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Molecular detection of *Lecanicillium fungicola* on white button mushroom (*Agaricus bisporus*), using species-specific primer set

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Abstract

The dry bubble disease (caused by *Lecanicillium fungicola*) is the most threatening disease of cultivated button mushrooms throughout the world. Most of the symptoms shown by the dry bubble are also shown by the wet bubble (*Mycogone perniciosa*) and they may even be confused. Early detection and precise monitoring of the diseases are very important for managing effective treatments. A PCR-based assay for a specific diagnosis of the disease in various stages of the disease development was developed. A primer pair designed based on ribosomal DNA generated two bands, 650 and 800 bp, specific for the detection of *L. fungicola*. Additionally, a simple and time-saving method for direct extraction of DNA from the affected sporophores is presented and the impact of sample pre-treatment on the efficiency of DNA isolation is highlighted. Here, we also report *Simplicillium lamellicola* from Iran, using the nuclear ribosomal internal transcribed spacer (ITS) region.

Keywords: DNA extraction, dry bubble disease, molecular markers, *Simplicillium lamellicola*, wet bubble

شناسایی مولکولی بیماری حباب خشک قارچ دکمه‌ای با استفاده از آغازگرهای اختصاصی

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خلاصه

بیماری حباب خشک (ناشی از قارچ *Lecanicillium fungicola*) یکی از مهلک‌ترین بیماری قارچ دکمه‌ای (*Agaricus bisporus*) در سراسر جهان است. بسیاری از علایم این بیماری با یکی دیگر از جدی‌ترین بیماری‌های قارچ دکمه‌ای (بیماری حباب تر ناشی از *Mycogone perniciosa*) مشابه است. تشخیص سریع و به موقع می‌تواند نقش تعیین‌کننده‌ای در مدیریت این بیماری ایفا نماید. برای تشخیص اختصاصی *L. fungicola* یک جفت آغازگر با توانایی تکثیر دو باند ۶۵۰ و ۸۰۰ جفت بازی از ناحیه rDNA طراحی شد که در هر مرحله‌ای از توسعه بیماری قادر به تشخیص آن می‌باشد. در این تحقیق همچنین، روش ساده‌ای برای استخراج مستقیم دی.ان.آ. از کلاهک‌های قارچ دکمه‌ای ابداع شد و تاثیر پیش‌تیمار بافت کلاهک بر کارایی این روش مورد بررسی قرار گرفت. به علاوه در این مقاله، گونه *Simplicillium lamellicola* با استفاده از آغازگرهای عمومی ناحیه آی.تی.اس. برای قارچ‌ها از ایران گزارش می‌شود.

واژه‌های کلیدی: استخراج دی.ان.آ.، بیماری حباب تر، نشانگر مولکولی، واکنش زنجیره‌ای پلیمراز، *Simplicillium lamellicola*

