TYPE Original Research
PUBLISHED 28 April 2023
DOI 10.3389/fcimb.2023.1134921

OPEN ACCESS

EDITED BY
Jianping Xu,
McMaster University, Canada

REVIEWED BY
Banu Metin,
Istanbul Sabahattin Zaim University, Türkiye
Tuhong Wang,
Institute of Bast Fiber Crops (CAAS), China

*CORRESPONDENCE
Dai Yu-cheng
yuchengd@yahoo.com
Chen Jia-jia
jiajiachen@jsafc.edu.cn

RECEIVED 31 December 2022
ACCEPTED 11 April 2023
PUBLISHED 28 April 2023

CITATION
Hong-min Z, Jian Y, Ying L, Yuan Y, Cui-ping W, Yu-cheng D and Jia-jia C (2023) Rapid detection of *Heterobasidion annosum* using a loop-mediated isothermal amplification assay. *Front. Cell. Infect. Microbiol.* 13:1134921. doi: 10.3389/fcimb.2023.1134921

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Rapid detection of *Heterobasidion annosum* using a loop-mediated isothermal amplification assay

Zhou Hong-min[1,2], Yu Jian[2], Liu Ying[2], Yuan Yuan[1], Wu Cui-ping[3], Dai Yu-cheng[1*] and Chen Jia-jia[2*]

[1]Institute of Microbiology, School of Ecology and Nature Conservation, Beijing Forestry University, Beijing, China, [2]College of Landscape Architecture, Jiangsu Vocational College of Agriculture and Forestry, Zhenjiang, China, [3]Animal, Plant and Food Inspection Center, Nanjing Customs, Nanjing, China

Heterobasidion annosum is one of the most aggressive pathogens of *Pinus* forests in Europe, causing considerable economic losses. To detect *H. annosum* for disease diagnosis and control, we developed a loop-mediated isothermal amplification (LAMP) reaction with a primer set designed from the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) DNA sequences of *H. annosum*. In our study, this LAMP assay was found to be capable of efficiently amplifying the target gene within 60 min at 63°C. In specificity tests, *H. annosum* was positively detected, and other species were negative. The detection limit of this assay was found to be 100 pg·µL^{-1}, and the assay was also successfully tested for use with basidiospore suspensions and wood samples. This study provides a rapid method for diagnosing root and butt rot caused by *H. annosum*, which will be of use in port surveillance of logs imported from Europe.

KEYWORDS
***Heterobasidion annosum*, pathogens of *Pinus*, LAMP assay, molecular diagnosis, port quarantine**

Introduction

Heterobasidion annosum (Fr.) Bref. sensu lato (s.l.) has been studied intensively over several decades, with interfertility studies showing that *H. annosum* s.l. is a species complex (Korhonen, 1978; Dai and Korhonen, 1999; Dai et al., 2003). Recently, species of *Heterobasidion* have been divided into three groups using multilocus phylogenetic approaches; furthermore, the pathogenic species *H. annosum* sensu stricto (s.s.) has been found to be a sister to *H. irregulare* Garbel. & Otrosina. Most taxa of *H. annosum* s.l. are distributed in the conifer forests of the northern hemisphere (Chen et al., 2015; Dai et al., 2021a, b; Yuan et al., 2021; Wu et al., 2022).

Heterobasidion annosum is one of the most aggressive pathogens in the destruction of pine plantations in Europe (Edmonds et al., 1989; Woodward et al., 1998; Dai and

Korhonen, 1999). The root and butt rot caused by *Heterobasidion* s.l. species can destroy the most valuable part of the tree (Korhonen and Stenlid, 1998; Niemelä and Korhonen, 1998; Seifert, 2007), depreciating the usability of the timber (Aza et al., 2021) and lowering the tree's resistance to strong winds (Oliva et al., 2008). Furthermore, *H. annosum* s.l. may remain active in residual stumps and roots for decades until the next rotation (Rishbeth, 1951; Greig and Pratt, 1976). Significantly, *H. annosum* s.l. grows more quickly in dead trees than in living trees (Bendz-Hellgren et al., 1999). Hence, poor thinning and logging operations may increase the incidence of annosum-related rot (Shaw et al., 1995; Morrison and Johnson, 1999). Dai et al. (2021a) proposed that the most aggressive conifer pathogens, *H. abietinum*, *H. annosum* s. s., *H. irregulare*, *H. occidentale*, and *H. parviporum*, should be identified as quarantine fungi, as they are not found in China. Therefore, effective detection of annosum-related rot is important.

