



MOLECULAR IDENTIFICATION OF *TRICHODERMA* STRAINS COLLECTED TO DEVELOP PLANT GROWTH-PROMOTING AND BIOCONTROL AGENTS

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ABSTRACT

Trichoderma strains that are beneficial to both the growth and health of plants can be used as plant growth-promoting fungi (PGPF) or biological control agents (BCA) in agricultural and horticultural practices. In order to select PGPF or BCA strains, their biological properties and taxonomy must be carefully studied. In this study, 104 strains of *Trichoderma* collected at geographically different locations in Poland for selection as PGPF or BCA were identified by DNA barcoding, based on the sequences of internal transcribed spacers 1 and 2 (ITS1 and 2) of the ribosomal RNA gene cluster and on the sequences of translation elongation factor 1 alpha (*tef1*), chitinase 18-5 (*chi18-5*), and RNA polymerase II subunit (*rpb2*) gene fragments. Most of the strains were classified as: *T. atroviride* (38%), *T. harzianum* (21%), *T. lentiforme* (9%), *T. virens* (9%), and *T. simmonsii* (6%). Single strains belonging to *T. atrobrunneum*, *T. citrinoviride*, *T. crassum*, *T. gamsii*, *T. hamatum*, *T. spirale*, *T. tomentosum*, and *T. viridescens* were identified. Three strains that are potentially pathogenic to cultivated mushrooms belonging to *T. pleuroticola* and *T. aggressivum* f. *europaeum* were also identified. Four strains: TRS4, TRS29, TRS33, and TRS73 were classified to *Trichoderma* spp. and molecular identification was inconclusive at the species level. Phylogeny analysis showed that three of these strains TRS4, TRS29, and TRS33 belong to *Trichoderma* species that is not yet taxonomically established and strain TRS73 belongs to the *T. harzianum* complex, however, the species could not be identified with certainty.

Key words: fungal barcoding, multilocus sequence analysis, phylogeny, species identification

INTRODUCTION

Trichoderma fungi occur worldwide and are present in different geographic regions and climatic zones (Hoyos-Carvajal et al. 2009). *Trichoderma* can be isolated from the air, water reservoirs, soils, plants, animals, and other fungi. Several species reveal high association with plants and are often isolated from different soils as the predominant species in the plant root ecosystem (Kredics et al. 2014). *Trichoderma* plays a significant role as a decaying wood decomposer and sometimes occurs as a plant endophyte (Gazis & Chaverri 2010; Chaverri et al. 2011). First interest in *Trichoderma* was related to its cellulolytic properties. Then, it was found that

the fungi of this genus may produce multiple enzymes that can be of great importance in several industries and can be used in agricultural practice as plant growth-promoting fungi (PGPF) or biocontrol agents (BCA) to control fungal diseases or nematodes (Hermosa et al. 2013; Stewart & Hill 2014; Monfil & Casas-Flores 2014). Plant pathogens can be limited due to the facility of mycoparasitism, antibiosis, and competition of nutrients and space with *Trichoderma*. There are also other ways in which *Trichoderma* may benefit plant cultivation, such as enhancing root system and plant development, inducing resistance, and increasing the availability of nutrients (Benítez et al. 2004; Harman et al. 2004; Harman 2006).

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Commercial products based on *Trichoderma* are available on the market and are used to protect plants or to stimulate plant growth. These products are based on strains belonging to the following species: *T. asperellum* (T-203), *T. atroviride* (SC1, P1, or IMI206040), *T. harzianum* (NF-9, T1, T22, or 2413), and *T. virens* (G-6, G-6-5, or G-11) (Hermosa et al. 2000; Kullnig et al. 2001; Dodd et al. 2003; Longa et al. 2009; Chaverri et al. 2015). However, since the application of beneficial microbes has become an integrated component of plant management in agricultural or horticultural practice, interest in developing new products based on *Trichoderma* is growing. Studies conducted at the Research Institute of Horticulture in Skierniewice are aimed at developing *Trichoderma* preparations with the use of agricultural wastes to be applied in the production of vegetables and other crops for plant growth promotion, protection, and enhanced yield quality (Kancelista et al. 2013; Smolińska et al. 2014a, b). The first step in such studies should be species identification of promising *Trichoderma* strains. Correct species identification is necessary due to *Trichoderma* similarity in morphology and complex taxonomy (Hermosa et al. 2000). Especially *Trichoderma* strains that are potentially dangerous to cultivated mushrooms (*T. aggressivum* f. *europaeum*, *T. aggressivum* f. *aggressivum*, *T. pleurotum*, and *T. pleuroticola*) should be excluded from PGPF or BCA strain selection (Komoń-Żelazowska et al. 2007). Morphology alone is insufficient in accurate *Trichoderma* species identification, particularly when such a group of species as the *T. harzianum* complex or *T. viridescens* complex are investigated, as they are genetically diverse and characterized by variable morphology (Chaverri et al. 2003; Druzhinina et al. 2010; Jaklitsch et al. 2013). Recently, Chaverri et al. (2015) re-evaluated the taxonomy of *T. harzianum*; they distinguished 14 species within the *T. harzianum* complex and reported that none of the *T. harzianum* BCA strains belong to *T. harzianum* sensu stricto.