Introduction

Dry bubble disease (caused by *Lecanicillium fungicola*) and wet bubble disease (caused by *Mycogone perniciosa*) are among the most threatening problems for commercial cultivation of white button mushroom (*Agaricus bisporus*) and *Lecanicillium fungicola* has two aggressive varieties. In Americas, *L. fungicola* var. *aleophilum* is the sole causal agent, whereas in Europe and Iran *L. fungicola* var. *fungicola* is responsible for the dry bubble disease (Gams & Zaayen 1982, Gams 1995, Bonnen & Hopkins 1997, Collopy *et al.* 2001). So far, *L. fungicola* var. *aleophilum* has not been reported from Iran (Zare & Khabbaz-Jolfaei 2005). Most of the symptoms of dry bubble disease are also shown by the wet bubble disease and they may even be confused by an experienced eye on mushroom growth bed especially in the initial stages of the disease development. Generally, the symptoms are varied from small necrotic lesions on the cap or stipe of the fruiting bodies to partially deformed fruiting bodies called stipe blowout or distorted and undifferentiated masses of mushroom tissue. Although, some of the symptoms are more common with one disease than the other, however, the frequency of the symptoms would greatly be affected by the environmental conditions (i.e., humidity, evaporation) and growth stages of the infected mushrooms. The primary source of the pathogens is contaminated casing soil especially peat carrying fungal propagules (Ware 1933, Beyer *et al.* 2005, Fletcher & Gaze 2007, Rokni *et al.* 2016). In Iran, imported peat from Europe forms only a fraction of the casing and most of the materials are provided from different domestic sources. Currently, control management of the bubble diseases relies mainly on chemical and mechanical methods (Rokni *et al.* 2016). Development of fungicide resistance in the pathogens, risks to human health and negative effects of many chemicals on *A. bisporus* are among the limitations on the use of chemicals for the control (Bollen & Van Zaayen 1975, Wuest *et al.* 1975, Fletcher & Yarham 1976, Gea *et al.* 2005, Mehrparvar *et al.* 2013). Therefore, early detection and precise monitoring of the diseases are very important for managing effective treatments. The polymerase chain reaction (PCR) sensitivity and accuracy have made it a fast and reliable method for the detection of fungi associated with the diversity of organisms or habitats. PCR-based techniques using fungal nuclear ribosomal DNA (rDNA) loci have proven rapid, sensitive, and specific by providing an alternative method for diagnosis and identification. Between coding regions (i.e., 18S, 5.8S, and 28S) of nuclear rRNA genes are the internal transcribed spacers 1 (ITS1) and 2 (ITS2) regions which evolve more rapidly and possess characteristics that are useful for the detection of pathogens at the species level (White *et al.* 1990, Hibbett 1992, Johannesson & Stenlid 1999). Using the sequence of a randomly amplified polymorphic DNA (RAPD)-PCR product, Romaine *et al.* (2002) developed a polymerase chain reaction (PCR)-based test specific for detection of *L. fungicola* var. *aleophilum* (Lfa) from mushroom infected fruit bodies. Zijlstra *et al.* (2008) used a set of primers and TaqMan MGB Probe Fluorescence Real-Time Quantitative PCR for detection of *L. fungicola* var. *fungicola* and *L. fungicola* var. *aleophilum* from allegedly trace amounts of target DNA extracted from casing soil or any other potential sources. Ten sets of primers that were designed from rDNA region for specific detection of *L. fungicola* by Piasecka (2010) amplified the same size products for other ascomycetous fungi. Taxon-selective ITS amplification has also been used for the detection of *Verticillium* spp. (Nazar *et al.* 1991). This study was initiated to develop a sensitive and reliable PCR assay for the selective detection of *L. fungicola* from the infected white button mushroom fruit bodies.

Materials and Methods

- Fungal isolates and biomass preparation

Four isolates of *Lecanicillium fungicola* (*Lf*-Jd, *Lf-B*, *Lf*-P, and *Lf*-M) with specific symptoms of dry bubble and an isolate (*Lf*-S) from infected mushrooms which did not exhibit the common symptoms of bubble diseases were collected from seven mushroom farms

located in Tehran, Karaj, and Dezful (Iran) in 2014. The isolates were selected according to different sources of casing materials. They were then examined both morphologically and physiologically to confirm their initial identification and purity by microscopic examination and tests of growth at 30 ˚C for intraspecific varietal distinctions (Zare & Gams 2008). Morphological studies were performed by measuring 40 conidia and colony growth across two diameters in 14 days of incubation at 22 ˚C in triplicates. Statistical analyses were carried out by one-way ANOVA model with statistical software SAS, Ver. 9.4, and the comparison of means by Duncan's multiple range tests. *Mycogone perniciosa* was also isolated from infected mushroom samples with typical symptoms of the wet bubble. Microscopic characterization was also performed using the description provided by Brady and Gibson (1976). Some prevalent fungal flora of casing soil were also isolated by using serial dilution method on water agar (WA) culture medium (supplemented with 2% glucose and 200 mg/L streptomycin sulfate). A soil sample was used for the purpose, made by a mixture of equal amounts of casings from different above-mentioned sources. Characterization and identification of the isolates were performed by microscopy following the mycological literature (Kiffer 2011). For fungal biomass of *A. bisporus*, fungal materials, commercial cultivar Syl-737 (Jolgeh-Dez Mushroom Agricultural Co., Dezful, Iran), were tissue-cultured under aseptic condition by placing small sections of internal mushroom cap tissue without surface sterilization on potato dextrose agar media (PDA) (supplemented with 5 g/L yeast extracts plus 200 mg/L streptomycin sulfate) (Rokni *et al*. 2015). The cultures were incubated for two weeks at 25 ˚C and then purified by subculturing on PDA and kept at 2–5 ˚C for further studies.