Over the past several decades, various methods for *H. annosum* detection have been developed, mainly focusing on morphological characters, mating tests, and molecular strategies. Traditionally, morphological identification of *H. annosum* has relied on macroscopic and microscopic observations (Rishbeth, 1951; Greig and Pratt, 1976; Tokuda et al., 2009; Aberg et al., 2016). However, once the basidiomata can be observed, it is already too late to protect the trees in question from decay (Garbelotto and Gonthier, 2013). Although mating tests are a relatively reliable method to determine compatibility with known species, they take time (Korhonen, 1978; Mitchelson and Korhonen, 1998; Dai and Korhonen, 1999; Dai et al., 2002). In fact, as all the classical diagnostic methods are complicated, time-consuming, and require professional skill, researchers have been investigating molecular methods of assay. A potential polymerase chain reaction (PCR) method offers great promise for detection of pathogenic fungi because of its speed and specificity (Schulze, 1999). Multiplex real-time PCR assay, with better resolution than traditional technology, has already been conducted by several researchers (Hietala et al., 2003; Ioos et al., 2019), and qPCR technology has been used to measure the distribution of species of *Heterobasidion* (Oliva et al., 2017).

Although PCR technology has already been applied in detection of *H. annosum* due to its sensitivity and specificity, long periods of time and expensive laboratory instruments are still required for these procedures. These intrinsic disadvantages prevent this method from being used in resource-limited regions. Loop-mediated isothermal amplification (LAMP) is an alternative method that amplifies target DNA sequences with high sensitivity and specificity under isothermal conditions (Notomi et al., 2000). The technology has previously been applied in pathogen detection (Sillo et al., 2017; Kong et al., 2020; Vettraino et al., 2021). So far, LAMP technology has been widely used in the medical field (Parida et al., 2005; Parida et al., 2007; Santiago, 2021), food science (Petersen et al., 2021), and plant protection (Franco Ortega et al., 2019; Enicks et al., 2020). The North American species *H. irregulare* Garbel. & Otrosina was detected by LAMP using a HirrSC3 gene within cytochrome P450 monooxygenase (Sillo et al., 2017). However, methods for rapid detection of *H. annosum* have rarely been reported.

LAMP utilizes a *Bst* DNA polymerase with stand-displacement activity, along with two inner primers (FIP, BIP) and two outer primers (F3, B3) that recognize six separate regions within a target DNA sequence (Notomi et al., 2000). Correct recognition of all six regions by the primers ensures the specificity of the assay. Positive reactions can be examined in the products, as follows: turbidity of magnesium phosphate increases (Mori et al., 2001); ladder-like bands can be observed on gel electrophoresis; and color changes can be induced in the reaction system through the addition of DNA-intercalating dyes (Goto et al., 2009). The metal ion hydroxynaphthol blue (HNB) is a reliable indicator of DNA amplification because of the low risk of cross-contamination along with sensitivity equivalent to that of SYBR green, and the results can easily be judged with the naked eye (Goto et al., 2009).

In this study, we aimed to develop a simple LAMP detection method for specific identification of *H. annosum* and to evaluate its accuracy in detecting wood decay caused by *H. annosum*.

Materials and methods

Culture conditions and DNA extraction

This study used forty-five cultures and specimens which were maintained at the Institute of Microbiology, the Beijing Forestry University (BJFC, Beijing, P.R. China), Jiangsu Vocational College of Agriculture and Forestry (JSAFC), and the Natural Resources Institute, Finland (Luke, Helsinki, Finland) (Table 1). Fungal strains were cultured on potato dextrose agar (PDA) (Caten and Jinks, 1968; Gams et al., 1998) in 90 mm petri dishes at 25°C for 28 days. In order to obtain abundant mycelia, fungal strains were cultured on potato dextrose agar for 7 days.

