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Development of loop mediated isothermal amplification for rapid species detection of *Armillaria ostoyae* using assimilating probe

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Abstract: We introduced here the first loop mediated isothermal amplification (LAMP) assay for the identification of honey fungus, *Armillaria ostoyae*, a basidiomycote playing an important role in spruce declines in the Palaearctic region. In total, 101 isolates, representing three *Armillaria* species, were used to develop a new LAMP assay to determine species specific identification. We have here described LAMP primers enhanced with fluorescent dye that are able to amplify *A. ostoyae* DNA and detect fungi in a fast single step reaction. The detection limit of LAMP was 1 pg of genomic DNA per reaction. We optimized a new LAMP assay for the rapid detection of *A. ostoyae* using the translation elongation factor 1-α (*tef1*) marker and fluorescence labelled oligonucleotide assimilating probe. The LAMP assay does not require any specialized equipment, hence it can be used in the field for the rapid detection of *A. ostoyae* even using the portable and mobile device. The specificity of the assay was confirmed by the use of *A. ostoyae* strains and *Armillaria cepistipes* and *Armillaria gallica* strains, respectively. In conclusion, the assay could be a rapid, specific, sensitive and low-cost tool for identification of *A. ostoyae* as well as the first step for expansion of this method in practical applications.

Keywords: detection of fungi; molecular diagnosis; forest pathogen; root rot fungi; spruce decline

Fungal pathogens play an important role in forest ecosystems. The complex *Armillaria/Desarmillaria* comprises approximately 41 species (Klopfenstein et al. 2017; Koch et al. 2017; He et al. 2019b) which are well-known pathogens causing root diseases on trees worldwide resulting in huge economic losses (Baumgartner et al. 2011). Norway spruce (*Picea abies*) is an important host of *Armillaria* sp. (honey fungi) in the Palaearctic region and has suffered a generalized decline enhanced by *Armillaria* in the last years (Cienciala et al. 2017; Holuša et al. 2018).

It is difficult to determine the abundance and species composition of honey fungus populations. Currently, with the progress in the development of molecular methods, *Armillaria* can be directly detected by PCR (Lochman et al. 2004; Antonín et al. 2009; Park et al. 2018). However, the use

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of this assay in real-time surveillance is not practicable as it is not directly applicable in the field due to operational constraints, including time consumption and costs. Therefore, cost-effective rapid DNA amplification methods able to rapidly screen a large number of samples are needed.

Loop mediated isothermal assays may provide a solution to real-time inspection including field use. This method was first described by Notomi et al. (2000). Advantages of LAMP assays include improved sensitivity and specificity, as well as fast reaction times. Thus, it has also become popular for rapid identification of plant pathogens, including fungi (Sillo et al. 2018; Aglietti et al. 2019; He et al. 2019a; Stehlíková et al. 2020). As compared to PCR-based assays, LAMP methods are inexpensive, fast, sensitive and they can be performed in a minimally equipped laboratory (Ghosh et al. 2015).

As far as we know, no report about the LAMP-based method for rapid *Armillaria* species identification has been published to date. The LAMP method has widely been applied to soil, mycorrhizal, and pathogenic fungi, but not yet to *Armillaria* spp. (Ortega et al. 2018; King et al. 2019; Panek, Frac 2019). In the present work, we accomplished several goals: raising awareness of this method useful in the differentiation of *Armillaria* spp., using this method as a preventive technique in soil screening against *Armillaria* pathogens and utilizing this method in forest ecology in general.

MATERIAL AND METHODS

A collection of 100 samples from locations in the eastern part of the Czech Republic and one sample from CCBAS218 (*Armillaria gallica* – Culture Collection of Basidiomycetes, Institute of Microbiology, Czech Academy of Sciences, Czech Republic, http://www2.biomed.cas.cz/ccbas/fungi.htm) were used to test the new LAMP assay (101 in total). The *Armillaria* rhizomorphs were collected from trees that were less than 50 years old. Parts of rhizomorphs were sampled in the basal part of the trunk, 5–15 cm below the soil and placed in separate plastic bags.

We have isolated DNA directly from collected rhizomorphs using the CTAB-PVP method (Porebski et al. 1997). The extracted DNA was suspended in 1X TE buffer and stored at –20 °C for further use. The DNA isolated directly from rhizomorphs was used for PCR and LAMP experiments.

