Species diversity of *Trichoderma* in Poland

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**Abstract** In the present study, we reinvestigate the diversity of *Trichoderma* in Poland utilizing a combination of morphological and molecular/phylogenetic methods. A total of 170 isolates were collected from six different substrata at 49 sites in Poland. These were divided among 14 taxa as follows: 110 of 170 *Trichoderma* isolates were identified to the species level by the analysis of their ITS1, ITS2 rDNA sequences as: *T. harzianum* (43 isolates), *T. aggressivum* (35), *T. citrinoviride* (11), *T. hamatum* (9), *T. virens* (6), *T. longibrachiatum* (4), *T. polysporum* (1), and *T. tomentosum* (1); 60 isolates belonging to the Viride clade were identified based on a fragment of the translation-elongation factor 1-alpha (*tef1*) gene as: *T. atroviride* (20 isolates), *T. gamsii* (2), *T. koningii* (17), *T. viridescens* (13), *T. viride* (7), and *T. koningiopsis* (1). Identifications were made using the BLAST interface in *TrichoKEY* and *TrichoBLAST* ([http://www.isth.info](http://www.isth.info)). The most diverse substrata were soil (nine species per 22 isolates) and decaying wood (nine species per 75 isolates). The most abundant species (25%) isolated from all substrata was *T. harzianum*.

**Keywords** Hypocreales · Molecular identification · ITS1, ITS2 rRNA · *tef1* · Phylogenetic analysis · Biogeography

**Introduction**

The fungal genus *Trichoderma* (Ascomycetes, Hypocreales) includes cosmopolitan soil-borne species that are frequently found also on decaying wood, compost, or other organic matter (Harman et al. 2004; Samuels 2006). Several *Trichoderma* species are significant biocontrol agents against fungal plant pathogens either through direct parasitism, competition with pathogens for nutrients, stimulators of plant health, or inducers of plant systemic resistance to pathogens (Hjeljord and Tronsmo 1998; Harman et al. 2004; Bailey et al. 2006). The ability for mycoparasitism in some species also has a negative economic impact in the commercial production of *Agaricus bisporus* (J.E. Lange) Imbach and *Pleurotus ostreatus* (Paulet) Rolland mushrooms, both of which are reported for Poland (Samuels et al. 2002; Krupke et al. 2003; Hatvani et al. 2007; Szczech et al. 2008). While *Trichoderma* is not pathogenic towards healthy mammals, there is a growing number of immunocompromised individuals who suffer opportunistic infections by some species (Kuhls et al. 1999; Kredics et al. 2003; Piens et al. 2004; Druzhinina et al. 2008), and volatile compounds produced by some *Trichoderma* species can cause allergic reactions (Tang et al. 2003; Caballero et al. 2007). *Trichoderma* species produce a wide diversity of metabolites, most notably commercially important cellulase and hemicellulases, antibiotics, peptaibiotics, as well as the toxins (such as trichodermamides) and trichothecenes that display *in vitro* cytotoxicity (Kubiček and Penttilä 1998; Sivasithamparam and Ghielmi 1998; Garo et al. 2003; Liu et al. 2005; Nielsen et al. 2005; Degenkolb et al. 2006, 2008).
Because of the intimate relationship between species of *Trichoderma* and human activity, there is a great need for the accurate identification of *Trichoderma* species. However, accurate species identification based on morphology is difficult at best because of the paucity and similarity of useful morphological characters (Druzhinina et al. 2005; De Respinis et al. 2010), and increasing numbers of morphologically cryptic species that can be distinguished only through their DNA characters are being described (Atanasova et al. 2010; Samuels et al. 2010). This has already resulted in incorrect identification and the propagation of errors for strains associated with the production of secondary metabolites (Humphris et al. 2002), with human diseases (Gautheret et al. 1995), and biological control (Kullig et al. 2001). However, with the advent of molecular methods and identification tools, which are based on sequence analysis of multiple genes (rDNA and genes encoding actin, calmodulin, endochitinase, RNA polymerase II, and translation-elongation factor 1-alpha [*tef1*]), it is now possible to identify every *Trichoderma* isolate and/or recognize it as a putative new species (Druzhinina et al. 2005; Samuels 2006; Kubicek et al. 2008).

At present, the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy lists 104 species, all of which have been characterized at the molecular level (http://www.isth.info). Seventy-five species of *Hypocrea* have been identified in temperate Europe, in particular, in Austria (Jaklitsch 2009). Nevertheless, the information about the diversity of *Trichoderma/Hypocrea* in Poland is scarce. A preliminary checklist of micromycetes in Poland reported 20 *Trichoderma* species (Muleńko et al. 2008). However, all of these species were identified between 1903 and 2002 based on morphological characters.

The objective of the present study was to document the occurrence and species diversity of *Trichoderma* collected from different substrata and locations in Poland.