Mycelia and basidiomata were ground in liquid nitrogen and subsequently collected in 1.5-mL microfuge tubes. Genomic DNA was extracted using the CTAB rapid plant genome extraction kit (Aidlab Biotechnologies Co., Ltd., Beijing, China) according to the manufacturer's instructions, with some modifications (Chen et al., 2015). The concentration of the extracted DNA was evaluated using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) following Kong et al. (2020); this was then diluted in 10-fold serial dilutions to produce concentrations from 10 ng·μL^{-1} to 10 fg·μL^{-1} and stored at –20°C. The cultures and specimens used were identified by morphological examination, and/or by sequencing Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chen et al., 2015) or the internal transcribed spacer (ITS) (Table 1).

Optimization of the LAMP reaction

The LAMP reaction was performed according to previously described methods (Niu et al., 2012; Duan et al., 2014; Kong et al., 2020; Vettraino et al., 2021). The final LAMP reaction (26 μL volume) was performed by combining 2.5 μL 10 × ThermoPol buffer, 1.6 μmol·L^{-1} forward inner primer (FIP) and backward inner primer (BIP), 0.2 μmol·L^{-1} B3 and F3 primers, 0.8 μmol·L^{-1} LB and LF primers, 5 mmol·L^{-1} Mg^{2+}, 0.8 mol·L^{-1} betaine, 1.4 mmol·L^{-1}

TABLE 1 Fungal isolates and basidiomata used in this study.

Species	Specimen No.	Host	Location	Result	GenBank accession		Reference or GenBank accessions
					ITS	GAPDH	
Albatrellus alpinus	Cui 17023	On ground in forest of *Pinus* sp. and *Quercus* sp.	China	–	MW534154	—	Zhou et al., 2021
Aleurocystidiellum disciformis	He 3159	*Quercus* sp.	China	–	KU559340	—	Liu et al., 2017
Aleurodiscus amorphus	Ghobad-Nejhad2464	*Abies* sp.	China	–	KU559342	—	Liu et al., 2017
Amylonotus labyrinthinus	Yuan 1475	Angiosperm	China	–	KM107860	—	Liu et al., 2017
Amylosporus succulentus	Dai 7802	Lawn	China	–	KM213669	—	Liu et al., 2017
Amylostereum orientale	He 479	*Cunninghamia lanceolata*	China	–	JX049987	—	Liu et al., 2017
Bondarzewia submesenterica	Cui 10345	*Podocarpus* sp.	China	–	KJ583204	—	Chen et al., 2016
Bondarzewia podocarpi	Cui 6380	*Podocarpus*	China	–	KJ583206	—	Chen et al., 2016
Dentipellis coniferarum	Cui 10063	*Abies* sp.	China	–	JQ349106	—	Chen et al., 2016
Echinodontium japonicum	Dai 7378	Angiosperm	China	–	KY172887	—	Liu et al., 2017
Echinodontium tinctorium	HHB 12866-Sp	*Tsuga* sp.	USA	–	KY172888	—	Liu et al., 2017
Heterobasidion annosum	06071/1	*Pinus pinea*	Italy	+	—	KJ651761	Chen et al., 2015
Heterobasidion annosum	06125/2	*Pinus sylvestris*	Russia	+	—	KJ651762	Chen et al., 2015
Heterobasidion annosum	06129/6	*Pinus sylvestris*	Russia	+	—	KJ651763	Chen et al., 2015
Heterobasidion annosum	09001/1	*Pinus* sp.	Italy	+	—	KJ651765	Chen et al., 2015
Heterobasidion annosum	93691/6	—	England	+	—	KJ651760	Chen et al., 2015
Heterobasidion annosum	Dai 6540	*Pinus* sp.	Italy	+	—	—	Chen, 2015
Heterobasidion annosum	Dai 14857	*Pinus* sp.	Poland	+	—	—	Chen, 2015
Heterobasidion abietinum	00051/1	*Picea* sp.	Italy	–	—	AJG42512	Chen et al., 2015
Heterobasidion amyloideum	L 1878	Gymnosperm	China	–	—	KJ651758	Chen et al., 2015
Heterobasidion araucariae	65008	*Araucaria* sp.	Australia	–	—	KJ651766	Chen et al., 2015
Heterobasidion armandii	Dai 17605	*Pinus* sp.	China	–	MT146482	—	Yuan et al., 2021
Heterobasidion australe	Y 05054/1	Gymnosperm	China	–	—	—	Chen, 2015
Heterobasidion insulare	Dai 15095	*Pinus* sp.	China	–	—	MT157728	Yuan et al., 2021

