Mycoscience (2009) 50:94–99
DOI 10.1007/s10267-008-0460-2

FULL PAPER

Kazuhiro Miyazaki · Yuki Tsuchiya · Toru Okuda

Specific PCR assays for the detection of *Trichoderma harzianum* causing green mold disease during mushroom cultivation

Received: January 25, 2008 / Accepted: September 13, 2008

Abstract We have developed a polymerase chain reaction (PCR)-based detection method for *Trichoderma harzianum*, which causes green mold disease in mushroom cultivation fields and facilities. Based on the sequence data of the internal transcribed spacer (ITS) region of *T. harzianum* strains and several other species, six primers consisting of three forward and three reverse primers were designed. Among the nine possible combinations of these primers, PCR with the pair THITS-F2 and THITS-R3 distinguished most *T. harzianum* strains from other *Trichoderma* species. The optimal annealing temperature for detection of *T. harzianum* strains was from 62° to 63°C with this primer combination. We designed new primers derived from THITS-F2 and THITS-R3. Annealing temperatures to detect *T. harzianum* ranged from 64° to 67°C using the new primers. The detection limit of *T. harzianum* DNA was 50 fg by nested PCR with THITS-F1 and LR1-1 for the first PCR and the new primers for the second PCR. *T. harzianum* was readily detectable in contaminated cultures of *Lentinula edodes* by this method.

Key words Nested PCR · Specific PCR assay · *Trichoderma harzianum*

Introduction

Several species of *Trichoderma* cause green mold disease in edible mushrooms in Japan, Europe, and North America, resulting in serious problems for mushroom farmers (Seaby 1987; Rinker 1993). Virulence depends on the *Trichoderma* species: it has been confirmed by a paired culturing test with mushroom strains that *Trichoderma harzianum* Rifai shows high pathogenicity against mushrooms (Tokimoto and Komatsu 1975; Tokimoto 1985; Miyazaki et al. 1995). In addition, the isolation frequency of *T. harzianum* from cultivated fields of *Lentinula edodes* (Berk.) Pegler in forests was higher than that of other species of *Trichoderma* (Tokimoto 1985).

A rapid detection method for the identification of *T. harzianum* is important for decreasing damage in mushroom cultivation. For characterization of this fungus, various molecular analyses, e.g., isozymes, sequencing of genes, and random amplified polymorphic DNA, have been applied (Zamir and Chet 1985; Grondona et al. 1997; Miyazaki and Tsunoda 2003). Recently, sequence information accumulated on the internal transcribed spacer (ITS) region of *Trichoderma* fungi has been used for online identification (Druzhinina et al. 2005).

Polymerase chain reaction (PCR)-based detection using specific primers is an efficient method to identify the target organism and has been used to identify several plant pathogenic fungi (Mazzaglia et al. 2001; Konstantinova et al. 2002). Specific PCR primers have been developed to detect the biocontrol strain *T. harzianum* 2413 in soil (Rubio et al. 2005); however, this method is only applicable to this particular strain.

In this study, we have developed both specific primers for PCR detection of a diverse range of *T. harzianum* strains based on the ITS region sequence and a nested PCR assay to directly detect *T. harzianum* infecting cultivated edible mushrooms.

Materials and methods

Fungal strains

Twelve *T. harzianum* isolates, 4 *T. atroviride* Bissett isolates, 1 *T. virens* (J.H. Mill., Giddens & A.A. Foster) Arx isolate, 1 *T. pseudokoningii* Rifai isolate, 1 *T. citrinoviride* Bissett isolate, 1 *T. longibrachiatum* Rifai isolate, 1 *Trichoderma* cf. *stramineum* isolate, 1 *T. polysporum* (Link) Rifai

K. Miyazaki (✉)
Kyushu Research Center, Forestry and Forest Products Research Institute, 4-11-16 Kurokami, Kumamoto 860-0862, Japan
Tel. +81-96-343-3948; Fax +81-96-344-5054
e-mail: miyazaki@affrc.go.jp