### Materials and methods

**Substrata, storage, and isolation of pure cultures**

Fungal isolates investigated in this study were collected from pieces of decaying wood, cultivated mushroom compost, samples of soil (garden, forest), and cereal grain (triticale, maize) at 49 sites in Poland (Table 1). Samples of decaying wood with white or brown rot were collected in parks and forests of the Wielkopolska region of Poland, placed in paper bags, dried at room temperature if wet, and stored until isolation. The pieces of decaying wood were plated on saltwater nutrient agar (SNA, Nirenberg 1976) and incubated at 20°C for 6 days. Putative *Trichoderma* colonies were purified by two rounds of subculturing on potato dextrose agar (PDA, Oxoid). Pure culture were transferred to the tube containing SNA and stored at −4°C for further study. *Trichoderma* spp. originated from other substrata were isolated according to the method described by Mańka (1974). Thirty-seven isolates originating from mushroom compost at mushroom farms in Poznań and in Skiermiewice, as well as from forest soil of the Wielkopolski National Park were kindly supplied by Profs. H. Kwasna and M. Mańka, Department of Forest Pathology, Poznań University of Life Sciences, and by Dr M. Szczech, Department of Plant Protection, Research Institute of Vegetable Crops, Skiermiewice.

**Morphological analysis**

Fungal colonies were grown on PDA and SNA at 25°C for 7 days. *Trichoderma* species were identified according to Gams and Bissett (1998) and Samuels et al. (2002, 2009; http://nt.ars-grin.gov/taxad Descriptions/keys/TrichodermaIndex. cfm).

**Isolation of DNA**

Mycelium for DNA extraction was obtained by inoculating Czapek-Dox broth (Sigma) with Yeast Extract (Oxoid) and streptomycin sulfate (50 mg/L, AppliChem), and after incubation at 25°C for 21 days on a rotary shaker (120 rpm). Mycelium was collected on filter paper in a Büchner funnel, washed with sterile water, frozen at −20°C, and freeze-dried.

Total DNA was extracted using the CTAB method (Doohan et al. 1998).

**PCR amplification and sequencing**

Primary identification was based on the sequencing of internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) of the rRNA gene cluster. In cases where ITS1 and ITS2 did not provide unambiguous identification, a fragment of the translation-elongation factor 1-alpha [*tef1*] gene was sequenced. The ITS region of the rDNA of 170 isolates was amplified using primers ITS4, ITS5 (White et al. 1990). The PCR reaction was carried out in a 25-μl reaction mixture containing the following: 1 μl 50 ng/μl of DNA, 2.5 μl 10×PCR buffer (50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl, pH 8.8, 0.1% Triton X-100), 1.5 μl 10 mM dNTP (GH Healthcare), 0.2 μl 100 mM of each primer, 19.35 μl MQ H2O, 0.25 μl (2 U/μl) DyNAzymeTM II DNA Polymerase (Finnzymes). Amplifications were performed in either a PTC-200 or PTC-100 thermocycler (MJ
| Culture code | Species                     | Sources/localization                        | Allelic group<sup>a</sup> | NCBI GenBank accession number |
|--------------|-----------------------------|---------------------------------------------|---------------------------|-------------------------------|
|               |                             |                                             | ITS1, ITS2 <br>tefl       | ITS1, ITS2 <br>tefl          |
| AN 13        | *T. atroviride*             | forest soil, WNP<sup>b</sup>                | cV3 AT1                   | HQ292784 HQ292961            |
| AN 14        | *T. atroviride*             | forest soil, WNP                            | cV3 AT1                   | HQ292785 HQ292962            |
| AN 19        | *T. atroviride*             | forest soil, WNP                            | cV3 AT1                   | HQ292786 HQ292963            |
| AN 21        | *T. harzianum*             | maize kernels, Radzików                     | cV3 AT2                   | HQ292787 HQ292953            |
| AN 22        | *T. viridescens*            | soil                                        | C1                        | HQ292839                     |
| AN 23        | *T. koningii*               | compost, Połana                             | HR7                       | HQ292866                     |
| AN 24        | *T. koningii*               | compost, Połana                             | VS3                       | HQ292943                     |
| AN 25        | *T. viridescens*            | forest soil, Malta, Połana                  | cV5 AT2                   | HQ292789 HQ292954            |
| AN 26        | *T. harzianum*             | compost, Połana                             | HR6                       | HQ292860                     |
| AN 27        | *T. harzianum*             | compost, Połana                             | HR5                       | HQ292867                     |
| AN 28        | *T. viridescens*            | forest soil, Malta Park, Połana             | cV5 VD3                   | HQ292927 HQ292995            |
| AN 29        | *T. harzianum*             | compost, Połana                             | HR3                       | HQ292873                     |
| AN 30        | *T. atroviride*             | compost, Połana                             | cV3 AT2                   | HQ292789 HQ292955            |
| AN 31        | *T. atroviride*             | compost, Połana                             | cV3 AT2                   | HQ292790 HQ292956            |
| AN 32        | *T. atroviride*             | compost, Połana                             | HR5                       | HQ292868                     |
| AN 33        | *T. koningii*               | compost, Połana                             | C1                        | HQ292842                     |
| AN 34        | *T. koningii*               | compost, Połana                             | C1                        | HQ292843                     |
| AN 35        | *T. koningii*               | compost, Połana                             | C2                        | HQ292848                     |
| AN 36        | *T. koningii*               | compost, Połana                             | C1                        | HQ292844                     |
| AN 37        | *T. koningii*               | compost, Połana                             | cV1 KO1                   | HQ292904 HQ292976            |
| AN 38        | *T. koningii*               | compost, Połana                             | cV1 KO1                   | HQ292905 HQ292977            |
| AN 39        | *T. koningii*               | compost, Połana                             | cV1 KO1                   | HQ292906 HQ292978            |
| AN 40        | *T. koningii*               | compost, Połana                             | cV1 KO1                   | HQ292907 HQ292979            |
| AN 41        | *T. koningii*               | compost, Połana                             | cV1 KO1                   | HQ292911 HQ292983            |
| AN 42        | *T. koningii*               | compost, Połana                             | cV1 KO1                   | HQ292912 HQ292984            |
| AN 43        | *T. koningii*               | compost, Połana                             | cV1 KO1                   | HQ292915 HQ292987            |