(Continued)

TABLE 1 Continued

Species	Specimen No.	Host	Location	Result	GenBank accession		Reference or GenBank accessions
					ITS	GAPDH	
Heterobasidion irregulare	01056	*Tsuga* sp.	Canada	–	—	KJ651780	Chen et al., 2015
Heterobasidion linzhiense	Dai 5408	*Abies* sp.	China	–	—	KJ651788	Chen et al., 2015
Heterobasidion occidentale	79034/VE	—	USA	–	—	AJG42548	Chen et al., 2015
Heterobasidion orientale	N 97011/7	—	China	–	—	KJ651794	Chen et al., 2015
Heterobasidion parviporum	04121/3	*Picea* sp.	Finland	–	—	KJ651800	Chen et al., 2015
Heterobasidion subinsulare	Li 140804-30	*Pinus* sp.	China	–	—	MT157733	Yuan et al., 2021
Heterobasidion subparviporum	Cui 6961	*Larix* sp.	China	–	—	KJ651809	Yuan et al., 2021
Heterobasidion tibeticum	I 04031/1	Gymnosperm	China	–	—	KJ651810	Chen et al., 2015
Larssoniporia incrustatocystidiata	Dai 13607	Angiosperm	China	–	KM107863	—	Liu et al., 2017
Laurilia sulcata	He 20120916-7	*Abies* sp.	China	–	KY172894	—	Liu et al., 2017
Lauriliella taxodii	FP-105464-Sp	*Taxodium distichum*	USA	–	KY172896	—	Liu et al., 2017
Peniophora erikssonii	Cui 11871	—	China	–	MK588771	—	Xu et al., 2023
Peniophora albobadia	He 2159	—	USA	–	MK588755	—	Xu et al., 2023
Peniophora bicornis	He3609	—	China	–	MK588763	—	Xu et al., 2023
Peniophora crassitunicata	He 3814	—	China	–	MK588770	—	Xu et al., 2023
Peniophora vietnamensis	He 5242	—	Vietnam	–	MK588760	—	Xu et al., 2023
Peniophora yunnanensi	CLZhao3978	—	China	–	OP380617	—	Xu et al., 2023
Perplexostereum endocrocinum	Dai 15998	Gymnosperm	China	–	KY172899	—	Liu et al., 2017
Pseudowrightoporia japonica	Dai 12086	Angiosperm	China	–	KJ513293	—	Chen, 2015
Wrightoporia subavellanea	Dai 11484	*Pinus*	China	–	KJ513295	—	Chen, 2015
Wrightoporiopsis amylohypha	Yuan 3460	Angiosperm	China	–	KM107875	—	Chen, 2015

dNTPs, 300 μmol·L^{-1} HNB, 8 U of *Bst* DNA polymerase, and 2 μL DNA template.

The LAMP reaction mixtures were heated at a range of reaction temperatures (viz., 61°C, 62°C, 63°C, 64°C, and 65°C) for 60 min to select the optimal temperature (Figure S1). Additionally, LAMP reactions were performed at the optimal reaction temperature (63°C) for 15 min, 30 min, 60 min, and 90 min in order to select the shortest viable reaction time (Figure S2). Runs were performed with positive controls (*H. annosum*), negative controls (14 *Heterobasidion* spp. and 24 other fungi), and controls consisting of distilled water without DNA. The assays were evaluated by observation of the HNB color change from violet to blue, which denotes positive amplification, while a negative assay remains violet. The optimum temperature and shortest viable time were identified as 63°C for 60 min. Each condition was repeated at least three times.