The internal transcribed spacer (ITS) and the translation elongation factor 1-α (*tef1*) regions were amplified from extracted DNA using AR1/AR2 and EF595F/EF1160R primers to confirm the species identification (Kauserud, Schumacher 2001; Lochman et al. 2004). PCR products were subjected to electrophoresis on a 2% agarose gel in 1X Tris/borate/EDTA buffer and were visualized by ethidium bromide staining. Successfully amplified samples were sequenced at SEQme s.r.o (Dobříš, Czech Republic). The sequences were assembled and analyzed by the use of Geneious (Version 8.1.9, 2015). Each sequence was parsed into the primer specific regions (AR1/AR2 and EF595F/EF1160R, respectively) and was then separately used to perform a blastn query against the GenBank database using the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence-based identities with a cutoff of > 98% were considered significant, i.e. belonging to the same species, and the best hit was defined as the sequence with the highest maximum identity to the query sequence.

A set of *Armillaria tef1* gene sequences of representatives of all major European species was obtained from the GenBank database and aligned to determine the pattern of sequence conservation. Sequences included those of target *A. ostoyae* (GenBank accession No. EU251401, MN580142, JN657489) and other related *Armillaria* species, including *A. cepistipes* (MN580151), *A. gallica* (EU251391), *A. ectypa* (EU251403) and *A. mellea* (EU251399). The sequences were imported into Geneious 8.1.9 and primers were determined based on *in silico* analysis using LAMP designer software (OptiGene Limited; Version 1.12, 2017) to design *A. ostoyae* specific primers.

Five *A. ostoyae* LAMP primers were proposed: outer primers F3 and B3, internal primers FIP (consisting of the F1c and F2 sequences) and BIP (B1c and B2), and one loop primer LB. Primer codes mentioned here have been used for convention since the development of LAMP, with primer codes Loop F and Loop B applied in later assays (Notomi et al. 2000). Additionally, we designed an assimilating probe labelled with the TAMRA fluorescent dye (5-carboxytetramethylrhodamin) at the 5' end. Primers, including the probe, were synthesized by Macrogen Inc. (South Korea) and their sequences are given in Table 1.

To determine a quick extraction method for LAMP assay, genomic DNA was extracted from

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Table 1. Designed LAMP primers and the assimilating probe for detection of *A. ostoyae*

Name of the primer	Sequence 5'–3'
F3	TACCTCCCAGGCTGATTG
B3	TCCACTGAGATAACAGTCAGA
FIP[a]	**TCGAGTCTGGCCGTCCTT**TGCCATTCTCATCATCGC
BIP[b]	**TTGGTGTCAGGCAGCTCATC**TAGATCTCGTACCTTGGTGG
LB[c]	TCGCCGTCAACAAGATGG
Probe_LF[d]	TAMRA[e] –ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGA CTTCAAATTCACCAGTTCCACC
Qstrand[f]	TCGGCATCCGCATCCGCATTCGCATCCGGGTCCTCAGCGT – BHQ[g]

[a]bold sequence – F1c; underlined sequence – F2; [b]bold sequence – B1c; underlined sequence – B2; [c]Loop B primer; [d]fluorescence strand of assimilating probe (underlined fragment acts as Loop F); [e]TAMRA – 5-carboxytetramethylrhodamine; [f]quench strand of assimilating probe; [g]BHQ – Black Hole Quencher-1

mycelia using the Plant Material DNA extraction kit (OptiGene Limited, the United Kingdom). All fungal cultivations were performed at 25 °C in the dark. As a medium, we used malt extract agar (48 g·L^{-1}, ME agar, Merck). After three weeks of mycelium growth, DNA was extracted according to the manufacturer's instructions.