Y. Tsuchiya · T. Okuda
Tamagawa University Research Institute, Tokyo, Japan

Table 1. *Trichoderma* and *Gliocladium* strains used in this study

Species name	Strain no.[a]	Location	Habitat	DDBJ/EMBL/GenBank accession no.
T. harzianum	KRCF 131	Kumamoto Pref., Japan	*Lentinula edodes*, block	AB249675
	KRCF 175	Kumamoto Pref., Japan	*L. edodes*, block	AB249676
	KRCF 180	Oita Pref., Japan	*L. edodes*, block	AB257269
	KRCF 220	Fukuoka Pref., Japan	*L. edodes*, block	AB257270
	KRCF 221	Fukuoka Pref., Japan	*L. edodes*, block	–
	KRCF 301	Kumamoto Pref., Japan	*L. edodes*, block	–
	KRCF 302	Kumamoto Pref., Japan	*L. edodes*, block	–
	KRCF 348	Kagoshima Pref., Japan	Wild mushroom	–
	KRCF 349	Kagoshima Pref., Japan	Wild mushroom	–
	KRCF 350	Kagoshima Pref., Japan	Wild mushroom	–
	KRCF 351	Kagoshima Pref., Japan	Wild mushroom	–
	KRCF 352	Kagoshima Pref., Japan	Wild mushroom	AB257268
T. atroviride	KRCF 222	Fukuoka Pref., Japan	*L. edodes*, block	AB249677
	KRCF 301	Kumamoto Pref., Japan	*L. edodes*, block	–
	KRCF 345	Oita Pref., Japan	*L. edodes*, block	AB257272
	KRCF 660	Fukuoka Pref., Japan	*Flammulina veltipes* (Curtis) Sing, bottle	AB249680
T. virens	KRCF 219	Fukuoka Pref., Japan	*L. edodes*, block	AB257271
T. pseudokoningii	KRCF 304	Miyazaki Pref., Japan	*L. edodes*, bed log	–
T. citrinoviride	KRCF 305	Fukuoka Pref., Japan	*Hypsizygus marmoreus* (Peck) H.E. Bigelow, bottle	AB249678
T. longibrachiatum	KRCF 306	Kumamoto Pref., Japan	*Pleurotus ostreatus* (Jacq.) P. Kumm, bottle	AB249679
Trichoderma cf. *stramineum*	KRCF 224	Fukuoka Pref., Japan	*L. edodes*, block	AB298692
T. polysporum	KRCF 347	Nagasaki Pref., Japan	*L. edodes*, bed log	AB298695
Trichoderma sp.	KRCF 307	Oita Pref., Japan	*L. edodes*, block	AB298694
G. viride	KRCF 353	Kagoshima Pref., Japan	Wild mushroom	–

[a]All the KRCF strains are maintained in the Kyushu Research Center, Forestry and Forest Products Research Institute, as the vouchers

isolate, 1 *Trichodema* sp. isolate, and 1 *Gliocladium viride* Matr. isolate were used in this study (Table 1). Details of each isolate are shown in Table 1. Individual strains were cultured on malt agar (MA) (2% malt extract, 1.5% agar) or modified malt agar (MMA) (1% malt extract, 0.1% yeast extract, 0.1% soytone, 1% glucose, 2% agar) slants at 20°–25°C, and the cultures were preserved in 10% (w/w) glycerol at –80°C.

Lentinula edodes MCR 14, stocked at Kyushu Research Center, Forestry and Forest Products Research Institute (Miyazaki and Neda 2004), was used for inoculation tests with *T. harzianum*.

DNA extraction for PCR detection

Mycelia cultured in SMY (1% saccharose, 1% malt extract, 0.4% yeast extract) liquid medium were filtered through Miracloth (pore size, 10 µm) (Calbiochem, San Diego, CA, USA). The mycelia were washed at least three times with distilled water followed by squeezing between dry paper towels. Approximately 100 mg mycelia of each sample was separated and preserved at –80°C until use.

Crude genomic DNA was prepared by the modified cetyl trimethyl ammonium bromide (CTAB) procedure of Murray and Thompson (1980). A dried pellet of crude DNA sample was resuspended in 49 µl TE buffer [10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5]. The DNA solution was treated with 1 µl RNase solution (1 mg/ml) (Wako, Osaka, Japan) in 49 µl TE buffer containing the DNA sample. After incubation at 37°C for 1 h, the solution was purified with an ELU-QUIK Kit (Schleicher & Shuell, Dassel, Germany), and the concentration of DNA was measured by UV spectroscopy (GeneQuant II instrument, Amersham Pharmacia, Little Chalfont, UK).