DNA extraction from basidiospore suspensions

Basidiospore suspensions were prepared by scraping four-week-old PDA-cultured mycelium with sterile distilled water. The concentration was determined using a hemacytometer and then adjusted in sterile water to obtain the desired final concentrations, containing 10^4, 10^3, 10^2, 50, 10, and 0 basidiospores per 1 µL. DNA was extracted from these basidiospore suspensions in order to evaluate the effectiveness of the LAMP assay in detecting basidiospores of *H. annosum*.

LAMP assay on wood samples

In order to evaluate the ability of LAMP to detect *H. annosum* in wood, trials were conducted following Li (2014). *Pinus sylvestris*, a cultivar highly susceptible to *Heterobasidion* spp., was selected for this experiment. Six pieces of *P. sylvestris* almost 20 cm long and 30–35 cm in diameter were prepared for this assay. Each piece was disinfected with 75% ethanol, wiped with distilled water, and air dried. Three pieces of wood were inoculated with strains of *H. annosum*; the other three were sprayed only with sterile distilled water. The pieces of wood were incubated in a partially darkened room for five weeks, with the surface kept moist during this period. DNA was extracted from each piece of wood and stored at −80°C until used.

Results

Design of LAMP primers

The primers were designed using the PRIMEREXPLORER V5 software program (http://primerexplorer.jp/lampv5e/index.html) based on GAPDH. Sequences were aligned using MAFFT 7 (https://mafft.cbrc.jp/alignment/server/). Regions conserved among all tested *H. annosum* populations but differentiating between closely related fungal species were selected for the design of LAMP primers (Figure 1). We designed a set of four primers to identify six regions of the target DNA, consisting of two inner primers (a forward inner primer FIP and a backward inner primer BIP) and two outer primers (a forward primer F3 and an outer backward primer B3). Additionally, we designed a loop forward primer (LF) and a loop backward primer (LB) to expedite the LAMP reaction. These primers were synthesized by Sangon Biotech. Nineteen sets of primers were designed for *H. annosum*; the set deemed suitable are listed in Table 2.

Specificity of the LAMP assay

DNA from the isolates and specimens of *Heterobasidion* and others, as listed in Table 1, were used to validate the specificity of the assay. The LAMP primers were found to detect the species of *H. annosum* accurately. A positive reaction is indicated by a color change from violet to blue in the presence of the HNB indicator (Figure 2). GAPDH primers were able to distinguish *H. annosum* from other *Heterobasidion* species, along with fungi commonly detected in wood samples. Based on visual detection using HNB, only samples of *H. annosum* displayed a blue color (Figure 3).

Sensitivity of the LAMP assay

LAMP sensitivity was tested using 10-fold serial dilutions of target genomic DNA prepared with distilled water (10 ng·µL^{-1}, 1 ng·µL^{-1}, 100 pg·µL^{-1}, 10 pg·µL^{-1}, 1 pg·µL^{-1}, 100 fg µL^{-1}, 10 fg·µL^{-1}). A Nanodrop spectrophotometer was used to measure DNA concentration. The results showed that a blue color could be detected up to the point where the DNA concentration was as low as 100 pg·µL^{-1}. However, the color remained violet when the DNA concentration was reduced further to 10 pg·µL^{-1}, 1 pg·µL^{-1}, 100 fg µL^{-1}, or 10 fg·µL^{-1} (Figure 4).

LAMP assay for basidiospore suspensions

The color changed from violet to blue in the suspension of basidiospores from the positive control and other treatments containing 10^4, 10^3, 10^2, 50, or 10 basidiospores per 1 µL.