The LAMP amplification reaction contained 2.4 µL LAMP primer mixture (0.8 µM FIP and BIP, 0.2 µM F3 and B3 each, and 0.4 µM LB), 15 µL Isothermal Master Mix without intercalating dye (Optigene, Inc., the United Kingdom), 2 µL LAMP probe mixture (Probe_LF and Quench strand at a final concentration 0.3 µM and 0.5 µM, respectively) and 3 µL DNA template (final concentration 2.5 ng·µL^{-1}), filled with DEPC water. The reaction was performed in a 0.2 mL tube at a final volume of 25 µL. The reaction was incubated at 65 °C for 30 min and finally at 80 °C for 2 min to termination. Sterilized water was used as a no template control (NTC). All reactions were performed in triplicates. Real-time LAMP assays were carried out on a QuantStudioTM 6 Flex Real-Time PCR System (Applied Biosystems, USA) and on a handheld device, Smart-DART (Diagenetix, USA).

To test the specificity, assays for *A. ostoyae* were initially tested using DNA samples from the isolates of *A. cepistipes* and *A. gallica*, respectively. To evaluate the sensitivity, the LAMP assays were performed using a 10-fold dilution series of genomic DNA from *A. ostoyae* ranging from 10 ng to 1 pg. Time to threshold (T_t) was calculated using manual baseline settings in QuantStudio Software (Version 1.3; 2015).

RESULTS

In total, 100 collected isolates and one *A. gallica* from CCBAS were tested to develop a new LAMP assay. Based on the ITS/*tef1* PCR amplification, 64 isolates of *A. ostoyae,* 33 isolates of *A. cepistipes* and three *A. gallica* strains were confirmed by the blast against the NCBI database. Together with CCBAS *A. gallica* was used in the LAMP assays.

The *tef1 A. ostoyae* LAMP assays gave unambiguous positive results when tested against DNA from *A. ostoyae* isolates collected at different sites. They produced clear amplification curves (Figure 1). No signal was obtained using either assay when screening other *Armillaria* species (*A. cepistipes* and *A. gallica*, respectively) (Figure 1). The *tef1*-specific *A. ostoyae* primers gave positive results within 14:12–20:41 minutes. No template controls (NTC – water) included in each test ran tested negatively, that is, no amplification curves were observed for *A. cepistipes* or *A. gallica*. When screened against serial dilutions of *A. ostoyae* DNA, the *tef1* LAMP assay could consistently detect down to 1.0 pg (Figure 2). The testing method enabled the fast quantitative estimation of target *A. ostoyae* DNA present in samples. The detection limit of LAMP was 1.6 pg·µL^{-1} of DNA per reaction (Figure 2). The detection limit of conventional PCR with F3/B3 primers was 0.1 ng·µL^{-1} (data not shown). As shown in Figure 1, the results indicated that a positive LAMP reaction occurred only in *A. ostoyae*, while the other species were negative.

This study also employed the handheld device Smart-DART for fast and clear low-cost detection

