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ORIGINAL ARTICLE

# Development of PCR-based DNA markers for identification and detection of *Trichoderma* species associated with the green mold disease of oyster mushroom

## 느타리버섯 푸른곰팡이병에 관여하는 *Trichoderma* 속균의 동정 및 검출을 위한 PCR 기반 DNA 마커 개발

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## ABSTRACT

*Trichoderma* is known as pathogen caused serious green mold disease on commercial production. *T. pleuroti* and *T. pleuroticola* were common species in various mushroom media. Many strains of *T. pleuroti*, known as aggressive species causing major economic losses in Korea, showed benomyl resistance. Accurate identification and detection of *Trichoderma* species associated with oyster mushrooms is very important for disease control. We developed species-specific primers for *T. pleuroticola*, *T. pleuroti*, *T. harzianum*, and *T. atroviride* based on species-specific fragments isolated from amplified fragment length polymorphism analysis. PCR products corresponding to the predicted fragment of 500bp, 230bp, 180bp, and 410bp were amplified from *T. pleuroticola*, *T. pleuroti*, *T. harzianum*, and *T. atroviride*, respectively. Multiplex PCR assay using species-specific primers quickly and accurately identified and detected *T. pleuroti* from mushroom media in which various species co-exist. Our results can be useful for the effective control of mushroom disease.

Key Words : *Trichoderma*, Sequence characterized amplified region, species-specific primer, *Pleurotus ostreatus*.

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## Introduction

The genus *Trichoderma* is one of the most common fungi from various environments such as soil, decaying wood and vegetable matters (Samuels 1996). They play important ecological roles as the biocontrol agents of plant pathogens (Blanchette and Walke 2019) and producer such as enzyme and antibiotics (Sivasithamparam and Ghisalberti, 1998). Some *Trichoderma* species are associated

with green mold disease of commercial mushroom, *Agaricus bisporus* (Samuels et al., 2002), *Lentinula edodes* (Wang et al., 2016), and *Pleurotus ostreatus* (Park et al., 2005a; Park et al., 2005b). The outbreak of green mold by *Trichoderma* caused serious impact on commercial production (Fletcher, 1990; Ospina-Giraldo et al., 1998). Although many *Trichoderma* spp. (*T. afroharzianum*, *T. atrobrunneum*, *T. atroviride*, *T. citrinoviride*, *T. guizhouense*, *T. koningii*, and *T. longibrachiatum*) have been isolated from mushroom substrate, aggressive species are known as *Trichoderma aggressivum* for *A. bisporus* and *T. pleuroti*, *T. pleuroticola*, *T. simmonsii*, and *T. guizhouense* for *Pleurotus* spp. (Castle et al., 1998; Park et al., 2005a; Allaga et al., 2021).

The accurate and rapid identification of pathogens is one of the most important factors in disease management. Disease diagnosis and pathogen identification by traditional methods of isolating and characterizing pathogens are labor intensive, time consuming and unacceptable when high-throughput diagnostics are required. Recently, molecular diagnostic methods based on polymerase chain reaction (PCR) have increasingly received attention as an alternative to conventional methods due to more specific, sensitive and rapid methods (Schaad et al., 2003 ; Ward et al., 2004). PCR-based methods have been developed to detect various plant pathogens such as *Bipolaris sorokiniana* (Aggarwal et al., 2011), *Colletotrichum falcatum* (Nithya et al., 2012), and *Phytophthora nicotianae* (Das et al., 2019). Detection of *Trichoderma* species based on PCR-based methods mainly focused on *T. atroviride*, *T. harzianum* and *T. hamatum*, known as potential biocontrol agents (Kredics et al., 2018).

Since the early 1980s, commercial production of oyster mushrooms has developed rapidly. Currently, it occupies the first place in production among mushrooms cultivated in Korea (Lee and Cho, 2021). The oyster mushroom production has been severely affected by green mold disease in Korea. Seven species have been reported: *T. atroviride*, *T. citrinoviride*, *T. harzianum*, *T. longibrachiatum T. pleuroti*, *T. pleuroticola*, and *T. virens* (Park et al., 2005 a; Park et al., 2005b). *T. pleuroti* showed relatively rapid colonization in the surface of mushroom compost (Park et al., 2005a) and benomyl resistance (data not shown). The objective of this study is to develop species-specific primers based on amplified fragment length polymorphism (AFLP) fragments for the detection and identification of aggressive species in oyster mushroom cultivation.

## Materials and methods

### Fungal cultures and DNA extraction

Fungal strains used in this study are listed in Table 1. Forty-four strains isolated from oyster mushroom farms in Korea were maintained on potato dextrose agar (PDA, Difco, Becton Dickinson, MD, USA) at 25℃.

