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ARTICLE

Genetic and biochemical diversity among *Trichoderma* isolates in soil samples from winter wheat fields of the Great Hungarian Plain

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ABSTRACT One hundred and sixteen *Trichoderma* isolates were collected from chopped roots of winter wheat of five agricultural fields in the Great Hungarian Plain. The isolates were identified by the sequence analysis of the internal transcribed spacer (ITS) region with *Trich*OKEY 2.0 and BLAST similarity searches. The *Trichoderma* species detected in the samples were *T. atroviride, T. brevicompactum*, *T. gamsii, T. harzianum*, *T. koningiopsis/T. ovalisporum, T. longibrachiatum/H. orientalis*, *T. pleuroticola, T. rossicum, T. spirale, T. tomentosum/T. cerinum* and *T. virens.* Beneficial taxa widely used as biocontrol agents against plant pathogenic fungi (e.g. *T. harzianum*, *T. virens*, *T. atroviride*) could be isolated from the samples examined during this study, indicating that the winter wheat rhizosphere may be a rich source of potential biocontrol isolates. Several species could be characterized with well-defined isoenzyme patterns during cellulose-acetate electrophoresis, suggesting that this method can be used for the analysis of biochemical diversity between and within particular species of the genus *Trichoderma.*

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KEY WORDS

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The genus *Trichoderma* (Ascomycota, Hypocreales, Hypocreaceae) involves promising biocontrol candidates with excellent antagonistic abilities against a number of plant pathogenic fungi. Modes of action proposed to play roles in biocontrol capabilities of *Trichoderma* species include competition for space and nutrients, antibiosis by the production of antifungal metabolites, mycoparasitism, induction of the defense responses in plants and plant growth promotion (Harman 2004).

During the early studies about the biodiversity of the genus *Trichoderma* (Danielson and Davey 1973; Widden and Abitbol 1980; Nelson 1982), identification of the species was based on morphological characters only, which is an uneasy task. Furthermore, a series of *Trichoderma* species were not yet described at the times these initial reports were published, therefore the results of these studies are not easy to interprete. Recent studies applied molecular methods including ITS (internal transcribed spacer) sequence-based identification with the aid of *Trich*Okey (Druzhinina et al. 2005) and BLAST simlarity searches performed with *Tricho*BLAST (Kopchinskiy et al. 2005) for the examination of *Trichoderma* communities at different habitats. The biodiversity of the genus *Trichoderma* has been examined by molecular methods in different natural ecosystems including soils from Russia, Nepal, northern India (Kullnig et al. 2000), south-east Asia (Kubicek et al. 2003), a mid-European, primeval floodplain-forest (Wuczkowski et al. 2003), Sardinia (Migheli et al. 2009) and South America (Hoyos-Carvajal et al. 2009). These studies reported about a series of new genotypes as well as new phylogenetic species of *Trichoderma*. On the other hand only a few studies were focusing on agricultural ecosystems (Gherbawy et al. 2004; Mulaw et al. 2010). However, the results of these studies demonstrated that – besides the natural ecosystems - the investigation of agricultural soils may also reveal interesting data about *Trichoderma* biodiversity. The practical impact of such studies is that the rhizosphere of agricultural soils is an ideal source of beneficial strains with biocontrol potential.

This study was aimed at the assessment of *Trichoderma* biodiversity in samples derived from the winter wheat rhizosphere at five locations of the Great Hungarian Plain.

Materials and Methods

Strains, isolation conditions and identification procedure

Soil samples with winter wheat seedlings were collected from five agricultural fields (Algyő, Deszk, Kunszentmiklós, Rúzsa and Tiszasziget) in the Great Hungarian Plain by a 5 cm x 5

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cm square sampler in random sampling order. Isolations were performed directly from the chopped roots of winter wheat on Rose Bengal medium (5 g l^{-1} peptone, 1 g l^{-1} KH_2PO_4, 10 g l^{-1} glucose, 0.5 g l^{-1} $MgSO_4X7H_2O$, 0.5 ml l^{-1} 0.2% dichloran-ethanol solution, 0.25 ml l^{-1} 5% Rose Bengal, 20 g l^{-1} agar supplemented with 0.1 g l^{-1}oxytetracyclin, 0.1 g l^{-1} streptomycin and 0.1 g l^{-1} chloramphenicol to inhibit bacteria). Monospore cultures were prepared from the isolates and deposited at the Microbiological Collection of the University of Szeged (SZMC; Table 1).

DNA isolation, PCR amplification of the internal transcribed spacer (ITS1-5.8S rDNA-ITS2) region, and automatic DNA sequencing were performed as described by Andersson et al. (2009). ITS sequences were analysed by the program *TrichO*Key 2.0 (Druzhinina et al. 2005) available online at www.isth.info. In the cases where *TrichO*key 2.0 was not able to identify the isolate at the species level, BLASTN homology searches (Zhang et al. 2000) were performed at the homepage of NCBI (National Center for Biotechnology Information). The validities of the BLASTN hits were checked with *TrichO*key 2.0 and literature searches. Sequences were deposited at the NCBI Genbank database, accession numbers are listed in Table 1.