DNA-based methods used to build the modern taxonomy of the *Trichoderma* genus and DNA barcoding are now routinely used in species identification. Accurate *Trichoderma* species discrimination

can be done based on DNA sequence analysis of internal transcribed spacers (ITS) 1 and 2 of the rDNA gene cluster and fragments of the *tefl*, *rpb2*, *chi18-5*, and actin (*act*) or calmodulin (*cal1*) genes (Kundermann et al. 1998; Dodd et al. 2003; Druzhinina et al. 2005, 2010; Jaklitsch et al. 2006; Samuels et al. 2006; Gal-Hemed et al. 2011; Błaszczuk et al. 2011; Atanasova et al. 2013; Jaklitsch & Voglmayr 2015). The taxonomy of *Trichoderma* is continuously adjusted and updated (Jaklitsch 2011; Atanasova et al. 2013; Jaklitsch & Voglmayr 2015; Chaverri et al. 2015). More and more strains are isolated from different geographic regions (Mulaw et al. 2010; Gal-Hemed et al. 2011) and novel DNA sequences are available, hence still more *Trichoderma* species and clades are being established. Recently, 228 species of *Trichoderma* and several not yet taxonomically characterized species were distinguished (Jaklitsch & Voglmayr 2015).

The aim of this study was to conduct the molecular identification of *Trichoderma* strains collected in order to select PGPF or BCA strains suitable for application in agricultural and horticultural production.

MATERIALS AND METHODS

Trichoderma strains

A total of 104 *Trichoderma* strains (Table 1) collected for the selection of PGPF or BCA strains were subjected to species identification. Based on colony morphology and microscopic observations, the strains were preliminarily recognized as *T. atroviride*, *T. harzianum*, and *T. virens* (Szczech et al., unpublished). *T. atroviride* strains were previously initially identified based on ITS1 and 2, and *tefl* sequences (Skoneczny et al. 2015). The reference strains were as follows: *T. atroviride* IMI 206040 (CABI Europe, UK), *T. afroharzianum* T22 (ATCC 20847, LGC Standards, Poland), *T. virens* Gv29-8 (FGSC 10586, Fungal Genetics Stock Center, School of Biological Sciences, University of Missouri, Kansas City, USA), and *T. aggressivum* f. *europaeum* (CBS100526T, Centraalbureau voor Schimmelcultures, the Netherlands). All strains were preserved in -80°C on PDA discs submerged in glycerol.

DNA isolation

Fungal liquid cultures were initiated in Potato Dextrose Broth (Fluka, Buchs, Switzerland) and maintained on a rotary shaker at 25 °C for one week. Fungal cells were collected by centrifugation and homogenized in liquid nitrogen with quartz sand (Sigma-Aldrich, St. Louis, MO, USA). DNA was isolated using the 2× CTAB method (Aldrich & Cullis 1993) and further purified using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with minor modifications. Polyvinylpyrrolidone (PVP) and polyvinylpolypyrrolidone (PVPP) were used according to Vandroemme et al. (2008), and proteinase K and RNase A were added to the lysis buffer. Both the purity and quantity of DNA were checked by agarose gel electrophoresis, determined with the NanoDrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the DNA concentration was adjusted to 10 ng·μl⁻¹.

PCR and sequencing

PCR was used to amplify the internal transcribed spacer regions 1 and 2 (ITS1 and 2) of the rRNA gene cluster and the translation elongation factor 1-alpha (*tef1*), chitinase 18-5 (*chi18-5*), and RNA polymerase II subunit (*rpb2*) gene fragments. The PCR mixture (20 μl) contained 1× PCR buffer, 1.5 mM MgCl₂, 0.25 mM dNTPs, 0.5 μM of each primer, 4 μg bovine serum albumin (BSA), 1 U of DreamTaq Polymerase (Fermentas, Vilnius, Lithuania), and 20 ng of DNA template. The primers used in this study are listed in Table 2. The ITS region and *chi18-5* gene fragment were amplified using the following cycling parameters: 3 min at 94 °C followed by 35 cycles each of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C and, finally, 10 min at 72 °C. For amplification of the *tef1* gene fragment, the following program was used: 1 min at 94 °C, followed by 35 cycles each of 1 min at 94 °C, 1 min at 59 °C, 50 s at 74 °C and, finally, 7 min at 74 °C (www.isth.info). For the *rpb2* gene fragment the following protocol was used: 3 min at 94 °C, 5 cycles each of 45 s at 94 °C, 45 s at 60 °C, and 2 min at 72 °C, followed by 5 cycles with the temperature decreasing by 1.0 °C per cycle from 58 °C to 54 °C, followed by 30 cycles at 54 °C, and, finally, 10 min