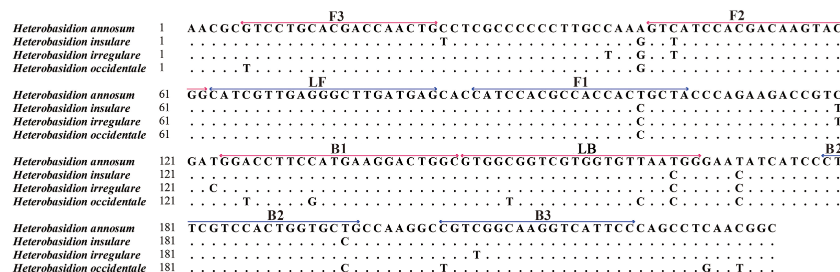

FIGURE 1
Genomic alignment between *Heterobasidion annosum* and *H. insulare*, *H. irregulare*, and *H. occidentale* at the locus selected for design of the LAMP primer sets. The locations of the designed primers (F3–B3) are shown: forward primer FIP includes the F1 and F2 regions; backward primer BIP includes the B1 and B2 regions; and loop primers include the LB and LF regions. (Red color represents the forward primers, and blue color represents the reverse primers).

TABLE 2 Primers used in this study.

Target name	Primer name	Primer sequences	Reference
GAPDH	GAPDH-F	YGGTGTCTTCACCACCACYGASSA	Johannesson et al. (2000)
	GAPDH-R	RTANCCCCAYTCRTTRTCRTACCA	
ITS	ITS5	GGAAGTAAAAGTCGTAAC AAG G	White et al. (1990)
	ITS4	TCCTCCGCTTATTGATATGC	
anno-GAPDH-5	BIP	GGACCTTCCATGAAGGACTGGC-CAGCACCAGTGGACGAAG	This study
	FIP	TAGCAGTGGTGGCGTGGATG-GTCATCCACGACAAGTACGG	
	B3	GGAATGACCTTGCCGACG	
	F3	GTCCTGCACGACCAACTG	
	LB	GTGGCGGTCGTGGTGTT	
	LF	CTCATCAAGCCCTCAACGATG	

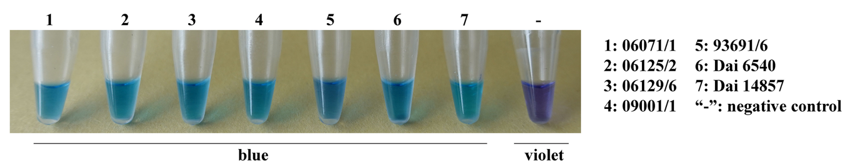

FIGURE 2
LAMP detection of the GAPDH gene in different isolates of *Heterobasidion annosum*. 1–7: *H. annosum* strains; "-": negative control.