Genomic DNA was extracted from fungal strains grown PDA using a modified cetytrimethylammonium bromide (CTAB) extraction protocol (Rogers and Bendich, 1994). Size and amount of extracted DNA was examined by electrophoresis through a 1% agarose gel in TAE buffer and visualized with UV light. DNA was stored at −20℃ until required.

### AFLP analysis

The AFLP analyses were performed as described by Vos et al. (1995) with some modifications. Genomic DNA (1 ㎍) was digested with restriction enzyme *EcoRI* and *MseI*. The restriction fragments were ligated to *MseI* and *EcoRI* adapter at 16℃ on overnight. The pre-selective amplification was carried out in a i-cycler (BIO-RAD, USA) for 20 cycles of 94℃ for 30 s denaturing, 57℃ for 1 min annealing, and 72℃ for 1 min extension. Amplification reactions contained 25 pmol of M00 and E00, 250 nM dNTP, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM MgCl2, 2U of Taq-DNA polymerase (Bioneer, Korea) and 4 ul of ligated DNA as a template in a final volume of 50 ㎕. The selective amplification was performed using three kinds of primer sets (M03/E13, M02/E32, M134/E03, M13/E13. The 25 ㎕ reaction mixture contained 25

**Table 1.** Strain information used in this study

| Species | Isolate No. | Collected site | Substrate |
|---|---|---|---|
| *T. atroviride* | CNU507 | Yeosu, Chonnam | Rice straw |
| | CNU511 | Yeosu, Chonnam | Rice straw |
| | CNU555 | Namwon, Chonbuk | Rice straw |
| | CNU572 | Inje, Kangwon | Rice straw |
| | CNU779 | Uiseong, Gyeongbuk | Rice straw |
| | CNU580 | Seosan, Chungnam | Sawdust |
| | CNU503 | Inje, Kangwon | Waste cotton |
| | CNU534 | Chongwon, Chungbuk | Waste cotton |
| | CNU683 | Kyeongju, Kyeongbuk | Waste cotton |
| *T. citrinoviride* | CNU627 | Yeongi, Chungnam | Waste cotton |
| *T. harzianum* | C40 | Buyeo, Chungnam | Rice straw |
| | C98 | Buyeo, Chungnam | Waste cotton |
| | CNU551 | Namwon, Chonbuk | Rice straw |
| | CNU554 | Namwon, Chonbuk | Rice straw |
| | CNU578 | Inje, Kangwon | Rice straw |
| | CNU556 | Yeongi, Chungnam | Waste cotton |
| | CNU625 | Yeongi, Chungnam | Waste cotton |
| | CNU662 | Taean, Chungnam | Waste cotton |
| | CNU663 | Taean, Chungnam | Waste cotton |
| | CNU681 | Kyeongju, Kyeongbuk | Waste cotton |
| *T. longibrachiatum* | CNU518 | Taean, chungnam | Rice straw |
| *T. pleuroti* | CNU545 | Namwon, Chonbuk | Rice straw |
| | CNU610 | Yeoju, Kyeonggi | Rice straw |
| | CNU729 | Hampyeong, Chunnam | Rice straw |
| | CNU501 | Chuncheon, Kangwon | Waste cotton |
| | CNU523 | Boeun, Chungbuk | Waste cotton |
| | CNU533 | Chongwon, Chungbuk | Waste cotton |
| | CNU537 | Jincheon, Chungbuk | Waste cotton |
| | CNU538 | Jincheon, Chungbuk | Waste cotton |
| | CNU558 | Juchon, Chonbuk | Waste cotton |
| | CNU797 | Annam, Chungbuk | Waste cotton |
| | CNU647 | Yeongi, Chungnam | Sawdust |
| *T. pleuroticola* | CNU571 | Inje, Kangwon | Rice straw |
| | CNU725 | Hampyeong, Chunnam | Rice straw |
| | CNU783 | Uiseong, Gyeongbuk | Rice straw |
| | CNU601 | Paju, Kyeonggi | Sawdust |
| | CNU618 | Paju, Kyeonggi | Sawdust |
| | CNU646 | Yeongi, Chungnam | Sawdust |
| | CNU647 | Yeongi, Chungnam | Sawdust |
| | CNU529 | Jecheon, Chungbuk | Waste cotton |
| | CNU682 | Jangseong, Chunnam | Waste cotton |
| | CNU795 | Uiseong, Gyeongbuk | Waste cotton |
| *T. virens* | C79 | Dangjin, Chungnam | Rice straw |
| | CNU639 | Yeongi, Chungnam | Waste cotton |