<sup>a</sup> indicates the allelic group number.

<sup>b</sup> WNP represents the Wzgórze Niedźwiedzie area.
| Culture code | Species             | Sources/localization          | Allelic group<sup>a</sup> | NCBI GenBank accession number |
|--------------|---------------------|-------------------------------|-----------------------------|--------------------------------|
|              |                     |                               | ITS1, ITS2                  |                               |
| AN 126       | *T. koningii*       | forest wood, Rusalka Park, Poznań | cV1                         | HQ292916 HQ292991             |
| AN 127       | *T. koningii*       | forest wood, Rusalka Park, Poznań | cV1                         | HQ292917 HQ292988             |
| AN 128       | *T. koningii*       | forest wood, Rusalka Park, Poznań | cV1                         | HQ292918 HQ292989             |
| AN 132       | *T. harzianum*      | forest wood, Rusalka Park, Poznań | HR5                         | HQ2928670 –                  |
| AN 133       | *T. harzianum*      | forest wood, Jezior, WNP      | HR4                         | HQ292874 –                   |
| AN 134       | *T. harzianum*      | forest wood, Jezior, WNP      | HR4                         | HQ292875 –                   |
| AN 135       | *T. harzianum*      | forest wood, Jezior, WNP      | HR4                         | HQ292876 –                   |
| AN 136       | *T. harzianum*      | forest wood, Jezior, WNP      | HR1                         | HQ292901 –                   |
| AN 137       | *T. harzianum*      | forest wood, Jezior, WNP      | HR4                         | HQ292877 –                   |
| AN 138       | *T. harzianum*      | forest wood, Jezior, WNP      | HR6                         | HQ292861 –                   |
| AN 141       | *T. viride*         | forest wood, Jezior, WNP      | cV6                         | HQ292922 HQ293008             |
| AN 142       | *T. viride*         | forest wood, Jezior, WNP      | cV8                         | HQ292920 HQ293009             |
| AN 143       | *T. koningiopsis*   | forest wood, Jezior, WNP      | cV4                         | HQ292929 HQ292992             |
| AN 144       | *T. koningii*       | forest wood, Jezior, WNP      | cV1                         | HQ292919 HQ292990             |
| AN 145       | *T. viridescens*    | forest wood, Jezior, WNP      | cV5                         | HQ292930 HQ292996             |
| AN 146       | *T. viridescens*    | forest wood, Jezior, WNP      | cV5                         | HQ292931 HQ292997             |
| AN 147       | *T. viridescens*    | forest wood, Jezior, WNP      | cV5                         | HQ292932 HQ292998             |
| AN 148       | *T. viridescens*    | forest wood, Jezior, WNP      | cV5                         | HQ292933 HQ292999             |
| AN 149       | *T. viridescens*    | forest wood, Jezior, WNP      | cV5                         | HQ292934 HQ293000             |
| AN 150       | *T. harzianum*      | forest wood, Jezior, WNP      | HR4                         | HQ292878 –                   |
| AN 152       | *T. atroviride*     | triticale kernel, Choryń       | cV3                         | HQ292792 HQ292957             |
| AN 153       | *T. atroviride*     | triticale kernel, Choryń       | cV3                         | HQ292793 HQ292958             |
| AN 155       | *T. hamatum*        | rye rizosphera, Lublin        | HM1                         | HQ292851 –                   |
| AN 171       | *T. aggressivum*    | mushroom compost, Skiermiewice | AG2                         | HQ292807 –                   |
| AN 172       | *T. aggressivum*    | mushroom compost, Skiermiewice | AG2                         | HQ292808 –                   |
| AN 176       | *T. viride*         | forest wood, Strzeszyn Park, Poznań | cV8                         | HQ292923 HQ293010             |
| AN 179       | *T. viride*         | forest wood, Strzeszyn Park, Poznań | cV8                         | HQ292924 HQ293011             |
| AN 182       | *T. atroviride*     | forest wood, Strzeszyn Park, Poznań | cV2                         | HQ292794 HQ292965             |
| AN 188       | *T. atroviride*     | mushroom compost, Skiermiewice | cV4                         | HQ292803 HQ292959             |
| AN 197       | *T. longibrachiatum*| mushroom factory, Skiermiewice | L1                          | HQ292780 –                   |
| AN 198       | *T. citrinoviride*  | mushroom factory, Skiermiewice | C1                          | HQ292845 –                   |
| AN 199       | *T. citrinoviride*  | mushroom factory, Skiermiewice | C1                          | HQ2929846 –                  |
| AN 201       | *T. citrinoviride*  | mushroom factory, Skiermiewice | C3                          | HQ292849 –                   |
| AN 203       | *T. harzianum*      | mushroom compost, Poznań       | HR4                         | HQ292879 –                   |
| AN 205       | *T. harzianum*      | mushroom compost, Poznań       | HR4                         | HQ292880 –                   |
| AN 206       | *T. atroviride*     | mushroom compost, Poznań       | cV4                         | HQ292804 HQ292960             |
| AN 207       | *T. harzianum*      | mushroom compost, Poznań       | HR4                         | HQ292881 –                   |
| AN 208       | *T. aggressivum*    | mushroom compost, Poznań       | AG1                         | HQ292805 –                   |
| AN 209       | *T. aggressivum*    | mushroom compost, Poznań       | AG1                         | HQ292882 –                   |
| AN 211       | *T. harzianum*      | mushroom compost, Poznań       | HR4                         | HQ292882 –                   |
| AN 212       | *T. atroviride*     | mushroom compost, Poznań       | cV3                         | HQ292795 HQ292966             |
| AN 213       | *T. longibrachiatum*| mushroom compost, Poznań       | L1                          | HQ292781 –                   |
| AN 215       | *T. atroviride*     | mushroom compost, Poznań       | cV3                         | HQ292796 HQ292967             |
| AN 216       | *T. aggressivum*    | mushroom compost, Poznań       | AG2                         | HQ292809 –                   |
| AN 223       | *T. harzianum*      | forest soil, WNP               | HR2                         | HQ292902 –                   |
| AN 225       | *T. hamatum*        | forest soil, WNP               | HM21                        | HQ292856 –                   |
| AN 226       | *T. viridescens*    | forest soil, WNP               | cV5                         | HQ292935 HQ293004             |
| Culture code | Species            | Sources/localization       | Allelic group<sup>a</sup> | NCBI GenBank accession number |
|--------------|--------------------|----------------------------|---------------------------|-----------------------------|
