In situ DNA barcoding of *Trichoderma* in soil reveals a narrow community of opportunistic species§

Martina A. Friedl & Irina S. Druzhinina*

Abstract: In this paper we report on the *in situ* diversity of the mycotrophic fungus *Trichoderma* (teleomorph *Hypocrea,* Ascomycota, Dikarya) revealed by a taxon-specific metagenomic approach and oligonucleotide DNA barcoding. We designed a set of genus-specific ITS1 and 2 rRNA primers and constructed a clone library containing 411 molecular operational taxonomic units (MOTUs). The overall species composition in soils of the two distinct ecosystems of the Danube floodplain consisted of 15 known species and 2 potentially new taxa. The latter taxa accounted only for 1.5 % of all MOTUs, suggesting that almost no hidden or uncultivable *Hypocrea/Trichoderma* species is present at least in these temperate forest soils. The species were unevenly distributed in vertical soil profiles although no universal factors controlling the distribution of all of them (chemical soil properties, vegetation type, and affinity to rhizosphere) were revealed. Our data suggest that only a relatively small portion of *Hypocrea/Trichoderma* species is adapted to soil as a habitat and that the interaction between these species should be considered in a screening for *Hypocrea/Trichoderma* agent(s) of biological control of pests.

Zusammenfassung: Die vorliegende Publikation zeigt Ergebnisse bezüglich der *in-situ* Diversität des mycotrophen Pilzes *Trichoderma* (teleomorph *Hypocrea*, Ascomycota, Dikarya) basierend auf einer artspezifichen, metagenomischen Studie. Dazu wurden gattungsspezifische rRNA Primer für ITS1 und und ITS2 entwickelt und damit eine umfassende Klonbibliothek (411 molecular operational taxonomic units = MOTUs) angelegt. Die Zusammensetzung der Arten in den beiden Bodenproben aus zwei verschiedenen Ökosystemen der Donauauen umfasste 15 bekannte Arten und zwei potentiell neue, wobei letztere nur 1,5 % alle MOTUs ausmachten. Daraus lässt sich ableiten, dass zumindest in Waldböden der gemäßigten Zone keine versteckten oder nicht kultivierbaren Arten von *Hypocrea/Trichoderma* existieren. Die verschiedenen Arten waren unterschiedlich in den vertikalen Bodenprofilen verteilt. Es konnte allerdings kein Zusammenhang zwischen globalen Einflussfaktoren wie den chemischen Eigenschaften des Bodens, der umliegenden Vegetation oder der Affinität zur Rhizosphäre nachgewiesen werden.

Key words: Oligonucleotide DNA barcode; *Hypocrea*, metagenomics, Danube floodplain, Nationalpark Donau-Auen, opportunistic species.

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INTRODUCTION

Trichoderma species are one of the most frequently isolated conidial fungi (teleomorph *Hypocrea*, Hypocreales, Ascomycota, Dikarya). They are known from an innumerable diversity of natural and artificial substrata what demonstrates their high opportunistic potential and adaptability to various ecological conditions (Druzhinina et al. 2011). The exploitation of the latter properties of *Trichoderma* in biotechnology and agriculture made the genus well studied and brought it in focus of numerous -*omic* studies [see Lorito et al. (2010) for the review] including the three complete genome sequences recently released for public access - *H. jecorina* (Martinez et al. 2008), *H. virens* and *H. atroviridis* (Kubicek et al. 2011). The comparative analysis of these genomes with each other and other Ascomycota revealed that the outstanding antagonistic ability of *Trichoderma* spp. against plant pathogenic fungi (known as necrotrophic hyperparasitism or mycoparasitism) is the ancestral state for the genus (Kubicek et al. 2011). Several studies have documented that *Trichoderma harzianum* sensu lato, *T. asperellum* and *T. asperelloides* are highly rhizosphere competent and are capable to stimulate growth and immune defense of plants (reviewed by Harmann et al. 2004).

The current diversity of the holomorphic genus *Hypocrea/Trichoderma* is reflected in approximately 160 species (Samuels 2005; Druzhinina et al. 2006; Kubicek et al. 2008, NCBI Taxonomy Browser for February 2011), the majority of which have been recognized on the basis of DNA sequence analysis and molecular phylogeny of pure cultures and/or herbaria specimens. A multigene phylogeny established in several studies [see Kubicek et al. 2008 for review and Jaklitsch (20011) for updates] allowed the development of reliable tools for molecular species identification based on DNA barcoding, i.e. the analysis of DNA sequence polymorphism in the internal transcribed spacers 1 and 2 (ITS1 and 2) of the rRNA operon and other loci (Druzhinina et al. 2005; Kopchinskiy et al. 2005, www.ISTH.info).

The complete inventory of teleomorph forming species in Central Europe resulted in 75 species (Jaklitsch 2009, 2001). In addition to it about a dozen of anamorphic species (for which no sexual stages are known) were reported in surveys of European soils and in taxonomic studies of Wuczskowski et al. 2003; Kraus et al. 2004; Samuels et al. 2006b; Jaklitsch et al. 2006; Komon-Zelazowskay et al. 2007; Hagn et al. 2007; Druzhinina et al. 2008; Migheli et al. 2009; Zachow et al. 2009 and Meincke et al. 2009. Thus, the total number of species *Hypocrea/Trichoderma* so far known in Europe may be about one hundred.

The major ecological niche for *Hypocrea/Trichoderma* may be deduced from the distribution of holomorphic species (when both teleomorphic and anamorphic stages observed), which constitute the major genetic pool for the genus: they are associated with dead wood on different stages of its decay and with sporocarps of other fungi (Jaklitsch 2009). *Trichoderma* species were initially believed to be among the dominant taxa inhabiting soil ecosystems (Klein and Eveleigh 1998). However this statement was not supported by the application of high-throughput sequencing methods to study the cultivation-independent fungal diversity in soil. These studies did not reveal any abundance of *Trichoderma* listing it among minor taxa (Búee et al. 2009; Lim et al. 2010).

Thus, molecular ecology and genomics of the genus indicate that presence of *Hypocrea/Trichoderma* in soil where it may be either a saprotroph or establish various associations with plants and animals (biotrophy) may be driven by the general mycotrophy including various forms of mycoparasitism, combined with broad environmental opportunism (Rossman et al. 2003; see Druzhinina et al. 2011 for the review).

The aim of this research was to use a taxon-specific metagenomic approach and oligonucleotide DNA barcode available for *Hypocrea/Trichoderma* to explore the diversity of the genus in a soil profile and to identify the factors which control the size/occurrence of its infrageneric communities.

METHODS

Sampling sites

Two sampling sites were chosen in the River Danube National Park "Nationalpark Donau-Auen" (Austria). They were located a few hundred meters apart representing the essentially different biotopes (Table 1). The beech forest site (+48° 9' 28 N, +16°32'19 W, alt. 162 m) belongs to the hard wood riparian forest, which is situated above the seasonal Danube overflow level (approximately 3 m above the water level). The aspen forest site (+45° 30' 43 N, +73° 32' 44 W, alt. 141 m) represents the softwood riparian forest regularly flooded during the seasonal Danube overflows. Detailed botanical description of both ecosystems is given in Table 1.

