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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 *Trich*OKEY and *Tricho*BLAST (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*.

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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 (Kubicek and Penttilä 1998; Sivasithamparam and Ghisalberti 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 (Kullnig 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 (Mułenko 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 Skierniewice, as well as from forest soil of the Wielkopolski National Park were kindly supplied by Profs. H. Kwaśna 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, Skierniewice.

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/taxadescriptions/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^{-1}, 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). A fragment of *tef1* gene containing the 4th and 5th introns was amplified using the primers Ef728M (Carbone and Kohn 1999) and tef1R (Kullnig-Gradinger et al. 2002). 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 $MgCl_2$, 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 H_2O, 0.25 µl (2 U/µl) DyNAzymeTM II DNA Polymerase (Finnzymes). Amplifications were performed in either a PTC-200 or PTC-100 thermocycler (MJ

Table 1 List of *Trichoderma* isolates included in this study

Culture code	Species	Sources/localization	Allelic group[a]		NCBI GenBank accession number	
			ITS1, ITS2	*tef1*	ITS1, ITS2	*tef1*
AN 13	*T. atroviride*	forest soil, WNP[b]	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. hamatum*	forest soil, WNP	HM1	–	HQ292850	–
AN 22	*T. gamsii*	forest soil, WNP	cV9	–	HQ292951	–
AN 35	*T. atroviride*	maize kernels, Radzików	cV3	AT2	HQ292787	HQ292953
AN 46	*T. citrinoviride*	soil	C1	–	HQ292839	–
AN 55	*T. polysporum*	soil	–	–	HQ292950	–
AN 59	*T. citrinoviride*	soil	C1	–	HQ292840	–
AN 61	*T. harzianum*	soil	HR7	–	HQ292866	–
AN 68	*T. virens*	compost, Puławy	VS3	–	HQ292943	–
AN 69	*T. virens*	compost, Puławy	VS3	–	HQ292944	–
AN 70	*T. virens*	compost, Puławy	VS1	–	HQ292947	–
AN 73	*T. virens*	compost, Puławy	VS3	–	HQ292945	–
AN 74	*T. virens*	compost, Puławy	VS3	–	HQ292946	–
AN 75	*T. virens*	compost, Puławy	VS1	–	HQ292948	–
AN 89	*T. citrinoviride*	garden soil, Poznań	C1	–	HQ292841	–
AN 90	*T. atroviride*	garden soil, Poznań	cV3	AT2	HQ292788	HQ292954
AN 91	*T. harzianum*	compost, Poznań	HR6	–	HQ292860	–
AN 92	*T. harzianum*	maize kernels, Radzików	HR5	–	HQ292867	–
AN 93	*T. viridescens*	forest soil, Malta, Poznań	cV5	VD3	HQ292927	HQ292995
AN 94	*T. harzianum*	forest soil, Malta Park, Poznań	HR3	–	HQ292873	–
AN 95	*T. atroviride*	compost, Poznań	cV3	AT2	HQ292789	HQ292955
AN 96	*T. atroviride*	compost, Poznań	cV3	AT2	HQ292790	HQ292956
AN 97	*T. citrinoviride*	forest wood, Wieluń	C1	–	HQ292842	–
AN 98	*T. citrinoviride*	forest wood, Wieluń	C1	–	HQ292843	–
AN 99	*T. citrinoviride*	forest wood, Wieluń	C2	–	HQ292848	–
AN 100	*T. koningii*	forest wood, Wieluń	cV1	KO1	HQ292903	HQ292975
AN 101	*T. harzianum*	forest wood, Wieluń	HR5	–	HQ292868	–
AN 102	*T. citrinoviride*	forest wood, Wieluń	C1	–	HQ292844	–
AN 104	*T. koningii*	forest wood, Dziewicza Góra, Poznań	cV1	KO1	HQ292904	HQ292976
AN 105	*T. koningii*	forest wood, Dziewicza Góra, Poznań	cV1	KO1	HQ292905	HQ292977
AN 106	*T. koningii*	forest wood, Dziewicza Góra, Poznań	cV1	KO1	HQ292906	HQ292978
AN 107	*T. koningii*	forest wood, Dziewicza Góra, Poznań	cV1	KO1	HQ292907	HQ292979
AN 108	*T. harzianum*	forest wood, Dziewicza Góra, Poznań	HR5	–	HQ292869	–
AN 111	*T. atroviride*	forest wood, Dziewicza Góra, Poznań	cV3	AT1	HQ292791	HQ292964
AN 113	*T. koningii*	forest wood, Żurawiniec Park, Poznań	cV1	KO1	HQ292908	HQ292980
AN 114	*T. koningii*	forest wood, Żurawiniec Park, Poznań	cV1	KO1	HQ292909	HQ292981
AN 115	*T. koningii*	forest wood, Żurawiniec Park, Poznań	cV1	KO1	HQ292910	HQ292982
AN 116	*T. koningii*	forest wood, Żurawiniec Park, Poznań	cV1	KO1	HQ292911	HQ292983
AN 117	*T. koningii*	forest wood, Żurawiniec Park, Poznań	cV1	KO1	HQ292912	HQ292984
AN 118	*T. hamatum*	forest wood, Rusałka Park, Poznań	HM2	–	HQ292854	–
AN 120	*T. hamatum*	forest wood, Rusałka Park, Poznań	HM2	–	HQ292855	–
AN 121	*T. koningii*	forest wood, Rusałka Park, Poznań	cV1	KO1	HQ292913	HQ292985
AN 122	*T. viridescens*	forest wood, Rusałka Park, Poznań	cV5	VD4	HQ292928	HQ292994
AN 124	*T. koningii*	forest wood, Rusałka Park, Poznań	cV1	KO1	HQ292914	HQ292986
AN 125	*T. koningii*	forest wood, Rusałka Park, Poznań	cV1	KO1	HQ292915	HQ292987