| AN 227       | *T. viridescens*   | forest soil, WNP           | cV5                       | HQ292936 HQ293001           |
| AN 229       | *T. viridescens*   | forest soil, WNP           | cV5                       | HQ292937 HQ293002           |
| AN 231       | *T. viridescens*   | forest soil, WNP           | cV5                       | HQ292938 HQ293003           |
| AN 232       | *T. hamatum*       | forest soil, WNP           | HM1                       | HQ292852 –                  |
| AN 234       | *T. tomentosum*    | forest soil, WNP           | –                         | HQ292949 –                  |
| AN 235       | *T. viride*        | forest soil, WNP           | cV7                       | HQ292921 HQ293013           |
| AN 238       | *T. hamatum*       | forest soil, WNP           | HR4                       | HQ292883 –                  |
| AN 257       | *T. harzianum*     | forest wood, Radojewo      | HR5                       | HQ292871 –                  |
| AN 259       | *T. harzianum*     | forest wood, Radojewo      | HR5                       | HQ292872 –                  |
| AN 260       | *T. harzianum*     | forest wood, Radojewo      | HR4                       | HQ292884 –                  |
| AN 261       | *T. harzianum*     | forest wood, Radojewo      | HR4                       | HQ292885 –                  |
| AN 262       | *T. citrinoviride* | forest wood, Radojewo      | C1                        | HQ292847 –                  |
| AN 263       | *T. longibrachiatum* | mushroom compost, Poznań | L1                        | HQ292782 –                  |
| AN 264       | *T. longibrachiatum* | mushroom compost, Poznań | L2                        | HQ292783 –                  |
| AN 266       | *T. viride*        | mushroom compost, Poznań   | cV8                       | HQ292925 HQ293012           |
| AN 273       | *T. harzianum*     | forest soil, Kórnik        | HR4                       | HQ292886 –                  |
| AN 274       | *T. harzianum*     | forest soil, Kórnik        | HR4                       | HQ292887 –                  |
| AN 275       | *T. harzianum*     | forest soil, Kórnik        | HR4                       | HQ292888 –                  |
| AN 276       | *T. harzianum*     | forest soil, Kórnik        | HR4                       | HQ292889 –                  |
| AN 277       | *T. hamatum*       | forest soil, Kórnik        | HM1                       | HQ292857 –                  |
| AN 278       | *T. harzianum*     | forest soil, Kórnik        | HR4                       | HQ292890 –                  |
| AN 279       | *T. hamatum*       | forest soil, Kórnik        | HR4                       | HQ292858 –                  |
| AN 281       | *T. atroviride*    | forest soil, Kórnik        | cV2                       | HQ292804 HQ292974           |
| AN 282       | *T. harzianum*     | forest soil, Kórnik        | HR4                       | HQ292891 –                  |
| AN 283       | *T. harzianum*     | forest soil, Kórnik        | HR4                       | HQ292892 –                  |
| AN 284       | *T. harzianum*     | forest soil, Kórnik        | HR4                       | HQ292893 –                  |
| AN 285       | *T. harzianum*     | forest soil, Kórnik        | HR4                       | HQ292894 –                  |
| AN 286       | *T. harzianum*     | forest soil, Kórnik        | HR4                       | HQ292895 –                  |
| AN 287       | *T. atroviride*    | forest soil, Radojewo      | cV3                       | HQ292798 HQ292969           |
| AN 288       | *T. viridescens*   | forest soil, Kórnik        | cV5                       | HQ292941 HQ293006           |
| AN 425       | *T. harzianum*     | forest wood, Radojewo      | HR4                       | HQ292896 –                  |
| AN 426       | *T. harzianum*     | forest wood, Radojewo      | HR4                       | HQ292897 –                  |
| AN 427       | *T. viridescens*   | forest wood, Radojewo      | cV5                       | HQ292942 HQ293007           |
| AN 430       | *T. viride*        | forest wood, Radojewo      | cV8                       | HQ292926 HQ293014           |
| AN 431       | *T. harzianum*     | forest wood, Radojewo      | HR4                       | HQ292898 –                  |
| AN 435       | *T. harzianum*     | forest wood, Radojewo      | HR4                       | HQ292899 –                  |
| AN 436       | *T. atroviride*    | forest wood, Radojewo      | cV3                       | HQ292799 HQ292970           |
| AN 437       | *T. harzianum*     | forest wood, Radojewo      | HR4                       | HQ292900 –                  |
| AN 550       | *T. gamsii*        | forest soil, Poznań        | cV9                       | HQ292952 –                  |
| AN 561       | *T. aggressivum*   | mushroom compost, Nowy Tomyśl | AG2                     | HQ292810 –                  |
| AN 562       | *T. aggressivum*   | mushroom compost, Ostróda  | AG2                       | HQ292811 –                  |
| AN 563       | *T. aggressivum*   | mushroom compost, Toruń    | AG2                       | HQ292812 –                  |
| AN 564       | *T. aggressivum*   | mushroom compost, Komiza   | AG2                       | HQ292813 –                  |
| AN 565       | *T. aggressivum*   | mushroom compost, Siemiatycze | AG2                     | HQ292814 –                  |
| AN 566       | *T. aggressivum*   | mushroom compost, Olszyn    | AG2                       | –                           |
| AN 567       | *T. aggressivum*   | mushroom compost, Tychy     | AG2                       | HQ292815 –                  |
Research, USA) under the following conditions: initial denaturation 5 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 58°C (for the ITS region), or 63°C (for the \textit{tef1} fragment), 1 min at 72°C, with the final extension of 10 min at 72°C. Amplification products were separated on 1.5% agarose gel (Invitrogen) in 1×TBE buffer (0.178 M Tris-borate, 0.178 M boric acid, 0.004 M EDTA) and stained with ethidium bromide. The 10-μl PCR products were combined with 2 μl of loading buffer (0.25% bromophenol blue, 30% glycerol). A 100-bp DNA Ladder Plus (Fermentas) was used as a size standard. PCR products were electrophoresed at 3 V cm⁻¹ for about 2 h, visualized under UV light, and photographed (Syngene UV visualizer). The 3-μl PCR products were purified with exonuclease I and shrimp alkaline phosphatase according to Chełkowski et al. (2003). Sequencing reactions were prepared using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit in 5 μl volume (Applied Biosystems, Switzerland). DNA sequencing was performed on an ABI PRISM 310 Genetic Analyzer (USA). Sequences were edited and assembled using Chromas v.1.43 (Applied Biosystems). CLUSTAL W (Thompson et al. 1994) and MUSCLE (Edgar 2004) were used to align the sequences; the resulting alignments were inspected and refined manually.