PCR detection

PCR was conducted in a mixture containing genomic DNA (5 µl 1 ng/µl DNA solution), 2.5 mM $MgCl_2$, 50 mM KCl, 16 µM dNTP, 0.5 µM each oligonucleotide primer, and 1.25 units *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) per 12.5 µl reaction mixture. The primers used in this study are shown in Table 2. LR1-1 was modified from LR1 described by Vilgalys and Hester (1990). Basic PCR was carried out under the following conditions: initial denaturation at 95°C for 1 min, followed by 30 cycles consisting of denaturation at 95°C for 30 s, annealing at 60°C for 1 min 30 s, and extension at 72°C for 2 min. A final extension at 72°C for 10 min followed. Under these basic conditions, the annealing temperatures were adjusted at 1°C intervals from 60° to 64°C to determine the optimum conditions for detection with primer THITS-F2 (for forward) and THITS-R3 (for reverse) (Table 2), and adjusted at 1°C intervals from 62° to 68°C with G-THITS-F2 (for forward) and CAA-THITS-R3 (for reverse) (Table 2).

In the nested PCR, after products of the first-step PCR were diluted 10^3 fold with ultrapurified water, 5 µl diluted solution was used for the second-step PCR. The sensitivity of the PCR assay was determined by testing a series of DNA concentrations from 5 ng down to 5 fg for the first-step PCR.

Table 2. Nucleotide sequences of oligonucleotide primers used in this study

Primer no.	Sequence (5′–3′)
THITS-F1	ACTGTTGCCTCGGCGGGATCTC
THITS-F2	CGGGTTTTTTATAATCTGAGCC
THITS-F3	GCCTTCTCGGCGCCTCTCG
THITS-R1	GGAGACGGCCACCCGCTAAGG
THITS-R2	CTGTATTTCGGAGACGGAC
THITS-R3	CATTCAGAAGTTGGGTG
G-THITS-F2	GCGGGTTTTTTATAATCTGAGCC
CAA-THITS-R3	CAACATTCAGAAGTTGGGTG
LR1-1	GGTTGGTTTCTTTTCC
ITS4	TCCTCCGCTTATTGATATGC
ITS5	GGAAGTAAAAGTCGTAACAAGG

DNA extraction and sequencing of the ITS region

DNA samples for sequencing were prepared with the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) from mycelial cultures grown on MA. Amplification of the ITS region was primed with the oligonucleotides ITS4 and ITS5 (Table 2) (White et al. 1990). PCR was conducted under the following conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 46°C for 1 min, and extension at 72°C for 1 min. A final extension at 72°C for 10 min followed.

The amplification products were purified with a High Purity PCR Purification Kit (Roche Diagnostics, Basel, Switzerland) and sequenced in both directions using ITS4 and ITS5 primers by the dideoxynucleotide chain termination procedure (Sanger et al. 1977) with an automated sequencer (ABI, Model 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA). Sequences were aligned using the CLUSTAL-W program (Thompson et al. 1994).

Inoculation of *L. edodes* cultivation block with *T. harzianum*

Lentinula edodes was cultured on a medium of 4:1 mixture of sawdust and rice bran (v/v) with a water content of 65% in a plastic bag for 2 months at 25°C. An agar disk 4 mm in diameter was removed using a cork borer from a *T. harzianum* (KRCF175) colony on MA. A lateral surface of the *L. edodes* cultivation block was inoculated with the culture disk of *T. harzianum* and the inoculation hole was sealed with adhesion tape. The inoculated block was then cultured at 30°C for 30 days. Five disks were sampled out with a cork borer at equal intervals across the diameter of the culturing block from the point of inoculation with *T. harzianum*. A cultivation block of *L. edodes* without *Trichoderma* was used as a control. Samples were homogenized in sterilized water. After allowing it to stand for about 10 min to settle, the upper overlaid solution was transferred to new microtubes for DNA extraction. DNA was then extracted by the described method for the nested PCR method. A sample of the remaining sediment was used to inoculate a new MA plate to observe the regeneration of hyphae under a microscope.