Protein extraction and enzyme electrophoresis

Protein extraction, CAE and staining protocols were performed as described by Láday and Szécsi (2001), Szekeres et al. (2006) and Hebert and Beaton (1993), respectively. Banding patterns of five enzymes, 6-phosphogluconate-dehydrogenase (6PGDH), glucose-6-phosphate dehydrogenase (G6PDH), glucose-6-phosphate isomerase (G6PI), peptidase B (Leu-Gly-Gly) (PEPB) and phosphoglucomutase (PGM) were selected for analysis. All samples were extracted and analysed on three occasions in separate runs. Bands were ordered alphabetically based on their relative mobility; the band located next to the anode was designated as 'A' for each enzyme assay.

Analysis of the data

The Shannon diversity index (Shannon, 1948) was applied for the characterization of the diversity of isolates at the specific sampling sites and for the whole sample set. Sequences were aligned with the program Clustal X 2.0.10 (Larkin et al. 2007). Alignments were inspected, manually corrected and analysed with Genedoc 2.7. In the case of the isoenzyme data, binary matrices were created based on the presence or absence of a band with a given mobility. Simple matching coefficients were calculated with the PHYLTOOLS v. 1.32 software package (Buntjer, 1997). Bootstrap values were collected from 1000 replications of the bootstrap procedure using PHYLTOOLS and the CONSENSE programs of the PHYLIP v. 3.57c software package (Felsenstein, 1985, 1995). During the parsimony analysis, matrices were analysed with the program PARS according to the Wagner-algorythm (Eck and Dayhoff 1966; Kluge and Farris 1969). The consensus phylogenetic tree was created with the program CONSENSE.

Results

Identification of the isolates based on oligonucleotide barcodes

One hundred and sixteen *Trichoderma* strains were isolated from 18 sampling sites of 5 agricultural fields in the Great Hungarian Plain (Table 1). Seventy-nine out of the 116 isolates could be identified with the aid of the barcoding-based program *TrichO*key 2.0 (Druzhinina et al. 2005), (Table 1): 40 as *T. harzianum*, 15 as *T. virens*, 5 as *T. rossicum*, 4 as *T. brevicompactum*, 3 as *T. atroviride*, 3 as *T. pleuroticola* and 1 as *T. spirale*. Further 5 and 3 isolates proved to belong to *T. longibrachiatum/H. orientalis* and *T. koningiopsis/T. ovalisporum*, respectively (an exact identification at the species level based on ITS-sequences only is not possible for isolates belonging to these species duplets). The method identified 12 isolates at the clade level only (all of them from clade Rufa), while 25 strains were recognised by *TrichO*key 2.0 as unidentified species of *Hypocrea/Trichoderma*.

ITS sequences of the isolates identified as *Trichoderma* sp., clade Rufa or diagnosed as unidentified *Trichoderma* sp. were further analysed by NCBI BLASTN similarity searches. However, as the NCBI database contains a large number of *Trichoderma* sequences under incorrect species names (Druzhinina and Kubicek 2005), the validities of the BLASTN hits were checked with *TrichO*key 2.0, *Tricho*-BLAST and literature searches. Among the 12 isolates identified as *Trichoderma* sp., clade Rufa, 6 proved to be *T. atroviride*, while based on the *TrichO*key 2.0 analysis of the closest BLASTN hits, the other 6 isolates were related with the species duplet *T. koningiopsis/T. ovalisporum*. However, the six isolates differed from the sequence characteristic for *T. koningiopsis* and *T. ovalisporum* in a T insertion at position 121 but proved to be identical or highly similar (0.2% mismatch) for the examined first 505 nucleotides of the ITS region with sequences of *T. gamsii* (Jaklitsch et al. 2006), a species which is not included in the barcode library of *TrichO*key 2.0. Among the 25 isolates diagnosed as unidentified *Trichoderma* sp., 16 proved to belong to *T. virens*, 5 to *T. rossicum*, 3 to the species duplet *T. tomentosum/T. cerinum* and 1 to *T. harzianum* (Table 1).

Distribution of the detected species among the samples

The number of isolated strains was the highest and lowest in the case of sample K1 (location: Kunszentmiklós, number of isolates: 15) and sample R2 (location: Rúzsa, number of isolates: 1), respectively. The average number of isolates per sampling site was 6.4.

Table 1. Isolation data, identification details and electrophoretic types of the examined *Trichoderma* strains.