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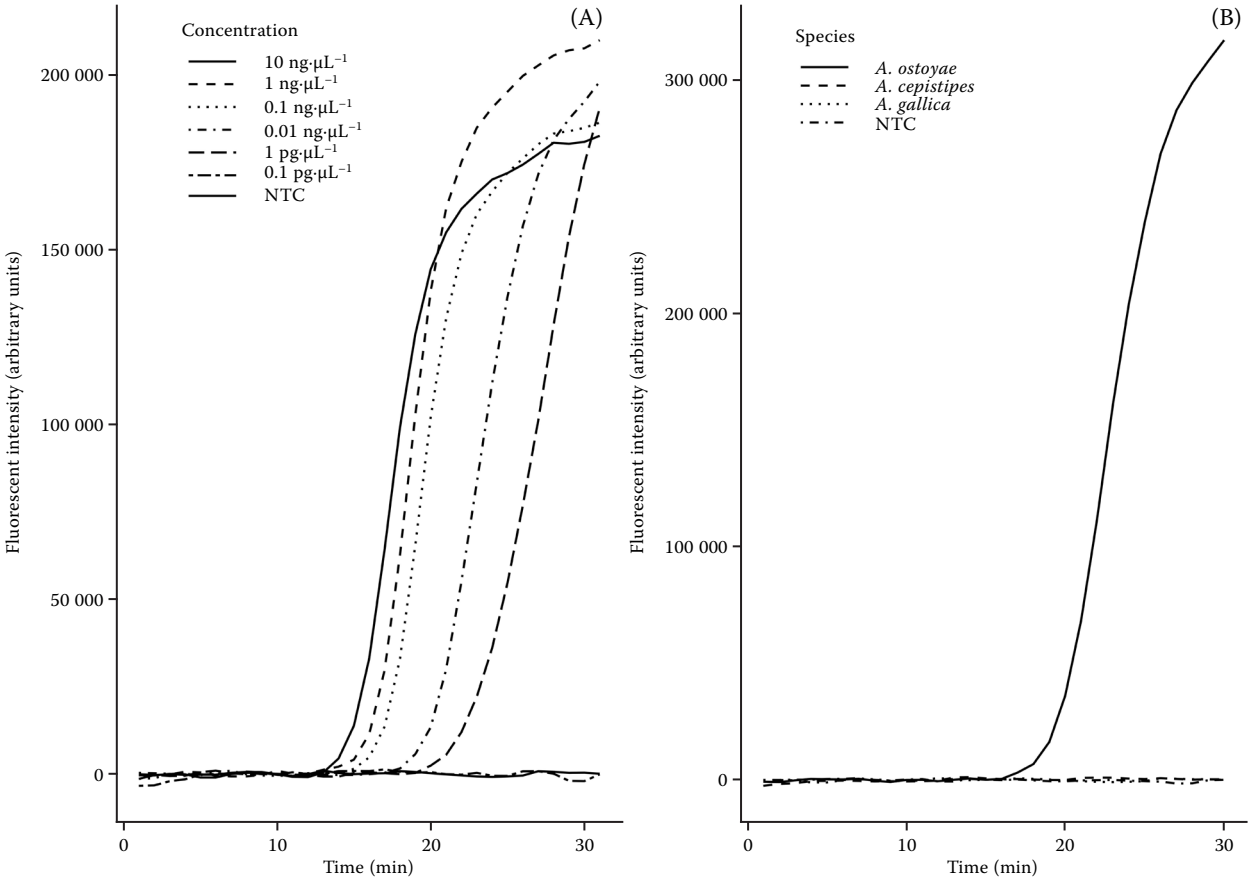

Figure 1. Representative results from screening of *A. ostoyae* with new LAMP primers (each dilution was run in three replicates); (A) specific LAMP amplification curves for serial dilutions of DNA extracted from *A. ostoyae* using the LAMP primers designed for this study for the *tef1* marker of *A. ostoyae*; (B) results of the specificity test of three different *Armillaria* spp.

NTC – no template control

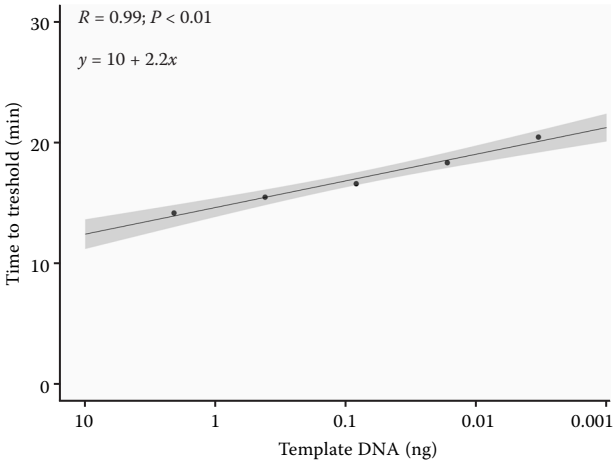

Figure 2. Sensitivity testing of EF1 – α LAMP primers developed in this study; screening against different amounts of *A. ostoyae* DNA [curve was generated by linear regression analysis, plotting the T_t (time to threshold) value in the y-axis vs. the logarithm of the starting DNA dilutions in the x-axis]

of amplified DNA. The results obtained by this system were consistent with those obtained by the Quant Real Studio. Since the detection can be accomplished in a closed system between mobile device and handheld equipment, there is no risk of contamination and this system can be used easily in field conditions.

DISCUSSION

To the best of our knowledge, this is the first report on the application of the LAMP assay for the detection of *A. ostoyae*. Relative to conventional PCR, the LAMP assay reported here is easier to perform, more rapid, and the results are easier to evaluate. The sensitivity advantage of the LAMP over conventional PCR is 10–100 times greater (Wang et al. 2015). Our results showed that the detection limits could be at the pg level for *A. ostoyae*

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genomic DNA. Compared to conventional PCR, the LAMP detection assay was more rapid and simple. The speed of the reaction was enhanced by the use of fluorescent dye in the assimilating probe. Additionally, the use of an assimilating probe with the LAMP assays allows sequence specific detection and it also allows detection in real-time (Kouguchi et al. 2010; Tanner et al. 2012).

