A Rapid and Efficient Loop-mediated Isothermal Amplification (LAMP) Assay for the Authentication of Food Supplements Based on Maitake (*Grifola Frondosa*)

Valeria Fochi[1] · **Fabiano Sillo[2]** · **Fabiano Travaglia[1]** · **Jean Daniel Coïsson[1]** · **Raffaella Balestrini[2]** · **Marco Arlorio[1]**

Abstract

Grifola frondosa ("Maitake") is an edible fungus with several nutraceutical properties, largely used in traditional medicine. The increased use of Maitake as a food supplements ingredient raised the need of accurate authentication methods since the morphological identification of *G. frondosa* is not feasible in formulated food supplements. We developed a diagnostic tool based on loop-mediated isothermal AMPlification (LAMP) for the detection of *G. frondosa* in food supplements. First, a modified CTAB protocol for DNA extraction from food supplements has been set up and it has been shown to be able to isolate amplifiable total genomic material from different types of commercial products. Subsequently, the LAMP assay confirmed high specificity and good analytical sensitivity, allowing to detect up to 0.62 pg of genomic DNA in less than 20 min. Ten related fungal species resulted negative, confirming the specificity of the assay. The presence of Maitake in commercial food supplements was confirmed, except for one, revealing a mislabeling (or a food fraud). This assay proved to be a rapid powerful tool for food authentication purposes and routine inspections at any level of the supply chain of Maitake-based products and it can be used as a model for other quality control assays of fungal food products.

Keywords Maitake · Mushroom food supplements · Food authentication · Loop-mediated isothermal amplification · Species identification

Introduction

Mushrooms are higher fungi belonging to Ascomycetes and Basidiomycetes species, able to produce fruiting bodies possessing nutraceutical properties and considered a potential source of novel natural molecules for exploitation in the pharmaceutical industry. Many of these mushrooms are edible and generally show low amount of calories and fats, as well as a high nutritional value (proteins, mineral salts, and vitamins), and for this they are considered ideal for a healthy diet. However, the main characteristic of higher fungi is represented by their richness in bioactive compounds, such as polysaccharides (mainly glucans), terpenoids, phenolic compounds, and lectins, making them nutraceutical valuable (Sharifi-Rad et al. 2020). The world production of mushrooms with nutraceutical value has been estimated to reach $18 billion in the first years of XXI century (Gargano et al. 2017). Currently, the main mushroom producer and consumer is China, covering almost the 75% of the whole-world production and meeting large external and internal market demands (Royse et al. 2017; Ferraro et al. 2020). Due to their putative antioxidant, anticancer, antimicrobial, and immunomodulating properties, mushrooms are included in hundreds of different food supplements called "mushroom pharmaceutical," successfully commercialized worldwide (Lindequist 2013; Gargano et al. 2017). Recently, the number of health-conscious consumers concerned with health benefits linked to the consumed food products has grown. The COVID 19 pandemic contributed to the rise in awareness about the food supplements with immunomodulatory effects to prevent health issues. The demand for functional foods with nutraceutical effects boosted the mushroom market, including those of fungal-based food supplements. In

✉ Marco Arlorio
marco.arlorio@uniupo.it

[1] Dipartimento Di Scienze del Farmaco & Drug and Food Biotechnology (DFB) Center, Università del Piemonte Orientale "A. Avogadro", Largo Donegani 2, 28100 Novara, Italy

[2] National Research Council - Institute for Sustainable Plant Protection (CNR-IPSP), Torino, Italy

the next future, the global mushroom market has been predicted to experience a significant expansion because of this rising awareness of a healthy nutritional lifestyle (Veljović and Krstić 2020).

Nowadays, one of the species, which has gained more and more attention and for which market has started to grow exponentially, is the fungus *Grifola frondosa* (Dicks.) Gray is a Basidiomycetes fungus belonging to the family of Meripilaceae and the order of Polyporales. In nature, as a wood decay agent, this fungus is found to associate to stumps of a wide range of broadleaf trees, and its distribution covers temperate forests in North America, Europe, and Asia (Wu et al. 2021). In several countries, particularly in Asia (e.g., Japan, China), the fungus is cultivated in bottle, bag, and outdoor bed cultures, in order to produce the high value fruiting bodies also know with the japanese name of *Maitake* and the chinese name "*hui-shu-hua*" (gray tree flower) (Mayuzumi et al. 1997). In the last years, this mushroom ranked 11th among all cultivated fungal species in the world in terms of annual output (Chang, 2006) and both fresh and dried Maitake basidiomata are commercialized in Asia, EU, USA, and Canada (Ferraro et al. 2020). Good taste and delicate aroma make this fungus as excellent to be consumed fresh; however, due to its peculiar biochemical profile, *G. frondosa* is not only used as a food ingredient, but also as nutraceutical component in food supplements based on mushrooms, generally in dried powder form (Wu et al. 2021). In fact, *G. frondosa* fruiting bodies are reported to harbor a wide range of bioactive polysaccharides and fractions, such as the β-glucan D-fraction, a complex with about 30% protein, MD-fraction, X-fraction $(1 \rightarrow 6)$-β-glucan with $(1 \rightarrow 4)$-α branches, Grifolan, and α-D-glucan (He et al. 2017). The plethora of effects of the different polysaccharide fractions isolated from *G. frondosa* has been documented, and included immunomodulation, antitumor, antiviral, antidiabetic, and anti-inflammation (He et al. 2017). In recent years, a link between *G. frondosa* polysaccharides and changes in gut microbiota, which play a role in maintaining immune homeostasis and may be related to antitumoral effects, has been also reported (Liu et al. 2019). Moreover, other compounds extracted from *G. frondosa* fruiting bodies and mycelia have shown promising medicinal values, such as the protein components, sterols, and phenolic compounds (Wu et al. 2021).

The high economic value, the growing interest, and the increased use of Maitake in food supplements highlighted the need of authentication methods of *G. frondosa* in mushroom-based products. Food authenticity is currently a subject of great concern to food authorities, since incorrect labeling of foods can represent a commercial fraud and it is subject to sanctions in several countries (Danezis et al. 2016). Moreover, the increased awareness of consumers regarding the composition of foods resulted in a growing interest in the