- DNA extraction

The efficiency of DNA isolation from filamentous fungi, intact and infected fruit bodies were assessed according to Doyle & Doyle (1990) and Safaie *et al*. (2005). The material used was either fresh mycelia of fungal samples grown in 50 ml potato dextrose broth for 5–10 days at 25 ˚C in a rotary shaker at 100 rpm, harvested by filtration through a piece of filter paper and rinsed with distilled water, or from intact/infected tissue of fruit bodies. The fungal materials (100 mg) were ground in liquid nitrogen, then, 500 µL of extraction buffer [Tris-HCl, 100 mmol/L, EDTA, 5 mmol/L (pH = 7.5–8.0), and NaCl, 1.4 mmol/L] or [2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA (NaOH pH 8), 100 mM Tris (HCl pH 8), 0.2% w/v ß-mercaptoethanol, 1% (w/v) PVP-40)] were added to the ground samples. The same subsequent steps were then performed for both methods. The samples were incubated at 65 ˚C in a water bath for 30 min and then placed on crushed ice for 10 min, then 500 µL of chloroform-isoamyl alcohol (24:1 w/v) was added and the tubes were mixed by gently shaking, then centrifuged for 10 min at 9000 g, and 300 µL of the aqueous phase was transferred into a new tube. Then, 210 µL of cold isopropanol was added and mixed by inverting the tubes 20–30 times. The tubes were then centrifuged for 15 min at 15600 g and 300 µL of 70% ethanol was added after discarding the supernatant. DNA was collected by centrifugation for 2 min at 15600 g. The DNA concentration was adjusted to approximately 200 ng/µL and the samples were stored at –20 ˚C. The aliquots (2 ml) of DNA from each sample were loaded on a 1% (w/v) agarose gel supplemented with DNA safe stain to check the quality.

- Molecular characterization of the isolates

The universal ITS (ITS 1 and ITS 4) primers (White *et al*. 1990) were used for DNA amplification of both *Lf*-Jd and *Lf*-S isolates. The sequencing of amplicons was performed by Takapou Zist Inc. (Tehran, Iran) and subsequently deposited in GenBank. Similarity checks were done at NCBI using the nucleotide BLAST tool. For further analysis, ITS sequences of closely related sequences were downloaded from NCBI, alignments were done by Clustal W implemented in MEGA 4.1 and the phylogenetic tree was reconstructed using the Maximum Likelihood method to estimate evolutionary distances between DNA sequences (Tamura

et al. 2007). The sequences of *Verticillium dahliae* were used as outgroup.