Results and discussion

Sequence analysis

The ITS region from six strains of *T. harzianum* isolates, three isolates of *T. atroviride*, a *T. citrinoviride* isolate, a *T. longibrachiatum* isolate, a *Trichoderma* cf. *stramineum* isolate, a *T. polysporum* isolate, and an isolate of *Trichoderma* sp. were sequenced; the accession numbers are given in Table 1. ITS sequences from each isolate were aligned by CLUSTAL-W software. On the basis of a consensus alignment, primers to recognize a common region of the *T. harzianum* isolates were synthesized: three forward primers, THITS-F1, THITS-F2, and THITS-F3, and three reverse primers, THITS-R1, THITS-R2, and THITS-R3. The target regions for the primers are shown in Fig. 1; their sequences are shown in Table 2.

Amplification with synthesized primers

We attempted amplification of the ITS region using all combinations of the synthesized primers. DNA fragments of the expected size were clearly observed by agarose gel electrophoresis for *T. harzianum* isolates when amplified with THITS-F2 and THITS-R3 (Fig. 2). Regarding the other combinations expected, DNA fragments were not amplified for the *T. harzianum* samples using the combinations THITS-F1 and THITS-R1, THITS-F1 and THITS-R2, THITS-F2 and THITS-R1, or THITS-F2 and THITS-R2. Several DNA fragments were amplified for the *T. harzianum* samples using the combinations THITS-F3 and THITS-R1 and THITS-F3 and THITS-R2: DNA fragments of the other species were clearly observed with THITS-F1 and THITS-R3 and THITS-F3 and THITS-R3 (data not shown). However, DNA fragments amplified with THITS-F2 and THITS-R3 were also faintly observed for *T. atroviride* KRCF 222 and *Gliocladium viride* KRCF353. We next evaluated the optimal annealing temperature for PCR with THITS-F2 and THITS-R3. We tested 60°, 61°, 62°, 63°, and 64°C at the annealing steps of the PCR reaction with THITS-F2 and THITS-R3. For KRCF 353, a DNA fragment was also faintly observed when the DNA samples were amplified at an annealing temperature of 61°C and absent when annealed at 64°C. For PCR detection with THITS-F2 and THITS-R3, the annealing temperature should be set at 62° or 63°C.

To stabilize the conditions for PCR amplification, we modified the 5′-ends of THITS-F2 and THITS-R3 to G-THITS-F2 and CAA-THITS-R3, respectively. To reevaluate the annealing temperature for PCR with G-THITS-F2 and CAA-THITS-R3, we tested annealing temperatures of 63°, 64°, 65°, 66°, 67°, and 68°C. When samples were amplified at 63°C for the annealing step, DNA fragments were amplified for samples in addition to *T. harzianum* strains (Fig. 3). DNA fragments were not observed at 68°C for annealing (Fig. 3). When G-THITS-F2 and CAA-THITS-R3 were used for PCR detection of *T. harzianum*, the annealing temperature that worked ranged from 64° to

*ACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTA*CCGAGTTTACAA
CTCCCAAACCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGATCTCT
THITS-F1
GCCCCGGGTGCGTCGCAGCCCCGGACCAAGGCGCCCGCCGGAGGACCA
ACCAAAACTCTTTTTGTATACCCCCTCGCGGGTTTTTTATAATCTGAGCCTT
THITS-F2
CTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCA*AAACTTTCAACAACG*
THITS-F3
GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA
TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGC
*CCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTT*CAACCCTCG
AACCCCTCCGGGGGGTCGGCGTTGGGGATCGGCCCTCCCTTAGCGGGTG
THITS-R1
GCCGTCTCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGT
THITS-R2
AGTTTGCACACTCGCATCGGGAGCGCGGCGCGTCCACAGCCGTTAAACAC
CCAACTTCTGAAATG*TTG*ACCT
THITS-R3

Fig. 1. Sequence of internal transcribed spacer (ITS) region of *Trichoderma harzianum* KRCF 131. The *underlined sites* represent the sequence corresponding to the primer first synthesized, and the *boxed sites* indicate the sequence corresponding to the primer synthesized second in this study. *Italics* indicate that the region is a small subunit, 5.8S or a large subunit of rRNA