| Culture code | Species          | Sources/localization       | Allelic group a | NCBI GenBank accession number |
|--------------|------------------|----------------------------|----------------|-------------------------------|
| AN 568       | \textit{T. aggressivum} | mushroom compost, Bytom   | AG2            | HQ292816                      |
| AN 569       | \textit{T. aggressivum} | mushroom compost, Losice   | AG2            | HQ292817                      |
| AN 570       | \textit{T. aggressivum} | mushroom compost, Biała Podlaska | AG2     | HQ292818                      |
| AN 571       | \textit{T. aggressivum} | mushroom compost, Międzychód | AG2     | HQ292819                      |
| AN 572       | \textit{T. aggressivum} | mushroom compost, Gorzów Wlkp. | AG2     | HQ292820                      |
| AN 573       | \textit{T. aggressivum} | mushroom compost, Przemyśl | AG2            | HQ292821                      |
| AN 574       | \textit{T. aggressivum} | mushroom compost, Siedle    | AG2            | HQ292822                      |
| AN 575       | \textit{T. aggressivum} | mushroom compost, Sokółów Podlaski | AG2     | HQ292823                      |
| AN 576       | \textit{T. aggressivum} | mushroom compost, Chojnice  | AG2            | HQ292824                      |
| AN 577       | \textit{T. aggressivum} | mushroom compost, Szczecinek | AG2            | HQ292825                      |
| AN 578       | \textit{T. aggressivum} | mushroom compost, Krosno Lubuskie | AG2     | HQ292826                      |
| AN 579       | \textit{T. aggressivum} | mushroom compost, Zielona Góra | AG2     | HQ292827                      |
| AN 580       | \textit{T. harzianum} | mushroom compost, Pszczyna | HR6            | HQ292862                      |
| AN 581       | \textit{T. harzianum} | mushroom compost, Marianów/Koło | HR6     | HQ292863                      |
| AN 582       | \textit{T. aggressivum} | mushroom compost, Turek     | AG3            | HQ292835                      |
| AN 583       | \textit{T. aggressivum} | mushroom compost, Czuchów   | AG3            | HQ292836                      |
| AN 584       | \textit{T. aggressivum} | mushroom compost, Piła      | AG3            | HQ292837                      |
| AN 585       | \textit{T. aggressivum} | mushroom compost, Skierniewice | AG2     | HQ292828                      |
| AN 586       | \textit{T. aggressivum} | mushroom compost, Świecie   | AG2            | HQ292829                      |
| AN 587       | \textit{T. aggressivum} | mushroom compost, Skierniewice | AG3     | HQ292838                      |
| AN 590       | \textit{T. harzianum} | mushroom compost, Piasek/Pszczyna | HR6     | HQ292864                      |
| AN 591       | \textit{T. aggressivum} | mushroom compost, Wolsztyn  | AG2            | HQ292830                      |
| AN 592       | \textit{T. aggressivum} | mushroom compost, Rzeszów   | AG2            | HQ292831                      |
| AN 593       | \textit{T. atroviride} | mushroom compost, Pszczyna  | cV3 AT1        | HQ292800 HQ292971             |
| AN 594       | \textit{T. aggressivum} | mushroom compost, Rakoniewice | AG2     | HQ292832                      |
| AN 595       | \textit{T. aggressivum} | mushroom compost, Wielichowo | AG2            | HQ292833                      |
| AN 596       | \textit{T. atroviride} | mushroom compost, Jarocin    | cV3 AT1        | HQ292801 HQ292972             |
| AN 597       | \textit{T. harzianum} | mushroom compost, Kalisz     | HR6            | HQ292865                      |
| AN 599       | \textit{T. aggressivum} | mushroom compost, Pszczyna  | AG2            | HQ292834                      |
| AN 600       | \textit{T. atroviride} | mushroom compost, Pszczyna  | cV3 AT1        | HQ292802                      |