Soil sampling and detection of soil properties

In both ecosystems, pits of about 100 cm of depth were made and soil samples of about 200 g were taken from each soil horizon using a sterile knife. The soil samples were immediately separated from roots and large particles, air dried and sieved through the metal mesh to collect fine particles < 2 mm ("fine earth fraction"). Thereafter each soil was spread into a sterile tray and divided into 2 x 2 equally sized fragments, two of which were discarded while the remaining two were thoroughly mixed and again spread on the

Table 1. Ecosystem description and soil properties.

		Beech forest site				Aspen forest site		
Types of ecosystem		Hard wood riparian forest patch				Clearing of the soft wood riparian forest		
Flooding frequency		rare to none				regular		
Botanical description								
Trees		tall, thick				moderately tall, thin		
	dominant	*Ulmus laevis, Populus canescens, Fraxinus excelsior*				*P. alba, P. nigra, P. canescens*		
	rare	*P. nigra, P. alba, Salix rubens*				*S. rubens*		
Shrub and regeneration		lighted				dense		
	dominant	*Crataegus monogyna*				*Rubus idaeus*		
	rare	*Populus* sp., *Acer* sp., *Salix* sp.				*Quercus robur, Populus* sp.		
Herbs		lighted				dense		
	dominant	*Aegopodium podagraria*				*Allium ursinum, Ae. podagraria*		
	rare	*Salvia glutinosa,* Poaceae, Cyperaceae				*Carex acuta, Urtica dioica, Epilobium* sp., *Salvia glutinosa, Solidago gigantea*		
Fallen trees		abundant				none		
Soil properties								
Litter layer		thick, well developed				thin, undeveloped		
Soil type (FAO)		cambic fluvisol				calcaic fluvisol with high level of sandy sediments		
Soil moisture content		moderate				high		
Siltation		moderate				high		
Vertical profile		well differentiated				well differentiated		
	horizon name	**A0**	**A**	**B**	**C**	**A**	**BC**	**C**
	thickness (cm of 1 m section)	5	10	40-50	20+	10	20-30	50-60
	soil color	very dark brown	dark grayish	grayish brown		very dark grayish brown	light olive brown	
	soil color code (Munsel)	10YR 2/2	10YR 4/2	2.5Y 5/2		2.5Y3/2	2.5Y 5/3	
	relative root density	no roots	very high	high	moderate	high	low	no roots
Chemical characteristics	pH	7.77	7.90	8.10	8.24	7.60	8.15	8.23
	C, %	8.16	2.38	1.82	2.50	6.52	1.04	2.51
	N, %	0.37	0.15	0.06	0.04	0.43	0.07	0.04
	C/N	22.05	15.87	30.33	62.50	15.16	14.86	62.75

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Table 2. Genus and species-specific PCR primers designed in this study.

Primer	Locus	Application	Suggested pair	Specificity	Direction	Primer sequence
Trirev1	ITS2	ciPCR	ITS5	genus, *Hypocrea* and *Trichoderma*	reverse	5'-CATTTC(A/C)GAAAGTTGGGGTG-3'
Trirev2	ITS2		ITS5			5'-CATTTC(A/C)GAAGTTTGGGGTG-3'
Trirev3	ITS2		ITS5			5'-CATTTC(A/C)GAAAGTTTGGGTG-3'
Trirev4	ITS2		ITS5			5'-CATTTC(A/C)GAAAGTTGGGTG-3'
Trirev5	ITS2		ITS5			5'-CATTTC(A/C)GAAGTTGGGTG-3'
Trirev6	ITS2		ITS5			5'-CATTTC(A/C)GAAGTTTGGTG-3'
citro1_64	*tef1*	ciPCR and qPCR	LLErev	species, *H. schweinitzii*	forward	5'-CGCTACTGCCTTCAGACCAC-3'
asp7_60	*tef1*		LLErev	species, *T. asperellum*	forward	5'-GCTTTGCCAGTCTACCTACC-3'

The table was originally published in Friedl & Druzhinina 2012, the special issue of Microbiology "*Trichoderma* - from Basic Biology to Biotechnology", January 2012

same tray for subsequent subsampling (Robertson 1999). Finally 50 g of each soil was stored at -20° C for further molecular and chemical investigations.

Soil classification and the detection of soil horizons were done directly at the two sampling spots. The soil color was defined using a standard color scale for soil science (Munsell Soil Color Charts, U.S. Dept. of Agriculture). The horizons were visually distinguished based on their color and later on differentiated based on their chemical properties. All chemical analyses were performed using the fine earth fraction. To measure pH, 1 g of soil was suspended in 100 ml of 1M KCl and shaked for one hour. Finally, pH was determined with a glass electrode. The total nitrogen content was determined according to the Kjeldahl method (see Batjes 1996 for a reference) on a Vapodest 30 (Gerhardt, Germany). The total organic carbon content was measured using the Liechtenfelder method (see Batjes 1996 for a reference), which oxidized carbon with potassium dichromate, and quantifies the generated Cr^{3+} photometrically (DIN 19684).

Development of genus-specific primers

The genus-specific primers were developed based on the master alignment of ITS 1 and 2 from 88 reference strains of *Hypocrea* and *Trichoderma* (Druzhinina et al. 2005) complemented by the new species described since that time (Jaklitsch et al. 2005; Jaklitsch et al. 2006a; Jaklitsch et al. 2006b; Overton et al. 2006; Samuels et al. 2006b; Komon-Zelazowska et al. 2007; Jaklitsch et al. 2008; Jaklitsch 2009, 2011). All primers are reverse primers and are complementary to the forward primer ITS 5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') (Table 2). The position of primers is shown on www.ISTH.info/metagenomics. The verification of primer specificity was done with reference cultures presented in Druzhinina et al. (2005). The general test of possible unspecific annealing was done by sequence similarity search against NCBI GenBank (May 2006) adjusted for short query sequences.

Isolation of DNA and ciPCR

A representative subsample of 1 g of fine soil fraction was thawed and dried over night in a drying chamber at 90°C. Afterwards, the individual soil samples were well homogenized and DNA was extracted using a FastDNA Spin kit for soil (MP Biobiomedicals, Germany). The DNA was then used as a template for ciPCR (culture independent PCR) with *Hypocrea/Trichoderma* specific rRNA primers as listed in Table 2. CiPCR products of all six primers were combined and purified using QiaQuick PCR purification kit (Qiagen, Germany).