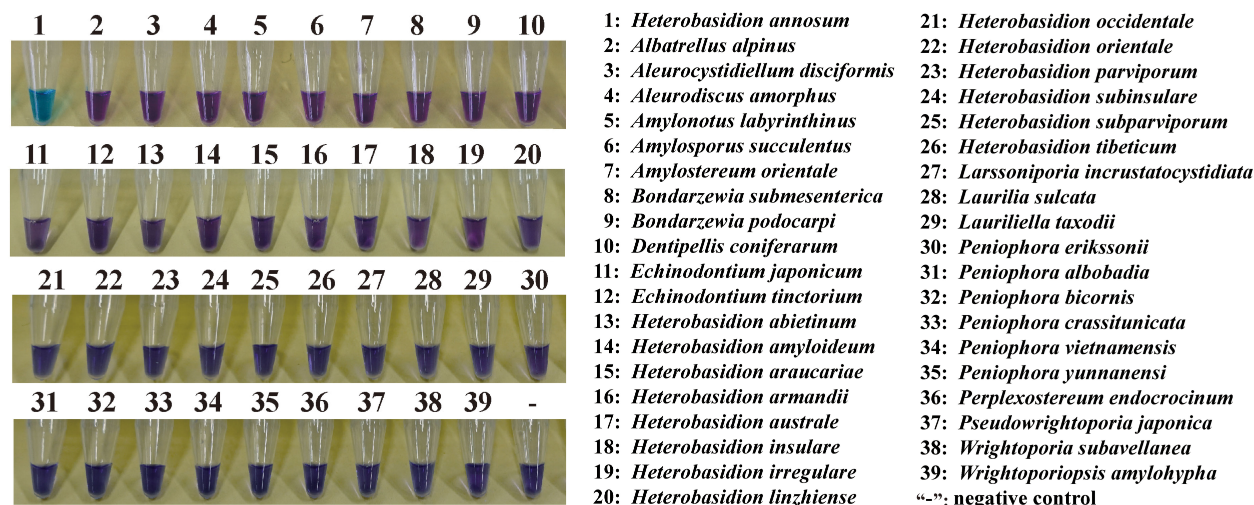

FIGURE 3
Ability of the LAMP assay to distinguish *H. annosum* from other species in Russulales. "-": negative control.

However, in the case of the treatment without basidiospores and the negative control, the color remained violet. This pattern indicated that the LAMP assay could detect the presence of at least ten basidiospores of *H. annosum* per 1 μL in suspension (Figure 5).

Detection in wood

The LAMP assay was applied to samples of wood infected with *H. annosum*. DNA was extracted from diseased pieces of wood

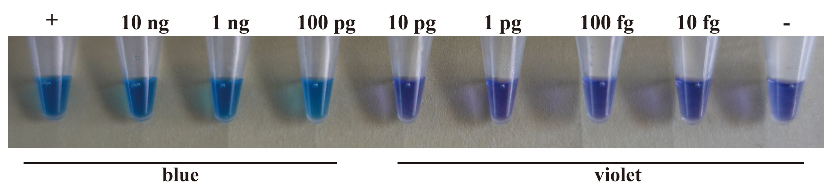

FIGURE 4
The sensitivity of the LAMP assay using a *H. annosum* s.s. 93961/6 DNA concentration gradient. "+": positive control; "-": negative control.

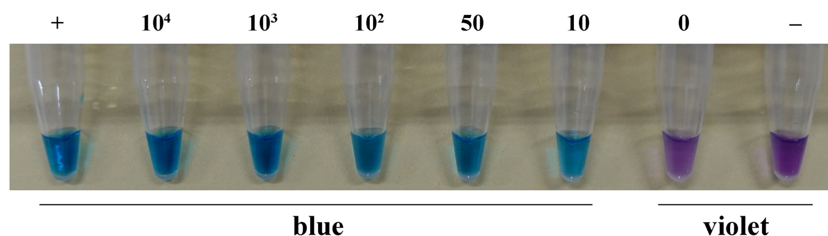

FIGURE 5
LAMP assay detection of *H. annosum* in basidiospore suspensions containing different numbers of basidiospores. "+": positive control; "-": negative control.

under simulated field conditions; as shown in Figure 6, *H. annosum* was successfully detected in diseased wood samples.

Discussion

Detection of wood decay based on symptoms is relatively difficult. Trees are usually asymptomatic for decades after infection by butt rot, much less for root rot. The external symptoms mostly occur after the sapwood of the tree has decayed (Greig, 1998). Omdal et al. (2004) suggested that aboveground variables can be used as reasonable indicators of root disease. However, the detection of infections caused by slow-growing wood pathogens and with less obvious outer symptoms, such as *H. annosum*, often requires considerable professional knowledge, especially to distinguish closely related species.