pmol of each primer, 250 nM dNTP, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM MgCl2, 2U of Taq-DNA polymerase (Bioneer, Korea) and 1 μl of 10 fold diluted pre-amplified DNA. The amplification was performed with the touch-down PCR thermal profile: 5 min at 94℃; 13 cycles of 30 s at 94℃, 20 s at 67℃ and 1 min at 72℃ (annealing temperature was lowered by 0.7℃ during each cycle); followed by 23 cycles of 30s at 94℃, 20 s at 57℃ and 1 min at 72℃; and a final extension of 20 min at 72℃ and cooling to 4℃ After completing the selective amplification, 4 μl of stop solution (95% formamide, 10 mM EDTA pH 8.0, 0.05% xylene cyanol and 0.05% bromo phenol blue) was added to 20 μl reaction mixture. Mixtures were heated for 2 min at 95℃ and chilled on ice. The amplified products were resolved on 4% denaturing polyacrylamide gel. Electrophoresis was performed at a constant 80 W for 3 hr. For DNA visualization, AFLP gels were silver-stained following the protocol described by Silverstar® Staining System (Bioneer, Korea).

## Isolation and sequencing of AFLP fragments

The specific band was cut from the dried gel using a sterile blade. The gel fragment was soaked 20 μl of TE buffer (pH 8.0) at 37℃ for 8 to 12 hr. After centrifugation for 10 min at 12,000 rpm, the supernatant was transferred to a fresh tube and 0.1 volume of 3 M sodium acetate and 2 volume of cold 95% ethanol were added, DNA was pelleted, washed with 70% ethanol, dried in air and then resuspended in 20 μl of TE buffer. The DNA (4 μl) was then PCR amplified using the same primer pairs that generated the AFLP fragment. The PCR products were run on 1.5% agarose gel. The PCR product purification was carried out using a Wizard PCR prep kit (Promega, Madison, WI, USA). DNA sequencing was performed in both forward and reverse directions with the same PCR primers at Macrogen, using an ABI Prism 3700 Genetic Analyzer (Life Technologies).

## Design of primer and PCR amplification

Four pairs of specific primer were designed from the sequence information of AFLP fragment using primer3_www.cgi v 0.2 (Rozen & Skaletsky, 2000) and were synthesized by Bioneer Inc. (Korea). These primers were used to amplify genomic DNA from *T. pleuroticola*, *T. pleuroti*, *T. harzianum*, and *T. atroviride* in a 50 μl reaction volume containing 25 pmol each primer, 250 nM dNTP, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM MgCl2, 2 U of Taq-DNA polymerase (Bioneer, Korea). PCR amplification was carried out in a i-cycler (BIO-RAD, USA) for 30 cycles of 94℃ for 30 s denaturing, 63℃ for 30 s annealing, and 72℃ for 40 min extension. Initial denaturing at 94℃ was extended to 5 min and the final extension was at 72℃ for 15 min.

## Specificity and sensitivity

Specificity of the PCR for *Trichoderma* species using each specific primer was tested on a collection of *T. pleuroticola*, *T. pleuroti*, *T. harzianum*, and *T. atroviride* and other *Trichoderma* species such as *T. longibrachiatum*, *T. citrinoviride*, and *T. virens*. In order to test for the ability to detect different s of each *T. pleuroticola*, *T. pleuroti*, *T. harzianum*, and *T. atroviride*, seven s of each species were checked for positive amplification. The presence or absence of the specific fragment band was visualized by gel electrophoresis.

A serial dilution of each genomic DNA of *T. pleuroticola*, *T. pleuroti*, *T. harzianum*, and *T. atroviride* as PCR template DNA was used to determine sensitivity of the PCR amplification. The amount of genomic DNA was from 10 ng to 10 pg.

## Multiplex PCR

All DNA samples were tested in multiplex PCR reations containing all four species-specific primer sets (K1-4146F/K1-4146R, K2-1311F/K1-1311R, Th1-16962F/Th1-16962R and Th3-3351F/Th3-3351R). PCR conditions were as above. PCR reactions were resolved by electrophoresis in 1.5% agarose gels in TAE buffer, stained with ethidium bromide and visualized with UV light. Each DNA

sample × primer × pair combination was repeated at least two times. *T. pleuroticola*, *T. pleuroti*, *T. harzianum*, and *T. atroviride* were then identified depending on the size of the amplified DNA fragment.