Table 1 (continued)

Culture code	Species	Sources/localization	Allelic group[a]		NCBI GenBank accession number	
			ITS1, ITS2	*tef1*	ITS1, ITS2	*tef1*
AN 126	*T. koningii*	forest wood, Rusałka Park, Poznań	cV1	KO2	HQ292916	HQ292991
AN 127	*T. koningii*	forest wood, Rusałka Park, Poznań	cV1	KO1	HQ292917	HQ292988
AN 128	*T. koningii*	forest wood, Rusałka Park, Poznań	cV1	KO1	HQ292918	HQ292989
AN 132	*T. harzianum*	forest wood, Rusałka Park, Poznań	HR5	–	HQ2928670	–
AN 133	*T. harzianum*	forest wood, Jeziory, WNP	HR4	–	HQ292874	–
AN 134	*T. harzianum*	forest wood, Jeziory, WNP	HR4	–	HQ292875	–
AN 135	*T. harzianum*	forest wood, Jeziory, WNP	HR4	–	HQ292876	–
AN 136	*T. harzianum*	forest wood, Jeziory, WNP	HR1	–	HQ292901	–
AN 137	*T. harzianum*	forest wood, Jeziory, WNP	HR4	–	HQ292877	–
AN 138	*T. harzianum*	forest wood, Jeziory, WNP	HR6	–	HQ292861	–
AN 141	*T. viride*	forest wood, Jeziory, WNP	cV6	V12	HQ292922	HQ293008
AN 142	*T. viride*	forest wood, Jeziory, WNP	cV8	V12	HQ292920	HQ293009
AN 143	*T. koningiopsis*	forest wood, Jeziory, WNP	cV4	–	HQ292929	HQ292992
AN 144	*T. koningii*	forest wood, Jeziory, WNP	cV1	KO1	HQ292919	HQ292990
AN 145	*T. viridescens*	forest wood, Jeziory, WNP	cV5	VD3	HQ292930	HQ292996
AN 146	*T. viridescens*	forest wood, Jeziory, WNP	cV5	VD3	HQ292931	HQ292997
AN 147	*T. viridescens*	forest wood, Jeziory, WNP	cV5	VD3	HQ292932	HQ292998
AN 148	*T. viridescens*	forest wood, Jeziory, WNP	cV5	VD3	HQ292933	HQ292999
AN 149	*T. viridescens*	forest wood, Jeziory, WNP	cV5	VD3	HQ292934	HQ293000
AN 150	*T. harzianum*	forest wood, Jeziory, WNP	HR4	–	HQ292878	–
AN 152	*T. atroviride*	triticale kernel, Choryń	cV3	AT2	HQ292792	HQ292957
AN 153	*T. atroviride*	triticale kernel, Choryń	cV3	AT2	HQ292793	HQ292958
AN 155	*T. hamatum*	rye rizosphera, Lublin	HM1	–	HQ292851	–
AN 171	*T. aggressivum*	mushroom compost, Skierniewice	AG2	–	HQ292807	–
AN 172	*T. aggressivum*	mushroom compost, Skierniewice	AG2	–	HQ292808	–
AN 176	*T. viride*	forest wood, Strzeszyn Park, Poznań	cV8	V13	HQ292923	HQ293010
AN 179	*T. viride*	forest wood, Strzeszyn Park, Poznań	cV8	V13	HQ292924	HQ293011
AN 182	*T. atroviride*	forest wood, Strzeszyn Park, Poznań	cV2	AT1	HQ292794	HQ292965
AN 188	*T. atroviride*	mushroom compost, Skierniewice	cV4	AT2	HQ292803	HQ292959
AN 197	*T. longibrachiatum*	mushroom factory, Skierniewice	L1	–	HQ292780	–
AN 198	*T. citrinoviride*	mushroom factory, Skierniewice	C1	–	HQ292845	–
AN 199	*T. citrinoviride*	mushroom factory, Skierniewice	C1	–	HQ2929846	–
AN 201	*T. citrinoviride*	mushroom factory, Skierniewice	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	AT2	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	AT1	HQ292795	HQ292966
AN 213	*T. longibrachiatum*	mushroom compost, Poznań	L1	–	HQ292781	–
AN 215	*T. atroviride*	mushroom compost, Poznań	cV3	AT1	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	VD1	HQ292935	HQ293004

