PMI). Results of both assays are shown in Figure 2. Results were mostly in accordance between the two assay formats which demonstrates that all LAMP assays can replace PCR for the detection of the major Esca species.

4 | DISCUSSION

The current study has demonstrated that LAMP can be a useful tool for the identification of the most important fungi related to the Esca disease of vine plants. Especially when testing woody sample materials, our study has shown that results can be obtained faster than with PCR because of the considerably shorter reaction time of LAMP and the application of the simple and rapid sample preparation protocol developed.

4.1 | Target selection and primer design

When designing LAMP primer sets for fungi, most commonly used target sequences are housekeeping genes or rRNA coding genes including adjacent spacer regions (Niessen, 2015). Due to the importance of such genes for establishing phylogenetic relationships, a high amount of sequence data are usually publically available for them. However, the degree of sequence variation of highly conserved genes is often insufficient as to provide the desired degree of assay specificity. Therefore, designing highly specific primer sets from such sequences can be a cumbersome task.

Even though in some cases, the targeted group of organisms directly relates to a unique gene of a specific metabolic pathway, for example in mycotoxin producers (Frisch & Niessen, 2019; Wigmann et al., 2020) or when genes for certain surface proteins are targeted (Denschlag et al., 2012; Frisch et al., 2021), very often no property-related genes are known or there is not enough sequence information available to ensure uniqueness to a respective targeted species. With the intention of providing an exemplary procedure that can be adapted to different species playing major roles for other GTDs, a comprehensible, systematic in silico target selection approach was chosen in the current study. The *RUCS* software (Thomsen et al., 2017) was used in order to determine DNA sequences that are unique to *P. chlamydospora*, *P. minimum* and *F. mediterranea*, respectively. The programme makes use of whole-genome-datasets, a

resource of increasing availability due to advances in sequencing techniques. It can be employed as a command-line or alternatively as a browser-operated version (https://cge.food.dtu.dk/services/RUCS/) in cases where computational resources are limited. Although the full programme includes automatic design of PCR primers, only the *fucs* (find unique core sequences) entry point was used in the current study, which provides multiple output sequences specific to the respectively queried genomes. *RUCS* has been applied previously to determine unique sequences for LAMP primer design by Sedaghatjoo et al. (2021). At the time of this study, for the majority of the GTD-related fungi, including the three targeted species FMED, PCH and PMI, only one genome per species was available in the GenBank database, which increases the risk of retrieving output sequences with a high degree of intra-specific variation. To address this problem, the CDS was included as a reference file in each run, because non-coding sequences are expected to include a great proportion of those highly variable sequences.

In contrast, the inter-specific variation of the output sequences that are used for primer design should be high enough to create a primer set that distinguishes between target and non-target species. The specificity of the *fucs*-output sequences depends on the k-mer size that was initially set for the analysis. In this study, a k-mer size of 17 was chosen as a trade-off between the number of obtained sequences and their specificity to the respective target species. By further processing in *R*, their number was reduced step-by-step using criteria facilitating the primer design process in the Primer Explorer V5 software such as sequence length, GC-content and complexity.

Before starting primer design, specificity of the finally selected sequences was checked by BLAST analysis. In all three cases, sequence variation between the target sequences and the second-best BLAST hit (data not shown), were sufficiently high (generally <75% identity at max. 70% query coverage) to be suitable for the design of highly specific primer sets. However, analysis of the obtained sequences (Table S2) revealed that the genes that they belonged to were not unique to the respective species since they included (putative) genes such as carboxypeptidase s1, the WD40 repeat-like protein and the 14-alpha sterol demethylase protein. This underlines that although partial sequences that are suitable for primer design can be obtained using the described procedure, it should not be expected to filter genes entirely specific to a certain target species.

4.2 | Specificity testing

Specificity could be confirmed for all selected primer sets as shown in Table 1. The few false positives that occurred belonged in each case to species that are closely related to the respective target species. The FMED primer set also tested positive for strains designated as *Fomitiporia punctata* (formerly *Phellinus punctatus*). *Fomitiporia* has been delimited as a separate genus from *Phellinus* (Fischer, 1996). Moreover, *Phellinus* spp. have been associated with Esca in the older literature (Baldacci et al., 1962; Chiarappa, 1959). In their survey, Cortesi et al. (2000) identified white rot basidiomycetes on Italian grapevine samples mostly as *F. punctata* based on a species concept that included the later described *F. mediterranea* (Fischer, 2002). The results of the current study indicate that the FMED specific primer set can be considered as sufficient for detection of both Esca-associated *Fomitiporia* spp., which is rather an advantage than a disadvantage from a phytopathological point of view. The PMI LAMP Primer set detected all tested *P. minimum* strains, but also yielded positive results for three additional *Phaeoacremonium* species, *P. iranianum*, *P. inflatipes* and *P. viticola*, while *P. krajdenii* resulted positive for the PCH primer set. *Phaeoacremonium* is a species complex with several of its members related to symptoms on grapevine plants associated with Petri or Esca disease. In a survey by Essakhi et al. (2008), 13 *Phaeoacremonium* spp. were isolated from diseased grapevine plants in Italy, of which 80% were identified as *P. minimum*. *P. chlamydospora* (formerly *Phaeoacremonium chlamydosporum*) is closely related to *Phaeoacremonium* spp. but was introduced as a new genus based on morphological and cultural characteristics (Crouse & Gams, 2000), which is supported by molecular data (Groenewald et al., 2001). All mentioned false positive *Phaeoacremonium* spp. are reportedly associated with Esca (Aroca et al., 2008; Essakhi et al., 2008; Mondello et al., 2019; Mostert et al., 2006), although less frequently occurring than *P. minimum*. These results are thus not likely to impede practical application of our assays because no species associated to other GTDs have tested positive for any of the three primer sets. However, for application in regions where species other than *P. minimum* are predominant, design of an alternative primer set might be necessary in the future.