Location	Sample number	Strain number	GenBank accession number of ITS	TrichOkey 2.0 diagnosis	Identification based on the closest valid NCBI BLAST hits	CAE electrophoretic type
Algyő	A1	SZMC 1600	DQ345793	*T. harzianum*		XIII
		SZMC 1601	DQ345794	*T. harzianum*		XIII
		SZMC 1602	DQ345795	*T. harzianum*		XIII
		SZMC 1603	DQ345796	unidentified *T.* sp.	*T. virens*	XXII
		SZMC 1604	DQ345797	*T. virens*		XXII
		SZMC 1605	DQ345798	T. virens		XXII
		SZMC 1606	DQ345799	*T. harzianum*		XIII
Deszk	D1	SZMC 1610	DQ345804	unidentified *T.* sp.	*T. tomentosum*/ *T. cerinum*	XVIII
		SZMC 1611	DQ345805	*T.* sp. clade Rufa	T. atroviride	VI
		SZMC 1612	DQ345806	*T. pleuroticola*		XVI
		SZMC 1615	DQ345809	*T.* sp. clade Rufa	*T. atroviride*	V
		SZMC 1616	DQ345811	unidentified *T.* sp.	*T. virens*	XXII
	D3	SZMC 0560	DQ118084	*T. harzianum*		XIII
		SZMC 1012	DQ345803	*T. longibrachiatum*/ *H. orientalis*		XV
		SZMC 1609	DQ345802	*T.* sp. clade Rufa	*T. atroviride*	V
		SZMC 1613	DQ345807	unidentified *T.* sp.	*T. tomentosum*/ *T. cerinum*	XVIII
		SZMC 1622	DQ345818	*T. harzianum*		XIII
	D4	SZMC 0559	DQ118087	*T. harzianum*		XIII
		SZMC 1607	DQ345800	*T.* sp. clade Rufa	*T. atroviride*	VI
		SZMC 1608	DQ345801	*T. pleuroticola*		XVI
		SZMC 1623	DQ345819	*T. harzianum*		XIII
		SZMC 1626	DQ345824	*T. harzianum*		XIII
	D5	SZMC 0886	DQ345821	*T. longibrachiatum*/ *H. orientalis*		XV
		SZMC 0887	DQ345823	*T. longibrachiatum*/ *H. orientalis*		XV
		SZMC 1159	DQ345812	*T. longibrachiatum*/ *H. orientalis*		XV
		SZMC 1624	DQ345820	unidentified *T.* sp.	*T. virens*	XXII
		SZMC 1625	DQ345822	unidentified *T.* sp.	*T. virens*	XXII
Rúzsa	R1	SZMC 0931	DQ118083	*T. virens*		XXII
		SZMC 1617	DQ345813	unidentified *T.* sp.	*T. tomentosum*/ *T. cerinum*	XVIII
		SZMC 1618	DQ345814	*T. rossicum*		XXV
		SZMC 1631	DQ345829	*T. rossicum*		XXV
		SZMC 1635	DQ345833	*T. harzianum*		XVII
		SZMC 1638	DQ345836	*T. harzianum*		XVII
		SZMC 1650	DQ345848	*T. harzianum*		XVII
		SZMC 1652	DQ345850	unidentified *T.* sp.	T. virens	XXII
		SZMC 1664	DQ345862	*T. harzianum*		XVII
		SZMC 1686	DQ345884	unidentified *T.* sp.	*T. virens*	XXII
		SZMC 2636	DQ345834	*T. harzianum*		XVII
	R2	SZMC 1667	DQ345865	*T. pleuroticola*		XVI
	R3	SZMC 1627	DQ345825	*T. atroviride*		VIII
		SZMC 1633	DQ345831	*T. harzianum*		XIII
	R4	SZMC 0930	DQ118086	*T. brevicompactum*		XIV
		SZMC 1614	DQ345808	*T.* sp. clade Rufa	*T. atroviride*	V
		SZMC 1628	DQ345826	*T. brevicompactum*		XIV
		SZMC 1649	DQ345847	*T. spirale*		XXIV
		SZMC 1656	DQ345854	*T.* sp. clade Rufa	*T. gamsii*	II
		SZMC 1657	DQ345855	*T.* sp. clade Rufa	*T. gamsii*	III
		SZMC 1659	DQ345857	*T.* sp. clade Rufa	*T. gamsii*	III
		SZMC 1661	DQ345859	unidentified *T.* sp.	*T. rossicum*	XX
	R6	SZMC 1619	DQ345815	*T. harzianum*		XIII
		SZMC 1620	DQ345816	*T. harzianum*		XIII
		SZMC 1629	DQ345827	*T. harzianum*		XIII

Table 1. Continued.