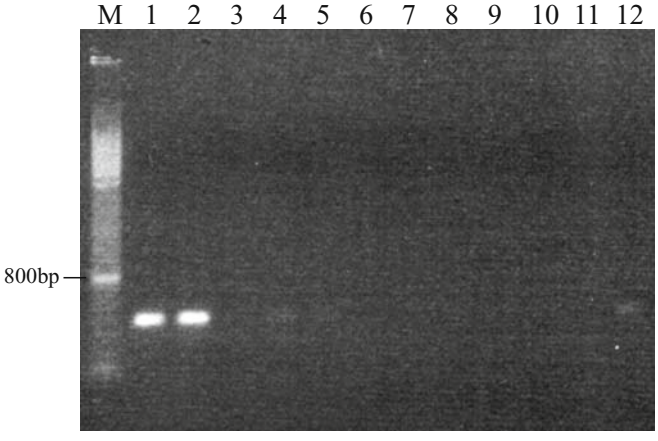

Fig. 2. Results of amplification by polymerase chain reaction (PCR) with THITS-F2 and THITS-R3. *M*, 100-bp ladder; *lanes 1*, *2*, *Trichoderma harzianum* isolates, KRCF 175 and 220; *lanes 3–5*, *T. atroviride* isolates KRCF 345, 222, and 301; *lane 6*, *Trichoderma* sp. isolate KRCF 307; *lane 7*, *Trichoderma* cf. *stramineum* isolate KRCF 224; *lane 8*, *T. citrinoviride* isolate KRCF 305; *lane 9*, *T. pseudokoningii* isolate KRCF 304; *lane 10*, *T. longibrachiatum* isolate KRCF 306; *lane 11*, *T. polysporum* isolate KRCF 347, *lane 12*, *Gliocladium viride* isolate KRCF 353. At an annealing temperature of 60°C, faint bands were observed for KRCF 222 (*lane 4*) and KRCF 353 (*lane 12*)

67°C and we therefore adopted 66°C. Only *T. harzianum* isolates yielded the expected fragment when amplified at an annealing temperature of 66°C using G-THITS-F2 and CAA-THITS-R3 (Fig. 4).

Application of nested PCR procedure to improve quantitation

The template DNA required for the PCR assay with G-THITS-F2 and CAA-THITS-R3 was more than 500 pg; however, a nested PCR method made it possible to reduce this amount to 50 fg using THITS-F1 and LR1-1 as primers for the first-step PCR (Fig. 5). The sensitivity was similar to that for the nested PCR detection of *Phytophthora nicotianae* Breda de Haan developed by Grote et al. (2002).

We analyzed a mixed zone of *L. edodes* infected with *T. harzianum* to confirm the ability to diagnose the presence of *T. harzianum* on the mushroom substrate. When the nested PCR procedure was conducted for samples from cultured blocks of *L. edodes* inoculated with *T. harzianum* KRCF 175, the target DNA fragments were amplified only in the samples from which *Trichoderma* was isolated (Fig. 6). Our data revealed that the nested PCR procedure using THITS-F1 and LR1-1 in the first step and G-THITS-F2 and CAA-THITS-R3 in the second step was useful for rapidly detecting *T. harzianum* from infected regions.

The PCR assay with G-THITS-F2 and CAA-THITS-R3 may also be useful for the identification of pure cultures of *T. harzianum*. In addition to the PCR assay with these primers, a nested PCR assay was effective to determine whether *T. harzianum* was the pathogenic fungus causing symptoms of infection during mushroom cultivation. The nested PCR method required 2 days for determination, i.e., a half-day for extraction, a half-day for purification, and 1