## Results

### Conversion of the AFLP marker into a species-specific primers

Different of *T. pleuroticola*, *T. pleuroti*, *T. harzianum*, and *T. atroviride* were analyzed for AFLP profiles with eight different primer pairs combination. Those primers generated genetic polymorphism between the different species were tested several times in order to confirm the reproducibility of their amplification data. Primer pair M03/E13 amplified a single band of approximately 500bp in all tested s of *T. pleuroticola*, but not from any of the other species tested (Fig. 1A). This PCR product was isolated from s of *T. pleuroticola* and this band was sequenced. The sequence has a G+C content of 52.5%. No significant homology could be detectedwith any sequences in GenBank. The sequence was used to design the species-specific primer pair K1-4146F/ K1-4146R (Table 2). Primer pair M02/E32 amplified a distinct fragment of approximately 230bp that was unique to all *T. pleuroti* (Fig. 1B). This PCR product was isolated from s of *T. pleuroti* and this band was sequenced. The sequence has a G+C content of 47.3%. No significant homology could be detected with any sequences in GenBank. The sequence was used to design the species-specific primer pair K2-1311F/ K2-1311R (Table 2). Primer pair M0134/E03 amplified a single band of approximately 180bp in all tested s of *T. harzianum*, but not from any of the other species tested (Fig. 1C). This PCR product was isolated from s of *T. harzianum* and this band was sequenced. The sequence has a G+C content of 47.9%. No significant homology could be detected with any sequences in GenBank. The sequence was used to design the species-specific primer pair Th1-16962F/ Th1-16962R

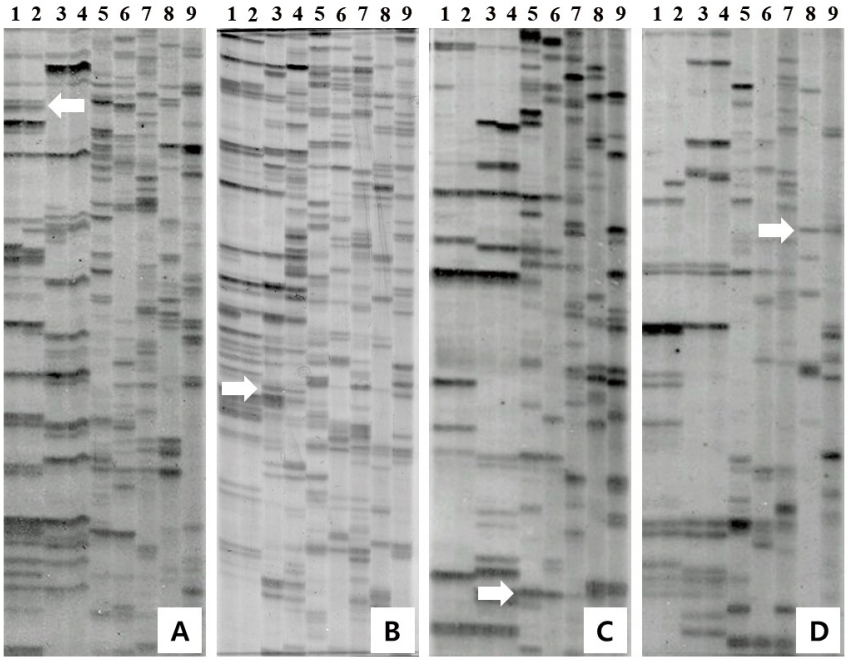


Fig. 1. Amplified fragment length polymorphism patterns of *T. pleuroticola* (lane 1 and 2), *T. pleuroti*, (lane 3 and 4), *T. harzianum* (lane 5 to 7) and *T. atroviride* (lane 8 and 9). The arrow point to unique fragment present only in profiles of each species

(Table 2). Primer pair M13/E13 amplified a distinct fragment of 410bp that was unique to *T. atroviride* (Fig. 1D). This PCR product was isolated from s of *T. atroviride* and this band was sequenced. The sequence has a G+C content of 49.5%. No significant homology could be detected with any sequences in GenBank. The sequence was used to design the species-specific primer pair Th3-3351F/ Th3-3351R (Table 2).