at 72 °C (www.isth.info). To confirm DNA amplification, 1 μl of each PCR reaction was electrophoresed with 1.5% agarose – 0.5 × Tris-borate-EDTA (TBE) gels containing ethidium bromide and visualized under UV light using the GelDoc-It System with VisionWorks LS 6.7.4 (UVP, Upland, CA, USA). Prior to sequencing, enzymatic ExoSAP clean-up was performed according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). PCR products were diluted with H₂O and mixed with 5 pmol of primer up to 6 μl, and sequenced by Genomed S.A. (Warsaw, Poland) or the DNA Sequencing and Oligonucleotide Synthesis Lab (IBB PAS, Warsaw, Poland). All amplicons were sequenced from both directions with primers used for amplification (Table 2). Additional sequencing primer *tef790* was used for sequencing *tef1* of the *T. harzianum* strains (Table 2). Amplification and sequencing of *chi18-5* for three strains (TRS4, TRS29, and TRS33) was done with *ech1f* and *ech1r* primers (Table 2). Sequences of the collected strains are available at the National Center for Biotechnology Information (NCBI) GenBank (accession numbers are provided in Table 1).

Bioinformatic analysis and *Trichoderma* species identification

The raw sequence reads of ITS1 and ITS2, *tef1*, *rpb2*, and *chi18-5* were checked for quality, trimmed, manually edited and assembled using CLC Genomics Workbench 7.5 (CLCBio, Aarhus, Denmark). Taxonomic identification based on ITS 1 and 2 was performed with *TrichoKEY2* (isth.info/tools/molkey/index.php, Druzhinina et al. 2005). Sequences of ITS 1 and 2, *tef1*, and *rpb2* were also analysed using *TrichoBLAST* (isth.info/tools/blast/blast.php; Kopchinskiy et al. 2005). To conduct taxonomic identification, intron 4 of the *tef1* gene was selected with *TrichoMARK* (isth.info/tools/blast/preblast.php) and compared with the International Subcommittee on *Trichoderma* and *Hypocrea* Taxonomy (ISTH) database (www.isth.info) using *TrichoBLAST*, and also with publicly available sequences deposited at NCBI (www.ncbi.nlm.nih.gov) by using a basic local alignment search tool (BLAST) (Altschul et al. 1997).

Table 1. Origin of the *Trichoderma* strains used in this study and sequences from NCBI GenBank accession numbers (species order according to general *Trichoderma* taxonomy)