verification of the labeling statements. Hence, it is pivotal to establish that species of high commercial value declared in product labels are not partially or entirely replaced by other species of lower value. This issue has been often reported for cult food based on high value fungal species (e.g., truffles, "porcini") or yeasts used as starter cultures (Arlorio et al. 1999; Mello et al. 2006; Rizzello et al. 2012). The taxonomic identification of fungal species used in commercial products is therefore a critical need, providing information on samples sold as food supplements, and ensuring quality, correct price value, correct labeling, and food safety (Raja et al. 2017; El Sheikha and Hu, 2018). However, traditional identification methods (e.g., morphology) are not feasible for processed fungi (e.g. dried, milled, lyophilized); also, chemical extraction might disrupt fungal mycelia and associated morphological characteristics (Raja et al. 2017). Molecular methods such as those based on analysis of DNA (e.g., rnd-point/real-time PCR) are now widely and routinely used in food authentication as they are generally efficient, sensitive allowing the fast detection and specific identification of a range of animal, plant, and fungal species in food (Böhme et al. 2019). Recently, DNA-based methods were demonstrated to be also suitable for the authentication of botanical ingredients in herbal food supplements (Grazina et al. 2020). Among them, loop-mediated isothermal amplification (LAMP) is a molecular tool offering fast, accurate, and cost-effective analysis. The LAMP is an isothermal amplification assay which formally works without thermocycler, requiring two sets of primers (external primers F3 and B3, and internal primers, FIP and BIP). The LAMP protocol requires DNA polymerase without exonuclease activity, with strand-displacing activities, in order to generate amplicons containing loop regions to which further primers (namely FL and BL) can bind (Notomi et al. 2000). The LAMP enzyme is rather tolerant to chemical inhibitors often found in environmental samples and complex food matrices compared to standard PCR polymerases, making LAMP assay as feasible for processing DNA extracted from complex raw materials (Tomlinson, 2013) like processed ingredients and foods. The application of LAMP-based assays in screening of food for authentication and safety has started to be popular, and it has been reported for different kinds of food products. Regarding food safety, much attention has been paid to the detection of foodborne pathogens (Zhong and Zhao 2018), genetically modified (GM) food contents (Singh et al. 2019) and allergenic ingredients such as pistachio (*Pistacia vera*) and peanut (*Arachis hypogea*) (Sheu et al. 2018; Mao et al. 2020). For the authentication of food products, the LAMP technique has been largely used for the identification of animal species in meat products (chicken, ostrich, buffalo, pork) (Tasrip et al. 2019), but also to discriminate some medicinal plant species ("botanicals"), like *Curcuma longa*, *Panax ginseng*, *Crocus sativus* (saffron), and *Hedyotis diffusa*, an

ingredient of popular herbal teas (Sasaki and Nagumo, 2007; Sasaki et al., 2008; Zhao et al. 2016; Li et al. 2013).

Here, we report on the development of a LAMP diagnostic assay for the detection of *G. frondosa* in food supplements. The assay has been evaluated for analytical specificity and sensitivity. A DNA extraction method optimized for mushroom-based food supplements has been developed; finally, the assay has been used to assess the presence of *G. frondosa* in several commercial food supplements with diverse chemical composition and different formulation.

Materials and Methods

Investigated Materials

Fungal Samples

In order to evaluate the sensitivity and the specificity of the LAMP assay, ten different fungal species were used. The target species, *Grifola frondosa*, was obtained from a fruiting body acquired in a local market, certified by expert mycologists, and by molecular assay (see below). Nine fungal species were selected as non-target controls: one species, *Ganoderma carnosum* Pat., selected as a closed representative of the Polyporaceae family, was collected in the wild and identified through molecular diagnostic assays. Eight species were selected as they are the most commercialized mushrooms and their carpophores were obtained in a local supermarket: *Agaricus bisporus* (J.E. Lange) Imbach (champignon), *Boletus* spp. (porcino), *Cyclocybe aegerita* (V. Brig.), Vizzini (poplar mushroom), *Lentinula edodes* (Berk.), Pegler (Shitake), *Pleurotus ostreatus* (Jacq.), P. Kumm. (oyster mushroom), *Saccharomyces cerevisiae* (Desm.) Meyen (yeast). Finally, two species of the most common fungi widely distributed in soil and indoor environments were selected, *Fusarium* sp. and *Cladosporium* sp. The two isolates were obtained from agar plates filled with MEA left open for 24 h, in an indoor environment. Isolates were identified as *Fusarium equiseti* and *Cladosporium ramotenellum* by molecular approach outlined below, according to the closest sequences present in NCBI database.

Food Supplements Containing Mushroom

Different commercial food supplements containing fungal ingredients were selected to be tested with the developed assay. Supplements were both commercial complex formulation and powdered mushroom blends. These products were acquired at local or online pharmacies and stores. Names, declared quality/quantity composition and formulation

(powder, capsules or compression tablets), concerning each sample, are listed in Table 1.

Genomic DNA extraction

Total genomic DNA extraction from all fungal samples as well as from commercial products was performed following the CTAB method described by Doyle and Doyle (1990) with some modifications. Briefly, powdered materials obtained from fungal carpophores and mycelia (approximately 20 mg), fungal supplements (60–80 mg), or dried-powdered fungal carpophores (20 mg) were mixed with 1 mL of CTAB extraction buffer (2% of CTAB (w/v), 0.1 mol L^{-1} of TrisHCl, 1.4 mol L^{-1} of NaCl, 0.02 mol L^{-1} of EDTA, pH 8.0), previously added with 2% PVP (w/v) and 1% PVPP (w/v). After the addition of 2% of β-mercaptoethanol, the mixture was incubated at 65 °C for 1 h in a thermal block under continuous stirring (400 rpm).

All samples were firstly purified by liquid–liquid extraction with phenol/chloroform/isoamyl alcohol (25:24:1 v/v) and later with chloroform/isoamyl alcohol (24:1 v/v) two times. DNA precipitation was performed with 0.7 volume parts of isopropanol and the solution was incubated for 1 h at 4 °C. DNA was dissolved in 50 µL of distilled water and all extracts were immediately kept at − 20 °C until further analysis. DNA concentration and purity were assessed using a NanoPhotometer® P-Class (Implen).

LAMP Primers Design

The primers set for the LAMP assay was developed using the PrimerExplorer V5 software (http://primerexplorer.jp/lampv5e/index.html), and the internal transcribed spacers of nuclear ribosomal DNA (ITS1-5.8S-ITS2) sequence was selected as a target region (hereafter referred as "ITS"). To obtain a specific region for *Grifola frondosa*, ITS sequences of this fungus (see below) were compared to 66 ITS sequences from closely related non-target species obtained from the National Center for Biotechnology Information (NCBI) GenBank database (Table S1). Fungal species belonging to the Polyporaceae family and closely related to *G. frondosa* have been identified thanks to the phylogenetic study of Justo et al. (2017). Six available ITS sequences of different isolates of *G. frondosa* (*GBPOL1291-13*, *GBPOL2259-14*, *GBPOL2668-15*, *GBPOL320-13*, *NOBAS1417-15*, *NOBAS1859-16*) were also selected from the Barcode of Life (BOLD) database (https://www.boldsystems.org/index.php/databases) to avoid intraspecific nucleotide diversity that could prevent an accurate detection of the target species. All selected sequences were aligned using the software MEGA X (Kumar et al. 2018) using ClustalW algorithm and a small region (222 bp) showing high nucleotide diversity

Table 1 Name, composition, and formulation of the commercial food supplements containing *G. frondosa* used in the LAMP assay