CiPCR reactions were carried out in a total volume of 50 µl containing 2.5 mM $MgCl_2$, 10 mM Tris- HCl pH 9.0, 50 mM KCl, 0.1 % (v/v) Triton X-100, 0.4 µM of each primer, 0.2 mM of each dNTP and 0.5 units of Taq-Polymerase (Promega, Madison, WI). The amplification program consisted of: 1 min initial denaturation (94°C), 30 cycles of amplification (1 min 94°C, 1 min 52° C,

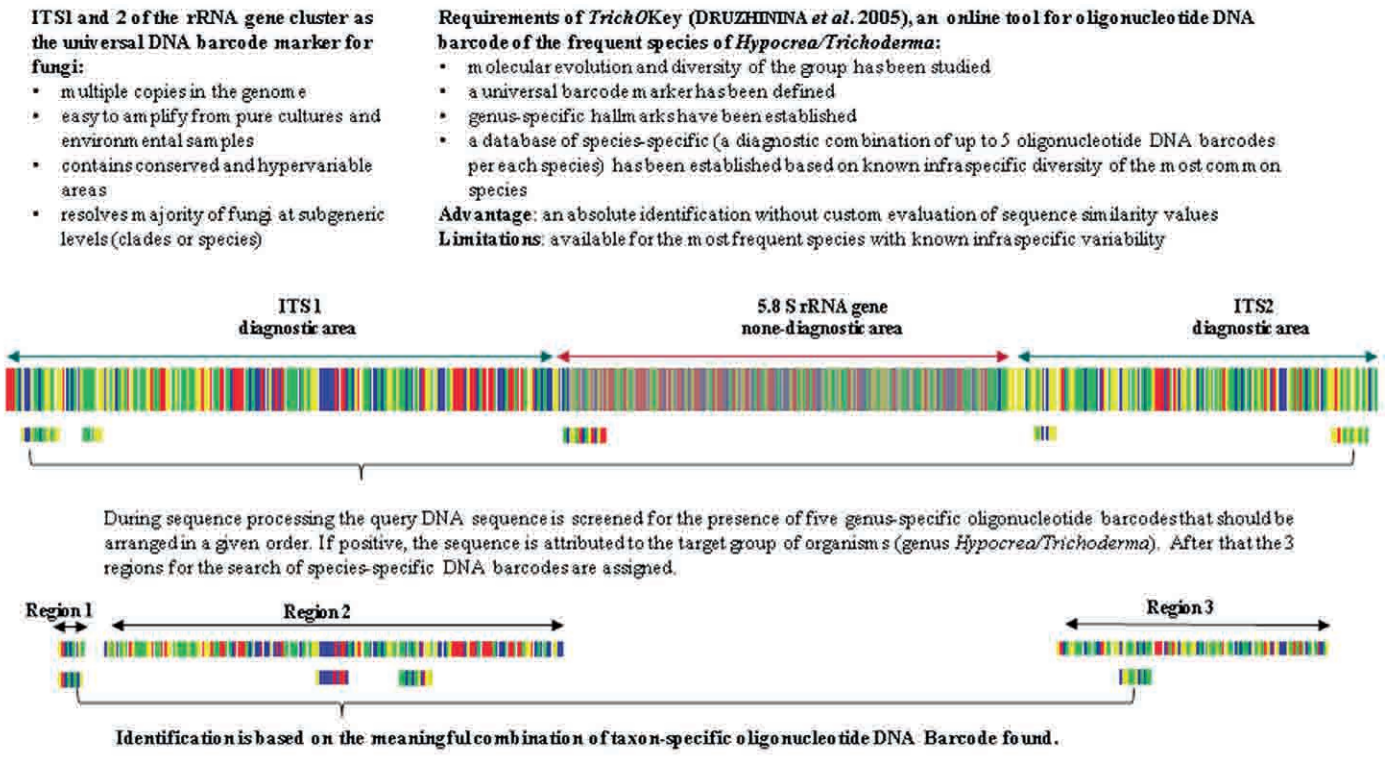

Fig. 1: Principle of the oligonucleotide DNA barcoding implemented in *Trich*OKey (Druzhinina et al., 2005)

1 min 72°C) and a final extension period of 7 min at 72°C.

Amplification products were visualized on 1% TAE agarose gels and the ciPCR amplicons at a size of 600 kb were excised, repurified using QiaQuick Gel excision kit (Qiagen, Germany) and suspended in 20 µl water.

Construction of clone libraries and sequencing

The ciPCR products were subcloned using the pGEMTeasy (Promega) standard procedure. At least 100 colonies were selected from each soil sample. Plasmids were extracted using a standard miniprep method. In order to control if the plasmid DNA was carrying the insert, the plasmid DNA was digested with 5u of the restriction enzymes *Not*1 and *Eco*R1 enzymes (Fermentas, Glen Burnie, USA). For positive samples 0.5 µg of the plasmid DNA was finally used for automated sequencing (Eurofins MWG, Ebersberg, Germany) from both directions.

Representative alleles for all species from each horizon have been deposited in the NCBI GenBank database (accession numbers. *T. harzianum* GQ981644, GQ981645, GU060097 - GU060105; *T. cerinum* GU060106, GU060107; *T.pleuroticola* GU060108, GU060109, *H. alni* GU060110; *H. virens* GU060111; *T.* sp. MOTU 1A 64 GU060112 - GU060117; *T. brevicompactum* GU060118 - GU060120; *H. pachybasiodes* GU060121; uncult. *H. pachypallida* GU060122 - GU060125; *T. longibrachiatum* GU060126 - GU060128; *H. schweinitzii* GU060129, *T. asperellum* GU060130 - GU060137; *T.* sp. MOTU 2B 48 GU060138, GU060139. The alignment matrix is available upon request.

Species identification by DNA barcode and diversity assessment

All sequences were aligned in GeneDoc 2.6 (Nicholas and Nicholas 1997) using the guidance for *Hypocrea/Trichoderma* ITS1 and 2 alignment provided by Druzhinina et al. (2005), available on-line at http://www.isth.info/tools/master.php. For species identification the complete set of sequences was submitted to the DNA oligonucleotide barcode program *Trich*OKey (http://www.isth.info/tools/molkey/index.php; Druzhinina et al. 2005). All sequences contained the set of five genus-specific hallmarks and were therefore attributed to *Hypocrea/Trichoderma*. Hence, tests for chimerical sequences were not required. All unusual ITS1 and 2 alleles have been further analyzed by

sequence similarity search against the NCBI GenBank, *Tricho*BLAST (Kopchinskiy et al. 2005) and the sequence database of the TUCIM the Collection of Industrial Microorganisms of the Vienna University of Technology that currently contains more than 4000 *Hypocrea*/*Trichoderma* strains with 5500 core nucleotide sequences including ITS1 and 2. Based on individual mismatches found in the otherwise conserved areas of ITS1 and 2 (for example, genus-specific hallmarks or 5.8S rRNA gene, see Druzhinina et al. 2005), 20 % of sequences have been diagnosed to contain single sequencing errors. Four sequences contained polymorphic sites in the diagnostic regions of both ITS1 and 2 and therefore have been diagnosed as potentially new alleles (*T.* sp. MOTU 1A 64 for Section *Longibrachiatum* and *T.* sp. MOTU 2B 48 for Section *Trichoderma*). The principle of the oligonucleotide DNA barcode is shown if Fig. 1.

Development and verification of species-specific *tef1* primers for qPCR

In order to design species-specific qPCR primers, representative sequences of the 4th large intron of *tef1* gene coding the elongation factor 1 alpha for the whole genus *Hypocrea*/*Trichoderma* were retrieved from the multilocus database of phylogenetic markers (www.ish.info) and automatically aligned in Clustal X (Thompson et al. 1997). The representative *tef1* sequence of a target species was submitted to NCBI sequence similarity search tool (blastn) and all homologous vouchered sequences (N≥20) attributed to the same species retrieved and added to the initial alignment. Subsequently, species-specific diagnostic regions were manually selected for each target species in a way that they contain the minimal level of infraspecific polymorphism. In parallel, the same alignments were used to design degenerate species-specific primers in HYDEN software (Linhart and Shamir 2005). The annealing temperature and secondary structure of oligonucleotides designed based on both approaches have been estimated using Gene Runner (Gene Runner 3.0 software) and SMS PCR Primer Stats tools http://www.bioinformatics.org/sms2/pcr_primer_stats.html. Specificity of selected primers was first tested against NCBI GeneBank database (automatically optimized for short queries) and then verified by PCR reactions (see Jaklitsch et al. 2006a for conditions) with reference DNAs from pure cultures of all genetically close and several members of the neighbor clades. The resulting potentially species-specific oligonucleotides were tested for the annealing efficiency by applying serial dilutions of a target DNA extract. Selectivity of designed primers was verified by subcloning the PCR product obtained from soil DNA extract. Around 40 oligonucleotides have been synthesized to hit the diversity of the most frequent temperate *Hypocrea*/*Trichoderma* species but only two primers targeting *T. asperellum* and *H. schweinitzii* (Table 2) showed high specificity, selectivity and appropriate efficiency (≥80%) to be applied to environmental samples.