Strain no.	Source	Origin in Poland	NCBI GenBank accession number			
			ITS1 and 2	<i>tefl</i>	<i>chi18-5</i>	<i>rpb2</i>
<i>T. atroviride</i> Bissett^a						
TRS1	mushroom compost	Maków	KJ786739	KJ786820	KP009492	KP009036
TRS2	dust, mushroom farm	composting factory, Łódź	KJ786749	KJ786830	KP009508	KP009052
TRS3	mushroom farm, floor	Maków	KJ786743	KJ786824	KP009507	KP009051
TRS5	mushroom compost	composting factory, Łódź	KJ786741	KJ786822	KP009502	KP009046
TRS6	mushroom compost	Sława	KJ786750	KJ786831	KP009509	KP009053
TRS7	mushroom farm	Bieruń	KJ786747	KJ786828	KP009472	KP009015
TRS9	mushroom farm, air	Maków	KJ786738	KJ786819	KP009506	KP009050
TRS10	not known	Turek	KJ786754	KJ786835	KP009515	KP009057
TRS11	not known	WU ^b	KJ786735	KJ786816	KP009483	KP009027
TRS12	not known	WU ^b	KJ786737	KJ786818	KP009485	KP009029
TRS13	not known	WU ^b	KJ786736	KJ786817	KP009484	KP009028
TRS14	mushroom compost	LMC ^c	KJ786725	KJ786806	KP009486	KP009030
TRS15	forest wood	Głuchów	KJ786724	KJ786805	KP009499	KP009043
TRS16	not known	not known	KJ786753	KJ786834	KP009512	KP009056
TRS17	mushroom compost	Skierniewice	KJ786734	KJ786815	KP009482	KP009026
TRS18	mushroom compost	Maków	KJ786757	KJ786839	KP009519	KP009061
TRS19	soil	not known	KJ786732	KJ786813	KP009480	KP009024
TRS20	mushroom farm, floor	Sierakowice Lewe	KJ786730	KJ786811	KP009489	KP009033
TRS21	soil	Topolowo near Sochaczew	KJ786727	KJ786808	KP009487	KP009031
TRS22	soil	Wiskitki	KJ786726	KJ786807	KP009491	KP009035
TRS24	mushroom farm, wall	Ignanie Nowe	KJ786722	KJ786803	KP009498	KP009042
TRS25	mushroom farm, wall	Ignanie Nowe	KJ786731	KJ786812	KP009490	KP009034
TRS26	soil	Lipniak near Kock	KJ786751	KJ786832	KP009510	KP009054
TRS28	mushroom farm, floor	Kolonia Bolimowska	KJ786721	KJ786802	KP009494	KP009038
TRS30	soil	Skierniewice	KJ786720	KJ786801	KP009493	KP009037
TRS31	soil	Balcerów	KJ786740	KJ786821	KP009501	KP009045
TRS32	mushroom farm, air	Maków	KJ786744	KJ786825	KP009504	KP009048
TRS34	not known	not known	KJ786719	KJ786800	KP009497	KP009041
TRS36	soil	Kątno	KJ786729	KJ786810	KP009488	KP009032
TRS38	soil	Myślubórz	KJ786733	KJ786814	KP009481	KP009025
TRS39	soil	Myślubórz	KJ786728	KJ786809	KP009479	KP009023
TRS40	organic manure	ecological farm, Rzeczyce	KJ786755	KJ786836	KP009516	KP009058
TRS42	soil	Radzanów	KJ786752	KJ786833	KP009511	KP009055
TRS43	forest soil	Wielkopolski National Park ^d	KJ786748	KJ786829	KP009473	KP009016
TRS44	not known	not known	KJ786756	KJ786837	KP009517	KP009059
TRS45	mushroom compost	Maków	KJ786718	KJ786799	KP009496	KP009040
TRS46	not known	not known	KJ786723	KJ786804	KP009495	KP009039
TRS47	not known	not known	KJ786746	KJ786827	KP009503	KP009047
TRS68	mushroom compost	Skierniewice	KJ786745	KJ786826	KP009505	KP009049
<i>T. gamsii</i> Samuels & Druzhinina						
TRS123	soil	Strobów	KP009330	KP008922	KP009528	KP009065
TRS125	soil	Wola Skromowska near Kock	KP009326	KP008919	KP009524	KP009062
<i>T. viridescens</i> (A.S. Horne & H.S. Williamson) Jaklitsch & Samuels						
TRS35	soil	Łękosz near Brodnica	KP009338	KP008930	KP009522	KP009074
<i>T. sp. = T. sp. 435</i> (Hoyos-Carvajal et al. 2009) and <i>T. sp. C.P.K. 3606</i> (López-Quintero et al. 2013)						
TRS4	mushroom farm	Kolonia Bolimowska	KP009339	KP008948	KP009542	KP009082
TRS29	mushroom farm, wall	Maków	KP009340	KP008949	KP009543	KP009083
TRS33	mushroom farm, floor	Skierniewice	KP009341	KP008950	KP009544	KP009084
<i>T. hamatum</i> (Bonorden) Bainier						
TRS121	forest wood	Głuchów	KP009342	KP008953	KP009537	KP009085
TRS127	not known	not known	KP009343	KP008954	KP009538	KP009086
<i>T. virens</i> (J.H. Miller, Giddens & A.A. Foster) Arx						
TRS106	mushroom compost	LMC ^c	KP009291	KP008854	KP009462	KP009093
TRS107	mushroom compost	LMC ^c	KP009297	KP008861	KP009469	KP009099
TRS108	mushroom compost	LMC ^c	KP009290	KP008857	KP009465	KP009092
TRS109	mushroom compost	Skierniewice	KP009299	KP008864	KP009463	KP009101
TRS110	mushroom compost	Skierniewice	KP009298	KP008863	KP009461	KP009100
TRS112	mushroom compost	Skierniewice	KP009296	KP008860	KP009468	KP009098
TRS114	soil	Wola Skromowska near Kock	KP009293	KP008862	KP009467	KP009095
TRS116	soil	Radzanów	KP009289	KP008856	KP009470	KP009091
TRS117	not known	not known	KP009294	KP008858	KP009466	KP009096