Name	Composition	Formulation	
I₁	**Maitake *(Grifola frondosa)* sporophore dry extract** titrated at 25% in polysaccharides, cellulose, magnesium salts of fatty acids, capsule casing (hydroxypropyl methylcellulose).	Capsule	
I₂	**Maitake (*Grifola frondosa*) sporophore dry extract** titrated at 30% in polysaccharides, **Maitake (*Grifola frondosa*) sporophore powder,** maize starch, capsule casing (hydroxypropyl methylcellulose).	Capsule	
I₃	**Sporophore dried extracts of Maitake (*Grifola frondosa*)** titrated to 25% in polysaccharides, Shiitake (*Lentinula edodes*) titrated at 10% in polysaccharides and Reishi (*Ganoderma lucidum*) titrated at 30% in polysaccharides; Rosehip (*Rosa canina*) cinorrodi dry extract titrated at 70% in vitamin C; Inulin, magnesium salts of fatty acids, silicon dioxide, capsule casing (hydroxypropyl methylcellulose).	Capsule	
I₄	**Maitake (*Grifola frondosa*) powder.**	Powder	
I₅	Reishi (*Ganoderma lucidum*) powder 40%, **Maitake (*Grifola frondosa*) powder 30%**, Shiitake (*Lentinula edodes*) powder 30%.	Powder	
I₆	***Grifola frondosa* biomass** (mycelium and young primordia) **powder**, microcrystalline cellulose, silica, vegetable magnesium sterate.	Tablet	
I₇	***Grifola frondosa* sporophore powder** ≥ 30% total polysaccharides, ≥ 15% β(1,3-1,6)-D-glucans, capsule casing (hydroxypropyl methylcellulose).	Capsule	
I₈	*Agaricus Blazei Murrill, Ganoderma Lucidum* (Reishi), ***Grifola Frondosa* (Maitake)**, *Lentinula Edodes* (Shiitake) **dry extracts** titrated in: polysaccharides ≥ 30%, triterpenes ≥ 5 mg, *Mapighia Glabra L.* (Acerola), *Polygomun Cuspidatum* (Fallopia japonica) dry extracts, polydatin, sorbitol, trehalose, xylitol, vegetable magnesium sterate, silicon dioxide.	Chewable tablet	
I₉	Maltodestrin, *Agaricus blazei Murril* sporophore, *Cordyceps sinensis* sporophore titrated at 7% in cordiceptic acid, **Maitake (*Grifola frondosa* Dicks. Gray) sporophore**, Reishi (*Ganoderma lucidum* Curtis P. Karst.) sporophore; Shitake (*Lentinuta edodes* Berk Pegler) sporophore, tartaric acid, isomalt, silicon dioxide, sodium carbonate, sucralose.	Powder in sachet	
I₁₀	**Maitake *(Grifola frondosa Dicks. Gray)* lyophilized biomass** (mycelium, primordia, sporophore), capsule casing (hydroxypropyl methylcellulose).	Capsule	

between species and no intraspecific diversity was selected for the primers design. A set of five oligonucleotide primers, including two outer primers (forward primer F3 and backward primer B3), two inner primers (forward inner primer FIP and backward inner primer BIP), and only one loop primer (forward loop primer LoopF) were developed following the standard parameter sets of the software. The key factors in the LAMP primer design are the Tm (about 65 °C for each region), the stability at the end of each primer, the GC content (between about 40 and 65%), and secondary structure. The distance between the different primers is also important: the region amplified by the LAMP method is considered to be between 120 and 180 bases, the distance for loop forming regions is 40–60 bp and the distance between F2 and F3 (and B2 and B3) is between 0 to 60 bases. Primers were synthesized by Metabion international AG and the sequences are listed in Table 2.

Table 2 Primers used in the present study

Target gene	Primer name	Sequences (5′-3′)
ITS1	Gf F3	ACGAGTTCAGAAAAGGGTT
	Gf B3	CGTTTTAATTATCGCAATGACATTC
	Gf FIP	AGTGCACAGGTGTGAGAGTG-GTA GCTGGCCTCAAATCC
	Gf BIP	TCTGTAGGTCGGTTCGGGAT-CTG AAGCGTTTGTGATTGT
	Gf loop	GGGTCGGGTTCTGTGCCTT

PCR Assays

Qualitative PCR amplification was performed to each DNA (i) to evaluate the suitability for amplification, particularly for DNA from food supplements, and (ii) for confirming the identity of the fungal species selected for the specificity test. The reaction was carried out in 25 µL of total volume containing 1.5 µL of DNA isolated from fungal carpophores or food supplements, 1×PCR Mix Plus (A&A Biotechnology) and 0.5 µM of each primer ITS1F–ITS2 (White et al. 1990; Gardes and Bruns 1993). PCR reactions were carried out in a T100 Thermal cycler (Bio-Rad, Milan, Italy) using the following program: denaturation step of 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C of 45 s, and a final extension at 72 °C for 5 min. The amplified products were then analyzed by electrophoresis on 1.5% TBE agarose gel (Bio-Rad, Milan, Italy), stained by Atlas ClearSight DNA Stain (Bio-atlas, Tartu, Estonia). The agarose gel was visualized under UV light using Multimage II-AlphaImager® HP (Alpha Innotech, CA, USA) and digital image was obtained with Alpha View—AlphaImager® HP software.

Real-Time LAMP Assay

LAMP reactions were performed in a total volume of 25 µL containing 0.25 µL of each external primers F3 and B3 (200 nM), 0.25 µL of each internal primers FIP and BIP (500 nM), 0.125 µL of the loop primer FL (500 nM), 15 µL of Isothermal Mastermix ISO-001 (OptiGene, UK), and 1 µL of fungal DNA template or 1.5 µL of commercial products DNA. LAMP assays were carried out using an ABI PRISM 7900 HT Sequence Detection System (Life Technologies, Monza, Italy) equipped with fluorescein (FAM) reading channel. All reactions were carried out at 65 °C for 40 min (with a read plate every minute) with a melting curve analysis step (ramp from 80 to 95 °C with a temperature increment of 0.1 °C and a read plate every 10 s).

The LAMP products were visualized as amplification curves in a plot where the C_t value of 1 corresponds to one min.

G. frondosa LAMP Primers Specificity Assay

The genomic DNA extracted from nine different fungal species, including the target one (*G. frondosa*), and non-target species, was used in the LAMP assay for specificity determination. DNA amplifiability of non-target samples was first assessed in PCR using ITS1F-ITS2 primers, following the procedure described above (section "*PCR Assays*"). For LAMP reactions, about 10 ng of DNA for each target and non-target species was used as template. Two technical replicates were performed for each biological sample.