- PCR primers

In this study, we used rDNA sequence analysis to design selective primers for *Lecanicillium fungicola* detection. Online internet tools were used for identifying primer sequences. Using the BLAST tool, FASTA format of nucleic acid sequences within the rDNA regions of the selected fungal species were retrieved from GenBank (www.ncbi.nlm.nih.gov). To ensure the adequacy of sample size, some common soil inhabitant fungi belonging to diverse taxonomic groups were added to the isolated species of this study (section 2.1). Multiple alignments of the target sequence (i.e., *L. fungicola*) with those of related fungal reference strains (i.e., *A. bisporus*, *M. perniciosa*, *Trichoderma* sp., *Cladobotryum dendroides*, *Aspergilus* sp., *Penicillium* sp., *Rhizopus* sp., *Mucor* sp., *Aphanocladium* sp., *Fusarium* sp., and *Geotrichum* sp.) were constructed with the program ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). Aligned sequences were manually searched for unique sequences appropriate for use as *L. fungicola* specific oligonucleotides. Through comparisons of the aligned sequences, several potential primer target sites were determined for *L. fungicola* with the least amounts of nucleotide similarity with other fungal genera. Primers were then evaluated for self- and hetero-dimerization, hairpins, as well as annealing temperatures and GC contents using Oligo analyzer 3.1 (http://www.idtdna.com). Ultimately, among several sets of primers evaluated both theoretically and practically for their selective amplification of the target sequence, PCR conditions for a set of primers designed were optimized and consequently, the working value of primers was assessed for specific detection of *L. fungicola* from the affected *A. bisporus* tissue. Primers Lec-F (5'-GTC GAA AGG CCA GC-3') and Lec-R (5'-TGC TTT AAT AAG CCA CTC AG-3') showed high efficiency for specific identification of *L. fungicola* from the affected mushroom tissue. While theoretical annealing temperatures were determined as 52 ˚C in the process of primer design, gradient temperatures between 50–54 ˚C were assessed to determine actual optimal annealing temperatures and primers specificity using gradient-capable head Bio-Rad thermal cycler. The amplicons were then migrated and visualized in 1% (w/v) agarose gel supplemented with DNA safe stain to confirm optimal temperature as determined by the brightest band. The efficiency and fidelity of the primers in producing a constant banding pattern were then validated by all the *L. fungicola* isolates. Ultimately, the practical value of primers was assessed for specific detection of *L. fungicola* in the infected tissue of *A. bisporus.* To verify the sensitivity of primers, DNA extractions were performed from fruit bodies at two different infection stages, i.e., initial and later stages of necrosis. Tissue sampled from non-infected caps served as healthy control.

- PCR amplifications

All primers were synthesized by Takapou Zist Inc. (Tehran, Iran). For polymerase chain reactions (PCR) each amplification (20 µL) contained a 100 ng DNA template and 10 pmol of each primer, 0.2 mmol dNTPs mix, 1 U high yield *Taq* DNA polymerase (Jena Bioscience), and 2 µL 10× PCR high-yield buffer. The amplification started with an initial denaturation step at 95 ˚C for 3 min, followed by 35 cycles, each consisting of 30s at 95 ˚C, 30s at appropriate annealing temperature for the primer pair being used, and 45s at 72 ˚C. A final extension step was performed at 72 ˚C for 5 min. The procedure was repeated several times to ensure the specificity, sensitivity, and reproducibility of the PCR using all the *L. fungicola* isolates. For ITS primers, the reaction started with 5 min of 95 ˚C followed by 35 cycles of denaturation at 95 ˚C for 30s, annealing at 62 ˚C for 30s and extension at 72 ˚C for 1 min. The reaction was finished by an extension step at 72 ˚C for 8 min. The quality of PCR was checked by electrophoresis in 1% (w/v) agarose gel supplemented with SYBR DNA Stain (Jena Bioscience). The fragment sizes of amplicons

were estimated based on a DNA ladder mix using UVIDOC Ver. 99.02.

Results

- Intraspecific varietal distinctions of *Lecanicillium fungicola* isolates

Both *Lecanicillium fungicola* var. *fungicola* and *L. fungicola* var. *aleophilum* have similar conidiophores and morphologically are indistinguishable. However, the only area of distinction is the physiological difference of the continuing growth of *L. fungicola* var. *aleophilum* at 30 ˚C (Zare & Gams 2008). As a result, except for *Lf-S*, the growth of all the tested isolates (i.e., *Lf*-Jd, *Lf-B*, *Lf*-P, and *Lf*-M) was stopped at 30 ˚C (Fig. 1 a,b).