Semi-quantitative PCR assessment

Quantitative PCR amplification was carried out with the iQ 5 Real-Time PCR detection system (Bio-Rad) in a 25 µl reaction containing 12.5 µl iQ SYBR Green Supermix (Bio-Rad), each primer at a concentration of 250 nM and sample corresponding to an initial concentration of 0.5 µl of total DNA. Amplification was carried out with the following PCR program: initial denaturation for 3 min at 95°C, followed by 45 cycles consisting of 95°C for 15 s, 54.0°C (*tef1*), respectively, for 20 s, and 72°C for 20 s. Successful amplification was verified by determination of the melting temperature and by agarose gel electrophoresis. For each species a series of dilutions was performed to assess the efficiency of the PCR. The results of the real-time PCR were analyzed with the iQ 5 optical system software (Bio-Rad). Using the PCR base line subtracted mode, the threshold cycle was calculated for all samples and the amplification efficiency for each primer was determined.

Statistical analysis

Cultivation-independent diversity and species richness of *Hypocrea*/*Trichoderma* was assessed in EstimateS 8.2. Rarefaction curves were computed for each soil profile. The number of species was quantified for 100 random combinations of 1 to N sequences and also by performing 100 bootstrap pseudoreplicates implemented in EstimateS (Colwell 2005).

Statistical analyses of metagenomic data and *in vitro* infrageneric interactions were done in Statistica 6.1 (StatSoft Inc., Tulsa, USA) software package using basic data exploration tools, correlation matrices, variance analyses and multifactorial techniques (factor analysis and cluster analysis). Discrete color plots representing the results of pair wise interactions between different *Hypocrea*/*Trichoderma* strains were constructed on the basis of a result of a two-way joining cluster analysis implemented in Statistica 6.1 with consequent reordering of both variables and cases in order to reflect phylogenetic groups inside the sample.

RESULTS

The pedogenesis of both sites is strongly influenced by the river

The two sampling sites representing essentially different biotopes (Table 1) in the riparian forest were selected in the River Danube National Park southeast of Vienna, Austria. We performed a 1 m deep vertical soil cut into both ecosystems in a place which is equally distanced from surrounding big trees and shrubs and which therefore does not represent an exclusive rhizosphere of any plant species.

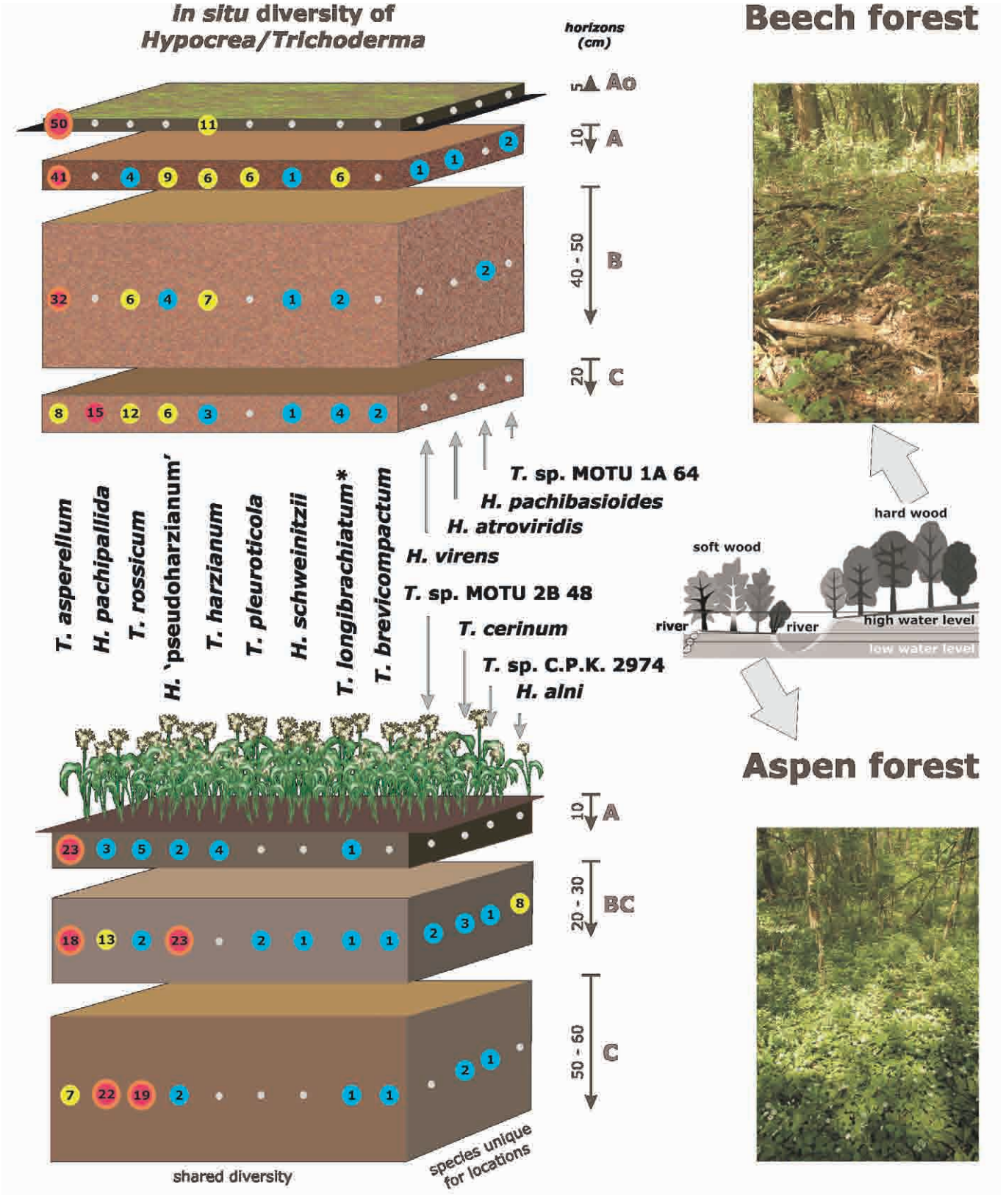

Fig. 2: *In situ* diversity of *Hypocrea/Trichoderma* in vertical profiles of two soil cuts in a hard wood beech and a soft wood aspen forest sides respectively. Numbers in colored cycles indicate the number of MOTUs of each species recovered; red, yellow and blue cycles correspond to dominant, sub-dominant and rare species, respectively. The middle right insert shows the schematic profile of the flood plain ecosystem of the river Danube and images of the sampling sites. * - *T. longibrachiatum – H. orientalis* species pair. The figure was originally published in Friedl & Druzhinina 2012, the special issue of Microbiology "*Trichoderma* - from Basic Biology to Biotechnology", January 2012 http://mic.sgmjournals.org/content/158/1/69.long.