Sensitivity Assessment of the LAMP Assay

The sensitivity of the LAMP assay was determined using a tenfold serial dilution of the genomic DNA isolated from a *G. frondosa* carpophore, ranging from 25 ng/µL to 25×10^{-5} ng/µL. Five technical replicates were tested for each dilution. To simulate the influence of the matrix effect in food supplements, the sensitivity of the assay was also assessed by diluting the genomic DNA of *G. frondosa* in an extract of mushrooms commercial product without Maitake between its ingredients instead of water, using a tenfold serial dilution as above. The LOD, calculated according to Shrivastava and Gupta (2011), was defined as follows: LOD = *3.3×(SD/s)*, where *SD* is the standard deviation of the *y* intercept and *s* is the slope of the curve. The coefficient 3.3 was used assuming a 95% confidence level (Shrivastava and Gupta 2011).

Purification, Sequencing and BLAST Analysis of PCR Products

In order to confirm the identity of fungal samples used in this study and to verify that the PCR products of the LAMP assay corresponded to the target species *G. frondosa*, Sanger sequencing of amplicons was performed. For this purpose, all the DNA samples were initially amplified using both the ITS1F/ITS2 and ITS1F/ITS4 primers. However, the primers pair with ITS4 (those usually used for the DNA barcoding of the ITS region in fungi) was unable to amplify the DNA isolated from the food supplements, probably depending on the partial degradation of the fungal DNA correlated to processing and on the "matrix effect" (excipients). So, only the PCR products obtained using ITS1F-ITS2 primers and with the external LAMP primers F3-B3 were analyzed, after being purified using the Wizard® SV Gel (Promega Corp., Madison, WI, USA) and PCR Clean-Up System (Promega) following the manufacturer's instructions. The purified DNA was eluted in 30 µL of nuclease free water and Sanger sequencing of the purified PCR products was performed at Sequencing Service of the Genomics Service Unit (Munich) using BigDye Terminator v3.1 cycle sequencing. The

sequences were obtained bidirectionally using both strands with a combination of the following primers: ITS1F (Gardes and Bruns 1993)/ITS1 and ITS2. For both sequencing reactions, approximately 5 µL of PCR template was used along with 3-µM sequencing primers. Sequences were assembled with Sequencher 5.2.3 (Gene Codes, Ann Arbor, MI, USA), optimized, and then corrected manually when necessary; the latter step was performed to ensure that proper base calls were assigned by the algorithm. Each sequence was subjected to an individual Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) search to verify nucleotide identity against a sequence database. BLASTn search was employed using nucleotide collection (nr/nt), excluding uncultured/environmental sample sequences.

Results

Development of Specific LAMP Primers for the G. frondosa Species

The multiple alignment of *G. frondosa* ITS1-5.8S-ITS2 nuclear ribosomal DNA sequences with the same genomic regions of other related fungal species allowed us to identify a region of 222 nucleotides with high interspecific variation and no intraspecific variation within the isolates of the target species. On this specific region of *G. frondosa* sequence, a set of LAMP primers were developed as shown in Fig. 1.

In order to evaluate the specificity of the new set of primers, DNA samples isolated from ten different fungal species, including *G. frondosa*, were used for the LAMP assay (Fig. 2a). Only the DNA of the target species *G. frondosa* was successfully amplified with the newly developed LAMP primers, confirming the specificity of the assay. No amplification curves were detected for all the nine non-target species (Fig. 2b) and melting curve analysis confirmed the absence of non-specific amplicons, both for non-target species and the negative control (Fig. 3).

Analytical Sensitivity and Limit of Detection (LOD) of the LAMP Assay

In order to calculate the analytical sensitivity of the LAMP assay, ten-fold serial dilutions were prepared starting from genomic DNA extracted from a *G. frondosa* carpophore (positive control) and were amplified with the LAMP primers. Results showed that *G. frondosa* detection time ranged from 7 to 13 min (Fig. 4a). The limit of detection (LOD) of the assay resulted 0.62 pg/µL.

To estimate the analytical sensitivity of the *G. frondosa* LAMP assay in commercial food supplements, the LOD was also calculated using target DNA diluted in a
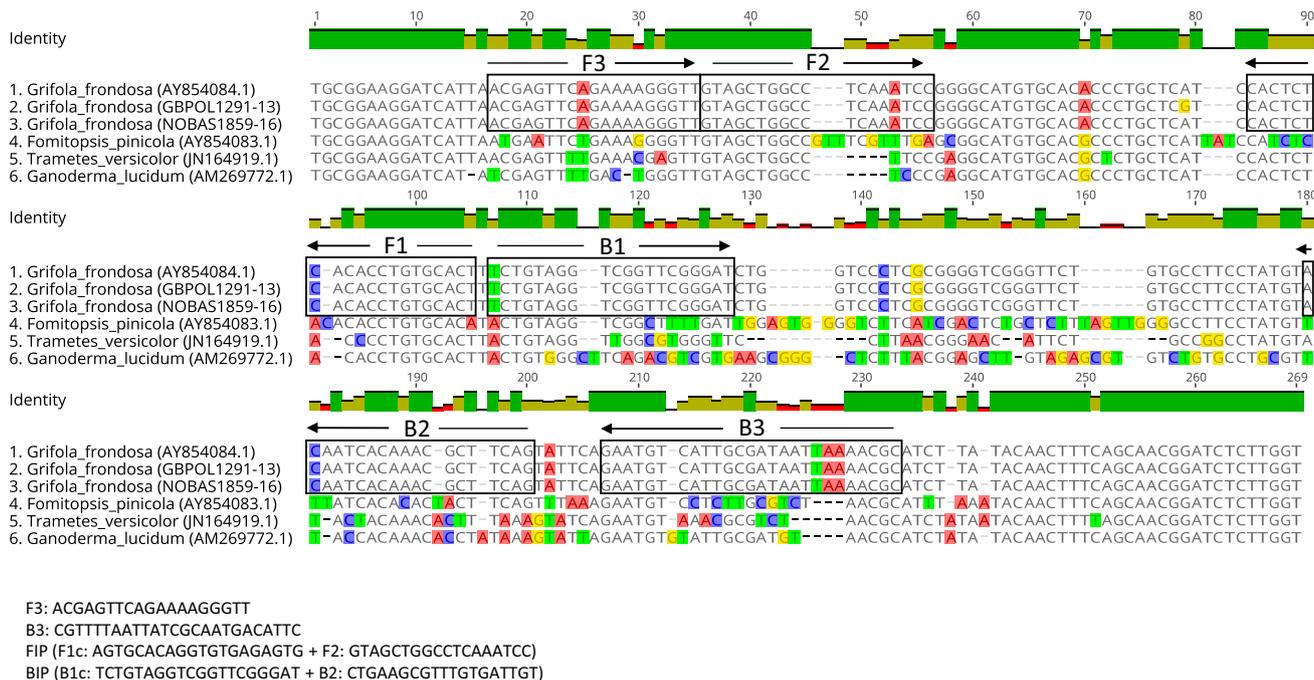

F3: ACGAGTTCAGAAAAGGGTT
B3: CGTTTTAATTATCGCAATGACATTC
FIP (F1c: AGTGCACAGGTGTGAGAGTG + F2: GTAGCTGGCCTCAAATCC)
BIP (B1c: TCTGTAGGTCGGTTCGGGAT + B2: CTGAAGCGTTTGTGATTGT)