<i>T. crassum</i> Bissett						
TRS113	soil	Wola Skromowska near Kock	KP009300	KP008865	KP009380	KP009102
<i>T. harzianum</i> Rifai (<i>T. harzianum sensu stricto</i>)						
TRS53	mushroom compost	Maków	KP009240	KP008808	KP009389	KP009110
TRS54	soil	not known	KP009217	KP008833	KP009408	KP009129
TRS55	mushroom farm	Maków	KP009211	KP008803	KP009400	KP009121
TRS56	mushroom compost	Częstochowa	KP009242	KP008810	KP009391	KP009112
TRS57	soil	Skierniewice	KP009241	KP008809	KP009390	KP009111
TRS58	soil	Wadowice	KP009214	KP008818	KP009403	KP009124
TRS59	soil	Balcerów	KP009237	KP008790	KP009386	KP009107
TRS61	mushroom compost	LMC ^c	KP009212	KP008804	KP009401	KP009122
TRS62	mushroom compost	Maków	KP009213	KP008817	KP009402	KP009123
TRS65	not known	Sokołów Podlaski	KP009247	KP008799	KP009396	KP009117
TRS69	mushroom farm	Maków	KP009216	KP008821	KP009406	KP009127
TRS71	soil	Janówek	KP009238	KP008806	KP009387	KP009108
TRS72	mushroom farm	Skierniewice	KP009236	KP008789	KP009385	KP009106
TRS83	soil	Nowy Białynin	KP009253	KP008820	KP009405	KP009126
TRS87	organic manure	ecological farm, Rzeszyce	KP009235	KP008812	KP009384	KP009105
TRS88	organic manure	ecological farm, Rzeszyce	KP009244	KP008796	KP009393	KP009114
TRS89	organic manure	ecological farm, Rzeszyce	KP009234	KP008813	KP009383	KP009104
TRS92	soil	Radzanów	KP009243	KP008811	KP009392	KP009113
TRS93	soil	Radzanów	KP009246	KP008798	KP009395	KP009116
TRS94	soil	Lipniak near Kock	KP009250	KP008802	KP009399	KP009120
TRS95	soil	Janowo	KP009249	KP008801	KP009398	KP009119
TRS122	mushroom compost	Skierniewice	KP009239	KP008807	KP009388	KP009109
<i>T. atroviride</i> F.B. Rocha, P. Chaverri & W. Jaklitsch, sp. nov.						
TRS60	mushroom compost	Balcerów	KP009260	KP008874	KP009432	KP009143
TRS86	soil	Rzeszyce	KP009254	KP008876	KP009431	KP009142
TRS91	soil	Wysoka	KP009261	KP008875	KP009438	KP009144
<i>T. lentiforme</i> (Rehm) P. Chaverri, Samuels & F.B. Rocha, comb. nov.						
TRS35x	soil	Lękosz near Brodnica	KP009231	KP008899	KP009436	KP009154
TRS58x	soil	Wadowice	KP009256	KP008901	KP009427	KP009157
TRS63x	soil	production field, Skierniewice	KP009230	KP008898	KP009435	KP009153
TRS64	not known	Pszczyna	KP009229	KP008897	KP009434	KP009152
TRS65x	not known	Sokołów Podlaski	KP009228	KP008896	KP009433	KP009151
TRS74	soil	Kątne	KP009258	KP008904	KP009429	KP009159
TRS76	soil	not known	KP009257	KP008903	KP009428	KP009158
TRS78	mushroom compost	Skórzec	KP009259	KP008905	KP009430	KP009160
TRS79	mushroom farm	Ignanie Nowe	KP009232	KP008902	KP009437	KP009155
<i>T. simmonsii</i> P. Chaverri, F.B. Rocha, Samuels, Degenkolb & W. Jaklitsch, sp. nov.						
TRS66	unknown	Parczew	KP009222	KP008867	KP009447	KP009135
TRS67	unknown	Radzyń Podlaski	KP009221	KP008866	KP009443	KP009134
TRS75	mushroom farm	Sierakowice Lewe	KP009223	KP008871	KP009448	KP009136
TRS77	mushroom farm, floor	Maków	KP009226	KP008869	KP009444	KP009139
TRS80	mushroom compost	Skierniewice	KP009225	KP008868	KP009445	KP009138
TRS85MO	mushroom farm, air	Sierakowice Lewe	KP009224	KP008872	KP009446	KP009137
<i>T. cf. harzianum</i>						
TRS73	mushroom farm	Kolonia Bolimowska	KP009263	KP008873	KP009442	KP009141
<i>T. aggressivum</i> f. <i>europaeum</i> Samuels & W. Gams						
TRS27	mushroom farm, air	Sierakowice Lewe	KP009302	KP008994	KP009440	KP009163
<i>T. pleuroticola</i> S.H. Yu & M.S. Park						
TRS70	forest wood	Gluchów	KP009264	KP008951	KP009450	KP009172
TRS120	forest wood	Gluchów	KP009265	KP008952	KP009451	KP009173
<i>T. tomentosum</i> Bissett						
TRS82	soil	Nowy Dwór	KP009287	KP008914	KP009455	KP009177
<i>T. spirale</i> Bissett						
TRS111	soil	Rudzieniec	KP009301	KP008963	KP009471	KP009182
<i>T. citrinoviride</i> Bissett						
TRS119	not known	WU ^b	KP009357	KP008895	KP009545	KP009183

^a *T. atroviride* strains identification is in agreement with the identification presented by Skoneczny et al. (2015), which included ITS and *tef1* sequences listed above

^b provided by Wrocław University of Environmental and Life Sciences, Poland

^c provided by the Laboratory of Mushroom Cultivars, Research Institute of Horticulture, Skierniewice, Poland

