Ann Microbiol (2015) 65:1591–1595
DOI 10.1007/s13213-014-0998-5

ORIGINAL ARTICLE

Multiplex PCR for detection and differentiation of diverse *Trichoderma* species

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Received: 12 August 2014 / Accepted: 29 October 2014 / Published online: 20 November 2014
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Abstract *Trichoderma* species are among the most common fungi frequently isolated as saprotrophs from free soil, soil litter, dead wood, and the rhizosphere of different crops. Four sets of species-specific primers were designed from the *tef1* and *rpb2* genes, in order to identify *Trichoderma asperellum* (*tef1* gene), *T. longibrachiatum* (*tef1* gene), *T. virens* (*tef1* gene), and *T. harzianum* (*rpb2* gene). Here, we report the development of a multiplex PCR assay to detect and distinguish each of these four most common *Trichoderma* species—viz., *T. asperellum*, *T. harzianum*, *T. longibrachiatum*, and *T. virens*—simultaneously in a single reaction through their distinct amplicons of 507, 824, 452, and 330 bp, respectively. The developed multiplex PCR technique will provide a rapid, simple, and reliable alternative to conventional methods and a new site for identification of different species of *Trichoderma* in a single reaction.

Keywords Cloning · Multiplex PCR · *rpb2* gene · ?*tef1* gene and *Trichoderma*

Introduction

The genus *Trichoderma* is cosmopolitan and typically soil-borne or wood-decaying fungi (Samuels 1996; Esposito and da Silva 1998). It is very difficult to differentiate *Trichoderma* species using morphological characteristics due to the increasing number species and the paucity of morphological characteristics (Błaszczyk et al. 2011; Prameeladevi et al. 2012a). In the mid-1990s, DNA-based sequencing methods became popular and provided high divergence through base pairs of particular genes (Samuels 2006). rRNA phylogenetic markers have limited applicability for in situ diversity studies using high-throughput methods (Esposito and da Silva 1998). Molecular markers demonstrate the variation in DNA sequences within and between the species and provide the basis for precise identification. Polymerase chain reaction (PCR) methods have found widespread use for pathogen identification, and a number of PCR-based assays have been developed for use in the diagnosis and characterization of *Trichoderma* species (Jaklitsch 2009). PCR methods are particularly promising in light of their simplicity, specificity, and sensitivity. Prameeladevi et al. (2011) reported genus-specific primers for detection of *Trichoderma* based on ITS region. Gene sequences of *tef1* are highly informative at the species level, as its small sequence renders easier and cheaper recovery of the sequences, and it also benefits from a paucity of repetitive regions that could produce misleading results owing to comparisons of non-orthologous sequence pairs. For these reasons, *tef1* has become the marker of choice for identification of *Trichoderma* (Prameeladevi et al. 2012b). Sequences of the most variable region of the *rpb2* gene (between domains 6 and 7) have proven important in studying the closely related species (Frøslev et al. 2005; Matheny 2005). *rpb2* is a single-copy gene of large size with a modest rate of evolutionary change, and provides better phylogenetic resolution in the Ascomycota. PCR priming within these highly conserved regions allows recovery of the *rpb2* genes from many different organisms for phylogenetic comparison (Liu et al. 1999). *rpb2* and *tef1* sequencing shows that the anatomy of the stroma is polyphyletic in nature (Chaverri and Samuels 2003). At present, the PCR method used for the identification of

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Trichoderma can detect only one species at a time. Hence, the objective of this study was to develop a multiplex PCR assay for simultaneous detection and differentiation of *Trichoderma* species by combining four species-specific primers in a single PCR reaction that could be used to detect species of *Trichoderma* under natural conditions.

Materials and methods

Biological material

Species of *Trichoderma* used in this study were obtained from the Culture Collection Centre (Indian Type Culture Collection [ITCC], New Delhi). They were grown in potato dextrose agar (PDA) slants and maintained at 4 °C for further study.

DNA isolation

A loopful of various species of *Trichoderma* was introduced separately into flasks containing potato dextrose broth. The cultures were incubated in a stationary state at 25 °C for 5–6 days. The mycelia were harvested after incubation, washed in sterile distilled water, and freeze-dried. The DNA of the individual fungus was extracted according to the method described by Culling (1992). The DNA pellet obtained was rehydrated by the addition of 100 μl TE buffer at 4 °C overnight. The quality and quantity of DNA were estimated using a NanoDrop spectrophotometer.