ANOVA results approved significant differences among colony sizes (Table 1) and mean comparisons once again ratified *Lf*-S in a separate group (Table 2). Furthermore, polyhedral crystals that are commonly present in agar culture of *L. fungicola* were not observed in the culture of *Lf*-S by microscopy and the size of conidia [2.9–(4.7)–7.7 × 1.2–(1.6)–2.1 µm] was slightly shorter than those described by Zare & Gams (2008). Although colony shape is not a discriminating factor for *Lecanicillium* species and they share many similarities, however, unlike flat colonies of the other isolates, those of *Lf*-S had a cottony, convex, and erumpent appearance (Zare & Gams 2008, Nonaka *et al*. 2013) (Fig. 1b). PCR amplification of all isolates using *L. fungicola* var. *aleophilum* specific primers (Romaine *et al.* 2002) did not amplify the expected fragment (Fig. 2a). DNA similarity searches of *Lf*-S and *Lf*-Jd ITS sequence regions identified them as *Simplicillium lamellicola* and *L. fungicola* var. *fungicola* with true homologies of 99% and 98%, respectively. Phylogenetic relationships of both isolates and the related species were reconstructed from maximum likelihood analysis of rDNA ITS region sequences (Fig. 2b). Sequences reported in this paper are deposited in the GenBank database.

- Efficiency of DNA extraction buffers

Both DNA extraction buffers were examined several times for isolation of DNA from filamentous fungi and intact/infected tissue of fruit bodies during the study. High quality DNA yield was obtained by using CTAB buffer from all samples which were prepared from biomass of examined fungi. An effective protocol for DNA isolation from intact *Agaricus bisporus* fruit bodies was described by Rokni *et al*. (2015). We concluded that, the method could be effectively used for DNA isolation from mushroom infected tissues. However, the reproducibility of the method significantly relies on sampling quality. For this purpose, an intact/infected sporophore is cautiously cut into halves, without surface sterilization. Using a sterilized sharp edge, some pieces of internal tissue are gently cut off and sliced into small pieces. The sliced tissues were then let to be semi-dried by keeping overnight at room conditions. Finally, 100 mg of the prepared sample was used for DNA extraction (Fig. 3). As a result, high-quality genomic DNA could be directly extracted from the intact/infected mushroom tissue with this sample preparation method following the protocol described by Rokni *et al*. (2015).

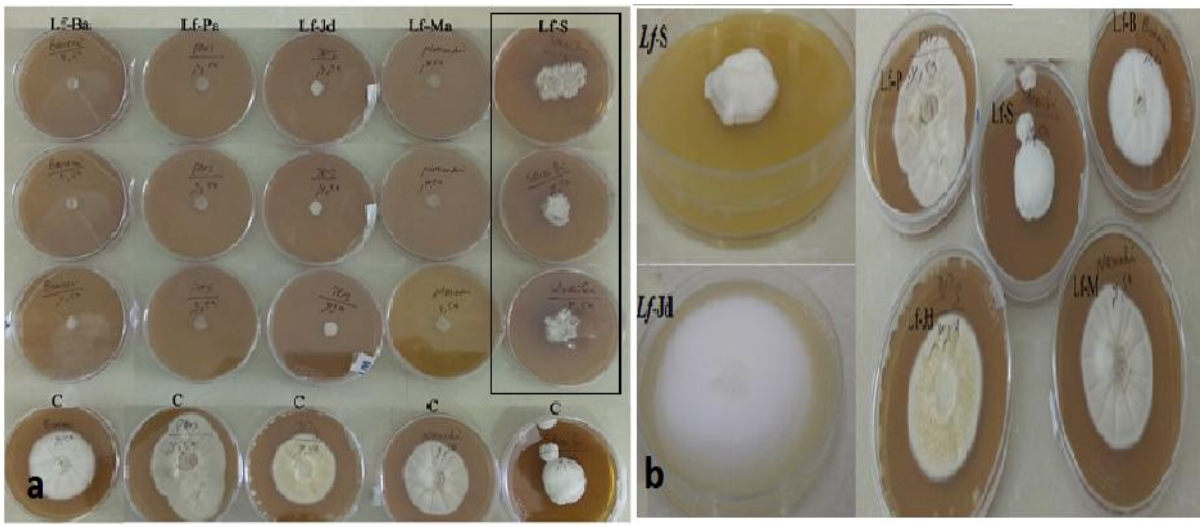

Fig. 1. The evaluation growth rate of *L. fungicola* isolates at 30 ˚C: a. The growth of all isolates, except for *Lf*-S was stopped at the given temperature. The bottom row (control treatment) along each column represents the normal growth of each isolate at 22 ˚C, b. Colony shape difference of the isolates. *Lf*-S: erumpent, convex, and cottony; others with a flat appearance on PDA culture media.