^d provided by the Poznań University of Life Sciences, Poland

Table 2. Primer names and sequences used for PCR amplification and sequencing of the ITS region and *tef1*, *chi18-5*, and *rpb2* gene fragments

Locus	Primer name	Sequence (5'-3')	Amplicon length (bp)	Reference
ITS1 and 2	ITS4	TCCTCCGCTTATTGATATGC	650	White et al. (1990)
	ITS6	GAAGGTGAAGTCGTAACAAGG		Cooke and Duncan (1997)
<i>tef1</i>	EF1-728F	CATCGAGAAGTTCGAGAAGG	1200	Carbone and Kohn (1999)
	TEF-LLErev	AACTTGCAGGCAATGTGG		Jaklitsch et al. (2006)
	tef790 ^a	GGGAGCGTCTGTGAATTG		This study
<i>chi18-5</i>	chit42-1af	AGCWAGCACSGATGCCAAC	800	Kullnig-Gradinger et al. (2002)
	chit42-2ar	AGGTTCTGAGTYGWTCCA		
	ech1f	CACTTCACCATGTTGGGCTTCCTC	1200	Fekete et al. (1996)
	ech1r	GATCTCTAGTTGAGACCGCTTCGG		
<i>rpb2</i>	RPB2-210up	TGGGGWGAYCARAARAAGG	1150	www.isth.info
	RPB2-1450low	CATRATGACSGAATCTTCCTGGT		

^a additional primer used for sequencing reactions of *T. harzianum* species complex strains

Phylogenetic analysis

For phylogenetic reconstruction, sequences of the *tef1* gene fragment (fourth and fifth intron, and a part of the sixth exon), generated in this study, published before by Hoyos-Carvajal et al. (2009), López-Quintero et al. (2013), Jaklitsch and Voglmayr (2015) and with NCBI GenBank taxonomic affiliation of *T. rogersonii*, *T. austrokonigii*, *T. pararogersonii*, *T. subeffusum* and as outgroups *T. strigosellum*, *T. strigosum* were used. Based on strain names or sequence accession numbers, sequences were retrieved from NCBI (www.ncbi.nlm.nih.gov/genbank) and compared using BLASTclust (toolkit.tuebingen.mpg.de/blastclust) with clustering of 100% identity sequence. A single GenBank sequence was selected for each sequence type (NCBI GenBank accessions are provided at Figure 1). Sequence alignments were prepared with ClustalX (Thompson et al. 1997) and manually edited using CLC Genomic Workbench 7.5 (CLCBio). Sequence sets of equal length were again processed with BLASTclust.

The obtained alignments were cleaned with a less stringent selection option using GBlocksServer (molevol.cmima.csic.es/castresana/Gblocks_server.html). The substitution model, nucleotide frequencies and substitution values were estimated with jModelTest (Darriba et al. 2012), with the AICc selection criterion (model HKY+G, -lnL = 1730.6888, K = 75, freqA = 0.2004, freqC = 0.2771, freqG = 0.2086, freqT = 0.3139, ti/tv = 1.5292, gamma = 0.387). Metropolis-coupled Markov chain Monte Carlo (MCMCMC) analysis was performed with 2 runs for 3 million generations with 4 chains with heating coefficient $\lambda = 0.2$ with MrBayes 3.2.2 x64 (Ronquist et al. 2012).

RESULTS

Among the 104 *Trichoderma* strains collected for PGPF or BCA selection for application in agricultural and horticultural production, 100 strains were clearly identified at the species level. PCR

amplification, sequencing, and sequence analysis of ITS 1 and 2 regions and the *tef1*, *chi18-5*, and *rpb2* gene fragments allowed for reliable identification of strains that belong to 15 *Trichoderma* species. The highest number of strains was identified as *T. atroviride* (39 strains), *T. harzianum* sensu stricto (22 strains), *T. lentiforme* (9 strains), *T. virens* (9 strains), and *T. simmonsii* (6 strains) (Table 1). Single strains were identified as: *T. atrobrunneum*

(3 strains), *T. gamsii* (2 strains), *T. hamatum* (2 strains), *T. pleuroticola* (2 strains), *T. aggressivum* f. *europaeum* (1 strain), *T. citrinoviride* (1 strain), *T. crassum* (1 strain), *T. spirale* (1 strain), *T. tomentosum* (1 strain), and *T. viridescens* (1 strain) (Table 1). Four loci-based identification of *T. atroviride* strains was in agreement with identification presented by Skoneczny et al. (2015).