Design of species-specific primers

Species-specific primers were designed based on the sequence data of the *tef1*and *rpb2* genes (Table 1), downloaded from the NCBI database, to specifically amplify *T. asperellum*, *T. longibrachiatum*, *T. virens* and *T. harzianum*. Gradient PCR with different annealing temperatures and different concentrations of $MgCl_2$, dNTPs, and Taq DNA polymerase was performed for the development of the individual pairs (Prameeladevi et al. 2012b).tgroup

Multiplex PCR condition

Four species-specific primers (Table 1) designed for identification of *T. asperellum*, *T. harzianum*, *T. longibrachiatum*, and *T. virens* individually were comixed for a multiplex PCR panel. The different concentrations of four pairs of primers (5, 10, 20, 30, 50, and 100 pmol), genomic DNA template (5, 10, 15, 20, 25 30 ng) and Taq DNA polymerase (0.5, 1, 1.5, 2U; Bangalore Genei, India) were used for optimization of the PCR reaction. The reaction mixture also contained 1.5 mM $MgCl_2$ and 0.2 mM dNTPs in a total volume of 25 μl. The reaction was carried out in a GenePro thermal cycler (Bioer Technology Co., Ltd., China) pre-equilibrated at 96 °C to provide a hot start. The gradient PCR reaction was optimized for the development of multiplex PCR, i.e., 94 °C for 1 min, followed by 30 cycles of 45 s at 94 °C, 45 s at 55–65 °C, and 1 min at 72 °C, with a final extension step for 7 min at 72 °C. PCR products were run on 1.5 % (*w/v*) agarose gel stained with ethidium bromide. The gel was visualized under UV radiation in a gel documentation system (Bio-Rad Laboratories, CA, USA) and digitally photographed (Canon, Tokyo, Japan).

Cloning and sequencing

To further confirm species-specific markers of each species, the amplified PCR products were eluted from agarose gels and cloned into the pGEM-T Easy Vector (Promega Corporation, WI, USA). Ligations, transformations of *Escherichia coli* XL blue, and plasmid amplifications were performed following standard procedures (Sambrook and Russell 2001). After cloning, positive colonies were selected, and each colony was cultured overnight in Luria-Bertani liquid medium containing ampicillin 100 mg/L. The size of cloned fragments was verified using the corresponding primers and digested with restriction enzymes in the multiple cloning sites of the vector. The recombinant plasmids were extracted using the alkaline lysis method (Ausubel et al. 2002), and the fragment was sequenced using an automated ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, USA) by Bangalore Genei (Bangalore, India) using M13 universal primers.

Molecular phylogenetic analysis

In order to identify the fungal isolates, *tef1*and *rpb2* gene sequences of four taxa of *Trichoderma* were downloaded from GenBank and aligned with our sequences for phylogenetic analysis. The nucleotide sequences were aligned using ClustalW multiple sequence alignment (Thompson et al. 1994). Phylogenetic analysis was carried out using the MEGA 5 software program (Tamura et al. 2011), and a neighbor-joining tree was constructed using the Kimura 2-parameter distance model (Kimura 1980). Finally, the CONSENSE program was used to construct the tree.

Results

Development of multiplex PCR

In order to increase the accuracy and efficiency of *Trichoderma* identification, a multiplex PCR assay was developed that could identify *T. asperellum*, *T. harzianum*, *T. longibrachiatum*, and *T. virens* based on the banding pattern

Table 1 Design of species-specific primers for the identification of *Trichoderma* sp. (Prameeladevi et al. 2012b)

Organism	Gene	Marker	Sequence (5′–3′)	Product size
T. asperellum	*tef1*	T2A FT2A R	5′- CTCTGCCGTTGACTGTGAACG -3′ 5′-CGATAGTGGGGTTGCCGTCAA -3′	507 bp 507 bp
T. longibrachiatum	*tef1*	Tl F T1R	5′- CCGTGAGTACACACCGAGCTT -3′ 5′- CGGCTTCCTGTTGAGGGGA -3′	452 bp 452 bp
T. virens	*tef1*	T2 F T2 R	5′- CCGTTTGATGCGGGGAGTCTA-3′ 5′- GGCAAAGAGCAGCGAGGTA-3′	330 bp 330 bp
T. harzianum	*rpb2*	Th1 F Th1 R	5′-TTGCATGGGTTCGCTAAAGG-3′ 5′-TCTTGTCAGCATCATGGCCGT-3′	824 bp 824 bp

of specific amplicons. In the multiplex PCR, four sets of primers (T2A F—T2A R, Th1 F—Th1 R, T1 F-T1 R, and T2 F-T2 R) were combined in a single tube for simultaneous identification of four different *Trichoderma* species. Under optimized conditions, multiplex PCR generated specific amplicons of expected size with their respective DNA templates (Fig. 1). The experiments were repeated a minimum of 10 times to ensure reproducibility, showing that the sensitivity of the multiplex PCR was similar to that of the single-primer-set PCR. Multiplex reactions did not produce any ambiguous or extra amplicons with non-target DNA. These results indicate that four different *Trichoderma* species can be identified simultaneously in a single PCR. PCR amplification was not significantly affected by changing concentrations of $Mgcl_2$ in PCR reactions. Using optimized reaction parameters, no cross-reactivity with non-target DNA was found. No possible cross-reactions were detected with other *Trichoderma* species, bacteria, or viral taxa, using BLAST analysis.

