Development of Species/Genus specific Primers for Identification of Three *Trichoderma* Species and for Detection of *Trichoderma* Genus

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Abstract

Trichoderma is a widely used bio-control agent, and has excellent ability to antagonize plant pathogens and promoting plant growth. It has been successfully used to control various plant diseases. The premise of using *Trichoderma* is accurate identification of it. In this study, four sets of species/genus-specific primers were designed based on the translation elongation factor 1-alpha (tef1) gene and internal transcribed spacer (ITS) region, in order to identify *Trichoderma harzianum*, *T. koningiopsis*, and *T. virens*, and detect the genus of *Trichoderma*. Here, the rapid, simple, and reliable PCR methods were development using the species-specific primers EHarF2/EHarR2, EKoisF/EKoisR, and EVireF/EVireR to produce 253 bp (tef1 gene), 255 bp (tef1 gene), and 263 bp (tef1 gene) DNA bands to identify *T. harzianum*, *T. koningiopsis*, and *T. virens*, respectively. The genus-specific primers ITricF/ITricR produces a single DNA band in the range of 103–113 bp (ITS region) to distinguish *Trichoderma* from other genera fungi, and the size of the band is related to the species of *Trichoderma*. In addition, ITricF/ITricR could use for real-time PCR amplification for detection the quantity of *Trichoderma* spp..

Introduction

Trichoderma is a widely used microorganism, which can be used not only for the production of industrial enzymes, but also for controlling plant diseases and promoting plant growth (Vinale *et al.* 2008; Nawrocka and Małolepsza 2013; Lehmann *et al.* 2016). At present, many pesticides and fertilizer products made of *Trichoderma* have been applied (Woo *et al.* 2014). Among *Trichoderma* species, *T. harzianum*, *T. virens*, *T. koningiopsis* have been widely exploited for prevention and control a variety of plant diseases (Moreno *et al.* 2009; Daguerre *et al.* 2014; Contreras-Cornejo *et al.* 2016).

Correct identification of *Trichoderma* species is a prerequisite for the use of *Trichoderma*. It is difficult to identify *Trichoderma* species by morphological characteristics (Błaszczuk *et al.* 2011; Sun *et al.* 2016; Kredics *et al.* 2018). The current method of identifying *Trichoderma* is mainly a combination of morphological and molecular characteristics (Jaklitsch 2009; Zhu and Zhuang 2015). The genes currently used for molecular identification of *Trichoderma* mainly include internal transcribed spacer (ITS), translation elongation factor 1-alpha (tef1) gene, RNA polymerase II subunit (rpb2) gene, and ATP citrate lyase (acl1) gene (Jaklitsch and Voglmayr 2015; Du Plessis *et al.* 2018). In a laboratory without a sequencing instrument, this is a time-consuming procedure for obtaining PCR products sequence information. In addition, identification of *Trichoderma* species based on BLAST searches may cause misidentification (Meincke *et al.* 2010). For the rapid identification of *Trichoderma* species, the ITS region, tef1 gene, and rpb2 gene are using to be the barcode locus for designing specific primers. So far, several species-specific primers have been developed for *Trichoderma* species identification (Friedl and Druzhinina 2012; Prabhakaran *et al.* 2015; Saroj *et al.* 2015).

Trichoderma are applied to the soil for controlling a variety of soil-borne plant diseases, such as tomato fusarium wilt and groundnut collar rot disease (Gajera *et al.* 2015; Taghdi *et al.* 2015). The amount (density) of *Trichoderma* in soil was related to the efficiency of inhibiting to soil-borne disease, high densities of *Trichoderma* spp. can effectively suppress pathogens in soil (Kataoka *et al.* 2010; Huang *et al.* 2011; Oskiera *et al.* 2017). However, the viability of *Trichoderma* in soil is affected by various factors such as soil water content, nutrients, and pH (Daryaei *et al.* 2016a; Daryaei *et al.* 2016b). So, monitoring the quantity of *Trichoderma* in soil can provide a scientific basis for increasing or no application of *Trichoderma* to soil. And, it is also important to determine *Trichoderma* where it extends in the soil. So, Hagn *et al.* (2007) developed genus-specific primers to identify and quantify *Trichoderma* spp. in soils.