Table 2. Species-specific primer for detecting *T. pleuroticola*, *T. pleuroti*, *T. harzianum*, and *T. atroviride* associated with oyster mushroom

| Target species | Primer name | Sequence (5'-3') | Length (bp) | GC (%) | Amplicon size (bp) |
|---|---|---|---|---|---|
| *T. pleuroticola* | K1-4146F | AGCGGTTTCAACCTCTTGC | 19 | 52.6 | 500 |
| | K1-4146R | CCAATTTTATCGACCAAAAGACC | 23 | 39.1 | |
| *T. pleuroti* | K2-1311F | CGCTCGACTACCAATAAGTGC | 21 | 52.3 | 230 |
| | K2-1311R | TCGTTTAGCTGGAAGCTGGT | 20 | 50.0 | |
| *T. harzianum* | Th1-16962F | CGTCGCCTTTCCTTCCTAGT | 20 | 50.0 | 180 |
| | Th1-16962R | GTCGTATCGTTTGGTGCAGA | 20 | 50.0 | |
| *T. atroviride* | Th3-3351F | AACTTGCTGGTGTCCTGGAT | 20 | 50.0 | 410 |
| | Th3-3351R | ATACTCGGGCAATTTCGATG | 20 | 45.0 | |

## Specificity and sensitivity

The optimized PCR condition for the specific amplification using each primer pair was 63ºC for annealing temperatures. all reactions were repeated at least two times, and results were consistent. *T. pleuroticola* s yielded a single fragment of 495bp when amplified with K1-4146F/K1-4146R. No cross reaction or additional fragment were observed for any other *Trichoderma* species (Fig. 2A). *T. pleuroti* s yielded a single fragment of 229bp when amplified with K2-1311F/K1-1311R, whereas no PCR products were amplified from other *Trichoderma* species (Fig. 2B). Only single PCR product was amplified from all s tested of *T. harzianum* isolated oyster mushroom farm when amplified with Th1-16962F/Th1-16962R. No amplification using primer pair Th1-16962F/ Th1-16962R was achieved with DNAs form any other *Trichoderma* species (Fig. 2C). *T. atroviride* was amplified a single fragment of 410bp when amplified with Th3-3351F/Th3-3351R. No PCR products were detected when DNAs of any other *Trichoderma* species were used in PCR with primer pair Th3-3351F/Th3-3351R (Fig. 2D). When the specific primers were tested with seven strains within *T. pleuroticola*, *T. pleuroti*, *T. harzianum*, and *T. atroviride*, respectively. The specific fragment of each species was amplified from all strains (Fig. 3)

To determine the minimum amount of genomic DNA that can be detected by the established PCR assay, reactions were set up with variable quantities of genomic DNA ranging from 10 pg to 10 ng. More than 500 pg of total genomic DNA of *T. pleuroticola* was sufficient to yield PCR products that could be visualized and faint band was detected when the amount was 200 pg (Fig. 4A). *T. pleuroti*-specific fragment was clearly amplified when the amount of DNA was 500 pg and faint band was detected when the amount was 100pg (Fig. 4B). A clear *T. harzianum*-specific fragment was produced when the amount of DNA was 200pg and faint band was detected when the amount was 100 or 50pg (Fig. 4C). A clear *T. atroviride*-specific fragment was produced when the amount of DNA was 500pg and faint band was detected when the amount was 200 or 100pg (Fig. 4D).