Multiplex PCR conditions

The multiplex PCR reaction contained 10 pmol of each species-specific primer and 10 ng of genomic DNA template of each species; 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.5 U of Taq DNA polymerase were added to the reaction in total volume of 25 μl. The PCR conditions were optimized as 94 °C for 1 min, followed by 30 cycles of 45 s at 94 °C, 45 s at 60 °C, and 1 min at 72 °C, with a final extension step for 7 min at 72 °C for amplification of species-specific markers for *T. asperellum*, *T. harzianum*, *T. longibrachiatum*, and *T. virens*.

Cloning and sequencing

The specific amplicons of the species *T. asperellum*, *T. harzianum*, *T. longibrachiatum*, and *T. virens* were eluted and cloned into the pGEM-T Easy Vector, and sequenced. A BLAST search showed that the amplified products from *T. asperellum* (507 bp) and *T. harzianum* (824 bp) were 100 % homologous with the respective sequences in the GenBank database. Homologies of the products derived from *T. longibrachiatum* (452 bp) and *T. virens* (330 bp) were 98 % and 99 %, respectively. The sequences were deposited in GenBank, and accession numbers were obtained (Table 2).tgroup

Phylogenetic analysis

Nucleotype distribution suggested a close relationship between the same species of *Trichoderma* and the uniqueness of each of the four species. The phylogenetic tree formed four separate groups of four species, viz., *T. asperellum*, *T. longibrachiatum*, *T. virens*, and *T. harzianum* (Fig. 2). Phylogenetic analysis of combined *tef1* and *rpb2* sequences

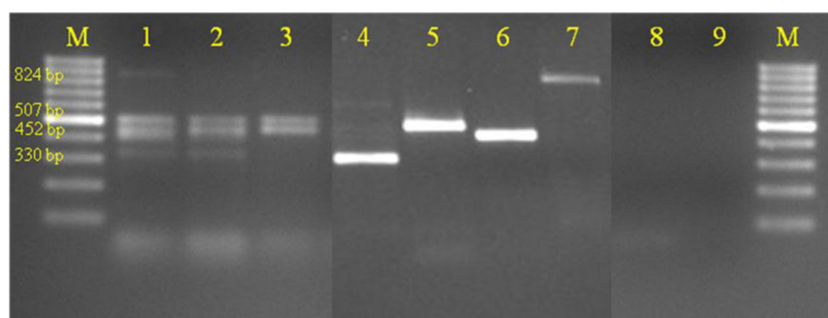

Fig. 1 Multiplex PCR showing amplified *tef1* and *rpb2* genes of *Trichoderma* species. Lanes: M−100 bp ladder, *(1) tef1* and *rpb2* genes of *T. virens*, *T. asperellum*, *T. longibrachiatum*, and *T. harzianum*; *(2) tef1* gene of *T. asperellum*, *T. longibrachiatum*, and *T. virens*; *(3) tef1* gene of *T. asperellum* and *T. longibrachiatum*; *(4) T. virens*; *(5) T. asperellum*; *(6) T. longibrachiatum*; *(7) T. harzianum*; *(8)* Other *Trichoderma* species (*T. hamatum*, *T. flavofuscum*, and *T. fasciculatum*); *(9)* Negative control

Table 2 GenBank accession numbers obtained from this study for *tef1* and *rpb2* genes of *Trichoderma*

S. No.	Organism	Gene	GenBank accession no.
1	*T. asperellum*	*tef1*	KF501572
2	*T. longibrachiatum*	*tef1*	KF501569
3	*T. virens*	*tef1*	KF501570
4	*T. harzianum*	*rpb2*	KF501571

showed the clear separation of each individual species, with 12 sequences with high phylogenetic affinity. Phylogenetic analysis proved the specificity of particular individual species for specific identification.