Fig. 1 Multiple alignment between the internal transcribed spacer (ITS) of *G. frondosa* and related species for the design of the specific LAMP primer sets. In the boxes are represented the six loci (F3, F2, F1, B3, B2, B1) of *G. frondosa* ITS where the primers are located. The identity between the ITS sequences of the selected species is indicated with a green bar and polymorphic sites are highlighted in colors. The arrows indicate the direction of the LAMP amplification

Fig. 2 Specificity of the LAMP primers for the identification of *G. frondosa*. **a** Amplicons obtained from genomic DNA extracted from carpophores of ten fungal species analyzed with ITS1-2 primers. **b** Plot showing the real-time amplification curve of the positive control (*G. frondosa* carpophore DNA), while no amplification is visible for non-target species (*A. bisporus*, *Boletus* spp., *Cladosporium* sp., *C. cylindracea*, *Fusarium* sp., *G. carnosum*, *L. edodes*, *P. ostreatus*, *S. cerevisiae*)

Non-target controls
Ab: *Agaricus bisporus*
Po: *Pleurotus ostreatus*
Sc: *Saccharomyces cerevisiae*
Gc: *Ganoderma carnosum*
Le: *Lentinula edodes*
Ca: *Cyclocybe aegerita*
Bsp: *Boletus spp.*
Fsp: *Fusarium sp.*
Csp: *Cladosporium sp.*
 - : Negative control

Gf: *Grifola frondosa*
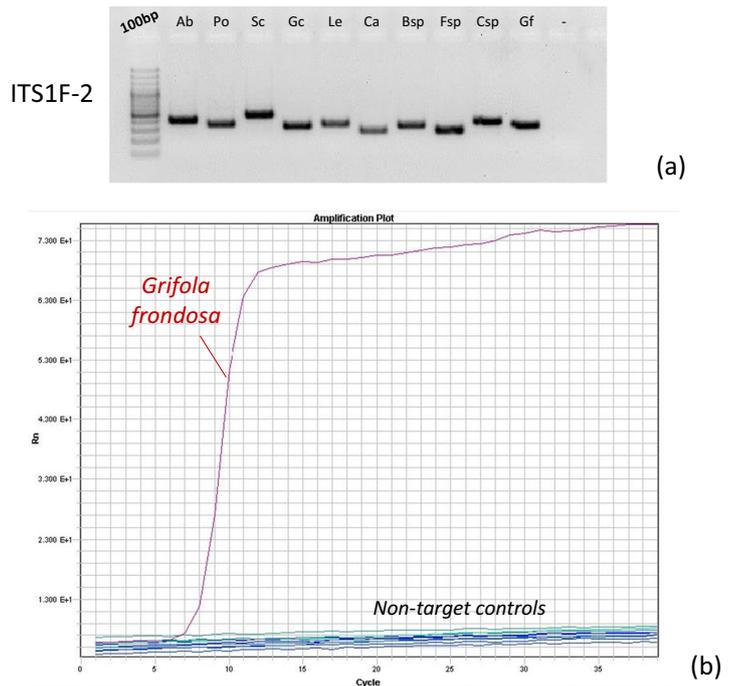
Fig. 3 Plot of the melting curve of *G. frondosa*
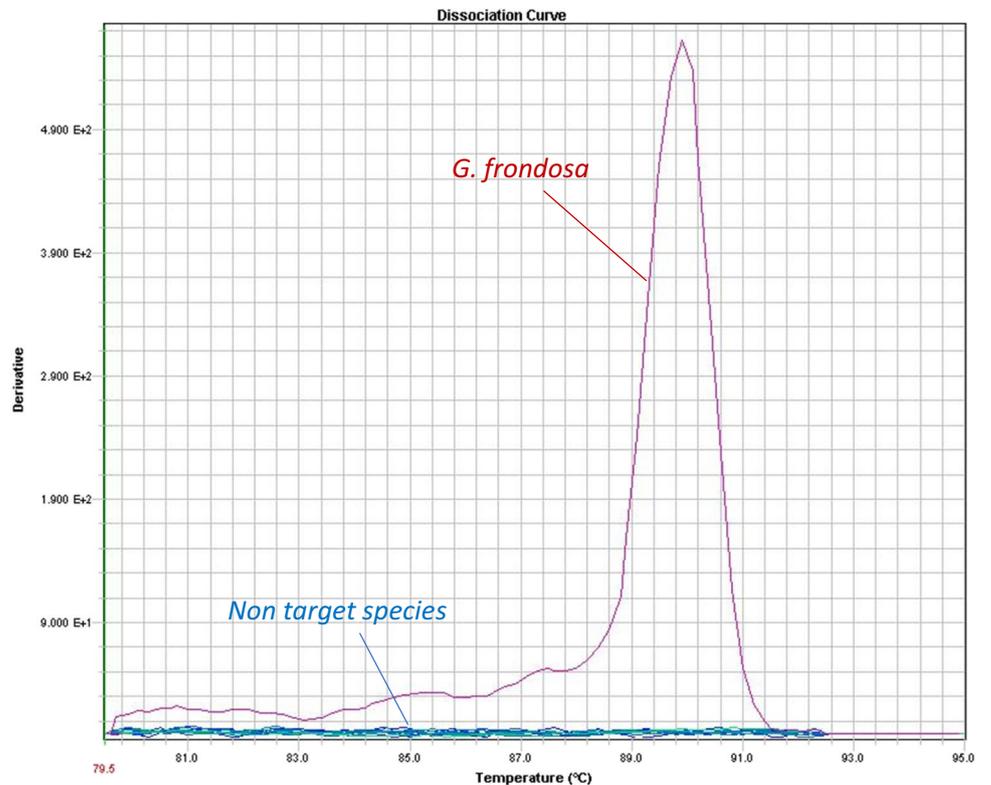
supplement extract. In this case, our method allowed the detection of about 2.5 pg of target DNA with a time of detection of 16 min (Fig. 4b). The LOD of the assay on DNA diluted in a commercial food supplement extract was slightly higher (0.70 pg/μL) compared to LOD resulted from sensitivity assay on water. Standard curves based on time of detection (C_t) and DNA concentration (pg) of analytical sensitivity assay on water and commercial food supplements showed R^2 of 0.99 and 0.95, respectively (Fig. 5).
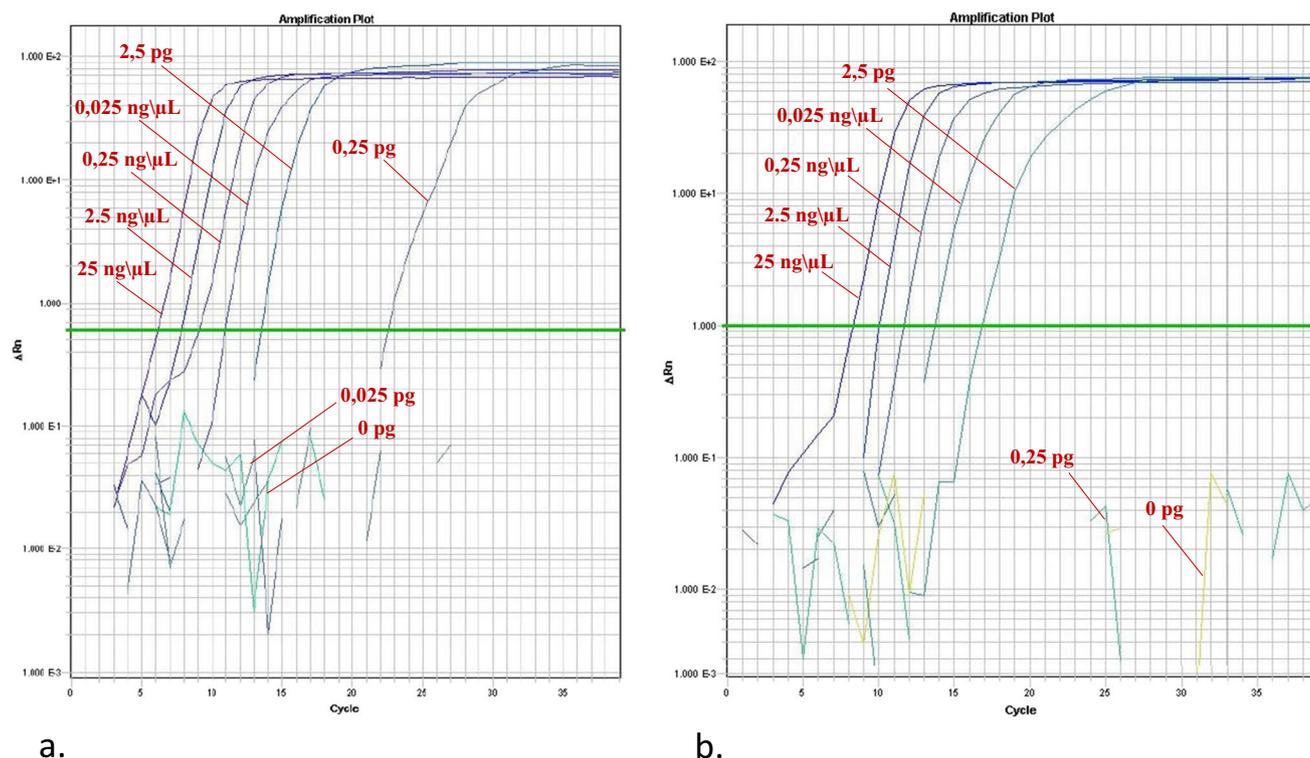
Fig. 4 Sensitivity of the LAMP assay for *G. frondosa* DNA. Amplification curves of a serial tenfold dilution of the genomic DNA extracted from a carpophore of *G. frondosa* (from 25 ng/μL to 0.2 pg/ μL). The target DNA was analyzed diluted in water (plot a) and in a supplement extract (plot b) to simulate the matrix effect of a food supplement