Several fragments of genes have been described for detection of species of *Heterobasidion* (Fabritius and Karjalainen, 1993; Kasuga and Mitchelson, 1993; Kasuga et al., 1993; Johannesson and Stenlid, 2003; Linzer et al., 2008; Chen et al., 2014, Chen, 2015; Shamoun et al., 2019; Pellicciaro et al., 2021; Yuan et al., 2021). Although the use of PCR techniques is more successful as a method of detection, it still requires specialized equipment and highly trained personnel, and it is difficult and time-consuming to implement the technique in remote areas and ports. A delay in the identification of wood pathogens causes a major threat to wood production and international trade in timber. We have developed a rapid, specific, and sensitive method of detecting wood decay caused by *H. annosum*, based on GAPDH sequences; furthermore we have evaluated the accuracy of this method in detecting this fungus directly on wood samples. The LAMP method is far more convenient and effective for detecting *H. annosum* in time- and resource- limited conditions. This fungus mostly infects pine (*Pinus* spp.), especially *Pinus sylvestris* (Chen et al., 2015), but also it can be associated with other conifer forests, such as *Abies* sp., *Larix* sp., and *Picea* sp. (Korhonen, 1978). Genetic evidence has confirmed the

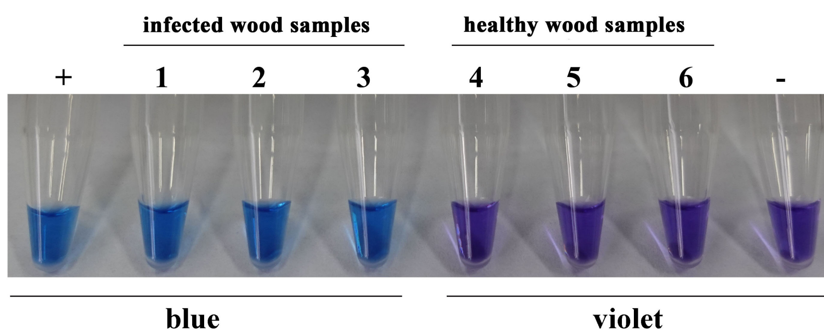

FIGURE 6
LAMP assay detection of *H. annosum* in wood samples. "+": positive control; "-": negative control.

major significance of stump infection by *H. annosum* s.l. (Swedjemark and Stenlid, 1993) in managed forests. The fungus infects freshly cut stumps through the spores and then progresses to the roots, and is able to spread to adjacent trees through root contact (Rishbeth, 1951; Wallis, 1962; Garbelotto and Gonthier, 2013). Thus, our assay may have value during thinning periods in conifer forests.

In general, most PCR amplifications are carried out with a DNA concentration of 20 ng/µL. Conventional PCR amplifications used to detect *Heterobasidion* species are carried out with a DNA concentration of 20 pg/µL (Shamoun et al., 2019). However, the LAMP assay tested in our study was found to detect *H. annosum* with a DNA concentration of 100 pg/µL. With adjustments to the temperature and time, the sensitivity of LAMP assay for detection of *H. annosum* failed to increase. This point necessitates further analysis.

When wood is infected with *H. annosum*, the pathogen may remain active in residual stumps and roots for decades until the next rotation (Rishbeth, 1951; Greig and Pratt, 1976). Significantly, *H. annosum* s.l. grows more quickly in dead trees than living trees. Thus, the method presented here is applicable to the analysis of samples stored for long periods or sent over long distances.

China is one of the biggest timber importers in the world, especially in regard to logs. Conifer logs account for a large proportion of wood imports, and this proportion has climbed from 68.8% to 78.5% since 2017 (Han, 2021). Economic losses to wood decay caused by *H. annosum* should not be ignored, and our LAMP assay provides a quarantine tool for reducing such losses through accurate testing of wood samples.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

Design of the research: ZH-M, YJ, CJ-J; performance of the research: ZH-M, LY; data analysis and interpretation: ZH-M, CJ-J, YJ; collect the materials: DY-C, YY, WC-P; writing and revising the manuscript: ZH-M, CJ-J, YJ, DY-C. All authors contributed to the article and approved the submitted version.

Funding

The research was supported by the National Natural Science Foundation of China (32161143013 & 32070022), the Science Fund of the Jiangsu Vocational College of Agriculture and Forestry (2020kj003 & 2021kj91), and the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (20KJB220003).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1134921/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The LAMP assay at different temperatures. The negative control was performed at 63°C for 60 min.

SUPPLEMENTARY FIGURE 2
The LAMP assay at different times. The negative control was performed at 63°C for 60 min.

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