Discussion

Trichoderma is one of the most successful biocontrol organisms of the ascomycetes family (Liu et al. 1999; Elad 2000a; 2000b; Consolo et al. 2012; Prameeladevi et al. 2012a; 2012b). Due to the inherent difficulties and inaccuracies associated with distinguishing *Trichoderma* species based on phenotypic characteristics, molecular markers have been used for authentic identification at the species level. Species-specific primers were developed for the identification of *T. asperellum*, *T. longibrachiatum*, and *T. virens* based on the *tef1* gene and for *T. harzianum* using the *rpb2* gene (Prameeladevi et al. 2012b). Identification of unknown *Trichoderma* biocontrol isolates at the species level may be important, as different species have variable resistance to multiple plant fungal diseases (Elad 2000a: 2000b; Consolo et al. 2012). In earlier studies, the *tef1* gene was used for identification of *Trichoderma* species (Jaklitsch 2009; Jaklitsch 2011; Friedl and Druzhinina 2012; Prameeladevi et al. 2012b), as its capacity for species differentiation is superior to that of the ITS rDNA region due to the variation in sequences (Samuels et al. 1998). In fact, the ITS and *tef1* sequences sometimes appeared contradictory, resulting in conflicting identification for many species complexes of fungal genera (Hoyos-Carvajal et al. 2009), and their phylogenetic markers have limited applicability for in situ diversity studies using high-throughput methods (Friedl and Druzhinina 2012). More recently, the applicability of more conserved markers such as *rpb2* and *chi18-5* are being tested for improved identification of fungal species complexes (Friedl and Druzhinina 2012).

In order to identify the four species of relevant interest for their biocontrol aspects, a multiplex PCR was performed using four pairs of primers. Compatible PCR conditions were standardized for the amplification of primers specific in size to four different biocontrol species, viz., *T. asperellum*, *T. longibrachiatum*, *T. virens*, and *T. harzianum*. This is the first study to combine four different species-specific primers in order to develop a multiplex PCR panel for rapid and accurate identification of multiple *Trichoderma* species.

Since the multiplex PCR was developed for only two *Trichoderma* biocontrol strains (Feng et al. 2011), an attempt was made to develop a multiplex PCR method to identify four different species of *Trichoderma*, as it was developed for the identification of different fungal species (Luo and Mitchell 2002; Logotheti et al. 2008).

The reconfirmation of specificity of each amplicon in a multiplex PCR is also an important step. In this study, the amplified regions for the identification of the above species were sequenced and reconfirmed. It is evident through these studies that the markers developed using *tef1* and *rpb2* genes could phylogenetically distinguish all four species, which is consistent with the results demonstrated by Chaverri and Samuels (2003). In the present study, molecular phylogenetic analyses supported the morphology of these four species (Prameeladevi et al. 2012a).

The multiplex PCR technique described here is a precise method of identification in that it has the ability to produce the same result given the same inputs and environmental conditions. This multiplex PCR assay reduces the length of time to obtain results of high quality, and offers high sensitivity and improved detection of the above four biocontrol strains of

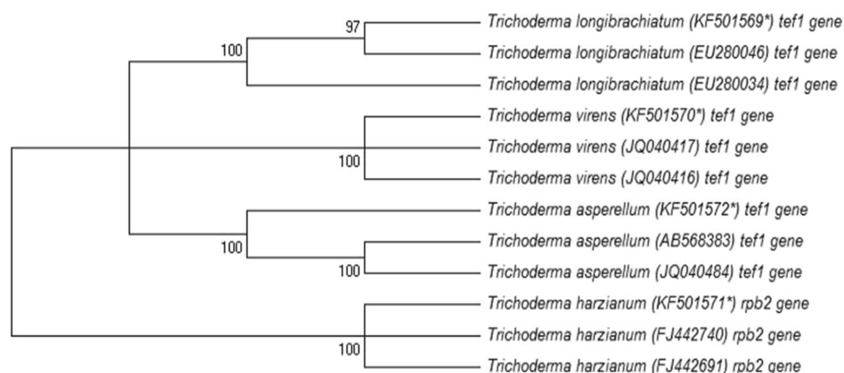

Fig. 2 Phylogenetic relationships of 12 isolates of four different *Trichoderma* spp. inferred by analysis of *tef1* and *rpb2* sequences. The neighbor-joining tree was constructed using the Kimura (1980) two-parameter model implemented in the MEGA 5.2 program. * Specific gene of a particular species used to design a specific molecular marker of individual species

Trichoderma. The appropriate combination of this new multiplex PCR assay may offer accurate and rapid identification of one or more of these four *Trichoderma* species in a single reaction. The technique is simple enough to be implemented in any molecular laboratory for detection of these *Trichoderma* species under natural conditions.

Acknowledgments The authors thank the Head of the Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi, for help in various aspects of this study. Financial support from the Department of Biotechnology of the Government Of India, New Delhi, is gratefully acknowledged.

Conflict of interest The authors declare that they have no conflict of interest.

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