		SZMC 1630	DQ345828	*T. harzianum*		XIII
		SZMC 1632	DQ345830	*T. brevicompactum*		XI
		SZMC 1640	DQ345838	unidentified *T.* sp.	*T. harzianum*	XIII
		SZMC 1641	DQ345839	*T. harzianum*		XIII
		SZMC 1644	DQ345842	*T. harzianum*		XIII
		SZMC 1655	DQ345853	*T. harzianum*		XIII
		SZMC 1665	DQ345863	*T. harzianum*		XIII
		SZMC 1668	DQ345866	*T. harzianum*		XIII
	R7	SZMC 1158	DQ345810	*T. longibrachiatum*		XV
		SZMC 1621	DQ345817	*T. virens*		XXII
Kunszentmiklós	K1	SZMC 1642		*T. harzianum*		XIII
		SZMC 1646	DQ345844	*T.* sp. clade Rufa	*T. atroviride*	IX
		SZMC 1647	DQ345845	*T. harzianum*		XIII
		SZMC 1654	DQ345852	unidentified *T.* sp.	*T. rossicum*	XXIII
		SZMC 1663	DQ345861	*T. atroviride*		IV
		SZMC 1670	DQ345868	unidentified *T.* sp.	*T. virens*	XXII
		SZMC 1671	DQ345869	unidentified *T.* sp.	*T. virens*	XXII
		SZMC 1672	DQ345870	unidentified *T.* sp.	*T. virens*	XXII
		SZMC 1674	DQ345872	*T. harzianum*		XIII
		SZMC 1684	DQ345882	unidentified *T.* sp.	*T. virens*	XXII
		SZMC 1687	DQ345885	*T. atroviride*		II
		SZMC 1693	DQ345891	*T. brevicompactum*		XIV
		SZMC 1662	DQ345860	unidentified *T.* sp.	*T. rossicum*	XIX
		SZMC 1682	DQ345880	*T.* sp. clade Rufa	*T. gamsii*	III
		SZMC 1701	DQ345899	*T. virens*		XXII
	K2	SZMC 1645	DQ345843	unidentified *T.* sp.	*T. rossicum*	XIX
		SZMC 1651	DQ345849	unidentified *T.* sp.	*T. virens*	XXII
		SZMC 1669	DQ345867	unidentified *T.* sp.	*T. rossicum*	XIX
		SZMC 1676	DQ345874	*T. harzianum*		XIII
		SZMC 1702	DQ345900	*T. rossicum*		XXVI
		SZMC 1703	DQ345901	*T. rossicum*		XXVI
Tiszasziget	T1	SZMC 1643		*T. harzianum*		XIII
		SZMC 1675	DQ345873	*T. virens*		XXII
		SZMC 1689	DQ345887	*T. harzianum*		I
		SZMC 1696	DQ345894	*T. virens*		XXII
		SZMC 2637	DQ345835	*T. harzianum*		XIII
	T2	SZMC 1653	DQ345851	*T.* sp. clade Rufa	*T. gamsii*	II
		SZMC 1673	DQ345871	*T. harzianum*		XIII
		SZMC 1681	DQ345879	*T. virens*		XXII
		SZMC 1685	DQ345883	*T. virens*		XXII
		SZMC 1690	DQ345888	*T. harzianum*		XIII
		SZMC 1695	DQ345893	unidentified *T.* sp.	*T. virens*	XXII
	T3	SZMC 0919	DQ118089	*T. harzianum*		XII
		SZMC 0566	DQ118088	*T. harzianum*		XII
		SZMC 1634	DQ345832	*T. harzianum*		XII
		SZMC 1639	DQ345837	*T. harzianum*		XIII
		SZMC 1648	DQ345846	*T. koningiopsis*/ *T. ovalisporum*		IX
		SZMC 1660	DQ345858	*T. koningiopsis*/ *T. ovalisporum*		X
		SZMC 1666	DQ345864	*T. koningiopsis*/ *T. ovalisporum*		VII
		SZMC 1678	DQ345876	*T. harzianum*		XIII
		SZMC 1679	DQ345877	*T.* sp. clade Rufa	*T. gamsii*	III
		SZMC 1698	DQ345896	*T. virens*		XXII
		SZMC 1699	DQ345897	unidentified *T.* sp.	*T. virens*	XXI
	T4	SZMC 0561	DQ118085	unidentified *T.* sp.	*T. virens*	XXII
		SZMC 1658	DQ345856	*T. rossicum*		XXV
		SZMC 1691	DQ345889	*T. virens*		XXII
		SZMC 1692	DQ345890	unidentified *T.* sp.	*T. virens*	XXII
		SZMC 1694	DQ345892	unidentified *T.* sp.	*T. virens*	XXII

Table 1. Continued.

T5	SZMC 1677	DQ345875	*T. harzianum*	XIII
	SZMC 1680	DQ345878	*T. harzianum*	XIII
	SZMC 1683	DQ345881	*T. virens*	XXII
	SZMC 1688	DQ345886	*T. virens*	XXII
	SZMC 1697	DQ345895	*T. virens*	XXII
	SZMC 1700	DQ345898	*T. virens*	XXI