Application of the LAMP Assay for the Detection of G. frondosa in Mushrooms Food Supplements

Ten different commercial products containing *G. frondosa* were selected and purchased to assess if the LAMP assay can be suitable for the detection and the authentication of food supplements containing Maitake mushrooms. Various types of commercial products were selected to evaluate the impact of different processing steps and formulations (especially pre-processing like drying or lyophilization, the composition of excipients as well as the presence of DNA of other fungal species) on the isolation of a DNA with sufficient quality to allow downstream applications. The CTAB modified method used in this study for the DNA extraction resulted to be a suitable protocol for the isolation of nucleic acids from mushroom-based food supplements. Prior to the LAMP amplification, the total genomic DNA extracted from all types of commercial products was tested in traditional PCR with the ITS1F-ITS2 primer set. All the DNA samples were successfully amplified with this primer set (Figure S1). Once the amplifiability of the DNA has been ascertained, the LAMP assay was performed on all samples. Using the ITS-based LAMP primers developed herein, *G. frondosa* was detected in

nine out of ten commercial products that were labeled to contain Maitake, confirming also the effectiveness of the DNA extraction method. An amplification plot for each type of mushroom product (capsule, powder, tablet, and chewable tablet) was created, including a positive control (*G. frondosa* DNA) (Fig. 6). Regarding the first type of supplements, the capsules, all analyzed products showed a positive signal in the LAMP assay within a time of detection of less than 20 min, except for the sample I2 that resulted as negative (Fig. 6a). All the samples in powder showed an amplification signal ranging from 8.8 to 35.3 min, with the sample I9 showing a time of detection of 35.3 min ± 0.85 (Fig. 6b). Finally, all tablet samples showed positive signals: the tablet (I6) showed a very low time of detection, around 10 min (Fig. 6c), while the chewable tablet (I8) showed a time of detection around 30 min (Fig. 6d). Sequencing of some representative amplicons (sample I6 and I7) confirmed that the positive signal obtained in food supplement samples belong to *G. frondosa*, after blastn analysis. The sequence obtained from the sequencing of ITS region from I2 sample, resulting negative to LAMP assay, showed the best match (100% nucleotide identity, *e*-value 4^{e-91}, query coverage 95%) with a sequence of a *Boletus* sp. isolate (accession number AB821462.1).