Table 1 (continued)

Culture code	Species	Sources/localization	Allelic group[a]		NCBI GenBank accession number	
			ITS1, ITS2	*tef1*	ITS1, ITS2	*tef1*
AN 227	*T. viridescens*	forest soil, WNP	cV5	VD5	HQ292936	HQ293001
AN 229	*T. viridescens*	forest soil, WNP	cV5	VD5	HQ292937	HQ293002
AN 231	*T. viridescens*	forest soil, WNP	cV5	VD2	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	VI1	HQ292921	HQ293013
AN 238	*T. hamatum*	forest soil, WNP	HM1	–	HQ292853	–
AN 257	*T. harzianum*	forest wood, Radojewo	HR4	–	HQ292883	–
AN 258	*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	VI3	HQ292925	HQ293012
AN 273	*T. harzianum*	forest wood, Kórnik	HR4	–	HQ292886	–
AN 274	*T. harzianum*	forest wood, Kórnik	HR4	–	HQ292887	–
AN 275	*T. harzianum*	forest wood, Kórnik	HR4	–	HQ292888	–
AN 276	*T. harzianum*	forest wood, Kórnik	HR4	–	HQ292889	–
AN 277	*T. hamatum*	forest wood, Kórnik	HM1	–	HQ292857	–
AN 278	*T. harzianum*	forest wood, Kórnik	HR4	–	HQ292890	–
AN 279	*T. hamatum*	forest wood, Kórnik	HM1	–	HQ292858	–
AN 281	*T. atroviride*	forest wood, Kórnik	cV2	AT3	HQ292804	HQ292974
AN 282	*T. harzianum*	forest wood, Kórnik	HR4	–	HQ292891	–
AN 283	*T. harzianum*	forest wood, Kórnik	HR4	–	HQ292892	–
AN 284	*T. harzianum*	forest wood, Kórnik	HR4	–	HQ292893	–
AN 285	*T. harzianum*	forest wood, Kórnik	HR4	–	HQ292894	–
AN 286	*T. harzianum*	forest wood, Kórnik	HR4	–	HQ292895	–
AN 287	*T. atroviride*	forest wood, Radojewo	cV3	AT1	HQ292798	HQ292969
AN 288	*T. viridescens*	forest wood, Kórnik	cV5	VD1	HQ292941	HQ293006
AN 425t	*T. harzianum*	forest wood, Radojewo	HR4	–	HQ292896	–
AN 426	*T. harzianum*	forest wood, Radojewo	HR4	–	HQ292897	–
AN 427	*T. viridescens*	forest wood, Radojewo	cV5	VD1	HQ292942	HQ293007
AN 430	*T. viride*	forest wood, Radojewo	cV8	VI1	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	AT1	HQ292799	HQ292970
AN 437	*T. harzianum*	forest wood, Radojewo	HR4	–	HQ292900	–
AN 550	*T. gamsii*	forest wood, 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, Łomża	AG2	–	HQ292813	–
AN 565	*T. aggressivum*	mushroom compost, Siemiatycze	AG2	–	HQ292814	–
AN 566	*T. aggressivum*	mushroom compost, Olsztyn	AG2	–	–	–
AN 567	*T. aggressivum*	mushroom compost, Tychy	AG2	–	HQ292815	–