The most abundant species was *H. lixii/T. harzianum* (35.3%) which could be found in 12 of the 18 samples. It was the most frequently occurring *Trichoderma* species in seven samples (A1, D3, D4, R1, R6, T1, T3) at four sampling sites (Algyő, Deszk, Rúzsa, Tiszasziget). *T. virens* and *T. rossicum* accounted for 26.7% and 8.6% of all isolates, being present in 11 and 5 samples, respectively. *T. virens* was the most abundant species in the agricultural fields examined at Tiszasziget with 45.5% of the isolates. *T. rossicum* was dominant in sample K2 and it could also be isolated from four further samples (R1, R4, K1 and T4). *T. atroviride* (7.8% of all isolates) and *T. gamsii* (5.17% of all isolates) could be found in 6 and 4 samples, respectively. The other taxa occurred with a frequency below 5%: *T. brevicompactum, T. longibrachiatum/H. orientalis*, *T. pleuroticola* and *T. tomentosum/cerinum* with 3.4%, 4.3%, 2.6% and 2.6%, respectively, each of them found in 3 samples at 2 locations; while *T. koningiopsis/T. ovalisporum* (2.6%) and *T. spirale* (0.9%) occured in single samples only (T3 and R4, respectively).

The highest diversity of *Trichoderma* species was detected in a sample from Rúzsa (sample R4: 5 species among 8 isolates, no *T. harzianum*) and Kunszentmikós (sample K1: *T. harzianum* and 5 further species among 15 isolates). Sample R5 was characterized with a large number of isolates from *T. harzianum* and a relatively poor biodiversity. Only two species were detected in 8 out of 18 samples (A1, D4, R3, R5, R6, T1, T4, T5), in these samples either one of the two most abundant species (*T. harzianum* or *T. virens*), or both of them (A1, T1, T5) were present.

No correlation was found between the occurrence of the species and the sites of isolation (p=95; Pearson: 0.199). The total biodiversity index (no. of species/no. of isolates) was 0.095. The Shannon diversity index of the isolates from winter wheat rhizospere samples was 1.86.

Intraspecific variability of ITS-genotypes

Among the *T. harzianum* isolates, 14 belonged to the ITS-genotype 1 described by Kullnig et al. (2000) for isolates deriving from Krasnoyarsk, Moscow and Vladimir, Russia. Eight and two isolates had identical sequences with the West-Uralian genotype 2a and the Nepalian genotype 2b, respectively (Kullnig et al. 2000). Ten isolates were similar to the genotype 5 while five proved to be related with genotypes 3 and 4, firstly reported for isolates from Nepal (Kullnig et al. 2000). The sequence of isolate SZMC 1633 proved to be identical for the examined first 521 nucleotides of the ITS region with that of strain *T. harzianum* C.P.K. 1936 isolated in Austria (FJ860767; Jaklitsch, 2009), but this ITS genotype is also known from the rhizosphere of rice fields in Iran (EU821781; Naeimi S and Kredics L, unpublished). Several Hungarian isolates represented new ITS genotypes of *T. harzianum* (Har I: strains SZMC 1635, 1638, 1650, 1664 and 2636, Har II: strains SZMC 1619, 1620, 1630, 1641, 1644, 1655 and 1668). Isolates of the group designated as Har I were related to the Ethiopian isolates C.P.K. 2615 and C.P.K. 2711 (Mulaw et al. 2010) with the characteristic difference of having C instead of A in position 193 of the ITS region, while Har II proved to be a genotype differing from the mushroom bed-derived isolate *T. harzianum* DAOM 222151 (Druzhinina et al. 2010) in the ITS positions 406-413 (sequences are 5'…TCTTTTTG…3' for Har II isolates while 5'…T-TT---G…3' for DAOM 222151).

In the case of *T. virens*, 16 isolates belonged to the same ITS-genotype as the ex-type strain DAOM 167651 (Kullnig et al. 2001) while 15 isolates were differing from it in a single T insertion at position 144 of the ITS-region (Vir I: SZMC 1603, 1616, 1624, 1625, 1651, 1652, 1670, 1671, 1672, 1684, 1686, 1692, 1694, 1695 and 1699). No representatives of the ITS-genotype containing a series of isolates from Siberia (Kullnig et al. 2000) could be found in the examined Hungarian winter wheat rhizosphere samples.

T. rossicum isolates could be divided into 2 distinct ITS-genotypes. Strain SZMC 1618, 1631, 1658, 1702 and 1703 (Ros I) proved to be identical with the ITS-genotype of *T. rossicum* strains MA2995 and MA2997 (Wuczkowski et al. 2003), while isolates SZMC 1645, 1654, 1661, 1662 and 1669 represented a new genotype (Ros II) differing from strain DAOM 233977 (Hojos-Carvajal et al. 2009) in 3 positions (A insertion in position 63, C to T substitution at position 73 and A to C substitution at position 164 of the ITS-region).

In the case of *T. atroviride*, three isolates (SZMC 1627, 1663, 1687) belonged to the same ITS-genotype as strain CBS 142.95, the epitype of *T. atroviride* (Dodd et al. 2003), while the other isolates shared identical ITS-sequences with strain *T. atroviride* MA 3643 (Wuczkowski et al. 2003), differing in a single T to C transition at position 71 of ITS 1.