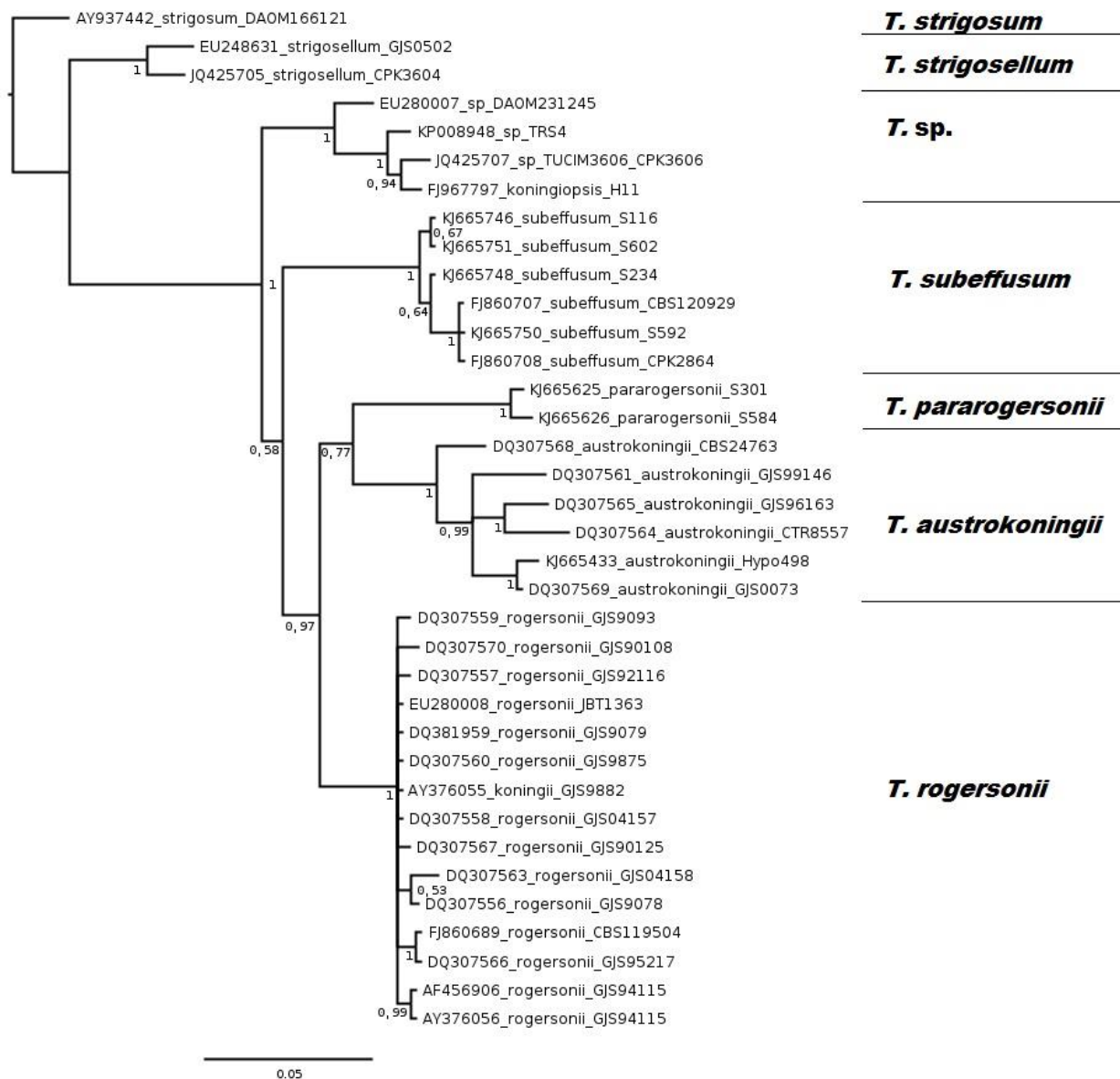


Fig. 1. Bayesian *tef1* 50% majority rule tree of the Rogersonii clade of *Trichoderma* with *T. strigosum* and *T. strigosellum* as outgroups. Node numbers indicate Bayesian posterior probability values. The names presented here indicate NCBI GenBank accession numbers and strain names. Species are grouped and names are listed on the right. TRS4, TRS29, and TRS33 *tef1* sequences are identical (NCBI GenBank Accession Numbers are: KP008948, KP008949, and KP008950)

Species identification performed with *Tricho*KEY and *Tricho*BLAST based only on ITS1 and 2, *tef1*, and *rpb2* sequences was not sufficient to identify *T. gamsii* and *T. viridescens*. Also all strains from *T. harzianum* species complex were identified as *T. harzianum*. ITS1 and 2, and *tef1* sequences only were not sufficient to identify *T. tomentosum* and *T. crassum*. Based on *tef1* and *rpb2* only, identification of *T. pleurotica* was not possible. The *tef1* sequences were insufficient to identify *T. citrinoviride* and *T. spirale*, and *T. atroviride* strains were not identified based on *rpb2*. NCBI GenBank BLAST analysis of all investigated loci fulfilled identifications, however, *chi18-5* sequences from *T. gamsii*, *T. crassum*, *T. spirale*, and *T. pleurotica* in GenBank were not present. The most conclusive identification was obtained after *Tricho*MARK selection of 4th intron from *tef1* sequences and NCBI GenBank BLAST search.