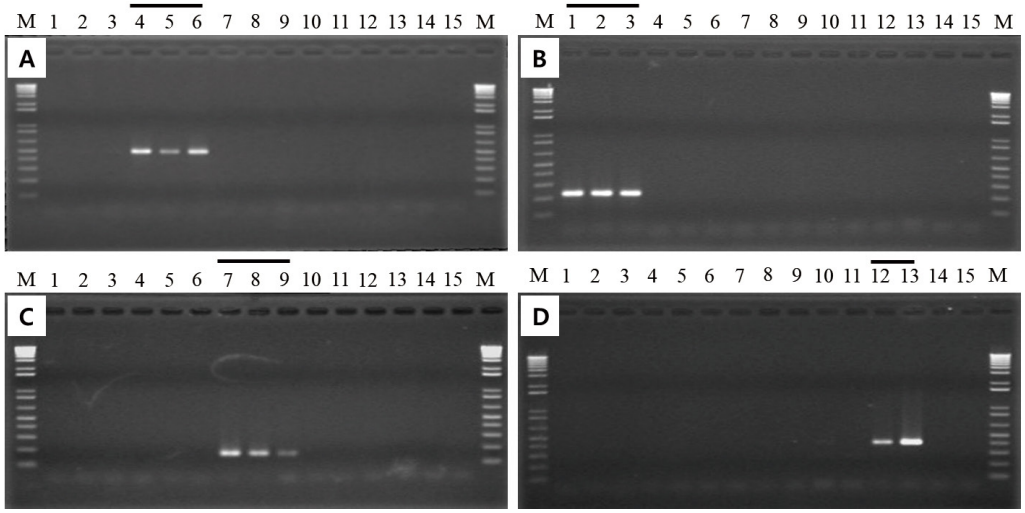


Fig. 2. PCR amplification of *Trichoderma* species using species-specific primer. A: *T. pleuroticola*-specific primer (K1-4146F/K1-4146R); B: *T. pleuroti*-specific primer (K2-1311F/K2-1311R); C: *T. harzianum*-specific primer (Th1-16962F/Th1-16962R); D: *T. atroviride*-specific primer (Th3-3351F/Th3-3351R). Size markers (M) are 50-1000bp DNA markers. lane 1 to 3: *T. pleuroti* CNU501, CNU538, and CNU523; lane 4 to 6: T.pleuroticola CNU571, CNU646, and CNU601; lane7 to 9: *T. harzianum* CNU578, CNU551, and CNU556; lane 10 and 11: *T. virens* C79 and CNU639; lane 12 and 13: *T. atroviride* CNU580 and CNU507; lane 14: *T. citrinoviride* CNU627; lane 15: *T. longibrachiatum* CNU518

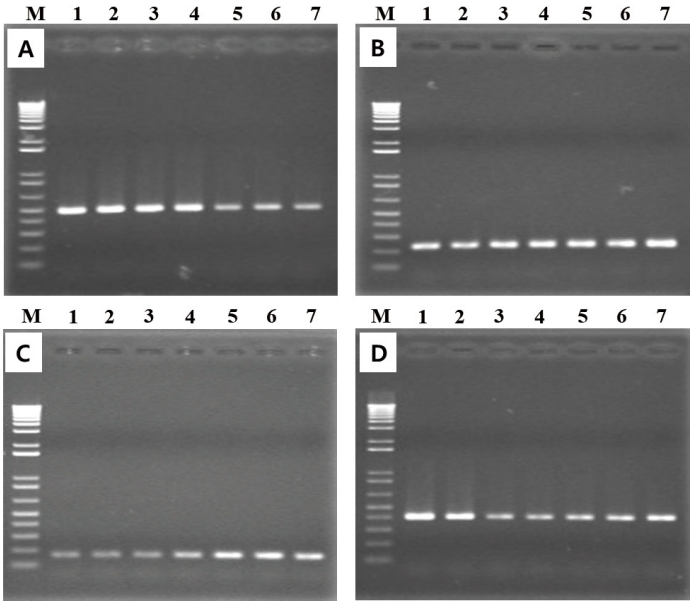


Fig. 3. PCR amplification of various isolates of *T. pleuroticola*, *T. pleuroti*, *T. harzianum*, and *T. atroviride* using species-specific primer. A: *T. pleuroticola*-specific primer (K1-4146F/K1-4146R); B: *T. pleuroti*-specific primer (K2-1311F/K2-1311R); C: *T. harzianum*-specific primer (Th1-16962F/Th1-16962R); D: *T. atroviride*-specific primer (Th3-3351F/Th3-3351R). Size markers (M) are 50-1000bp DNA markers. A: lane 1 to 7: CNU529, CNU618, CNU647, CNU682, CNU725, CNU783, CNU795; B: lane 1 to 7: CNU533, CNU537, CNU545, CNU558, CNU610, CNU729, CNU797; C: lane 1 to 7: CNU554, CNU625, CNU662, CNU663, CNU681, C40, C98; D: lane 1 to 7: CNU503, CNU511, CNU534, CNU555, CNU572, CNU693, CNU779

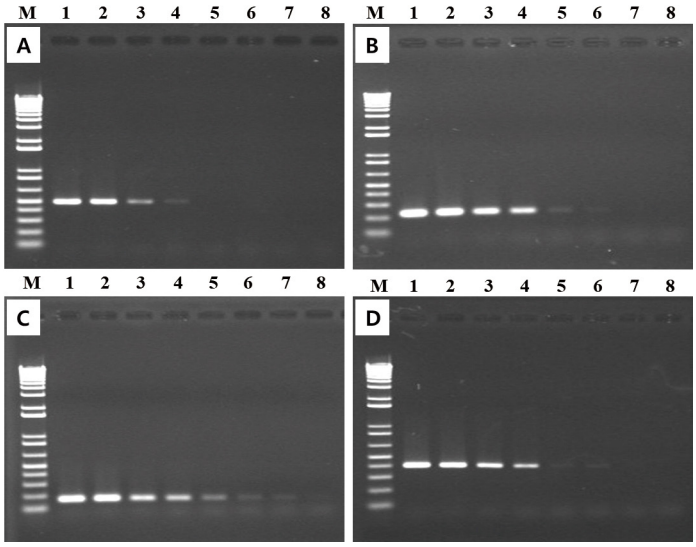


Fig. 4. Sensitivity of PCR amplification using species-specific primer. A: *T. pleuroticola*-specific primer (K1- 4146F/K1-4146R); B: *T. pleuroti*-specific primer (K2-1311F/K2-1311R); C: *T. harzianum*-specific primer (Th1-16962F/Th1-16962R); D: *T. atroviride*-specific primer (Th3-3351F/Th3-3351R). Size markers (M) are 50-1000bp DNA markers. lane 1 to 8: 10 ng, 5 ng, 1 ng, 500 pg, 200 pg, 100 pg, 50 pg and 10 pg