4.3 | Sensitivity testing

All primer sets showed good sensitivities with a detection limit of 10–100 pg/reaction using pure genomic DNA which corresponds to <2000 genome copies per reaction for each of the three species. Overall, this is in accordance with results obtained with LAMP assays by other authors (Niessen, 2015), although at best performance sensitivities of <10 pg/reaction can be achieved for assays detecting fungi of comparable genome size (Frisch & Niessen, 2019; Luo et al., 2012; Niessen & Vogel, 2010). The PCH primer set was the most sensitive in this study with 10 pg/reaction, surpassing the detection limit of 16 pg of the corresponding PCR assay (Groenewald et al., 2000), whereas the FMED and PMI primer sets could not match common PCR sensitivities of <100 copies.

4.4 | Sample preparation method

Aside from its rapidness and easy handling, the low proneness to inhibitory components is a major advantage of LAMP (Francois et al., 2011; Kaneko et al., 2007). Therefore, the use of target DNA should be possible with LAMP that has undergone only a minimum of sample preparation, obviating cumbersome DNA extraction and

purification. During the current study, a protocol was set up for the rapid preparation of DNA extracts from infected wood samples that showed sufficient purity for the LAMP reaction. Compared to a conventional DNA extraction, which often takes up to several hours, the described sample preparation for LAMP can be achieved in less than 30 min, depending on the number of samples to be processed.

The protocol was based on the procedure described by Sillo et al. (2018) for the detection of the pine-infecting fungus *Heterobasidion irregulare*. By processing wood samples with a metal rasp, a homogenous material was obtained that could be subjected to both the rapid sample preparation method as well as to conventional DNA extraction and purification for a performance comparison of both methods. A similar approach has been used for the LAMP-based detection of *Eutypa* and *Botryosphaeria* species by Baaijens et al. (2021).

Although in this study, a rasp served well for a limited number of pre-cut samples, other options may be more suitable for sampling at a larger scale since sterilizing the rasp after each operation is time-consuming and bears the risk of inter-sample contamination if not properly performed. As an alternative, wood samples can also be acquired using a drill which is especially applicable when wooden tissue is to be analysed for fungal infection from living plants (Sillo et al., 2018).

During further processing of the shavings in alkaline PEG-buffer, a Fastprep® homogenizer was used in the present study to apply maximum forces to ensure homogenous mixing of the samples with the buffer. In addition, bead-beating aimed to release more fungal tissue from the wood matrix into the buffer solution. However, due to its cost and size, the Fastprep instrument may not be the optimal choice for an in-field application that is supposed to suffice with minimal equipment. Therefore, it should be mentioned that the rapid sample preparation procedure can likely be modified by the application of manual shaking or vortexing, as demonstrated by Sillo et al. (2018) and Niessen et al. (2022) with comparable LAMP assays.

The results obtained with the rapid sample preparation protocol and subsequent LAMP assay largely match the results obtained with conventional DNA extraction and PCR in the three target species (Figure 2). Sample S1 resulted positive in the PMI LAMP assay but could not be confirmed by PCR. This may be attributed to low target quantities unevenly distributed in the sample material. Further differences were observed in samples S4 and R3, in which faint bands were observed with PCR primers PmaleoF/PmaleoR, although no positive result could be shown with the respective LAMP assay. This indicates a slight loss of sensitivity in the PMI specific LAMP assay due to the rapid sample preparation protocol in these particular samples. However, considering the enormous time saving, a certain reduction in sensitivity may be acceptable.

Overall, the current study has demonstrated that the LAMP assays for the detection of PCH, PMI and FMED can be run with minimally processed sample materials obtained from a difficult matrix such as wood. We therefore assume that the LAMP technology is a viable alternative to the more laborious PCR approach.

The rapid and specific diagnosis of fungi in wood samples is an important aspect in research and control of GTDs such as Esca. The current study provides a proof-of-principle for the application of LAMP assays for the detection of the three major Esca-associated species. Making use of the robustness of the LAMP method, the protocol takes less than 2 h from sample to result and offers high potential for large-scale monitoring. Moreover, the protocol used for target sequence selection and primer design may be adapted to a variety of other wood-inhabiting fungi and diseases for which currently no LAMP assays are available.

Although wood cuttings from mature infected plants were used in the current study for the validation of the assays in natural materials, a major application would be in commercial nurseries, where grafting is performed at a larger scale and a great number of plants are propagated. The early detection of latent pathogens in buds and rootstocks with the developed LAMP assays would aid to sort out infected plant material aiming at the reduced production of defective plants. This would also prevent the dispersal of disease plant material to clients, benefitting both vendors and recipients. Due to its rapidness and simplicity, the LAMP assay detection allows the use of molecular detection at a larger scale and can therefore be a viable option for public authorities or research institutes conducting quality control and disease surveillance in viticulture.

In the context of applied research, LAMP-based detection can also be a time-saving alternative when it comes to measuring the success of chemical or biological treatment of adult plants in the vineyard. In addition, the assay usage can potentially be extended to environmental samples of different types to investigate propagation of the fungi *on site* by testing potential inoculum sources other than plant material, such as tools and pruning equipment in vineyards and nurseries or monitoring seasonal and local differences in fungal spore densities via the use of spore traps as described by Billones-Baaijens et al. (2018) and Molnar et al. (2020), who used PCR-based assays. Here, direct detection of fungal spores in LAMP assays without any pre-treatment can be applied, as it was demonstrated for different fungal species (Frisch et al., 2021; Wigmann et al., 2020).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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