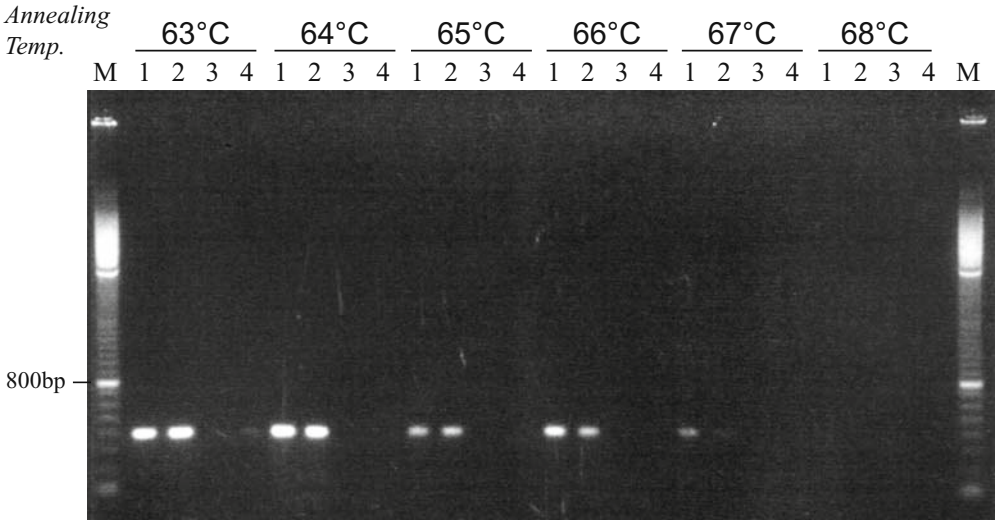

Fig. 3. Checking of annealing temperature to detect *Trichoderma harzianum* by PCR with G-THITS-F2 and CAA-THITS-R3. *Lanes 1*, *2*, *T. harzianum* isolates KRCF 175 and 220; *lane 3*, *T. atroviride* isolate KRCF 222; *lane 4*, *Gliocladium viride* isolate KRCF 353. At an annealing temperature of 63°C, faint DNA fragments were observed from nontargeted samples. At 68°C, DNA fragments were not observed from the targeted samples