### Multiplex PCR by species-specific primers

Multiplex PCR reactions were performed by four pairs of species-specific primers (K1-4146F/K1-4146R, K2-1311F/K1-1311R, Th1-16962F/Th1-16962R and Th3-3351F/ Th3-3351R) and were tested with isolates representing *T. pleuroticola*, *T. pleuroti*, *T. harzianum*, and *T. atroviride*. Results presented in Fig. 5 show that specificity of primers was not affected by a multiplex PCR. Single PCR products corresponding the expected fragment of 500bp, 230bp, 180bp and 410bp were amplified *T. pleuroticola*, *T. pleuroti*, *T. harzianum*, and *T. atroviride*, respectively, when all four primer pairs were used together in multiplex reaction. Four species-specific products were produced in multiplex reaction when a mixture of DNA from *T. pleuroticola*, *T. pleuroti*, *T. harzianum*, and *T. atroviride* was amplified. However, No PCR products were produced in any of the other *Trichoderma* species isolated from oyster mushroom farm under multiplex PCR conditions. All reactions were repeated at least three times, and results were consistent.

## Discussion

The recent severe problems of green mole disease on cultivated oyster mushroom in Korea has been attributed to the emergence of new species of pathogen designated *T. pleuroticola* and *T. pleuroti* (Park et al., 2006). Green mold was more sever and difficult to control, as more than 50% isolates of *T. pleuroti* exhibited benomyl resistance. Therefore, a rapid and accurate method for identification of *T. pleuroticola* and *T. pleuroti* is important to disease control.

AFLP analysis is a powerful tool in molecular fingerprinting and for studying relationships among isolates of various organisms including bacteria and fungi (Avrova et al., 2002; Janssen et al., 1996). It is a robust and reliable technique that can be used to detect genetic differences at the isolate level. AFLP

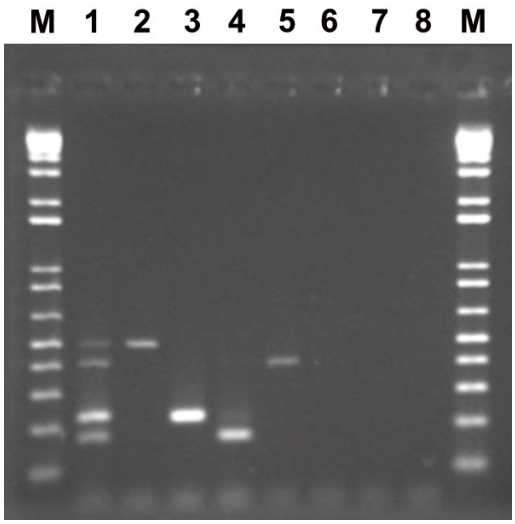


**Fig. 5. Multiplex PCR using species-specific primer. Size markers (M) are 50-1000bp DNA markers. lane 1: DNA mixture of *T. pleuroticola*, *T. pleuroti*, *T. harzianum*, and *T. atroviride*, lane 2: *T. pleuroticola*; Lane 3: *T. pleuroti*; lane 4: *T. harzianum*; lane 5: *T. atroviride*; lane 6: *T. virens*; lane 7: *T. citrinoviride*; lane 8: *T. longibrachiatum***

markers easily identified species-specific fragment, it can be converted into species-specific markers. The species-specific markers have been developed for identification and detection of plant pathogen (Casasnovas et al., 2013; Song et al., 2014). Eight AFLP primer pairs had to be screened for the successful development of diagnostic species-specific fragments for *T. pleuroticola*, *T. pleuroti*, *T. harzianum*, and *T. atroviride*, suggestion that this approach was relatively efficient. Four other primer pairs were designed that amplified species-specific markers that could resolved isolates of *Trichoderma* species associated with oyster mushroom. When the species-specific markers developed in this study were used in multiplex PCR assay, they produced reliable amplification pattern depended on amplicon size that were characteristic of *Trichoderma* species. In Korean, *Trichoderma* species associated with oyster mushroom were easily distinguished in a single PCR reaction. species-specific markers in multiplex PCR assay is very useful for identifying Korean type population in areas, where a mixed population of *T. pleuroticola* and *T. pleuroti* is suspected to occur.