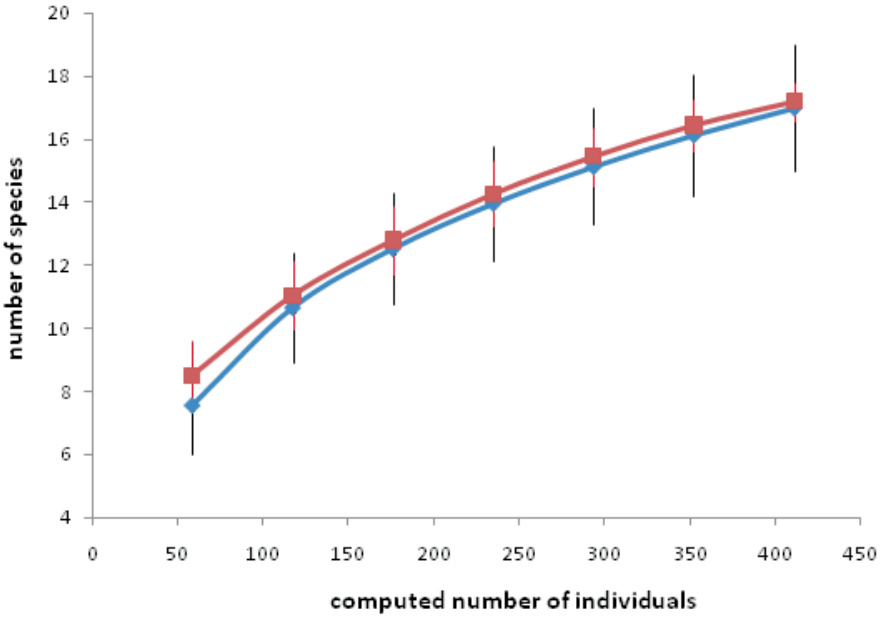

Fig. 3: Rarefaction curve estimating the total *Hypocrea/Trichoderma* community diversity as a function of clone library size. The curve was computed from 100 bootstrap replicates (blue) and the expected richness function (red) is the number of species estimated (random sampling without replacement) from our sequences dataset. Vertical bars show the standard deviation. The figure was originally published in Friedl & Druzhinina 2012, the special issue of Microbiology "*Trichoderma* - from Basic Biology to Biotechnology", January 2012 http://mic.sgmjournals.org/content/158/1/69.long.

The major difference between the two soil cuts was in the presence of the thick (min 5 cm) soil litter layer (horizon A0, FAO) in the beech site which was absent in the aspen forest. Instead, the latter site was covered by the dense herbal layer dominated by the blossoming *Alnus urcinum* with several co-dominated herbal species (see Table 1). Correspondingly the beech forest soil profile was evenly penetrated by roots from different plants while in the aspen forest a high root density was observed only in the upper horizon.

The pedogenesis of both sites is strongly influenced by the river and results in formation of fluvisols (FAO classification: J) with a high level of calcaric soil material and sedimentation. The flooded soil in the aspen forest contains significantly more moisture compared to the beech forest (Table 1). Both sites were characterized by well developed soil profiles and presence of at least three horizons in FAO nomenclature: the soil of the beech forest was characterized by three clear horizons A, B and C; at the aspen forest the B layer contained little alteration products and was mixed with the parent material, therefore the horizons at this sampling spot were classified as A, BC and C (Table 1). The chemical properties of the soil are detailed in Table 1.

DNA barcode revealed restricted diversity of *Hypocrea/Trichoderma* in temperate soil

We designed the set of genus-specific ciPCR (culture independent PCR) reverse primers Trirev (1 to 6) 5'-CATTTCMG[A_2/A_3]G[T_2/T_3][G_2/G_3/G_4]TG-3' (Tm 61°C) (Table 2) in the 3 prime end of the internal transcribed spacer 2 (ITS2) of the rRNA gene cluster (Table 2). The position of primers is illustrated on http://www.isth.info/methods/method.php?method_id=12. When applied together with ITS5 forward primer (White et al. 1991), these primers amplified a 510 - 540 bp fragment covering the complete diagnostic area of ITS1 and 2 including all five genus-specific hallmarks (Druzhinina et al. 2005).

Application of ITS5/Trirev primer pair to ciPCR with DNA extracts from soils samples showed different results for individual primers and also unequal efficiency of Trirev primers in different horizons. The highest affinity of Trirev primers was detected for the horizon A followed by the two other soil horizons of the beech forest site. PCR efficiency in soil of aspen forest was essentially lower. The pooled ciPCR products of ITS5/Trirev obtained for each soil horizon were used for subsequent clone libraries.

In total 411 ITS1 and 2 rRNA molecular taxonomic units (MOTUs) were recovered (Fig. 2). All sequences had diagnostic genus-specific hallmarks (Druzhinina et al. 2005) suggesting high selectivity of Trirev primers. All MOTUs were identified as 15 known species and two putatively new taxa (Table 3). The rarefaction curve suggested that the species richness was close to saturation as additional 100 MOTUs could reveal only one or two additional species (Fig. 3).

MOTUs frequencies were used to calculate the diversity index as an indirect quantitative measure of community composition. The dominant species in both sites (54 and 28.6 % for beech and aspen forests respectively) was *T. asperellum* sensu stricto (Samuels et al. 2009).

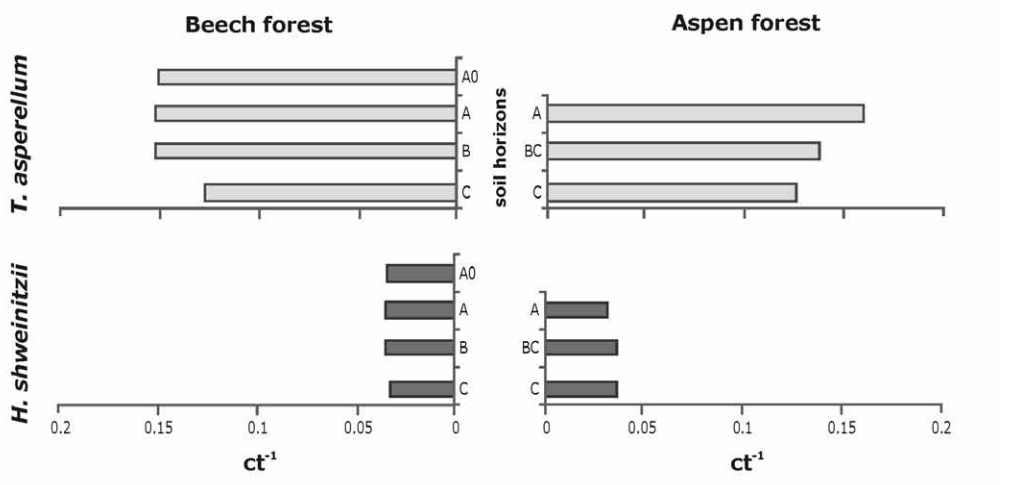

Fig. 4: Results of the semi-quantitative PCR analysis based on species-specific *tef1* primers developed for *T. asperellum* and *H. schweinitzii* respectively. As the efficiency of primers binding the environmental (soil) DNA was slightly different between these species only semi-quantitative comparison is possible. ct^{-1} (the number of threshold cycles/1) corresponds to abundance of the target DNA in the sample. The figure was originally published in Friedl & Druzhinina 2012, the special issue of Microbiology "*Trichoderma* - from Basic Biology to Biotechnology", January 2012 http://mic.sgmjournals.org/content/158/1/69.long.