Table 1. ANOVA results for comparison of colony sizes of *Lecanicillium fungicola* isolates after incubation for 14 days at 22 ˚C

Source	DF	Sum of squares	Mean square	F value	Pr > F**
Treatment	4	984.990667	246.247667	**34.01	< 0.0001
Error	10	72. 40667	7.240667	-	-
Total	14	1057.397333	-	-	-

Accession Nos: KT898392 and KT898393

Table 2. Mean comparison of colony sizes of *Lecanicillium fungicola* isolates after incubation for 14 days at 22 ˚C

Isolate	N	Mean (mm)	Duncan grouping at P ≤ 0.01
Lf-JD	4	59.067	A
Lf-M	3	58.833	A
Lf-P	3	58.833	A
Lf-B	4	54.333	A
Lf-S	3	38.00	B

Means with the same letter are not significantly different from each other

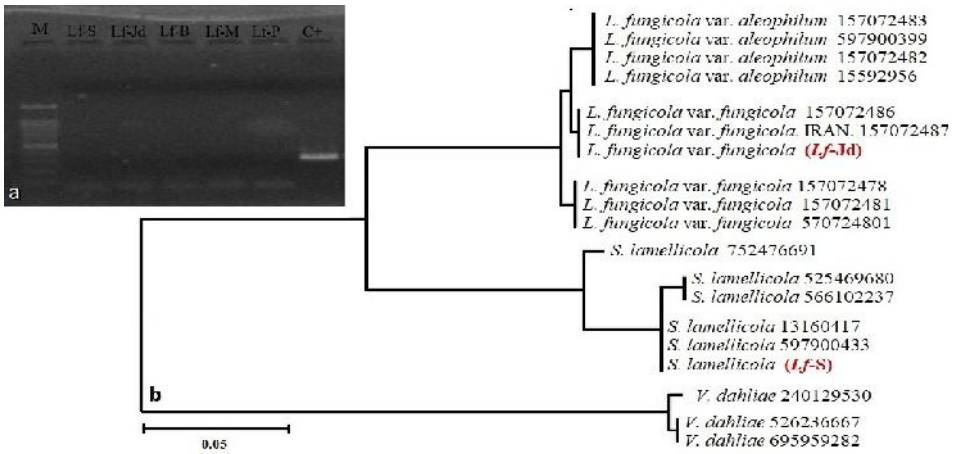

Fig. 2. a. PCR amplification of fungal isolates using specific primers designed by Romaine *et al.* (2002) did not generate the expected fragment, indicating that, none of the tested isolates belonged to *Lecanicillium f.* var. *aleophilum*, b. Phylogenetic tree for the isolates and related species of *Simplicillium lamellicola* and *L. fungicola* var. *fungicola* drawn from the Maximum likelihood analyses of the partial sequences of ITS region performed in MEGA 4.1. *Verticillium dahliae* was used as outgroup. The numbers indicate GenBank accessions for each taxon.

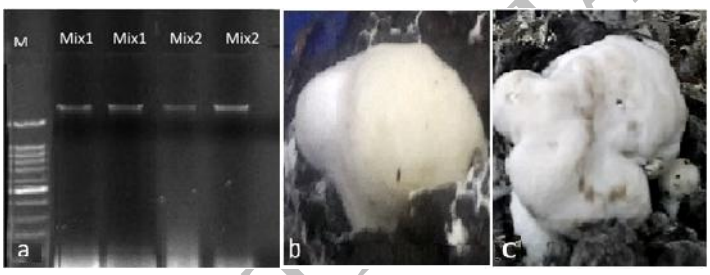

Fig. 3. Electrophoretic profile of DNA extraction from mushroom infected tissue: a. Integrity of DNA and reproducibility of the results by the isolation method described by the authors for PCR amplification using primers Lec-F and Lec-R, b. From the tissue in necrotic spots on the early (Mix1), c. Late (Mix2) stages of disease development.