The principal advantage of our species-specific markers is that it is offers an easy and reliable means to distinguish the highly aggressive species; *T. pleuroticola* and *T. pleuroti* causing major crop loss from isolates of *Trichoderma* species that are widespread in mushroom farm in Korea (Park et al., 2015a). These primers can be used routinely to determine a give *Trichoderma* population in oyster mushroom farm. Moreover, rapid and accurate identification of *T. pleuroticola* and *T. pleuroti* from *Trichoderma* species associated with oyster mushroom using this tool is very useful in effective disease control.

## Referances

1. Avrova AO, Hyman LJ. Toth RL and Toth IK. (2002). Application of amplified fragment length polymorphism fingerprinting for taxonomy and

identification of the soft rot bacteria *Erwinia carotovora* and *Erwinia chrysanthemi*. Appl Environ Microbio 68: 1499-1508.
2. Bunbury-Blanchette AL and Walker AK. (2019). *Trichoderma* species show biocontrol potential in dual culture and greenhouse bioassays against *Fusarium* basal rot of onion. Bio Control 130: 127-135.
3. Casasnovas F, Fantini EN, Palazzini JM, Giaj-Merlera G, Chulze SN, Reynoso MM and Torres AM. (2013). Development of amplified fragment length polymorphism (AFLP)-derived specific primer for the detection of *Fusarium solani* aetiological agent of peanut brown root rot. J Appl Microbiol. 114: 1782-1792.
4. Fletcher JT. (1990). *Trichoderma* and *Penicillium* diseases of *Agaricus bisporus*. A literature review for the Horticultural Development Council. London: ADAS.
5. Janssen P, Coopman R, Huys G, Swings J, Bleeker M, Vos P, Zabeau M and Kersters K. (1996). Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. Microbiology, 142: 1881-1893.
6. Lee SW and Cho YS. (2021). Historical and current perspective of oyster mushroom (*Pleurotus ostreatus*) cultivation in South Korea. Asian j adv agric. 17: 33-41.
7. Ospina-Giraldo MD, Royse DJ, Thon M R, Chen X & Romaine CP. (1998). Phylogenetic relationships of *Trichoderma harzianum* causing mushroom green mold in Europe and North America to other species of *Trichoderma* from world-wide sources. Mycologia, 90(1), 76-81.
8. Park MS, Bae KS, and Yu SH. (2006). Two new species of *Trichoderma* associated with green mold of oyster mushroom cultivation in Korea. Mycobiology, 34: 111-113.
9. Park MS, Seo GS, Lee KH, Bae KS and Yu SH. (2005a). Morphological and cultural characteristics of *Trichoderma* spp. associated with green mold of oyster mushroom in Korea. Plant Pathol J. 21: 221-228.
10. Park MS, Seo GS, Bae KS and Yu SH. (2005b). Characterization of *Trichoderma* spp. associated with green mold of oyster mushroom by PCR-RFLP and sequence analysis of ITS regions of rDNA. Plant Pathol J. 21: 229-236.
11. Rogers SO and Bendich AJ. (1994). Extraction of total cellular DNA from plants, algae and fungi. In: Gelvin S. B. and R. A. Schilperoort, editors. Plant molecular biology manual. Dordrecht: Springer Netherlands. pp. 183-190.
12. Rozen S. and Skaletsky H. (2000). Primer3 on the WWW for general users and for biologist programmers. In Bioinformatics methods and protocols. Humana Press, Totowa, NJ. pp. 365-386.
13. Samuels GJ. (1996). *Trichoderma*: a review of biology and systematics of the genus. Myco Res. 100: 923-935.
14. Samuels GJ. Dodd SL, Gams W, Castlebury LA and Petrini O. (2002). *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. Mycologia, 94: 146-170.
15. Sivasithamparam K and Ghisalberti EL. (1998). Secondary metabolism in *Trichoderma* and *Gliocladium*. pp 139-191 in: *Trichoderma* and *Gliocladium*, Vol. 1. C. P. Kubicek
16. Song ES, Kim SY, Noh TH, Cho H, Chae SC and Lee BM. (2014). PCR-based assay for rapid and specific detection of the new *Xanthomonas oryzae* pv. *oryzae* K3a race using an AFLP-derived marker. J Microbiol Biotechnol. 24: 732-739.
17. Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J and Kuiper M . (1995). AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23: 4407-4414.