Table 1 (continued)

Culture code	Species	Sources/localization	Allelic group[a]		NCBI GenBank accession number	
			ITS1, ITS2	*tef1*	ITS1, ITS2	*tef1*
AN 568	*T. aggressivum*	mushroom compost, Bytom	AG2	–	HQ292816	–
AN 569	*T. aggressivum*	mushroom compost, Łosice	AG2	–	HQ292817	–
AN 570	*T. aggressivum*	mushroom compost, Biała Podlaska	AG2	–	HQ292818	–
AN 571	*T. aggressivum*	mushroom compost, Międzychód	AG2	–	HQ292819	–
AN 572	*T. aggressivum*	mushroom compost, Gorzów Wlkp.	AG2	–	HQ292820	–
AN 573	*T. aggressivum*	mushroom compost, Przemyśl	AG2	–	HQ292821	–
AN 574	*T. aggressivum*	mushroom compost, Siedlce	AG2	–	HQ292822	–
AN 575	*T. aggressivum*	mushroom compost, Sokołów Podlaski	AG2	–	HQ292823	–
AN 576	*T. aggressivum*	mushroom compost, Chojnice	AG2	–	HQ292824	–
AN 577	*T. aggressivum*	mushroom compost, Szczecinek	AG2	–	HQ292825	–
AN 578	*T. aggressivum*	mushroom compost, Krosno Lubuskie	AG2	–	HQ292826	–
AN 579	*T. aggressivum*	mushroom compost, Zielona Góra	AG2	–	HQ292827	–
AN 580	*T. harzianum*	mushroom compost, Pszczyna	HR6	–	HQ292862	–
AN 581	*T. harzianum*	mushroom compost, Marianów/Koło	HR6	–	HQ292863	–
AN 582	*T. aggressivum*	mushroom compost, Turek	AG3	–	HQ292835	–
AN 583	*T. aggressivum*	mushroom compost, Człuchów	AG3	–	HQ292836	–
AN 584	*T. aggressivum*	mushroom compost, Piła	AG3	–	HQ292837	–
AN 585	*T. aggressivum*	mushroom compost, Skierniewice	AG2	–	HQ292828	–
AN 586	*T. aggressivum*	mushroom compost, Świecie	AG2	–	HQ292829	–
AN 587	*T. aggressivum*	mushroom compost, Skierniewice	AG3	–	HQ292838	–
AN 590	*T. harzianum*	mushroom compost, Piasek/Pszczyna	HR6	–	HQ292864	–
AN 591	*T. aggressivum*	mushroom compost, Wolsztyn	AG2	–	HQ292830	–
AN 592	*T. aggressivum*	mushroom compost, Rzeszów	AG2	–	HQ292831	–
AN 593	*T. atroviride*	mushroom compost, Pszczyna	cV3	AT1	HQ292800	HQ292971
AN 594	*T. aggressivum*	mushroom compost, Rakoniewice	AG2	–	HQ292832	–
AN 595	*T. aggressivum*	mushroom compost, Wielichowo	AG2	–	HQ292833	–
AN 596	*T. atroviride*	mushroom compost, Jarocin	cV3	AT1	HQ292801	HQ292972
AN 597	*T. harzianum*	mushroom compost, Kalisz	HR6	–	HQ292865	–
AN 599	*T. aggressivum*	mushroom compost, Pszczyna	AG2	–	HQ292834	–
AN 600	*T. atroviride*	mushroom compost, Pszczyna	cV3	AT1	HQ292802	–

[a] The group of isolates possessing identical alleles in the locus of ITS or *tef1*, analyzed in the present study(Figs. 1 and 2)

[b] WNP: Wielkopolski National Park

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 *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 Vcm^{-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.