Isolates of *T. brevicompactum, T. longibrachiatum/H. orientalis, T. tomentosum/T. cerinum, T. pleuroticola*, *T.*

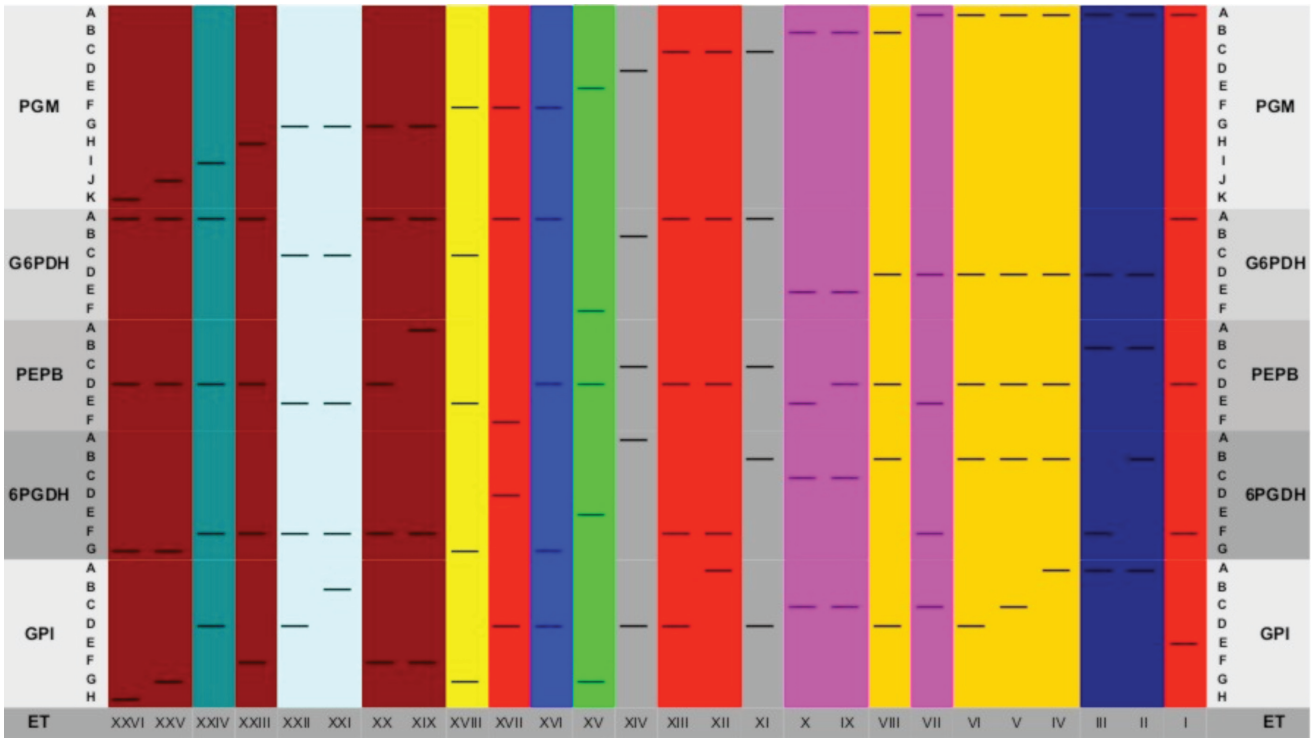

Figure 1. Schematic illustration of patterns belonging to the particular electrophoretic types. Different colors show electrophoretic types containing *Trichoderma* isolates identified as *T. harzianum* ■, *T. pleuroticola* ■, *T. tomentosum/T. cerinum* ■, *T. virens* ■, *T. rossicum* ■, *T. spirale* ■, *T. longibrachiatum/H. orientalis* ■, *T. brevicompactum* ■, *T. atroviride* ■, *T. gamsii* ■, *T. koningiopsis/T. ovalisporum* ■.

koningiopsis/T. ovalisporum and *T. gamsii* represented single ITS-genotypes of the respective taxa.

Isoenzyme analysis of *Trichoderma* strains isolated from winter wheat rhizosphere

The different patterns belonging to the particular enzymes are summarized in Table 1 and Figure 1. The most frequent electrophoretic type in the examined population was ET XIII with 32 isolates of *T. harzianum*. The remaining 9 *T. harzianum* isolates belonged to ETs I (1 isolate), XII (3 isolates) and XVII (5 isolates). The 3 isolates of *T. pleuroticola* exhibited a distinct electrophoretic type (ET XVI, 3 isolates). *T. virens* isolates belonged to the second most frequent electrophoretic type, ET XXII (29 isolates) with the exception of two isolates in ET XXI. ET XV involved the 5 *T. longibrachiatum/H. orientalis* isolates, while the *T. atroviride* isolates were dispersed among ETs IV, V, VI and VIII.