The beech forest soil was co-dominated by *T. harzianum* sensu stricto (Druzhinina et al. 2010b), *T. rossicum*, *H.* 'pseudoharzianum' (Druzhinina et al. 2010b, widely known as *T. harzianum* complex) and *H. pachypallida* (Jaklitsch 2011) which contributed 11, 9, 8 and 6 % of the total diversity at this location. Sequences of *T. longibrachiatum - H. orientalis* species pair (Druzhinina et al. 2008) added 5 per cents and could be considered as a subdominant taxon (Fig. 2). *T. pleuroticola* (2.5%), *H. schweinitzii* (1.2%), *T. brevicompactum* (0.8%), *H. pachybasioides* (0.8%) and taxonomic units of a putative new species from Section *Longibrachiatum T.* cf. sp. nov. MOTU 1A 64 (0.8%) were rare. MOTUs of *H. atroviridis* and *H. virens* were recovered with a frequency of less than 0.5 %.

A qualitatively similar but quantitatively different species composition was observed in the aspen forest (Fig. 2): the dominant *T. asperellum* was followed by co-dominant *H. pachypallida* (23%), *H.* 'pseudoharzianum' (16%) and *T. rossicum* (15.5%). The subdominant species *H. alni* (5%) and *T. cerinum* (3%) were significantly less present. *T. harzianum* s. s. (2.4%), *T. longibrachiatum - H. orientalis* species pair (1.8%), *T. pleuroticola* (1.2%), *T. brevicompactum* (1.2%), *T.* sp. C.P.K. 2974 (I. Druzhinina, unpublished data) (1.2%) and a putative new species from Section *Trichoderma T.* sp. nov. MOTU 2B 48 were rare; the ITS sequence of *H. schweinitzii* was recovered only once.

Thus, among 17 recovered taxa, we detected nine species present in both sites and four species unique for each ecosystem. In total, soils in both forest sites supported the community of at least 13 co-existing *Hypocrea/Trichoderma* species although these communities were unequal in two locations.

Highest *Hypocrea/Trichoderma* diversity was detected in the moist horizon of aspen forest soil that has a low root density and poor carbon content

The analysis of *Hypocrea/Trichoderma* species distribution in the vertical soil profile showed that *T. asperellum* was the most abundant in the soil litter layer of the beech forest (82 %) and also dominated in A and B horizons (53 and 59 % respectively) while in the horizon C it was only the third most abundant taxon (16 %). A similar distribution pattern was observed in the aspen forest soil (Table 3). The correlation analysis showed a significant negative correlation of *T. asperellum* abundance with pH values (Table 3) that decreases with depth, indicating that this species is associated either with the

Table 3. In situ diversity of *Hypocrea/Trichoderma* in vertical soil profile and its correlation with soil properties.

Species	total freq., %	beech forest/243* local freq., %	beech forest/243* soil horizons A0 61§	A 77	B 54	C 51	aspen forest/168 local freq., %	aspen forest/168 soil horizons A 38	BC 70	C 55	correlation coefficients soil horizon	pH	C%	N%	C/N	root density	depth (cm)
T. asperellum	43.6	53.9	0.206#	0.169	0.132	0.033	28.6	0.137	0.107	0.042	**-1.0**	**-0.8**	-	-	**-0.8**	-	**0.9**
H. pachypallida	12.9	6.2	-	-	-	0.062	22.6	0.018	0.077	0.131	**0.8**	-	-	-	-	-	-
T. rossicum	11.7	9.1	-	0.016	0.025	0.049	15.5	0.030	0.012	0.113	-	-	-	-	**0.8**	-	-
H. 'pseudoharzianum'	11.2	7.8	-	0.037	0.016	0.025	16.1	0.012	0.137	0.012	-	-	-	-	-	-	-
T. harzianum s.s.	7.5	11	0.045	0.025	0.029	0.012	2.4	0.024	-	-	-	-	-	-	-	-	-
T. longibrachiatum - H. orientalis	3.6	5	-	0.025	0.008	0.016	1.8	0.006	0.006	0.006	-	-	-	-	-	**0.9**	-
H. alni	1.9	-	-	-	-	-	4.8	-	0.048	-	-	-	-	-	-	-	-
T. pleuroticola	1.9	2.5	-	0.025	-	-	1.2	-	0.012	-	-	-	-	-	-	-	-
T. cerinum	1.2	-	-	-	-	-	3.0	-	0.018	0.012	-	-	-	-	-	-	-
T. brevicompactum	1.0	0.8	-	-	-	0.008	1.2	-	0.006	0.006	***0.9***	***0.8***	-	-	-	-	***-0.8***
H. schweinitzii	1.0	1	-	0.004	0.004	0.004	0.6	-	0.006	-	-	-	***-0.8***	-	-	-	-
T. sp. C.P.K. 2974	0.5	-	-	-	-	-	1.2	-	0.006	0.006	-	-	-	-	-	-	-
T. sp. MOTU 2B 48 sect. Trichoderma	0.5	-	-	-	-	-	1.2	-	0.012	-	-	-	-	-	-	-	-
H. pachybasioides	0.5	0.8	-	-	0.008	-	-	-	-	-	-	-	-	-	-	-	-
T. sp. MOTU 1A 64 sect. Longibrachiatum	0.5	0.8	-	0.008	-	-	-	-	-	-	-	-	-	-	-	-	-
H. atroviridis	0.2	0.4	-	0.004	-	-	-	-	-	-	-	-	-	-	-	-	-
H. virens	0.2	0.4	-	0.004	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	100	100					100										
Diversity Index	1.0	0.8	0.15	0.77	0.34	0.62	0.8	0.46	0.92	0.62	0.5	0.5	-0.7	-0.5	0.0	0.4	-0.3

* - total size of the clone library for the sampling site; § size of the horizon-specific clone library, # diversity index calculated as ratio between a number of taxon attributed MOTUs (as given in **Fig. 2**) to a total number of MOTUs per soil horizon; bold font indicates significant product-moment correlation coefficients $P<0.05$, ***bold*** x ***italics*** indicates the significant coefficients for repressed species. The table was originally published in FRIEDL & DRUZHININA 2012, the special issue of Microbiology "*Trichoderma* - from Basic Biology to Biotechnology", January 2012 http://mic.sgmjournals.org/content/158/1/69.long.

soil litter layer or with the upper organic horizon A.

The distribution of the next most frequent species *H. pachypallida* in the vertical soil profile is reverse proportional to that of *T. asperellum* (Fig. 2, Table 3): in the beech forest soil it dominated the deepest C horizon (30 %) and was not detected in others while in the aspen forest it was found in all horizons but with increasing frequency from top to down. The significant correlation of its occurrence with the horizon (Table 3) but not with other factors (carbon content, pH and depth) suggests the presence of yet another parameter which controls the distribution of this species and which was not monitored in this study.