At presently, a number of species/genus-specific primers have published for using to identify *T. harzianum*, *T. virens* and *Trichoderma* genus (Kredics *et al.* 2018). However, after our tests, it was found that not all published specific primers can accurately identify the target species of *Trichoderma*, and some genus specific primers of *Trichoderma* produce non-specific amplification products, so these primers can't be used to detect the amount of *Trichoderma* in environmental samples.

Therefore, in order to obtain more alternative specific primers for identification of *T. harzianum*, *T. virens*, *T. koningiopsis*, and for detection the quantity of *Trichoderma* genus. The aims of the study were to: 1) establish easy and reproducible PCR methods with species-specific primers base on *tef1* gene for rapid identification *T. harzianum*, *T. virens*, and *T. koningiopsis*; 2) utilizing the ITS region to design a genus-specific primers used for future detecting, monitoring and quantifying *Trichoderma*.

Materials And Methods

Fungal strains DNA extraction

11 *Trichoderma* species and 13 Non-*Trichoderma* strains utilized in this study were obtained from Environment and Plant Protection Institute, Chinese Academy of Tropical Agricultural Sciences, and Department of Plant Pathology, College of Agriculture, Sichuan Agricultural University (Table 1). The GenBank numbers of the strains see Table 1. Total DNA of these strains was extracted from pure cultures using the method as described by Thambugala *et al.* (2015). DNA concentrations were adjusted to 50 ng/μl and 10 ng/μl.

Table 1
Organisms for primers specificity tests

Isolates	Species	GenBank accession no.		
		ITS	tef1	rbp2
<i>Trichoderma</i>				
Tafum2	<i>T.afroharzianum</i>	MT102402	MT081432	MT081442
Tasum66	<i>T.asperellum</i>	MT102403		
Tghse7	<i>T.ghanense</i>	MT102398		MT118247
Thaum12	<i>T.harzianum</i>	MT102390	MT081433	MT118248
Thaum14	<i>T.harzianum</i>	MT102391	MT081434	MT118249
Tkoi25	<i>T.koningii</i>	MT102397		
Tkoi1	<i>T.koningiopsis</i>	MT102394	MT081435	MT081443
Tkoi2	<i>T.koningiopsis</i>	MT102395	MT081436	MT118250
Tloum3	<i>T.longibrachiatum</i>	MT102396	MT081437	MT118251
Tpyle24	<i>T.pyramidale</i>	MT102399	MT081438	MT118255
Tsise129	<i>T.sinense</i>	MT102400	MT081439	MT118252
Tvien3	<i>T.virens</i>	MT102392	MT081441	MT118253
Tvien6	<i>T.virens</i>	MT102393	MT081440	MT118254
JZ-67	<i>Trichoderma</i> sp.	HQ637333		
Non- <i>Trichoderma</i>				
JZ-5	<i>Penicillium montanense</i>	HQ637346		
JZ-26	<i>Mortierella minutissima</i>	HQ637328		
JZ-62	<i>Aspergillus versicolor</i>	HQ637361		
Mht-5	<i>Lasiodiplodia theobromae</i>	JQ658976		
Mht-18	<i>Botryosphaeria dothidea</i>	JN662929		
XCSY1	<i>Cladosporium tenuissimum</i>	MG873077		
HNCS015	<i>Colletotrichum scovillei</i>	KX673575		
Di7	<i>Diaporthe</i> sp.	MT102322		
Gif5	<i>Gibberella fujikuroi</i>	MT102320		
Glt16	<i>Gloeotinia temulenta</i>	MT102321		
Nio6	<i>Nigrospora oryzae</i>	MT102319		
Pem9	<i>Pestalotiopsis mangiferae</i>	MT102323		
Phc3	<i>Phyllosticta capitalensis</i>	MT102324		

Table 2

Design of species/genus-specific primers for the identification of three *Trichoderma* species and *Trichoderma* genus fungi

