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# 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 (*tef*1), chitinase 18-5 (*chi*18-5), and RNA polymerase II subunit (*rpb*2) 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 *tef*1, *rpb*2, *chi*18-5, and actin (*act*) or calmodulin (*cal*1) genes (Kindermann 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; Błaszczyk 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 *tef*1 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 (*tef*1), chitinase 18-5 (*chi*18-5), and RNA polymerase II subunit (*rpb*2) gene fragments. The PCR mixture (20 µl) contained 1× PCR buffer, 1.5 mM $MgCl_2$, 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 *chi*18-5 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 *tef*1 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 *rpb*2 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 $H_2O$ 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 *tef*1 of the *T. harzianum* strains (Table 2). Amplification and sequencing of *chi*18-5 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, *tef*1, *rpb*2, and *chi*18-5 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 *Trich*OKEY2 (isth.info/tools/molkey/index.php, Druzhinina et al. 2005). Sequences of ITS 1 and 2, *tef*1, and *rpb*2 were also analysed using *Tricho*BLAST (isth.info/tools/blast/blast.php; Kopchinskiy et al. 2005). To conduct taxonomic identification, intron 4 of the *tef*1 gene was selected with *Tricho*MARK (isth.info/tools/blast/preblast.php) and compared with the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (ISTH) database (www.isth.info) using *Tricho*BLAST, 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 | *tef*1 | *chi*18-5 | *rpb*2 |
| ***T. atroviride* 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 | Kątne | KJ786729 | KJ786810 | KP009488 | KP009032 |
| TRS38 | soil | Myślibórz | KJ786733 | KJ786814 | KP009481 | KP009025 |
| TRS39 | soil | Myślibórz | 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 |
| ***T. gamsii* Samuels & Druzhinina** | | | | | | |
| TRS123 | soil | Strobów | KP009330 | KP008922 | KP009528 | KP009065 |
| TRS125 | soil | Wola Skromowska near Kock | KP009326 | KP008919 | KP009524 | KP009062 |
| ***T. viridescens* (A.S. Horne & H.S. Williamson) Jaklitsch & Samuels** | | | | | | |
| TRS35 | soil | Lękosz near Brodnica | KP009338 | KP008930 | KP009522 | KP009074 |
| ***T.* sp. = *T.* sp. 435 (Hoyos-Carvajal et al. 2009) and *T.* sp. C.P.K. 3606 (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 |
| ***T. hamatum* (Bonorden) Bainier** | | | | | | |
| TRS121 | forest wood | Głuchów | KP009342 | KP008953 | KP009537 | KP009085 |
| TRS127 | not known | not known | KP009343 | KP008954 | KP009538 | KP009086 |
| ***T. virens* (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 |

| | | | | | | |
|---|---|---|---|---|---|---|
| ***T. crassum* Bissett** | | | | | | |
| TRS113 | soil | Wola Skromowska near Kock | KP009300 | KP008865 | KP009380 | KP009102 |
| ***T. harzianum* Rifai (*T. harzianum* sensu stricto)** | | | | | | |
| 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, Rzeczyce | KP009235 | KP008812 | KP009384 | KP009105 |
| TRS88 | organic manure | ecological farm, Rzeczyce | KP009244 | KP008796 | KP009393 | KP009114 |
| TRS89 | organic manure | ecological farm, Rzeczyce | 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 |
| ***T. atrobrunneum* F.B. Rocha, P. Chaverri & W. Jaklitsch, sp. nov.** | | | | | | |
| TRS60 | mushroom compost | Balcerów | KP009260 | KP008874 | KP009432 | KP009143 |
| TRS86 | soil | Rzeczyce | KP009254 | KP008876 | KP009431 | KP009142 |
| TRS91 | soil | Wysoka | KP009261 | KP008875 | KP009438 | KP009144 |
| ***T. lentiforme* (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 | Sokołow 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 |
| ***T. simmonsii* 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 |
| ***T.* cf. *harzianum*** | | | | | | |
| TRS73 | mushroom farm | Kolonia Bolimowska | KP009263 | KP008873 | KP009442 | KP009141 |
| ***T. aggressivum* f. *europaeum* Samuels & W. Gams** | | | | | | |
| TRS27 | mushroom farm, air | Sierakowice Lewe | KP009302 | KP008994 | KP009440 | KP009163 |
| ***T. pleuroticola* S.H. Yu & M.S. Park** | | | | | | |
| TRS70 | forest wood | Głuchów | KP009264 | KP008951 | KP009450 | KP009172 |
| TRS120 | forest wood | Głuchów | KP009265 | KP008952 | KP009451 | KP009173 |
| ***T. tomentosum* Bissett** | | | | | | |
| TRS82 | soil | Nowy Dwór | KP009287 | KP008914 | KP009455 | KP009177 |
| ***T. spirale* Bissett** | | | | | | |
| TRS111 | soil | Rudzieniec | KP009301 | KP008963 | KP009471 | KP009182 |
| ***T. citrinoviride* 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 *tef*1 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 *tef*1, *chi*18-5, and *rpb*2 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) |
| *tef*1 | EF1-728F | CATCGAGAAGTTCGAGAAGG | 1200 | Carbone and Kohn (1999) |
| | TEF-LLErev | AACTTGCAGGCAATGTGG | | Jaklitsch et al. (2006) |
| | tef790[a] | GGGAGCGTCTGTGAATTG | | This study |
| *chi*18-5 | chit42-1af | AGCWAGCACSGATGCCAAC | 800 | Kullnig-Gradinger et al. (2002) |
| | chit42-2ar | AGGTTCTGAGTYGWGTCCA | | |
| | ech1f | CACTTCACCATGTTGGGCTTCCTC | 1200 | Fekete et al. (1996) |
| | ech1r | GATCTCTAGTTGAGACCGCTTCGG | | |
| *rpb*2 | 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 *tef*1 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. austrokoningii*, *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 *tef*1, *chi*18-5, and *rpb*2 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).