The phylogenetic analysis of isoenzyme patterns was performed by parsimony analysis (Figure 2). When the *T. longibrachiatum/H. orientalis* strains (all belonging to ET XV) were used as the outgroup, the examined isolates were separated between two main clusters, one of them containing two subclades. The upper subclade contained all isolates from clade Virens (ETs XXI, XXII) along with ET XVIII (the *T. tomentosum/T. cerinum* isolates) while the lower subclade was corresponding with clade Rufa (*T. atroviride*: ETs IV, V, VI and VIII; *T. gamsii*: ETs II and III; *T. koningiopsis/T. ovalisporum*: ETS VII, IX and X). The *T. harzianum* (ETs I, XII, XIII and XVII), *T. pleuroticola* (ET XVI), *T. rossicum* (ETs XIX, XX, XXIII), *T. brevicompactum* (ETs XI and XIV) and *T. spirale* (ET XXIV) isolates were in the other main cluster.

Discussion

All three sections of the recently accepted *Trichoderma/Hypocrea* taxonomy (Druzhinina and Kubicek, 2005) were represented in the examined sample set. From section Longibrachiatum, five isolates proved to belong to the clinically important species duplet *T. longibrachiatum/H. orientalis* (Druzhinina et al. 2008), while other representatives of this section could not be isolated.

From section Trichoderma, clade Rufa, three taxa, *T. atroviride* (a species widely used as biocontrol agent), *T. gamsii* and *T. koningiopsis/T. ovalisporum* were found in the winter wheat rhizosphere samples. *T. gamsii*, a recently described species (Jaklitsch et al. 2006) has been shown to have a widespread distribution (Hoyos-Carvajal et al. 2009, Migheli et al. 2009) and it was the exclusive species found in a sample