Organism	Gene	Primers	Sequence(5'–3')	Product size
<i>T.harzianum</i>	tef1	EHarF2/EHarR2	TCTGCTTCGCTCTTACTGC/TGTGTGAAGTTGATGGAAAG	253 bp
<i>T.koningiopsis</i>	tef1	EKoisF/EKoisR	CAAAATGCACTCRTTCAA/TGATTTGACATGTCAAGAGCCAGG	255 bp
<i>T.virens</i>	tef1	EVireF/EVireR	ACCTCGCTGCTCTTTGCCATCGT/GCTGGATGAATGGGACGAGA	263 bp
<i>Trichoderma</i> spp.	ITS	ITricF/ITricR	GAACCCCTCCGGGGGGGHC/TGTGCAAACACTACTGCGCAKGA	103–113 bp

Primer Designing

Species-specific primers were designed based on the sequences data of the *tef1* genes, the reference *tef1* sequences of 80 different *Trichoderma* species which are according to previous publications and 50 different Non-*Trichoderma* genera were obtained from NCBI (<http://www.ncbi.nlm.nih.gov/>) (Jaklitsch 2009; Samuels *et al.* 2012; Jaklitsch and Voglmayr 2015; Dou *et al.* 2018; Du Plessis *et al.* 2018). The genus-specific primers of *Trichoderma* were designed based on the sequences of rDNA ITS region, the reference sequences of ITS region of 50 different *Trichoderma* species which are according to previous publications and 65 different Non-*Trichoderma* genera were retrieved from NCBI (<http://www.ncbi.nlm.nih.gov/>) (Jaklitsch 2009; Jaklitsch and Voglmayr 2015; Dou *et al.* 2018; Du Plessis *et al.* 2018). All fungal *tef1* or ITS sequences alignment was performed using ClustalX (1.7) software to find the polymorphic areas of target *Trichoderma* species or *Trichoderma* spp. relative to other fungi, and manually design primers based on the polymorphic areas. The species/genus-specific primers see Table 2. Primer specificity was first analyzed online in the Primer-BLAST tool of NCBI.

Pcr Conditions

PCR with different denaturation and annealing temperatures, and different extension time was performed for different specific primers pairs.

The conventional PCR reaction mixtures (25 µl) contained: 9.5 µl of sterile deionized water, 12.5 µl of 2 × Taq PCR Mastermix (0.05 units/µl Taq DNA polymerase ; 4 mM MgCl₂, and 0.4 mM dNTPs) (Sangon Biotech, Shanghai, China), 1 µl of genomic DNA (50 ng/µl or 10 ng/µl), 1 µl of each primer (10 Mm).

The PCR parameters of EVireF/EVireR and EKoisF/EKoisR were 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 58 or 66 °C for 30 s (EKoisF/EKoisR is 58 °C; EVireF/EVireR is 66 °C), and 72 °C for 30 s. The PCR parameters of EHarF/EHarR were 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 61 °C for 30 s, 72 °C for 30 s, and the final extension step was at 72 °C for 5 min. The PCR parameters of ITricF/ITricR were 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 68 °C for 60 s, 72 °C for 90 s, and the final extension step was at 72 °C for 5 min. The amplified products were resolved by electrophoresis on 1.5% agarose gel, and stained with GoldView, and visualized under UV light.

If the specific primer does not produce non-specific products in conventional PCR amplification, the primer will be used for quantitative PCR amplification to further test its specificity. The real-time PCR were performed in the QuantStudio™ 6 Flex real-time PCR system (Life Technologies), and the reaction mixtures (25 µl) contained: 9 µl of nuclease-free water, 12.5 µl of SYBR-green PCR Master mix (Tiangen Biotech, Beijing, China), 1 µl of genomic DNA (10 ng/µl), 1 µl of each primer (10 Mm), 0.5 µl of 50X ROX dye.

The real-time PCR parameters of ITricF/ITricR were 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 62 °C for 30 s, and a melting curve of 95 °C for 15 s followed by 1 min at 60 °C with a final ramp to 95 °C with continuous data collection

to test for primer dimers and non-specific amplification.

Results

In this study, three sets species-specific primers were designed based on *tef1* genes for rapid identification of *Trichoderma harzianum*, *T. virens*, and *T. koningiopsis*. The genus-specific primers were designed based on ITS sequences for detection and quantification of *Trichoderma* spp. under soil or other natural conditions.