Fig. 5 Standard curves generated by LAMP assay targeting *G. frondosa* in the analytical sensitivity assay, for water (blue line) and food supplements (red line) matrices. Values of R^2, slope, and intercept are reported
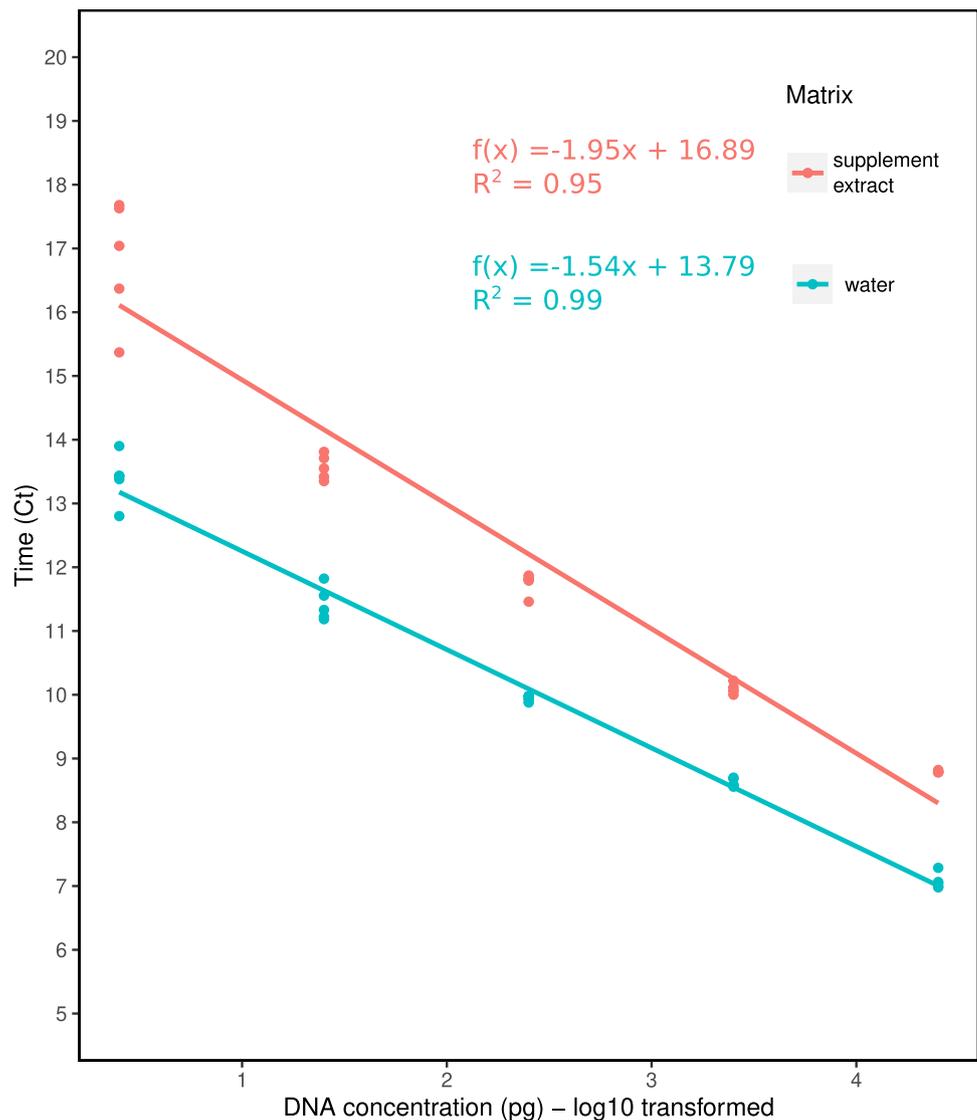
Discussion

Thanks to their richness in bioactive compounds and their traditional use in Asian medicine, fungi are increasingly used for health purposes, namely in food supplements area, and they have become an important source of bioactive compounds and nutraceuticals (Cateni et al., 2021). As the market for mushroom-based products grows, so does the need for more rapid and accurate controls to prevent fraud. Little attention has been put on this problem and very few works take advantage of molecular, DNA-based techniques to authenticate nutraceuticals containing mushrooms (Loyd et al., 2018; Raja et al., 2017; Zhang et al., 2020).

The goal of this study was the set-up and validation of a rapid and specific LAMP assay, coupled with an optimized protocol for DNA extraction from food supplements, aimed

to quickly detect and authenticate Maitake mushroom in fungal-based food supplements.

Extraction of DNA from these complex products, in which either mycelium and fruiting bodies of mushrooms are present in the form of extracts or dried biomass, was successfully achieved by the optimization of a CTAB-based method. It should be highlighted that the treatments to which the mushrooms are subjected to prepare the final product (food supplement), as well as the presence of excipients, might significantly affect both quantity and integrity of fungal DNA, and its subsequent amplification. The CTAB modified method used in this work was demonstrated to be effective in the extraction of DNA from all the different type of mushroom-based products. Compared to the commercially available DNA purification kits, the protocol allowed the processing of a larger quantity of starting sample (up to 80 mg), resulting in a satisfying extraction yield. As
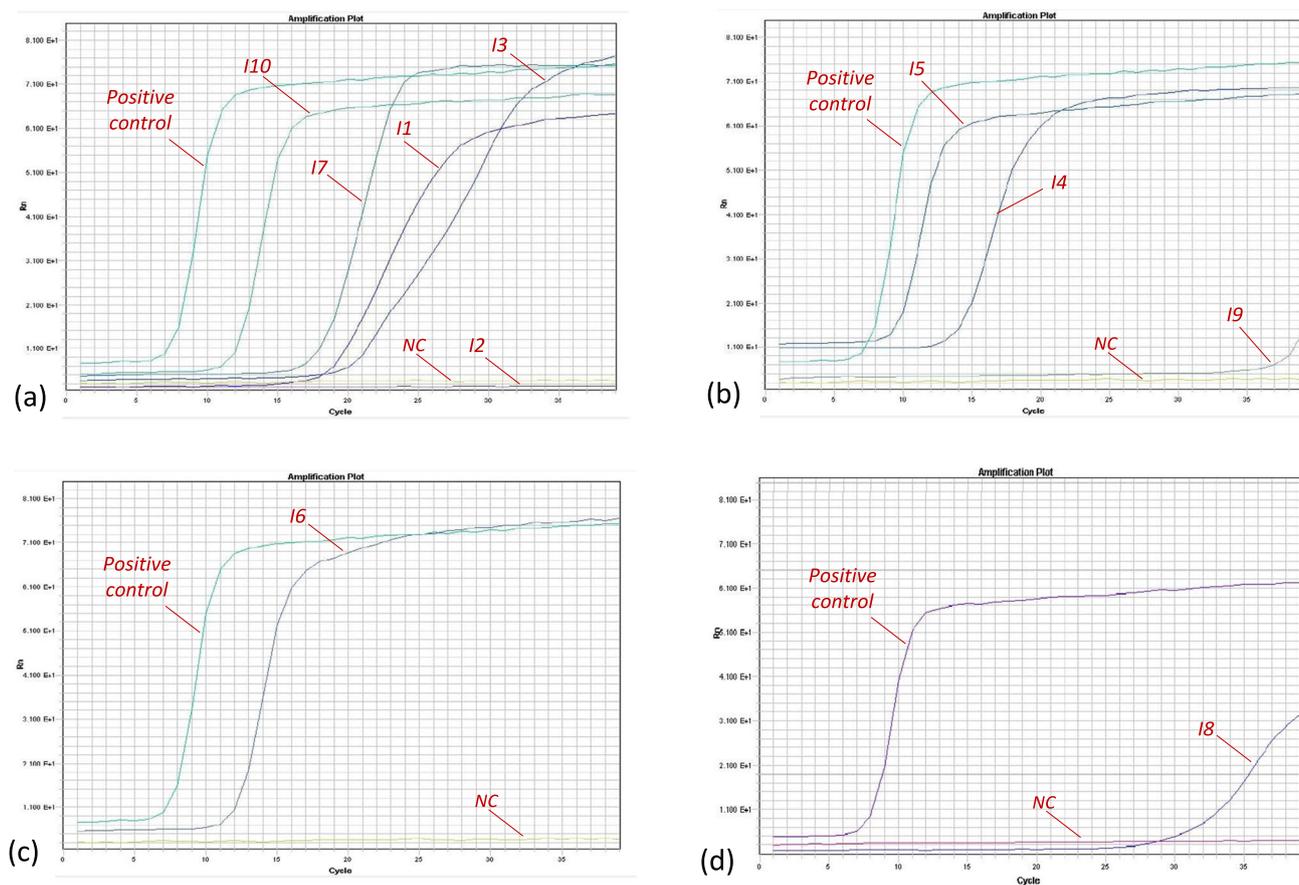
Fig. 6 Application of LAMP assay for the detection of *G. frondosa* in mushroom food supplements. An amplification plot was generated for each type of supplement: **a** capsule—*I1*, *I2*, *I3*, *I7*, *I10*; **b** powder— *I4*, *I5*, *I9*; **c** tablet—*I6*; **d** chewable tablet—*I8*. *G. frondosa* DNA isolated from a carpophore was used as positive control in each plot. *NC*, negative control