T. rossicum - one of the co-dominant species in both locations – is also more frequent in the deeper soil horizons showing a significant positive correlation with the C/N ratio (Table 3). MOTUs of the *T. longibrachiatum - H. orientalis* species pair were the most frequent in the A horizon of the beech soil profile which has the highest density of roots (r=0.8, P<0.05).

No correlations were detected for *H.* 'pseudoharzianum' and *T. harzianum* s. s.

The highest diversity of *Hypocrea/Trichoderma* (12 coexisting species) was found in the moist BC horizon of aspen forest soil, which has a low root density and the lowest carbon content of this study. The next most diverse infrageneric community (10 species) was in the topsoil horizon A in the beech forest, which has the highest root density. As these communities share six species but have no similar frequencies, consequently, no significant correlations between species richness and soil properties were detected.

Since the genetic diversity of *Hypocrea/Trichoderma* in individual soil profiles appeared unexpectedly high we applied several statistical techniques to reveal possible correlations between the distributions of MOTUs from different species. The product-moment correlation analysis confirmed the significant (P<0.05) negative correlation between *T. asperellum* on one hand and *H. pachypallida* and *T. rossicum* on the other. No other significant correlations between dominant (co- and sub-) species were revealed suggesting that these species have different responses to the microecological conditions.

Quantitative PCR confirms the vertical distribution of *T. asperellum*

In order to have a second means of testing species distribution in soil profile we designed species-specific primers for quantitative PCR (qPCR) based on the polymorphic fragment of the *tef1* gene. Although 40 primers were tested (see Methods for details), only those for *T. asperellum* and *H. schweinitzii* showed to be highly selective and efficient when applied to soil DNA extract (Table 2, Fig. 4). Using them, we detected a higher abundance of *T. asperellum* and a relatively small amount of *H. schweinitzii* DNA in all seven samples, which is in accordance with the metagenomic data. It was also possible to detect a reduction of *T. asperellum* biomass with depth while the distribution of *H. schweinitzii* remained constant.

DISCUSSION

To study the generic community of *Hypocrea/Trichoderma* in soil we selected a riparian forest in the River Danube National Park ("Nationalpark Donau-Auen") southeast of Vienna, Austria. This ecosystem is unique, as it still resembles the original European river floodplain landscape free from anthropogenic loadings (Wuczskowski et al. 2003). The forest is characterized by high biodiversity including several otherwise rare plant species (Tockner et al. 1998).

Temperate soil supports a limited diversity of highly opportunistic *Hypocrea/Trichoderma* spp.

Although the biodiversity of Higher Fungi is considered to be largely unknown (Hawksworth 1991), and studies using cultivation-independent methods should thus result in the identification of a high percentage of unknown taxa, this is not the case with *Hypocrea/Trichoderma*. Our data suggest that at least in soils of temperate climate there is almost no hidden diversity of *Hypocrea/Trichoderma*: among 411 MOTUs 407 were safely attributed to 15 existing species or to putatively new taxa that have previously been sampled. The diversity of *Hypocrea/Trichoderma* in Europe consists of at least 75 holomorphic species (Jaklitsch 2009) and 10 - 20 anamorphic species (see refs. in Introduction), in summary approaching one hundred taxa. Our finding of only a minor portion of potentially expected diversity (roughly 15 %) is in agreement with the previous hypothesis that soil itself is not the primary ecological niche for the genus (Druzhinina et al. 2011). The similar outcome was also obtained by the pioneering metagenomic studies of *HypocreaTtrichoderma*: Hagn et al. (2007) used ITS1 fragment and found only about a dozen species in arable soil. Meincke et al. (2010) used a partial ITS1 and 2 and detected about 20 *Hypocrea/Trichoderma* taxa in rhizosphere of *Solanum tuberosum*, although no undoubtful species identification was made. This view is also in agreement with studies that applied high-throughput sequencing to reveal the actual *in situ* diversity of high fungi in soil: in these studies, *Hypocrea/Trichoderma* MOTUs were found only at minor portions compared to other dominating groups of Ascomycota (Búee et al. 2009; Lim et al. 2010).

The perception of *Hypocrea/Trichoderma* as a common soil fungus is based on the abundant isolations from soil samples world-wide. However the qualitative analysis of the diversity revealed in such samples shows

the dominance by the same 15 - 20 highly opportunistic species such as *T. asperellum, T. asperelloides, T.* cf. *harzianum*, *T. hamatum*, *T. atroviride, T. virens, T. longibrachiatum, T. gamsii, T. citrinoviride, T. koningiopsis, T. spirale, T. koningii* complex etc. (see http://www.isth.info/materials/topic.php?material_id=42 for details) which likely obtained the ability to saprotrophic growth in soil due to their general outstanding opportunistic potential as suggested based on genomes of *T. atroviride* and *T. virens* (Druzhinina et al. 2011; Kubicek et al. 2011). Moreover the antifungal activity of *Hypocrea/Trichoderma* spp. favors their detection, as they are able to suppress other fungi. The view of soil as the main determinant of *Hypocrea/Trichoderma* ecological niche does not find its support.

The qualitative composition of *Hypocrea/Trichoderma* community reveals that soil is inhabited by highly opportunistic species with cosmopolitan distribution as all taxa, except the 3 MOTUs of putatively new species, are common and known from multiple isolates from numerous substrata (including soil) from temperate ecosystems world-wide.

The occurrence of *Hypocrea/Trichoderma* species in different soil profiles is not determined by root density

The PCR efficiency with *Hypocrea/Trichoderma*-specific primers was essentially higher in the moist beech forest (all horizons except the litter layer A0) compared to aspen site, while there was no visual difference between both sites when general fungal primers were applied (data not shown). This finding indirectly suggests that the beech forest contains more *Hypocrea/Trichoderma* biomass compared to the aspen soil.

Moreover, not all species known to be abundant in this region have been detected in soil profiles. For instance, the most frequent teleomorphic *Hypocrea/Trichoderma* species in Central Europe - *H. minutispora* (Jaklitsch 2009) - was not found in our study at all. Despite of this, *H. minutispora* was abundantly found in air samples in nearly the same region (within a few km^2) by the same methodology as here (M. A. Friedl, I.S. Druzhinina, unpublished), thus supporting the assumption that there is no methodical bias in our experimental procedures. Similar, many other very common local *Hypocrea* species (*H. viridescens*, *H. rufa*, *H. pulvinata*, *H. strictipilosa* etc., Jaklitsch et al. 2006; Overton et al. 2006) were not detected in Danube floodplain soils indicating other ecological niches for their anamorphic stages.

The observed distribution of MOTUs in the vertical soil profile also suggests the existence of ecological factors that determine the proliferation of *Trichoderma*. *T. asperellum*, which was the dominant species in this study, was reproducibly associated with upper soil horizons, but its abundance did not correlate with the carbon and nitrogen content of the soil or pH of soil solution. Also, the highest number of MOTUs for *T. asperellum* was found in the root-free A0 litter layer of the beech site, and we therefore suspect that the rhizosphere is not its prime habitat. However, it might be that the species is following other fungi highly abundant in soil litter and upper soil horizons. In contrast to that, *H. pachypallida* and *T. rossicum* were almost exclusively detected in the deepest soil horizons characterized by the sufficient organic carbon and the nearly complete absence of plant roots. It suggests predominantly saprotrophic nutrition for the later two species in deep soil horizons. Moreover, these species should be capable of growing well under conditions of nitrogen starvation as the amount of nitrogen in their habitat is one order of magnitude lower than that in the surface soil. Both the high species richness of the deepest mineral soil horizons (8 species in each sampling site) and their unique species compositions compared to upper soil layers suggest competitive relations between tribal relatives (and/or with other myco- and microbionts) rather than their associations with abiotic characteristics of these soil horizons.