- Selective primers for identification of *Lecanicillium fungicola*

In contrast to our expectation, none of the designed primer sets, either theoretically or those tested practically, were able to specifically amplify the estimated fragment. This was the case for primers Lec-F and Lec-R by producing an expected ~100 bp amplification product for *Lecanicillium fungicola*, but fragments of the same size were also produced by DNAs isolated from *Agaricus bisporus* and *Mycogone perniciosa*, and some other ascomycetous fungi tested. However, we found that, this primer set was also able to amplify two specific ~ 650 and ~800 bp fragments for *L. fungicola*. Reproducibility of the results was approved by running several independent tests by consistently producing the same amplicons using *L. fungicola* isolates. The optimal annealing temperature was determined at 50.5 ˚C by gradient search. Competency of the primers was also approved by consistent detection of *L. fungicola* after expanding the range of tests to some other Ascomycetes, Zygomycetes and Oomycetes. PCR amplification involving primers Lec-F and Lec-R readily detected the presence of expected amplicons from necrotic lesions at both early and late stages of disease development on fruit bodies. In contrast to the banding patterns of healthy mushrooms, the 550 bp diagnostic amplicon for *A. bisporus* was not generated with amplifications from infected tissue (Fig. 4 a,b). In conclusion, *L. fungicola* can be readily detected by primers Lec-F and Lec-R in any stages of the disease development from infected fruit bodies of the button mushroom.

Discussion

Fungal species belonging to the genus *Simplicillium* and *Lecanicillium* are a major part of the species traditionally classified in *Verticillium* sensu lato. They have similar morphological characteristics and this remarkable resemblance may have so far resulted in frequent misidentifications. However, using a combination of morphological characteristics and phylogenetic traits, these fungi can be clearly distinguished from related fungal genera (Sung *et al*. 2007, Kouvelis *et al.* 2008). In this study, different physiological and morphological characteristics of the so-called *Lf*-S isolate (*S. lamellicola*) from other isolates encouraged us to evaluate the molecular traits. *Simplicillium lamellicola* is a soil inhabitant species as well as a well-known mycopathogen causing gill mildew and brown spots on *A. bisporus* (Zare & Gams 2001), an entomopathogen (Fernandes & Bittencourt 2008), a parasite of cysts and eggs of nematodes (Gams 1988) and possess both antibacterial and antifungal activities (Le Dang *et al*. 2014). We isolated *S. lamellicola* from mushroom infected tissue showing brown spot symptoms which were intermingling with other infected ones showing dry bubble disease symptoms. Interestingly, the related casing soil was from a southwestern warm region of the country (Fars province). According to the literature, this is the first report of *S. lamellicola* from Iran. Usually DNA template quality greatly affects the reliability and reproducibility of amplification-based DNA fingerprinting of micro-organisms (Honore-Bouakline *et al*. 2003). Comparisons of two sample preparation methods, i.e., fresh and semi-dried mushroom tissue following the same extraction steps, confirmed the impact of the technique on the reproducibility of the DNA extraction method from healthy and infected mushroom tissues. In recent years, small-scale production of button mushroom is being developed and has become a good source of income for the household economies in the country. However, the threat of diseases, especially bubbles, is of the main concerns of the producers and is considered a high risk for investment. Molecular biological tools can be used to screen a large number of samples from different regions as a convincing way to understand the extent, frequency, and development of diseases, and subsequently to manage the disease using documentary data. However, the main challenge is to find a specific region for distinguishing the target pathogen among a large number of closely related fungi. Although the rDNA region is an attractive target for PCR-based detection methods, the fact that variability in this region is not very high between different fungi, should not be ignored (Geisen 2007). It seems, all the attempts for designing specific primers from the rDNA region for detection of *L. fungicola* have so far remained inconclusive (Largeteau *et al*. 2007, Piasecka 2010). Our results with other reports consistently support the idea that, the rDNA, as a conserved region in ascomycetes (White *et al*. 1990, Bruns *et al*. 1991, Richard *et al*. 2008), could be such a challenging target for designing selective primers for *L. fungicola*.