\(a\) The group of isolates possessing identical alleles in the locus of ITS or \textit{tef1}, analyzed in the present study(Figs. 1 and 2)

\(b\) WNP: Wielkopolski National Park
Molecular identification and phylogenetic analysis

For species identification, ITS1 and ITS2 sequences were submitted to the BLAST interface in TrichoKEY (http://www.isth.info; Druzhinina et al. 2005; Druzhinina and Kubicek 2005). In ambiguous cases, the result was re-checked using the TrichoBLAST program based on tef1 gene sequences (Druzhinina and Kopchinskiy 2004a, b). All positions containing gaps and missing data were eliminated from the dataset. Phylogenetic analyses were performed in MEGA4 (Tamura et al. 2004). Both ITS1, ITS2 and tef1 gene sequences were analyzed using the maximum parsimony (Eck and Dayhoff 1966) approach of close-neighbor-interchange algorithm with search level 3 (Nei and Kumar 2000), in which the initial trees were obtained with the random addition of sequences (10,000 replicates). In total, there were 48 parsimony informative positions retained from an initial alignment of 368 for the ITS1, ITS2 sequences and 491 positions in the final dataset, of which 118 were parsimony informative for tef1 gene sequences. In both cases, to infer the consensus, phylogenetic trees bootstrapping with 10,000 data replicates was conducted (Felsenstein 1985).