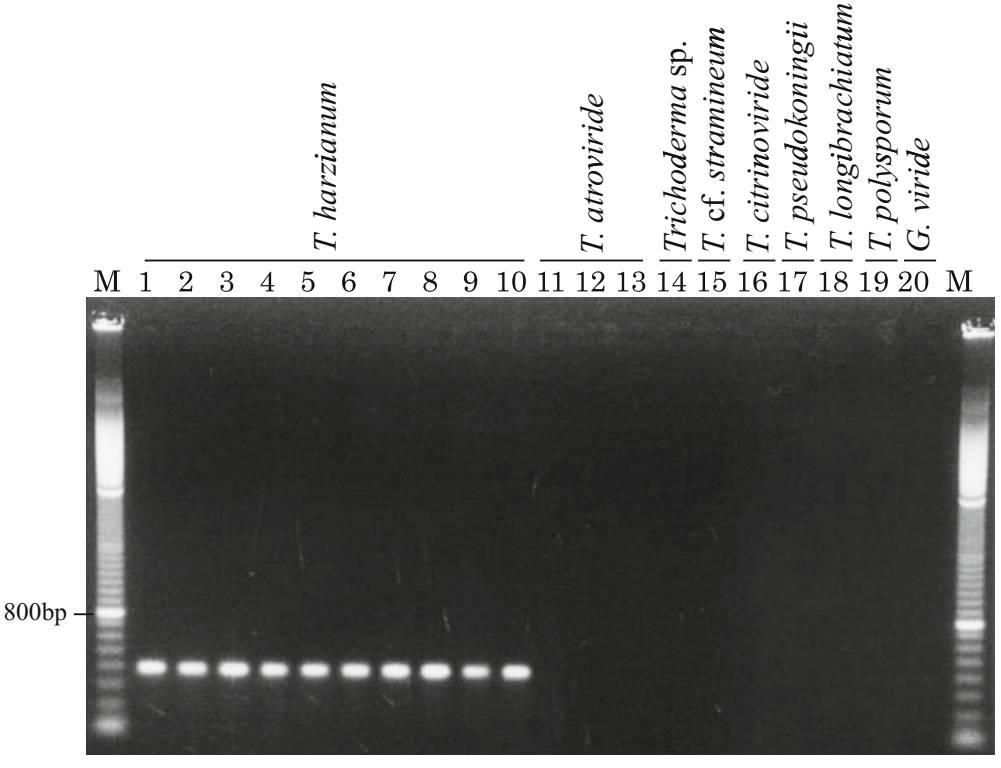

Fig. 4. Confirming the specificity of detection with G-THITS-F2 and CAA-THITS-R3. *M*, 100-bp ladder; *lanes 1–10*, *Trichoderma harzianum* isolates KRCF 175, KRCF 302, KRCF 131, KRCF 220, KRCF 221, KRCF 352, KRCF 348, KRCF 350 KRCF 349, and KRCF 351; *lanes 11–13*, *T. atroviride* isolates KRCF 345, 222, and 301; *lane 14*, *Trichoderma* sp. isolate KRCF 307; *lane 15*, *Trichoderma* cf. *stramineum* isolate KRCF 224; *lane 16*, *T. citrinoviride* isolate KRCF 305; *lane 17*, *T. pseudokoningii* isolate KRCF 304; *lane 18*, *T. longibrachiatum* isolate KRCF 306; *lane 19*, *T. polysporum* isolate KRCF 347, *lane 20*, *Gliocladium viride* isolate KRCF 353

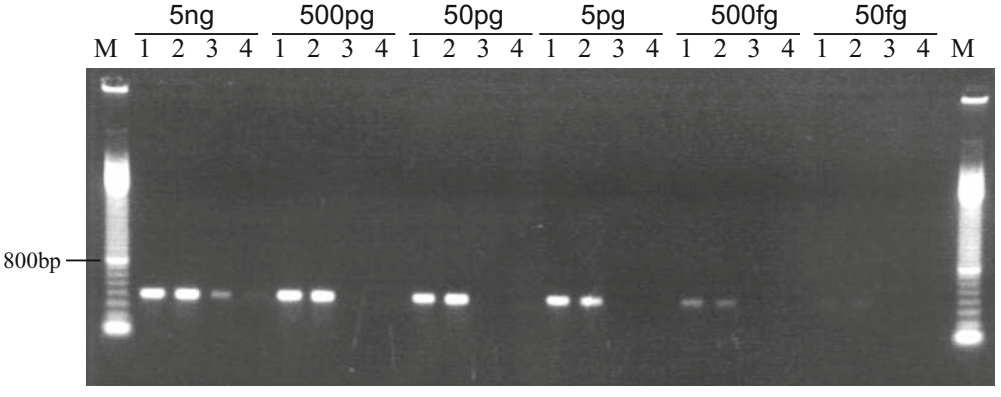

Fig. 5. Test of the effectiveness of the nested PCR method with LR1-1 versus THITS-F1 (for first step) and G-THITS-F2 versus CAA-THITS-R3 (for second step). *M*, 100-bp ladder; *lane 1*, *Trichoderma harzianum* isolate KRCF 175; *lane 2*, *T. harzianum* isolate KRCF 220; *lane 3*, *T. atroviride* isolate KRCF 222; *lane 4*, *Gliocladium viride* isolate KRCF 353. The quantity of genomic DNA used for the first-step PCR is indicated *above* the lane number

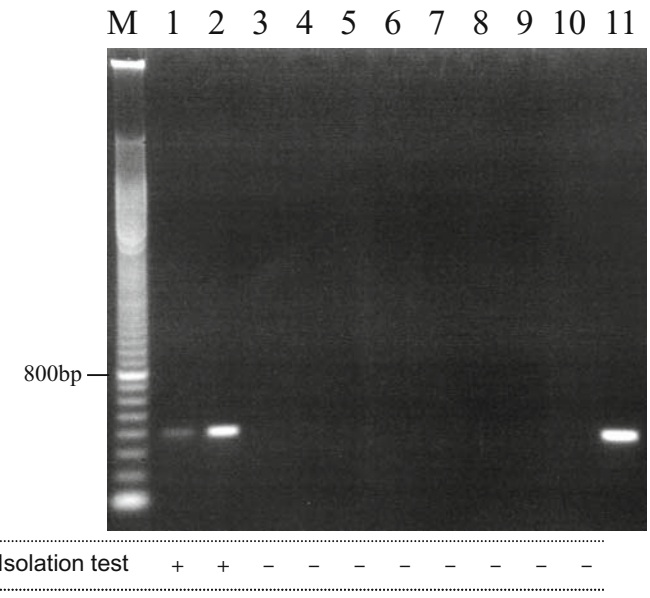

Fig. 6. Detection of *Trichoderma harzianum* (KRCF 175) from a culturing block of *Lentinula edodes* (MCR 14) infecting *T. harzianum* by the nested PCR method. *M*, 100-bp ladder; *lanes 1–5*, samples extracted from the inoculation point to the end of the sampling site on the block culture of *L. edodes* (MCR 14) inoculated with *T. harzianum* (KRCF 175); *lanes 6–10*, samples extracted from the block culturing only *L. edodes*; *lane 11*, genomic DNA of *T. harzianum* (KRCF 175). The result of isolation tests is indicated *below* a picture of the corresponding agarose gel on electrophoresis. "+", mycelium of *Trichoderma* sp. was recovered; "–", mycelium of *Trichoderma* sp. was not recovered