Molecular species identification was inconclusive for four strains (TRS4, TRS29, TRS33, and TRS73). It was found that strain TRS73 belongs to the *T. harzianum* species complex (*T. cf. harzianum*), however, the species could not be identified. Based on *tef1* phylogeny, strains TRS4, TRS29, and TRS33, isolated from mushroom farms, clustered together with the previously described strains DAOM231245 and TUCIM3606 which represents separate species from *T. rogersonii*, *T. austrokonigii*, *T. pararogersonii* and *T. subeffusum* in the *Rogersonii* clade (Fig. 1, Table 1).

DISCUSSION

In this study we identified *Trichoderma* strains collected in order to select the most suitable ones for application as PGPF or BCA with organic wastes in vegetable production. The preliminary classification of these strains to *T. atroviride*, *T. harzianum*, and *T. virens*, which was based on colony morphology and microscopic observations, was insufficient to reliably determine species. Within the analysed strains, several of them appeared to have been misidentified.

We used the comparative sequence analyses of ITS1 and ITS2, *tef1*, *chi18-5*, and *rpb2* loci to prepare a detailed molecular identification of strains. The main group of isolates was identified as members of three species, that is, *T. atroviride* (39 strains), *T. harzianum* sensu stricto (22 strains), and *T. virens* (9 strains). In recent decades, these species have often been recognised as the most commonly proposed candidates for biocontrol. Surprisingly, the latest studies on strains present in commercially available BCA preparations, which were previously defined as *T. harzianum*, showed that these strains were recognised as: *T. afroharzianum* in Canna AkTRIVator® and Trianium®, *T. guizhouense* in Promot® WP, and *T. simmonsii* in WP Trichosan® and Vitalin®. All of these species belong to the *T. harzianum* species complex, however, they are not *T. harzianum* sensu stricto (Chaverri et al. 2015). This suggests that during PGPF/BCA strain selection, more attention should be paid to the *T. harzianum* species complex than to *T. harzianum* sensu stricto, which is highly clonal and represents low genetic diversity (Druzhinina et al. 2010). In the strain collection analysed in this study, the *T. harzianum* species complex was represented by *T. atroviride* (3 strains), *T. lentiforme* (9 strains), *T. simmonsii* (6 strains) and one strain TRS73, which possibly represents an unknown lineage (Table 1).

Within the strains used in this study, three strains belonging to *T. aggressivum* (TRS27) and *T. pleurotica* (TRS70 and TRS120) were detected. These are morphologically similar to *T. harzianum*, however, they are pathogenic to cultivated mushrooms. Therefore, these strains will be excluded from further studies aimed at PGPF/BCA strain selection for vegetable cultivation. On the other hand, Komoń-Żelazowska et al. (2007) mentioned that two strains of *T. pleurotica* were selected as biofungicides against soil-borne plant diseases. Such fungal agents can be potentially risky for use in vegetable cultivation because they represent a potential source of mushroom pathogens. Our study confirms the necessity of careful molecular species identification

of microorganisms used for agricultural and horticultural applications.

Recently, Jaklitsch and Voglmayr (2015) recognised a new species, *T. pararogersonii*, in the Rogersonii clade that includes *T. rogersonii*, *T. austrokonigii* and *T. subeffusum*. Our study suggests that the Rogersonii clade comprises one more undescribed species represented by three *Trichoderma* strains, that is, TRS4, TRS29, and TRS33, (this study) and strains isolated from Central America DAOM 231245 and C.P.K. 3606. It has been proposed that DAOM 231245 and C.P.K. 3606 represent a putative new *Trichoderma* species described as *T. sp. 435* (Hoyos-Carvajal et al. 2009) and *T. sp. C.P.K. 3606* (López-Quintero et al. 2013). The phylogeny reconstruction that was performed in this study supports this observation (Fig. 1).

Our study confirmed the usefulness of multi-locus-based molecular identification of *Trichoderma* species. Such an identification, based on 4 loci (ITS1 and 2, *tef1*, *chi18-5*, and *rpb2*), was successful for 100 out of the 104 (96%) *Trichoderma* strains that were analyzed in this study. Based on such species identification, a selection of strains that could be potentially useful as PGPF or BCA in vegetable production could be improved and better focused. Strains belonging to *Trichoderma* species pathogenic to cultivated mushrooms can be eliminated from such programs. Also, strains belonging to taxonomic groups characterised by biocontrol properties can be distinguished, for example, in strains belonging to the *T. harzianum* species complex. The study also showed gaps in *Trichoderma* taxonomy and the existence of new, unestablished species. For even more successful *Trichoderma* species identification, further adjustment of *Trichoderma* taxonomy is necessary.

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