demonstrated by Lu et al. (2018), the DNA extracted from botanical extracts is often fragmented (fragments of less than 300 bp), implying that DNA amplifications were possible only with the use of primer pairs producing small amplicons (such as the ITS1F-ITS2 primer couple, designed on the repeated ribosomal genomic DNA). This important aspect was taken into account for the selection of the *G. frondosa* species-specific locus prior to LAMP primers design, and a short region (222 bp) of the ITS sequence was used for the purpose. Internal Transcribed Spacer (ITS) region of nuclear DNA was selected as candidate locus because it is one of the recognized universal barcode markers for fungal kingdom and has become the most sequenced region to identify fungal taxonomy at species level (Schoch and Seifert 2012). Our results showed that the newly designed set of ITS-based primers allows a specific identification of Maitake DNA in commercial products based on fungal ingredients. Both the forward primers (F3 and FIP) and the backward ones (BIP and B3) were designed to cover a region with high number of polymorphic sites between species, allowing specific detection of the target species (*G. frondosa*) and avoiding

any potential cross reaction with nine selected fungal species, as demonstrated by the specificity assay. The LAMP assay coupled with real-time PCR detection system allowed to obtain clear positive amplification results for the target control *G. frondosa* in less than 20 min. Our data showed that the minimum concentration of target DNA detected by the LAMP assay was 0.62 pg/µL and that a putative matrix effect due to the composition of a supplement (excipients and presence of other species) could affect negatively the sensitivity of the assay only to a small extent (0.70 pg/µL). As already demonstrated in several studies, the LAMP technique, compared with the conventional PCR assay, shows a higher sensitivity and is more tolerant to well-known PCR inhibitors, demonstrating to be an ideal molecular technique for identifying botanical ingredients (Zhao et al., 2016; Mao et al., 2020). In Table 3, we summarize the pros and cons of both LAMP and traditional DNA barcoding of ITS region.

In this study, we have successfully developed and applied LAMP technique for the identification of Maitake ingredient in different types of commercial food supplements. In nine out of ten products, it was possible to detect the DNA of *G.*

Table 3 Pros and cons of LAMP and traditional DNA barcoding methods

	PROS	CONS
LAMP	Target approach LAMP doesn't require thermal cyclers *Bst* DNA polymerase has higher tolerance to sample inhibitors compared to traditional TAQ polymerase LAMP assay is fast (less than 30 min) High sensitivity and specificity LAMP can be carried out as colorimetric assay and the detection can be also done with naked eyes by measuring the turbidity or by using specific dyes	Primer design and optimization constraints Require specific reagents and equipments (e.g., detection system) Only one (or few) target species can be detected in the assay Unsuitable for unknown fungi
DNA barcoding of ITS region	Untargeted approach DNA Barcoding can be used to identify unknown fungi Several species can be detected	Time-consuming It generally requires DNA of higher quality compared to LAMP assay Require access to sequencing facilities/services

frondosa, confirming what was declared on the label. The time of the detection varied according to the type of treatment that the fungus has undergone: products containing Maitake-dried carpophore were detected earlier than those with fungal extract, and lyophilized mushrooms showed a time of detection lower than mushrooms subjected to other types of drying. The most difficult products to analyze were those containing excipients and composed from different fungal species (I3, I8, I9). In these, the DNA of *G. frondosa* was successfully detected beyond 30 min. Only one sample (I2), a food supplement labeled to contain Maitake as the only fungal ingredient, resulted as negative after the LAMP assay. Sequencing of ITS sequence amplified from DNA of sample I2 revealed that the amplified fungal DNA belong to *Boletus* spp., an edible cultivated fungus, and more common (at least in Europe, where the food supplements I2 was produced) than *G. frondosa*. According to our LAMP assay and the sequencing of fungal DNA of the sample, *G. frondosa* DNA resulted absent in the I2 sample; however, we cannot exclude a presence of *G. frondosa* DNA below the LOD of the assay (0.62 pg). On the other hand, the presence of *Boletus* spp. suggests that this fungus replaced *G. frondosa* as main ingredient of I2 sample, or that the fungus contaminated the sample during transformation processes (e.g., during milling). Others authors have shown that DNA-based approaches can be useful to detect species substitutions in consumer products containing fungi: for example, Raja et al. (2017) found that many of the products labeled with *Cordyceps sinensis* and *Ganoderma lucidum* (Reishi) contained other fungal species. These studies confirm the usefulness of the molecular analysis in order to detect and fight food frauds in food supplements area, or simply to detect involuntary mislabeling and cross-contamination of fungal food ingredients. In fact, our results demonstrated that LAMP is an efficient tool to detect mushroom ingredients in food supplements and a novel molecular technique is provided for the rapid identification of *G. frondosa*, the famous Maitake mushroom. Given the importance of the correct use of ingredients for the quality assurance of food and food supplements, this strategy—which combines the speed and efficiency of LAMP and the accuracy of ITS barcode—may be extended to other nutraceutical products that need to be certified at the species level, and it can be used for routine inspections at any level of the production chain. In addition, as already demonstrated in plant pathogen studies (Sillo et al. 2018), LAMP assay can be also performed using in-field portable equipment, such as the Genie® II from OptiGene, becoming a possible practical solution for surveillance and monitoring throughout the food production chain also in laboratories not equipped with qPCR instruments.

Conclusions

In the present study, a LAMP assay based on the ITS gene was developed for the specific, sensitive, and rapid identification of *Grifola frondosa*, also known as Maitake mushroom. With the developed LAMP primer set, less than 1 pg of *G. frondosa* DNA could be detected without cross-amplification. This novel molecular approach can be applied for heat-processed Maitake products and for commercial food supplements to perform their authentication and prevent food fraud and adulteration.

Declarations

Conflict of Interest Valeria Fochi declares that she has no conflict of interest. Fabiano Travaglia declares that he has no conflict of interest. Jean Daniel Coïsson declares that he has no conflict of interest. Raffaella Balestrini declares that she has no conflict of interest. Marco Arlorio declares that he has no conflict of interest.

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