Results

Species identification

A total of 170 isolates were obtained from the six different substrata at 49 localities in Poland. Of these 170 Trichoderma isolates, 110 were identified at the species level by morphological characters and analysis of their ITS1, ITS2 nucleotide sequences as: T. harzianum Rifai (43 isolates), T. aggressivum Samuels & W. Gams (35), T. citrinoviride Bisset (11), T. hamatum (Bonord.) Bainier (9), T. virens (J.H. Mill., Giddens & A.A. Foster) Arx (6), T. longibrachiatum Rifai (4), T. polysporum (Link) Rifai (1), and T. tomentosum Bissett (1). In case of the remaining 60 Trichoderma isolates, where ITS1 and ITS2 did not provide unambiguous identification, the fragment of the tef1 gene was sequenced. Thereby, the following species were identified: T. atroviride P. Karst. (20 isolates), T. gamsii Samuels & Druzhin. (2), T. koningii Oudem. (17), T. viridescens (A.S. Horne & H.S. Will.) Jaklitsch & Samuels (13), T. virens Pers. (7), and T. koningiopsis Oudem. (1). The identification, origin, and NCBI GeneBank accession numbers of all isolates are given in Table 1.

Phylogenetic analysis

The result of the phylogenetic analysis based on the ITS sequences of 170 Trichoderma isolates is shown in Fig. 1.

In the ITS tree, the Harzianum clade, with T. harzianum, T. aggressivum, and T. tomentosum, the Longibrachiatum Clade, with T. longibrachiatum and T. citrinoviride, and the species T. virens, T. hamatum, and T. polysporum were distinguished in a single moderately supported branch with bootstrap support of 79%. Forty-three strains were identified as T. harzianum, but this species is known to include several ITS alleles (Hermosa et al. 2004; Migheli et al. 2009) and is considered to be a species complex (Chaverri et al. 2003). In the present research, seven haplotypes of T. harzianum were found (HR1, HR2, HR3, HR4, HR5, HR6, and HR7, according to Table 1 and Fig. 1). With bootstrap support of only 53%, these seven haplotypes of T. harzianum formed a moderately well-supported (75%) clade with T. aggressivum and an unresolved polytomy with T. tomentosum. Two groups were distinguished within the Longibrachiatum clade with moderate to good bootstrap support. One group, with a bootstrap value of 70%, contains four strains of T. longibrachiatum. The second group, with a bootstrap value of 93% includes 11 strains of T. citrinoviride. Sixty isolates of Trichoderma, belonging to the Viride clade, formed a polytomy. A phylogenetic analysis based on tef1 sequences was performed for them (Fig. 2). As a result of this, the six species (T. koningii, T. atroviride, T. virens, T. viridescens, T. gamsii) were resolved with high bootstrap support.

Species diversity

Fourteen species of Trichoderma were identified among 170 isolates collected from six different substrata and 49 localities in Poland, using both morphological and molecular analysis. The highest diversity of Trichoderma species was detected in the set of 22 isolates originating from soil, which included nine species (T. atroviride, T. citrinoviride, T. gamsii, T. hamatum, T. harzianum, T. polysporum, T. tomentosum, T. virens, T. viridescens). Most of the isolates were collected from decaying wood (75), but among them, only nine species were found (T. atroviride, T. citrinoviride, T. gamsii, T. hamatum, T. harzianum, T. koningii, T. koningiopsis, T. virens, T. viridescens). The single strains of T. polysporum and T. tomentosum were isolated from soil, whereas all 17 strains of T. koningii were isolated from forest wood at several sites. The 58 isolates from mushroom compost and mushroom farms comprised seven species: T. aggressivum, T. atroviride, T. citrinoviride, T. harzianum, T. longibrachiatum, T. virens, and T. viride. In the limited samples from grains of Zea mays and Triticosecale Wittm. ex A. Camus as well as from garden compost, only three species were identified: T. atroviride, T. harzianum, and T. virens. T. harzianum was the most abundant species (25%) and was isolated from all substrata. It was the most common species isolated from pieces of decaying wood (40%, 30 isolates). After T. harzianum, T. atroviride, T. koningii, T.
viridescens, and T. citrinoviride were the most abundant (respectively, 12%, 15%, 12%, and 7% of 112 isolates) Trichoderma species collected from soil, compost, forest wood, and cereal grains, respectively. The most common species isolated from mushroom compost was T. aggressivum (60% of isolates originated from mushroom compost and 20% of all isolates from the collection). T. hamatum, T. virens, T. viride, T. longibrachiatum, T. gamsii, T. koningiopsis, T. polysporum, and T. tomentosum were the most scarcely identified species of the genus (≤5% of all isolates from the collection).