Molecular identification and phylogenetic analysis

For species identification, ITS1 and ITS 2 sequences were submitted to the BLAST interface in *Trich*OKEY (http://www.isth.info; Druzhinina et al. 2005; Druzhinina and Kubicek 2005). In ambiguous cases, the result was re-checked using the *Trich*oBLAST 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

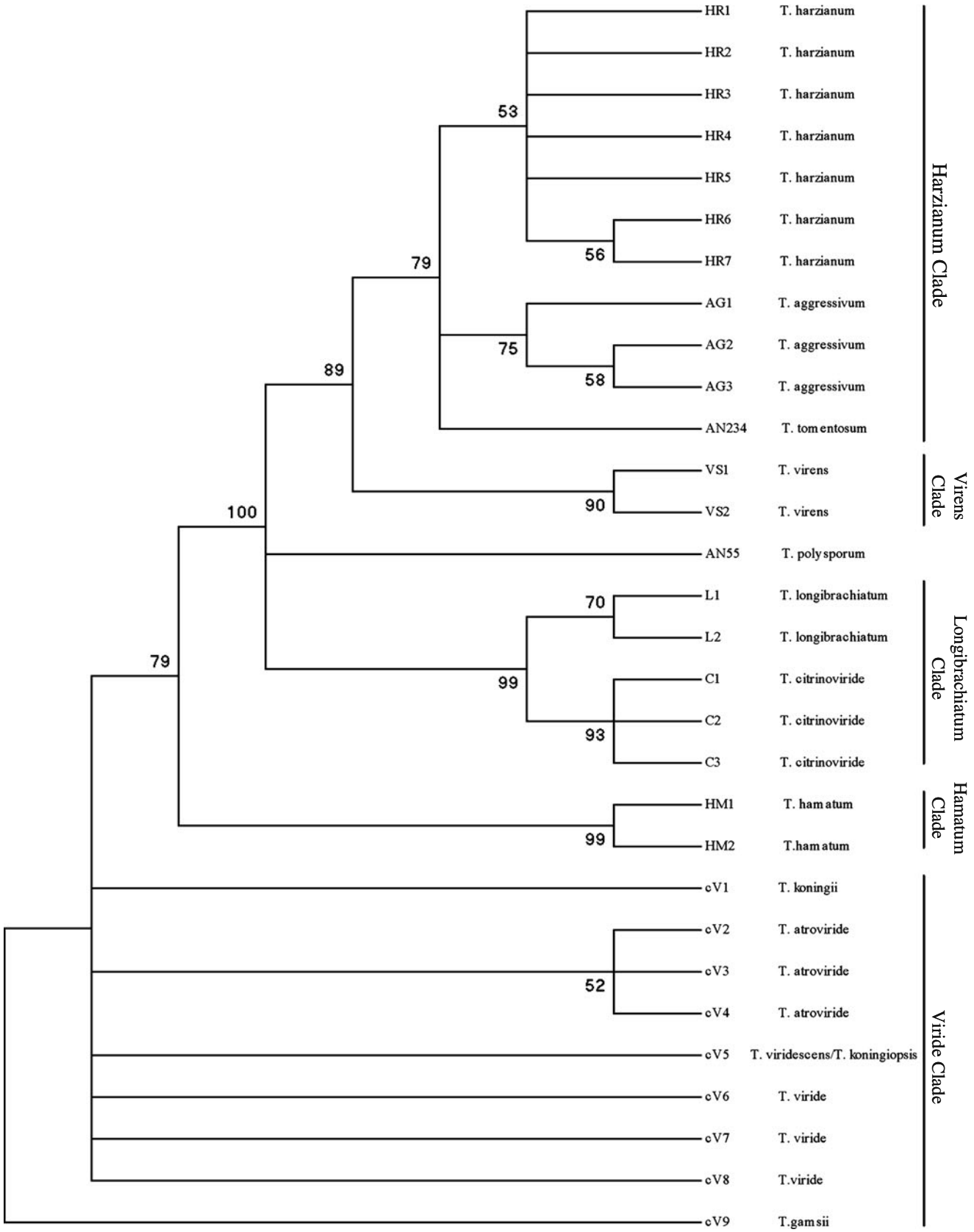
A total of 170 isolates were obtained from the six different substrata at 49 localities in Poland. Of theses 170 *Trichoderma* isolates, 110 were identified at the species level by morphological characteristics and analysis of their ITS1, ITS2 nucleotide sequences as: *T. harzianum* Rifai (43 isolates), *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. viride* 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. viride*, *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. viride*, *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. viride*, *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.*