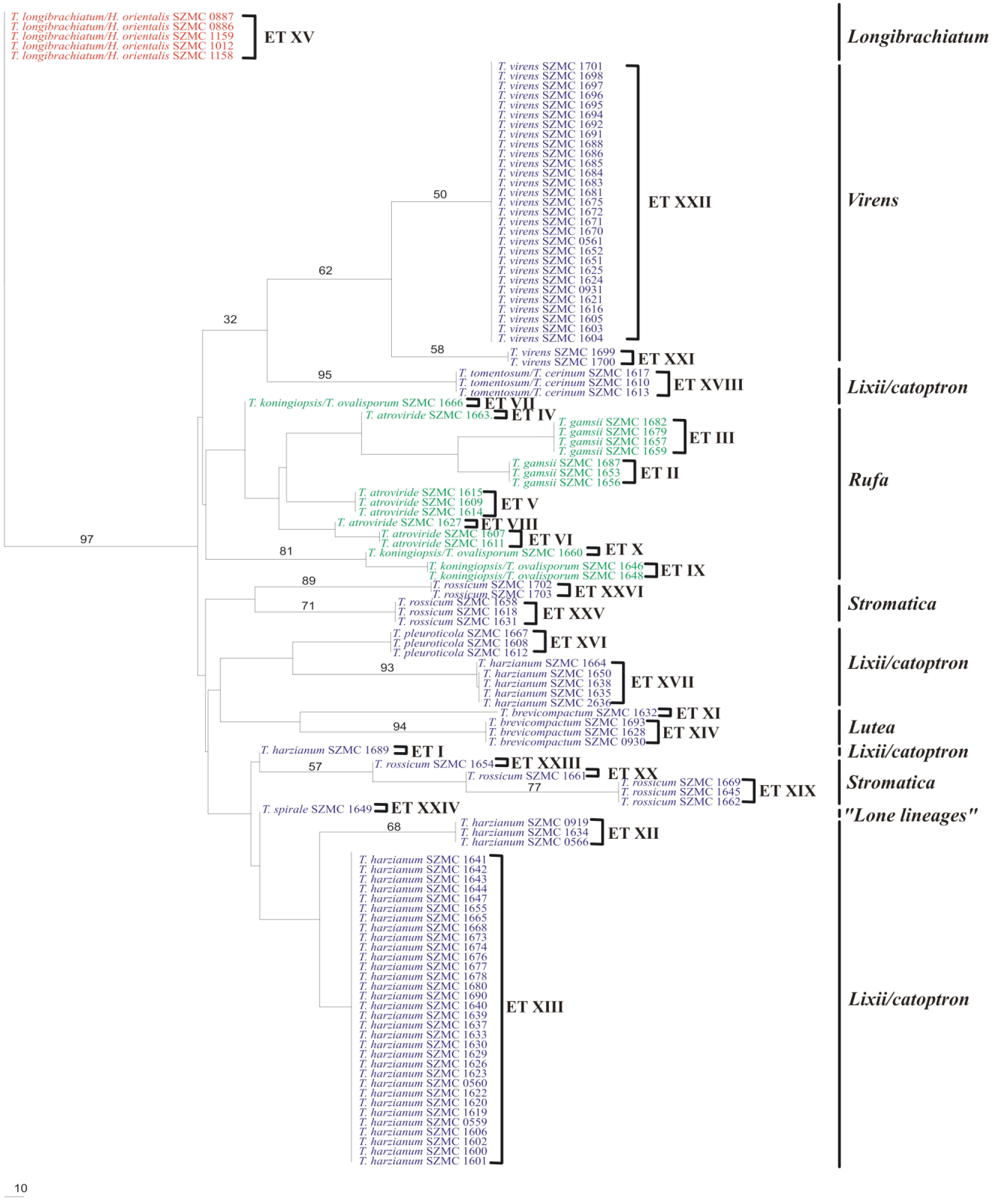

Figure 2. Parsimony dendrogram of the isolated *Trichoderma* strains from the data deriving from cellulose acetate electrophoresis-based isoenzyme analysis. Numbers on branches are bootstrap values. Electrophoretic types are indicated with Roman numbers. Sections Longibrachiatum, Pachybasium B and Trichoderma of the genus are shown in red, purple and green, respectively, names of clades are indicated in full.

from a shrub land and dominated in a sample from a grassland in Sardinia (Migheli et al. 2009). Interestingly, *T. hamatum*, a species which was found to be subdominant in several Sardinian soils (Migheli et al. 2009) and *T. asperellum*, a predominant species of neotropic regions (Hoyos-Carvajal et al. 2009) could not be isolated during this study.

The most frequent species isolated from the winter wheat rhizosphere samples was *T. harzianum* from clade Lixii/catoptron of section Pachybasium B, which is in congruence with previous data (Kullnig et al. 2000, Wuczkowski et al. 2003, Migheli et al. 2009). *T. harzianum* has been known for a long time as a species complex (Rifai 1969). Certain taxa that were previously belonging to this complex have already been described as separate species, e.g. the mushroom pathogenic *T. aggressivum* (Samuels et al. 2002), *T. pleurotum* and *T. pleuroticola* (Park et al. 2006; Komon-Zelazowska et al. 2007), while further species have been proposed recently by Druzhinina et al. (2010). The results of this recent study indicated that *T. harzianum* has a complex speciation history which revealed overlapping reproductively isolated biological species, recent agamospecies and numerous relict lineages with unresolved phylogenetic positions. The other representative of the Lixii/catoptron clade found during this study was the oyster mushroom pathogenic species *T. pleuroticola*, which is known to be present in the natural environment in several countries including Canada, the United States, Europe, Iran and New Zealand (Komon-Zelazowska et al. 2007) as well as on the natural substrates of oyster mushroom grown in the wild (Kredics et al. 2009). *T. virens*, a species of biocontrol significance from clade Virens proved to be the second most frequently isolated species in this study. Regarding *T. rossicum* from clade Stromatica of section Pachybasium B, the species was originally isolated in Krasnoyarsk, Russia (Bissett et al. 2003), but later it has been shown to have a widespread distribution, occurring also in Central Europe (Wuczkowski et al. 2003). The five isolates representing the ITS-genotype Ros II are mentioned in this study as *T. rossicum*, however, it is possible that they belong to a new, yet undescribed phylogenetic species of this clade, which needs further investigations. Clade Lutea and the group named „Lone lineages", both of them having ambiguous phylogenetic placements within the genus *Trichoderma* (Druzhinina and Kubicek 2005), were represented in the examined sample set by the species *T. brevicompactum* (Kraus et al. 2004) and *T. spirale*, respectively. Only a single isolate of *T. spirale* was found in this study, while it was shown to be the dominant *Trichoderma* in the carbon-rich forest soil of Badde Salighes in Sardinia (Migheli et al. 2009).

Further data about the biodiversity of *Trichoderma* in wheat rhizosphere of different geographic locations would be needed in order to find out, whether the new ITS-genotypes of *T. harzianum* and *T. rossicum* found in this study are endemic to the Great Hungarian Plain or maybe widespread but specialized to this type of habitat.

The biochemical diversity of the full sample set was examined by isoenzyme analysis. Although the CAE-based dendrogram shown in Figure 2 does not reflect the accepted phylogenetic relationships within the genus, most of the species could be characterized with well-defined CAE-patterns (Figure 1). Accordingly, the CAE method can be used for the analysis of biochemical diversity between and within particular species of the genus *Trichoderma*, as it has been demonstrated in a previous study involving clinical isolates from *Trichoderma* section Longibrachiatum (Szekeres et al. 2006).

In conclusion, the *Trichoderma* community of the winter wheat rhizosphere in the examined regions of the Great Hungarian Plain proved to be highly diverse. Beneficial taxa widely used as biocontrol agents against plant pathogenic fungi (*T. harzianum*, *T. virens* and *T. atroviride*) could be isolated from the samples examined during this study, indicating that the winter wheat rhizosphere may be a rich source of potential biocontrol isolates. On the other hand, *Trichoderma* species known as potential opportunistic pathogens in humans (*T. longibrachiatum/H. orientalis*) and as causal agents of the green mould disease in mushroom cultivation (*T. pleuroticola*) could also be detected in the examined samples. The development of biocontrol products from isolates of these potentially harmful species should be avoided.

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