PCR with the EHarF2/EHarR2 primers gave a single band of 253 bp for *T. harzianum* (Fig. 1a), and other *Trichoderma* species and fungi failed to amplify band on this set. But, the EHarF2/EHarR2 could amplify a more than 500 bp band from *T. ghanense* (Fig. 1a, Lane 7). The EKoisF/EKoisR primers could amplify a target amplicons of 255 bp from only the *T. koningiopsis* (Fig. 1b). However, the primers also could produce clear and obvious DNA bands of more than 500 bp from *Cladosporium tenuissimum*, *Colletotrichum scovillei*, and *Diaporthe* sp. (Fig. 1b, Lane 18, Lane 19 and Lane 20). The *T. virens*-specific primers EVireF/EVireR gave an amplicon of 263 bp for *T. virens*, and no amplicon was observed with the other representative strains on this set (Fig. 1c), but, this primer pair also amplify a more than 500 bp band from *T. sinense* (Fig. 1c, Lane 9).

The experimental results show that although EHarF2/EHarR2, EKoisF/EKoisR and EVireF/EVireR can only amplify target bands on the target *Trichoderma* species, however these primers can also amplify non-target bands on other fungi. Therefore, these three primers pairs can only be used for species identification and not for quantitative analysis.

The ITricF/ITricR primers could amplify a single band of 103–111 bp for 11 *Trichoderma* species, and none of these other fungal taxa produced any visible band (Fig. 2a). The melting curve analysis showed that there was only a single peak at Real-Time PCR amplification with ITricF/ITricR primers pair (Fig. 2b). The Real-Time PCR products were checked by agarose gel electrophoresis, and no band was observed with the no-*Trichoderma* genus fungi. The result was shown that the ITricF/ITricR primers could amplify DNA only from *Trichoderma* genus, and could use for Real-Time PCR amplification.

Using Primer-BLAST online tool analyzes the cross-reactivity of primers with fungi group to confirm primers specificity (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The results shown that the EHarF2/EHarR2, EKoisF/EKoisR, EVireF/EVireR, and ITricF/ITricR primers pairs were no cross-reactivity with DNA of non-target species (see Additional file 1: Figs.S1, S2, S3, and S4).

Discussion

Most *Trichoderma* species were as biocontrol fungus widely using to control soil-borne diseases. In order to rapidly identify *Trichoderma* species, species-specific primers pairs such as Th1F/Th1R for *T. harzianum*, Z04_2F/Z04_2R for *T. atroviride*, and Tc_RIF/ T_RIR for *T. citrinoviride* were developed in recently years (Prabhakaran *et al.* 2015; Skoneczny *et al.* 2015; Saroj *et al.* 2015).

In the present study, we designed 3 specific primer pairs EVireF/EVireR for *T. virens*, EHarF2/EHarR2 for *T. harzianum* and EKoisF/EKoisR for *T. koningiopsis* based on the *tef1* gene, and designed primers ITricF/ITricR according to the ITS region which can amplify all species of *Trichoderma* but no other genera. These primers were analyzed by a primer-blast online tool and found to be cross-reactivity with non-target species/genera. The operation is simple, and only one routine PCR is needed to obtain a stable experimental effect, which provides a possibility for rapid identification of *T. harzianum*, *T. virens*, *T. koningiopsis* and *Trichoderma* genus, respectively. High densities of *Trichoderma* spp. can effectively suppress pathogens in soil (Qiu *et al.* 2012; Oskiera *et al.* 2017). So, monitoring the quantity of *Trichoderma* in soil can provide a scientific basis for increasing or no to apply *Trichoderma* on soil. At present, Real-Time PCR is a popular and economical tool to quantify microorganism in samples. In this study, ITricF/ITricR primers produced only one single peak at RT-PCR reaction, which

indicating that a system for detecting the quantity of *Trichoderma* in soil or other environment can be developed in the future using the primers and its RT-PCR reaction conditions.

In conclusion, the species-specific primers EHarF2/EHarR2, EKoisF/EKoisR and EVireF/EVireR can meet the requirement for rapid identification the *T. harzianum*, *T. koningiopsis* and *T. virens*. Moreover, on future application, ITricF/ITricR primers can use to development a method for detection the quantity of *Trichoderma* in artificial or natural environment.