The limited diversity of *Hypocrea/Trichoderma* in studied soils may be attributed to the relatively high pH values (around 8) which could potentially prevent the development of other species. However the same cosmopolitan and opportunistic species were also dominated mole acidic soils on Sardinia (pH =~ 5, Migheli et al. 2009) and were present in rhizospheric soil of *Coffea arabica* in Ethiopian highland forest (pH =~ 5.5, Mulau et al. 2010) suggesting that this is not the controller of the diversity.

In situ diversity confirms the sympatric speciation within *T. harzianum* sensu lato and related taxa

In most previous studies that used cultivation-dependent methods to quantify *Hypocrea/Trichoderma* in various habitats, *T. harzianum* sensu lato represented the most dominantly occurring species (Druzhinina et al. 2005; Migheli et al. 2009; Zachow et al. 2009 and Druzhinina et al. 2010b for more references). Also in this study, we detected a remarkable diversity of genetically sibling species from Harzianum - Catoptron Clade (Chaveri et al. 2003; Druzhinina et al. 2010b) in nearly all soil samples. Interestingly, in all mineral soil horizons of the beech forest site *T. harzianum* s.s. coexisted with a member of *H.* 'pseudoharzianum' (Druzhinina et al. 2010b) while in the aspen forest site the later one was found together with other members of the Harzianum - Catoptron clade: *T. cerinum*, *T. pleuroticola* and *H. alni*. This finding suggests the sympatric speciation of *T. harzianum* s. s. and *H.* 'pseudoharzianum' (Druzhinina et al. 2010b) what is also proposed for other pairs of sister species in the genus *Hypocrea/Trichoderma* such as *H. jecorina* and *T. parareesei* (Druzhinina et al. 2010a, Atanasova, Jaklitsch et al. 2010) or *T. longibrachiatum* and *H. ori-*

entalis (Druzhinina et al. 2008). It is common to argue against the assignment of spatial speciation modes to fungi as, although they may be distributed in overlapping ranges, they can occupy different microhabitats in those areas and therefore may be still spatially isolated (Burnett 2003). Our findings show the non-random presence of MOTUs attributed to both *T. harzianum* s. s. and *H.* 'pseudoharzianum' in at least four soil habitats.

Biocontrol formulations may benefit from synergistic action of highly opportunistic Hypocrea/Trichoderma spp.

A community of highly opportunistic *Hypocrea/Trichoderma* has been detected in previous cultivation-dependent studies: Migheli et al. (2009) showed that among 16 species isolated from highly disturbed non-rhizosphere soils in Sardinia (Italy) *H.* 'pseudoharzianum, *T. spirale*, *T. gamsii*. *T. hamatum* or *H. koningiopsis* consistently co-occurred. A similar result was shown in the pioneering metagenomic study on *Trichoderma* in agricultural soils when representatives of Harzianum - Catoptron and Hamatum Clades, which cover species known for their antagonistic potential, were recovered from the same samples (Hagn et al. 2007). Zachow et al. (2009) applied metagenomic methods and traditional cultivation techniques to characterize the diversity of fungi in rhizosphere of endemic plant species of Tenerife (Canary Islands) and showed the co-existence of extraordinarily highly antagonistic strains of *Hypocrea/Trichoderma*.

From a practical point of view, this demonstrates that the knowledge about infrageneric communities and interactions will be important for screening for *Hypocrea/Trichoderma* strains to be used for the biological control of soil borne plant pathogenic fungi. Several strains showing synergism with one another may be combined in certain biocontrol formulations, while on the other hand, the indigenous *Hypocrea/Trichoderma* should not have antagonistic properties against the introduced biocontrol strain(s).

In this study we investigated one of the last remaining undisturbed ecosystems in Central Europe. However the diversity found in the national park largely resembles *Hypocrea/Trichoderma* species composition in disturbed and agricultural soils (Migheli et al. 2009, Hagn et al. 2006). This finding indicates that the local highly opportuninistic species (from those listed above) should be among the major taxa screened for the best *Trichoderma* biocontrol strains.

Both ITS rRNA and *tef1* phylogenetic markers have limited applicability for *in situ* diversity studies using the high-throughput methods

Hagn et al. (2007) designed *Hypocrea/Trichoderma*-specific primers for ITS1 fragment of the rRNA gene cluster. However, later studies showed that ITS1 is not sufficiently diagnostic as many species share the same allele. Meincke et al. (2009) set up genus-specific primers with the reverse primer located in a still polymorphic and indel-rich area of ITS2 30 bp upstream of the last genus-specific hallmark what makes several species undetectable. The six Trirev primers presented in this study amplify the entire diagnostic region of ITS1 and 2 of all members of the genus. We have demonstrated the high specificity and selectivity of these primers as no MOTUs belonging to other fungi were recovered but the two novel alleles of ITS1 and 2 were detected.

Hoyos-Carvajala et al. (2009) asserted the presence of paralogous copies of ITS1 and 2 in some *Hypocrea/Trichoderma* species. In order to test it we constructed a clone library for a randomly selected strain of *T. asperellum* (N=30) and found only one allele (data not shown) of ITS1 and 2 confirming the absence of different alleles within a single genome.

The applicability of ITS1 and 2 for larger metagenomic studies using high throughput sequencing methods remains questionable. On one hand, ITS-based quantification of species abundance in environmental samples will depend on the number of ITS copies in the respective genome what may vary between different species. Furthermore, the growing number of *Hypocrea/Trichoderma* species share the same allele of ITS making these taxa indistinguishable by this locus (Samuels et al. 2006; Jaklitsch et al. 2006, Druzhinina et al. 2008; Atanasova, Jaklitsch et al. 2010). Another disadvantage of ITS1 and 2 comes from the fact that it is not appropriate for a design of species-specific qPCR primers as its most diagnostic zones are surrounded by long mononucleotide stretches and/or have an intolerable GC content (L. Bodrossy, I.S. Druzhinina, unpublished). Gazis et al. (2011) compared the three communities of endophytic fungi and revealed that ITS alone usually underestimates the number of loci predicted by other loci. Sequences for the highly polymorphic 4th large intron of *tef1* gene are also available for the majority of *Hypocrea/Trichoderma* species making it a good alternative to ITS1 and 2. This gene has a single copy in the genome and therefore becomes appropriate for quantitative assessments. Yet the current public database of Hypocreales *tef1* sequences does not yet allow design genus-specific primers. In this study we attempted to develop species-specific *tef1* primers for those taxa, which we either detected among MOTUs or which we could expect to be present in temperate soils (based, for example, on Jaklitsch 2009, 2011). *In vitro* tests showed that only two out of 40 *in silico* designed oligonucleotides are selective for target taxa while others demonstrated unspecific affinity to DNA of non-target species likely due to the polymorphic secondary structures of the fragment. These results suggest that *tef1* intron alone is

also not appropriate for a large scale metagenomic analysis of the genus due to its hypervariablity. The applicability of more conserved markers such as *rpb2* or *chi18-5* are currently tested in author's laboratory.

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