Besides PCR and LAMP, quantitative real-time PCR (qPCR) is another molecular technique implemented to identify and quantify fungi (Aslam et al. 2017). This method allows detection and quantification of DNA sequences and is mostly used to detect phytopathogenic fungi in infected plant tissues (Hariharan, Prasannath, 2021). The real-time detection based on species-specific primers and fluorescent dyes is used to scan the reaction steps. Similar to our LAMP experiment with *tef1* primers the quantitative PCR was reported to amplify *tef1* sequences from *Armillaria* species in the northern hemisphere (Baumgartner et al. 2010). Nevertheless, qPCR is a tool for the quantification of nucleic acid in biological samples and is not widely used for the species identification from isolated DNA. For qPCR-based analysis a well-equipped laboratory with fluorescence detection modules is needed. However, in this work the newly developed LAMP assay was reported to identify *Armillaria* species from isolated DNA by the handheld device that can be used in the field.

PCR amplification and sequencing of the *tef1* gene requires an expensive instrument for the thermal reaction, extra time and cost for gel electrophoresis; the species identification period using sequencing of *tef1* usually takes several days and additional cost is needed. For LAMP, the identification only requires a water bath, and the detection can be completed and judged by a pending handheld device, connected to a mobile phone within several hours (Jenkins et al. 2011). Therefore, LAMP detection is faster and requires a lower cost than PCR (He et al. 2019a).

Several publications reported the detection of *Armillaria* fungi using specific primers including the IGS-1 or ITS1/ITS2 regions (Anderson, Stasovski 1992; Chillali et al. 1998). Although these gene regions were successfully used in various phylogenetic studies (Keča et al. 2006; Antonín et al. 2009; Klopfenstein et al. 2017), it was recognized that some of these primer pairs failed to differentiate among *Armillaria* species (Sicoli et al. 2003). Later on, several studies have shown that alternative loci should be considered to distinguish different *Armillaria* species, including these *tef1*, *β-tubulin* or *rpb2* genes (RNA polymerase) (Matheny et al. 2007; Guo et al. 2016; Koch et al. 2017). Therefore for a primer and probe design, we used the *tef1* gene because it provides sufficient species resolution and differentiates *Armillaria* species (Maphosa et al. 2006; Mulholland et al. 2012; Coetzee et al. 2018). However, with PCR amplification, *A. ostoyae* and other related *Armillaria* species are nearly indistinguishable on agarose gels. Their differentiation is complicated and direct sequencing of PCR products takes a long time (Lochman et al. 2004; Kim et al. 2006). Using the LAMP assays with fluorescent probe we were able to differentiate *A. ostoyae* from other species within 14 minutes.

Using the assimilating probe in LAMP reaction was described on different isolates of the pathogen *Ralstonia solanacearum* (Kubota et al. 2011), which enhanced the specificity and speed of LAMP reaction. The LAMP reaction might be facilitated by the addition of assimilating probes to the primers. In the present study, we used LAMP primers together with primers carrying a fluorescent marked probe (Table 1). This improved the reaction time and efficiency.

The results presented in this study demonstrated the use of LAMP to rapidly detect *A. ostoyae* using genomic DNA under controlled *in vitro* conditions, as was described previously for *A. cepistipes* (Stehlíková et al. 2019). Further steps should include testing the specificity of the method described here under more challenging conditions of soil and root material from the environment.

CONCLUSION

In conclusion, we have found that a LAMP assay combined with assimilating probe is simple, rapid, sensitive and specific. Because our LAMP assay does not require any specialized equipment, it can be used in the field for the rapid detection of *A. ostoyae* in connection with mobile device and handheld Android-based equipment. This is the first report on the use of LAMP assay for the detection of *A. ostoyae*. It is a promising assay for the rapid diagnosis of honey fungus infection in the laboratory and it will be very useful for monitoring the *Armillaria* complex in the field prior to suggesting management strategies.

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