Abbreviations

tef1
translation elongation factor 1-alpha
ITS
internal transcribed spacer
rpb2
RNA polymerase II subunit
acl1
ATP citrate lyase
T. harzianum
Trichoderma harzianum
T. virens
Trichoderma virens
T. koningiopsis
Trichoderma koningiopsis

Declarations

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Authors' contributions

Resources, methodology, funding acquisition, writing—review and editing: YZ; Resources, data curation, writing—original draft: JW; Formal analysis, validation: LY; Data curation, formal analysis: LG; Formal analysis, writing—original draft: SH; Validation, writing—review and editing, funding acquisition: WZ; Conceptualization, supervision, project administration: JH. All authors read and approved the final manuscript.

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Availability of data and materials

All data and material generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

This article does not contain studies with human participants or animals by any of the authors.

Consent for publication

The authors declare that they agree for publication.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

Figure 1

Amplification of DNA from *Trichoderma* species and other fungi by specific primers. a, Amplification of DNA from *T. harzianum* and other fungi by specific primers EHarF2/EHarR2; b, Amplification of DNA from *T. koningiopsis* and other fungi by specific primers EKoisF/EKoisR; c, Amplification of DNA from *T. virens* and other fungi by specific primers EVireF/EVireR. a, Lane 1, *T. harzianum* (Thaum12); Lane 2, *T. harzianum* (Thaum14); Lane 3, *T. virens*; Lane 4, *T. koningiopsis*; b, Lane 1, *T. koningiopsis* (Tkois1); Lane 2, *T. koningiopsis* (Tkois2); Lane 3, *T. harzianum*; Lane 4, *T. virens*; c, Lane 1, *T. virens* (Tvien3); Lane 2, *T. virens* (Tvien6); Lane 3, *T. harzianum*; Lane 4, *T. koningiopsis*. The 5-24 Lane of a, b and c are same, Lane 5, *T. longibrachiatum*; Lane 6, *T. koningii*; Lane 7, *T. ghanense*; Lane 8, *T. pyramidale*; Lane 9, *T. sinense*; Lane 10, *T. afroharzianum*; Lane 11, *T. asperellum*; Lane 12, *Trichoderma* sp.; Lane 13, *Lasiodiplodia theobromae*; Lane 14, *Phyllosticta capitalensis*; Lane 15, *Penicillium montanense*; Lane 16, *Mortierella minutissima*; Lane 17, *Aspergillus versicolor*; Lane 18, *Cladosporium tenuissimum*; Lane 19, *Colletotrichum scovillei*; Lane 20, *Diaporthe* sp.; Lane 21, *Gibberella fujikuroi*; Lane 22, *Gloeotinia temulenta*; Lane 23, *Nigrospora oryzae*; Lane 24 *Pestalotiopsis mangiferae*.

Figure 2

Specific detection of primer ITricF/ITricR. a, Amplification of DNA from *Trichoderma* spp. and other fungi by specific primers ITricF/ITricR; b, RT-PCR melt curve of the amplifying products from *Trichoderma* spp. by genus-specific primers ITricF/ITricR. a: Lane 1, *T. harzianum*; Lane 2, *T. virens*; Lane 3, *T. koningiopsis*; Lane 4, *T. longibrachiatum*; Lane 5, *T. koningii*; Lane 6, *T. ghanense*; Lane 7, *T. pyramidale*; Lane 8, *T. sinense*; Lane 9, *T. afroharzianum*; Lane 10, *T. asperellum*; Lane 11, *Trichoderma* sp.; Lane 12, *Lasiodiplodia theobromae*; Lane 13, *Botryosphaeria dothidea*; Lane 14, *Phyllosticta capitalensis*; Lane 15, *Penicillium montanense*; Lane 16, *Mortierella minutissima*; Lane 17, *Aspergillus versicolor*; Lane 18, *Cladosporium tenuissimum*; Lane 19, *Colletotrichum scovillei*; Lane 20, *Diaporthe* sp.; Lane 21, *Gibberella fujikuroi*; Lane 22, *Gloeotinia temulenta*; Lane 23, *Nigrospora oryzae*; Lane 24 *Pestalotiopsis mangiferae*.

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