day for nested PCR. In contrast, it takes a minimum of 10 days for determination by the conventional morphological approach. The application of PCR detection with primers developed in this study is expected to improve the accuracy of *T. harzianum* determination and reduce the time necessary to evaluate potential damage caused by infection.

Acknowledgments We thank Dr. Mitsutoshi Tsunoda (Forestry and Forest Products Research Institute) for supplying several valuable strains used for this study. This work was supported by a grant for research projects that utilize advanced technologies in agriculture, forestry, and fisheries (No. 1742).

References

Druzhinina IS, Kopchinshiy AG, Komon M, Bissett J, Szakacs G, Kubicek CP (2005) An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. Fungal Genet Biol 42:813–828

Grondona R, Hermosa R, Tejada M, Gomis MD, Bridge PD, Monte E, Garcia-Acha I (1997) Physiological and biochemical characterization of *Trichoderma harzianum*, a biological control agent against soilborne fungal plant pathogens. Appl Environ Microbiol 63:3189–3198

Grote D, Olmos A, Kofoet A, Tuset JJ, Bertolini E, Cambra M (2002) Specific and sensitive detection of *Phytophthora nicotianae* by simple and nested-PCR. Eur J Plant Pathol 108:197–207

Konstantinova P, Bonants P, Gent-Pelzer M, Zouwen P, Bulk R (2002) Development of specific primers for detection and identification of *Alternaria* spp. in carrot material by PCR and comparison with blotter and plating assay. Mycol Res 106:23–33

Mazzaglia A, Anselmi N, Gasbarri A, Vannini A (2001) Development of a polymerase chain reaction (PCR) assay for the specific detection of *Biscogniauxia mediterranea* living as an endophyte in oak tissues. Mycol Res 105:952–956

Miyazaki K, Neda H (2004) Evaluation of the use of outbred lines for screening of genetic markers in Shiitake (*Lentinula edodes*). Breed Sci 54:75–78

Miyazaki K, Tsunoda M (2003) Application of DNA markers to research on *Trichoderma* in mushroom facilities of Japan (1): RAPD, SSCP marker. Mushroom Sci Biotechnol 11:65–70

Miyazaki K, Sunagawa M, Neda H, Tsunoda M (1995) The distinction of pathogenicity of the genus *Trichoderma* and *Penicillium*: the distinction by paired culture with glass straw (in Japanese). Bull Kyushu Branch Jpn For Soc 48:233–234

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight DNA. Nucleic Acids Res 8:4321–4325

Rinker D (1993) Disease management strategies for *Trichoderma* mould. Mushroom World 4:3–5

Rubio MB, Hermosa MR, Keck E, Monte E (2005) Specific PCR assay for the detection and quantification of DNA from the biocontrol strain *Trichoderma harzianum* 2413 in soil. Microbial Ecol 49:25–33

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 74:5463–5467

Seaby D (1987) Infection of mushroom compost by *Trichoderma* species. Mushroom J 179:355–361

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Tokimoto K (1985) Physiological studies on antagonism between *Lentinus edodes* and *Trichoderma* spp. in bedlogs of the former (in Japanese). Rep Tottori Mycol Inst 23:1–54

Tokimoto K, Komatsu M (1975) Effect of carbon and nitrogen sources in media on the hyphal interference between *Lentinus edodes* and some species of *Trichoderma*. Ann Phytopathol Soc Jpn 45:261–264

Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J Bacteriol 172:4238–4246

White TJ, Bruns T, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp 315–322

Zamir D, Chet I (1985) Application of enzyme electrophoresis for the identification of isolates in *Trichoderma harzianum*. Can J Microbiol 31:578–580