Our primers (Lec-F and Lec-R) which unexpectedly were able to amplify two specific fragments, offer a specific and rapid alternative for culture-based detection and identification of *L. fungicola* that requires prolonged incubation time on selective media (Rinker *et al*. 1993, Piasecka *et al.* 2011). We tried to find an explanation for the loss of the expected 550 bp amplicon in the banding pattern of diseased sporophores. It has been well-documented that, *A. bisporus* responds to *L. fungicola* invasion by the production of extracellular phenol oxidases, peroxidases, and antibiotics (Score *et al*. 1997, Savoie *et al*. 2004, Largeteau *et al*. 2006). Therefore, the disappearance of the 550 bp amplicon could be further attributed to induction of apoptotic DNA damage and necrotic cell death, a response of *Agaricus* similar to the hypersensitive reaction in plants in which cells in infected tissue die to deprive the pathogens of a supply of food and confine them to initial infection point (Greenberg *et al*. 1994, Berendsen *et al*. 2010, Foulongne-Oriol *et al*. 2012). In conclusion, using both

the PCR banding patterns and typical symptoms of bubble-infected mushrooms, the primers could be used for rapid and early detection of both *M. perniciosa* and *L. fungicola* in mushroom infected tissue. Moreover, the PCR test can also be used for reliable estimation of the true incidence rates by screening a large number of samples on individual farms or a region to create effective data-dependent disease management programs.

Acknowledgments

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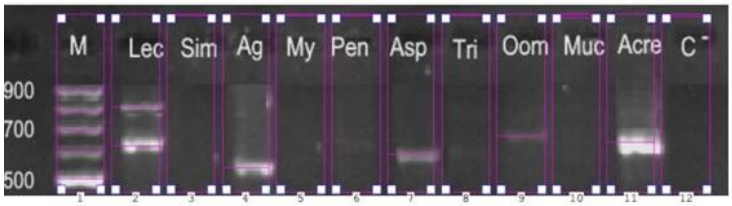
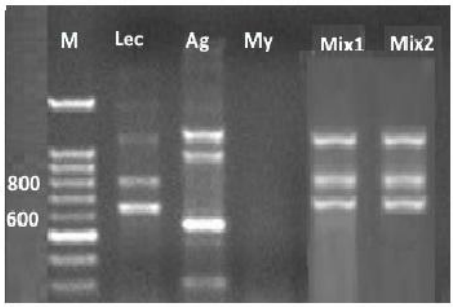


a

1	2	3	4	5	6	7	8	9	10	11	12
M	*Lec*	*Sim*	*Ag*	*My*	*Pen*	*Asp*	*Tri*	*Oom*	*Muc*	*Acre*	*C-*
900	800	-	550	-	-	600	-	700	-	600	-
800	650										
700											
600											
500											



b

Fig. 4. Electrophoretic profile of PCR products: a. Specificity of primers Lec-F and Lec-R for identification of *Lecanicillium fungicola* by amplification of two ~ 650 and 800 bp fragments. Lane 1, 100 bp molecular marker; Lane 2, *L. fungicola* var. *fungicola*; lane 3, *Simplicillium lamellicola*; Lane 4, *Agaricus bisporus*; lane 5, *Mycogone perniciosa*; Lane 6, *Penicillium* sp.; Lane 7, *Aspergillus* sp.; Lane 8, *Trichoderma* sp.; lane 9, *Oomycete*; Lane 10, *Mucor* sp.; Lane 11, *Acremonium* sp.; Lane 12, Negative control, b. Detection of *L. fungicola* on diseased fruit bodies. DNAs for PCR amplifications with primers Lec-F and Lec-R were extracted from the tissue of healthy fruit bodies and necrotic spots in the early and late stages of the disease development. Lanes indicate: M, 100 bp molecular marker; Lec, *L. fungicola* DNA control; Ag, *A. bisporus* DNA control; Mix1, DNA extracted from necrotic spots on early and Mix2, late stage of disease development. In contrast to *Ag* DNA control, the expected amplicon was not observed in 550 bp regions of both DNAs from Mix1 and Mix2 which could be attributed to the DNA damage-induced cell death response. Note the 1100bp band resolution from control Ag, which produced by both Ag and Lec, and compare it with the resolutions from Mix samples.

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