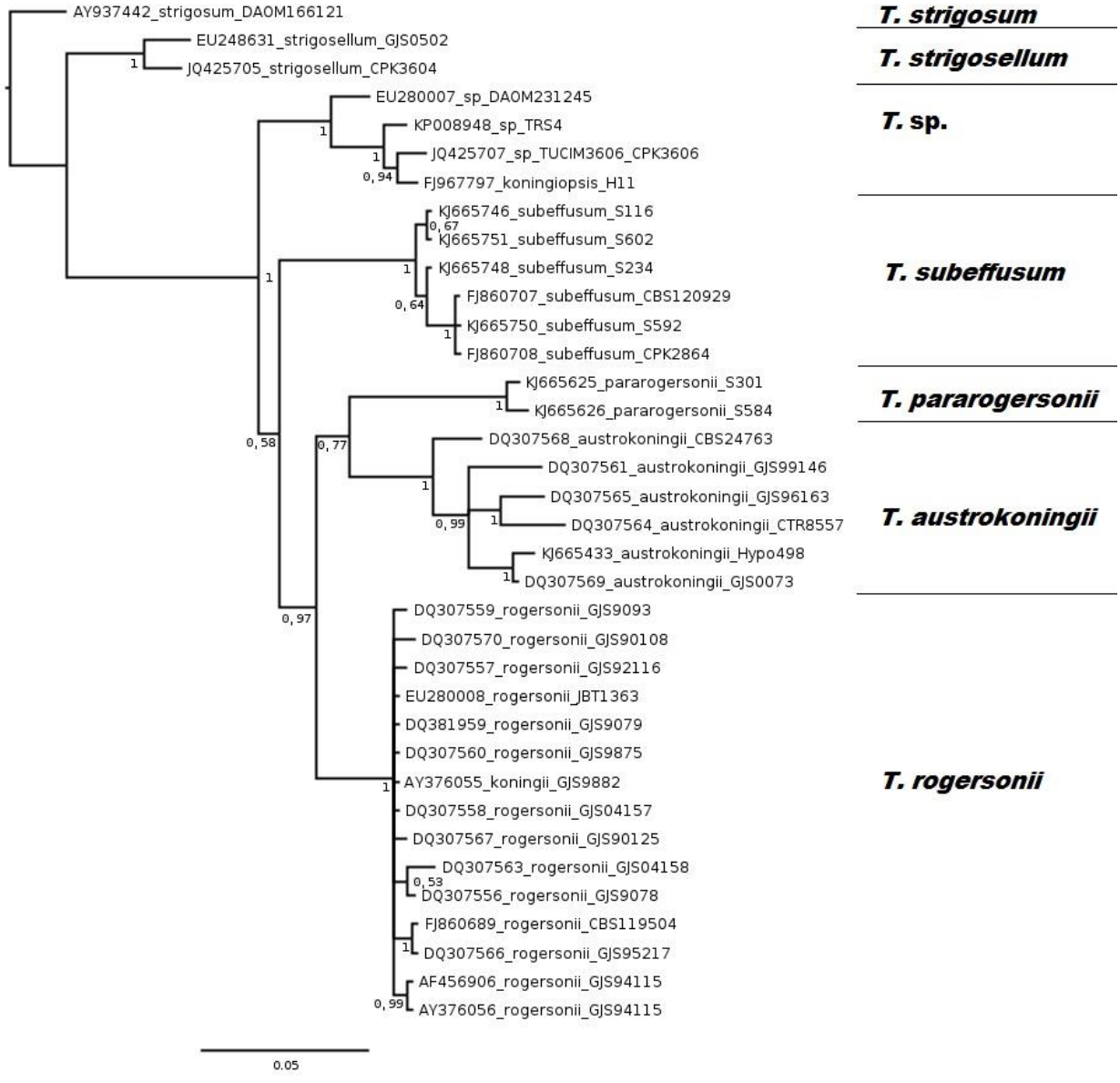


Fig. 1. Bayesian *tef*1 50% majority rule tree of the Rogersonii clade of *Trichoderma* with *T. strigosum* and *T. strigosellum* as outgroups. Node numbers indicate Bayesian posterior probability values. The names presented here indicate NCBI GenBank accession numbers and strain names. Species are grouped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

Species identification performed with *Trich*OKEY and *Tricho*BLAST based only on ITS1 and 2, *tef*1, and *rpb*2 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 *tef*1 sequences only were not sufficient to identify *T. tomentosum* and *T. crassum*. Based on *tef*1 and *rpb*2 only, identification of *T. pleuroticola* was not possible. The *tef*1 sequences were insufficient to identify *T. citrinoviride* and *T. spirale*, and *T. atroviride* strains were not identified based on *rpb*2. NCBI GenBank BLAST analysis of all investigated *loci* fulfilled identifications, however, *chi*18-5 sequences from *T. gamsii*, *T. crassum*, *T. spirale*, and *T. pleuroticola* in GenBank were not present. The most conclusive identification was obtained after *Tricho*MARK selection of 4th intron from *tef*1 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 *tef*1 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. austrokoningii*, *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, *tef*1, *chi*18-5, and *rpb*2 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 Trianum®, *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. atrobrunneum* (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. pleuroticola* (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. pleuroticola* were selected as biofungicides against soilborne 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. austrokoningii* 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 multilocus-based molecular identification of *Trichoderma* species. Such an identification, based on 4 *loci* (ITS1 and 2, *tef*1, *chi*18-5, and *rpb*2), 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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