Discussion

The present study is a preliminary domestic assessment of Trichoderma diversity in Poland. A collection of 170 isolates obtained from six different substrata and 49 localities in Poland were identified by phenetic observations and by analysis of the ITS 1, ITS 2 region of rRNA gene cluster and/or a fragment of the tef1 gene. A wide diversity of Trichoderma isolates was found (14 species were identified among 170 isolates) in comparison with the studies on the biodiversity of Trichoderma in South-East Asia (Kubicek et al. 2003), in Austria (Wuczkowski et al. 2003), in South America (Druzhinina et al. 2005), in China (Zhang et al. 2005), and on Sardinia (Migheli et al. 2009). The highest diversity of Trichoderma was found in Colombia, Mexico, Guatemala, Panama, Peru, Ecuador, and Brazil (Hoyos-Carvajal et al. 2009). Hoyos-Carvajal et al. (2009) recorded almost twice as many species from a comparably sized sample of 183 isolates collected in these neotropical regions.

Here and in a previous study, T. harzianum was the predominant taxon (Kubicek et al. 2003; Wuczkowski et al. 2003; Druzhinina et al. 2005, 2010; Zhang et al. 2005; Migheli et al. 2009). T. harzianum is the most commonly reported species in the genus, occurring in diverse ecosystems and ecological niches. However, it must be borne in mind that the name ‘T. harzianum’ applies to a species complex within which several morphologically cryptic phylogenetic species—haplotypes—are found (results presented here) and these ‘haplotype species’ may be seen to comprise a multiplicity of species when subjected to multilocus phylogenetic analysis (Chaverri et al. 2003; Gherbawy et al. 2004; Zhang et al. 2005; Druzhinina et al. 2010). In the present research, seven haplotypes (HR1–HR7) were evident in the analysis of ITS sequences for T. harzianum isolates. Haplotypes HR1, HR3, HR4, HR5, HR6, and HR7 correspond with ITS haplotypes, which are very common in Europe (Jaklitsch 2009, Chaverri et al. [unpublished]; Woo et al. [unpublished]). Haplotype HR2 (isolate AN 223) corresponds to the ex neotype strain of T. harzianum CBS 226.95, and, thus, represents T. harzianum sensu stricto. T. harzianum sensu stricto is also a species with a broad north temperate distribution, including at least North America, Europe, and Asia (Zhang et al. 2005; Chaverri and Samuels [unpublished]).
The second abundant species identified in the present study and the most prevalent species from mushroom compost was *T. aggressivum* (35 isolates). This result corresponds with the previous study of Szczec et al. (2008), who showed that, between 2004 and 2006, *T. aggressivum* was the most frequently isolated species of the genus identified in Polish mushroom farms. *T. aggressivum* has been isolated from mushroom compost used for *A. bisporus* cultivation in Europe and North America (Samuels et al. 2002). This species has only been isolated once from soil in Kenya (Samuels and Szakacs [unpublished]). It is not yet known whether this species also occurs in natural environments.

Other species identified in the present study were: *T. atroviride* (20 isolates), *T. koningii* (17), *T. viridescens* (13), *T. citrinoviride* (11), *T. hamatum* (9), *T. viride* (7), *T. virens* (6), *T. longibrachiatum* (4), *T. gamstii* (2), *T. koningiopsis* (1), *T. polysorum* (1), and *T. tomentosum* (1). These species are representative of a temperate *Trichoderma* biota (Kubicek et al. 2008). *T. viride*, *T. viridescens*, *T. koningii*, *T. citrinoviride*, *T. aggressivum*, *T. tomentosum*, and *T. polysorum* are rather restricted to temperate regions. However, *T. longibrachiatum*, *T. virens*, *T. koningiopsis*, *T. hamatum*, and *T. atroviride* were also found in the neotropical study (Hoyos-Carvajal et al. 2009).

The current results suggested that the most diverse habitats were soil (nine species per 22 isolates) and decaying wood (nine species per 75 isolates) gathered in parks and forests of the Wielkopolska region of Poland. The decaying wood was also the substrata from which the most isolates of *Trichoderma* (75) were collected. In this connection, we will continue to analyze the genetic and metabolic biodiversity of *Trichoderma* isolates originating from Polish mountains and isolated from forest wood with decay symptoms.

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