HR1
HR2
HR3
HR4
HR5
HR6
HR7
AG1
AG2
AG3
AN234
VS1
VS2
AN55
L1
L2
C1
C2
C3
HM1
HM2
cV1
cV2
cV3
cV4
cV5
cV6
cV7
cV8
cV9
T. harzianum
T. aggressivum
T. tomentosum
T. virens
T. polysporum
T. longibrachiatum
T. citrinoviride
T. hamatum
T. koningii
T. atroviride
T. viridescens/T. koningiopsis
T. viride
T.viride
T.gamsii
53
56
79
75
58
89
90
100
70
99
93
79
99
52
Harzianum Clade
Virens Clade
Longibrachiatum Clade
Hamatum Clade
Viride Clade

◀ **Fig. 1** Phylogenetic tree of the 170 *Trichoderma* isolates inferred by parsimony analysis of ITS1, ITS 2 sequences. Sequences obtained during this study are listed by their GenBank numbers in Table 1. The numbers given over branches indicate bootstrap coefficient >50%. The symbols given on the right (HR, AG, L, C, HM, cV) indicate the allelic groups of isolates, forming on the basis of ITS sequences identity. The isolates belonging to individual allelic groups are listed in Table 1

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 neo type 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]).

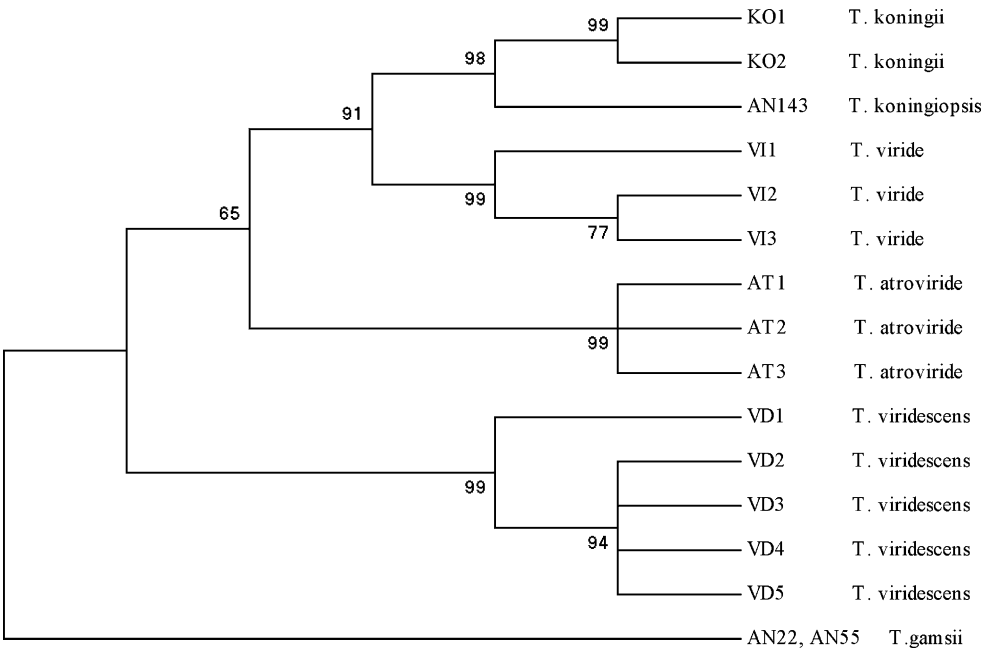


Fig. 2 Phylogenetic tree of the 60 *Trichoderma* isolates inferred by parsimony analysis of *tef1* sequences. Sequences obtained during this study are listed by their GenBank numbers in Table 1. The numbers given over branches indicate bootstrap coefficient >50%. The symbols given on the right (KO, VI, AT, VD) indicate the allelic groups of isolates, forming on the basis of *tef1* sequences identity. The isolates belonging to individual allelic groups are listed in Table 1

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 Szczech 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. gamsii* (2), *T. koningiopsis* (1), *T. polysporum* (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. polysporum* 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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