Diversity **2010**, *2*, 527-549; doi:10.3390/d2040527

OPEN ACCESS

diversity

ISSN 1424-2818

www.mdpi.com/journal/diversity

Article

The Rhizosphere of *Coffea Arabica* in Its Native Highland Forests of Ethiopia Provides a Niche for a Distinguished Diversity of *Trichoderma*

Temesgen Belayneh Mulaw, Christian P. Kubicek and Irina S. Druzhinina *

Research Area Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9-1665, A-1060 Vienna, Austria;
E-Mails: temub@yahoo.com (T.B.M.); ckubicek@mail.zserv.tuwien.ac.at (C.P.K.)

* Author to whom correspondence should be addressed; E-Mail: druzhini@mail.zserv.tuwien.ac.at; Tel.: +43-1-58801-172202; Fax: +43-1-58801-17299.

Received: 25 February 2010; in revised form: 24 March 2010 / Accepted: 24 March 2010 / Published: 5 April 2010

Abstract: The southwestern highlands forests of Ethiopia are the origin of the coffee plant *Coffea arabica*. The production of coffee in this area is affected by tracheomycosis caused by a soil-born fungus *Gibberella xylarioides*. The use of endemic antagonistic strains of mycoparasitic *Trichoderma* species would be a nature conserving means to combat this disease. We have used molecular methods to reveal that the community of *Trichoderma* in the rhizosphere of *C. arabica* in its native forests is highly diverse and includes many putatively endemic species. Among others, the putative new species were particularly efficient to inhibit growth *of G. xylarioides*.

Keywords: biocontrol; coffee; diversity; DNA barcode; Ethiopia; *Hypocrea*; molecular phylogeny; rhizosphere

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1. Introduction

Coffee (*Coffea arabica* L.) is a tropical crop, which today contributes 70% of the world's commercial coffee market, and is currently grown in 75 countries with a total production close to 108

million ton per year [1,2]. For the African country of Ethiopia, coffee is the major export crop, accounting for over 60% of total value of exports [3]. It originated in the former Province of Kaffa in the southwest of the country [4-6] rendering Ethiopia the origin and center of infraspecific genetic diversity for *C. arabica*.

The production of coffee is today severely affected by fungal wilt diseases including tracheomycosis (coffee wilt disease) caused by *Fusarium xylarioides* Steyaert (teleomorph: *Gibberella xylarioides* Heim and Saccas) [7-10]. The disease is responsible for a reduction in the production of coffee beans and is also accompanied by severe damage and death of millions of coffee bushes [7].

Currently, attempts to control coffee wilt disease are fundamentally based on the breeding of resistant plants, plant and environmental management, and synthetic fungicides [11]. The high cost of pesticides, the emergence of fungicide-resistant pathogen biotypes and other social and health-related impacts of conventional agriculture on the environment have however recently led to an increased interest in agricultural sustainability and biodiversity conservation [12]. Thus, there is a need for new solutions of plant disease problems that provide effective control while minimizing cost and negative consequences for human health and the environment [13].

Biological control—*i.e.*, the antagonism and eventual killing of plant pathogens by other living organisms, which are themselves not harmful to the plants—could present an attractive alternative for combating wilt disease. Species of the anamorphic genus *Trichoderma* (teleomorph: *Hypocrea*, Ascomycota) have been proven as effective biocontrol agents of soil-borne plant diseases [14,15]. *Trichoderma* would be especially suitable for combating coffee wilt disease because many of its species are rhizosphere competent [16,17], and the coffee roots are the first target for the attack by pathogens [18,19]. In support of this hypothesis, *Trichoderma* spp. have already been applied successfully to suppress *Fusarium* spp. causing *Asparagus* root rot [20], bean root rot [21], and carnation wilt [22].

Yet the antagonistic efficacy of *a biocontrol agent* in the field will also depend on the environment where it will be applied [23], and therefore—ideally—screening for appropriate biocontrol agents should be done in the same environment. This would also avoid the introduction of invasive microorganisms and conserve the microbial composition of the respective biotopes. To date, information about the diversity and abundance of *Trichoderma* in natural for coffee forest of Ethiopia is unknown.

The objective of this study was to survey the diversity of *Trichoderma* inhabiting the rhizosphere of *C. arabica*; and to compare two different coffee habitats, native populations and plantations in major coffee growing regions of Ethiopia with respect to their abundance and distribution of *Trichoderma* species.

2. Results

2.1. Habitats of C. arabica and the Sampling Strategy

C. arabica is a relatively small evergreen shrub (normally up to 5 m tall), which grows in shady places in subtropical forests. It usually inhabits the highland areas around 1500 m above the mean sea

level, but can be found up to 2800 m. *C. arabica* can grow best on deep, free-draining, loamy soils, with a good water holding capacity and a slightly acid soil.

The four major coffee growing regions of Ethiopia lie in the south, south-western and south-eastern parts of the country corresponding to Wellega, Jimma, Hararghe and SNNP (Southern Nations, Nationalities and Peoples) regions, respectively (Figure 1). The samples were taken during the rainy season of 2006 (August) on the basis of a road survey. More than 160 soil samples were collected along the main roads every several hundred meters in a way that (with the exception of Hararghe) both undisturbed native forests and disturbed semi-forests were equally represented. The latter ecosystem is common in Ethiopia. It is formed by coffee growers, when they intentionally thin out the forest in a way that the coffee plants get more sunlight but at the same time still have enough of large trees to provide adequate shade. In addition in semi-forests farmers also regularly slash the weeds to facilitate harvesting of the coffee beans. In extreme cases the artificial semi-forest may form after the primary burning of the native forest and subsequent introduction of fast growing large trees, which provide shade to coffee shrubs. The native forest is not disturbed by any anthropogenic pressure. Neither coffee plantations nor gardens were sampled.

As wild coffee inhabits a specific ecological niche it is not surprising that the soils sampled from the rhizosphere of *C. arabica* are similar in their physicochemical properties (Table 1). The representative samples from four studied regions were similar in carbon (approximately 2.3% in average) and nitrogen (approximately 0.3%) content. The pH of soil solutions was around 5 reaching a maximum of 6.5 in samples from the native forest in Hararghe.

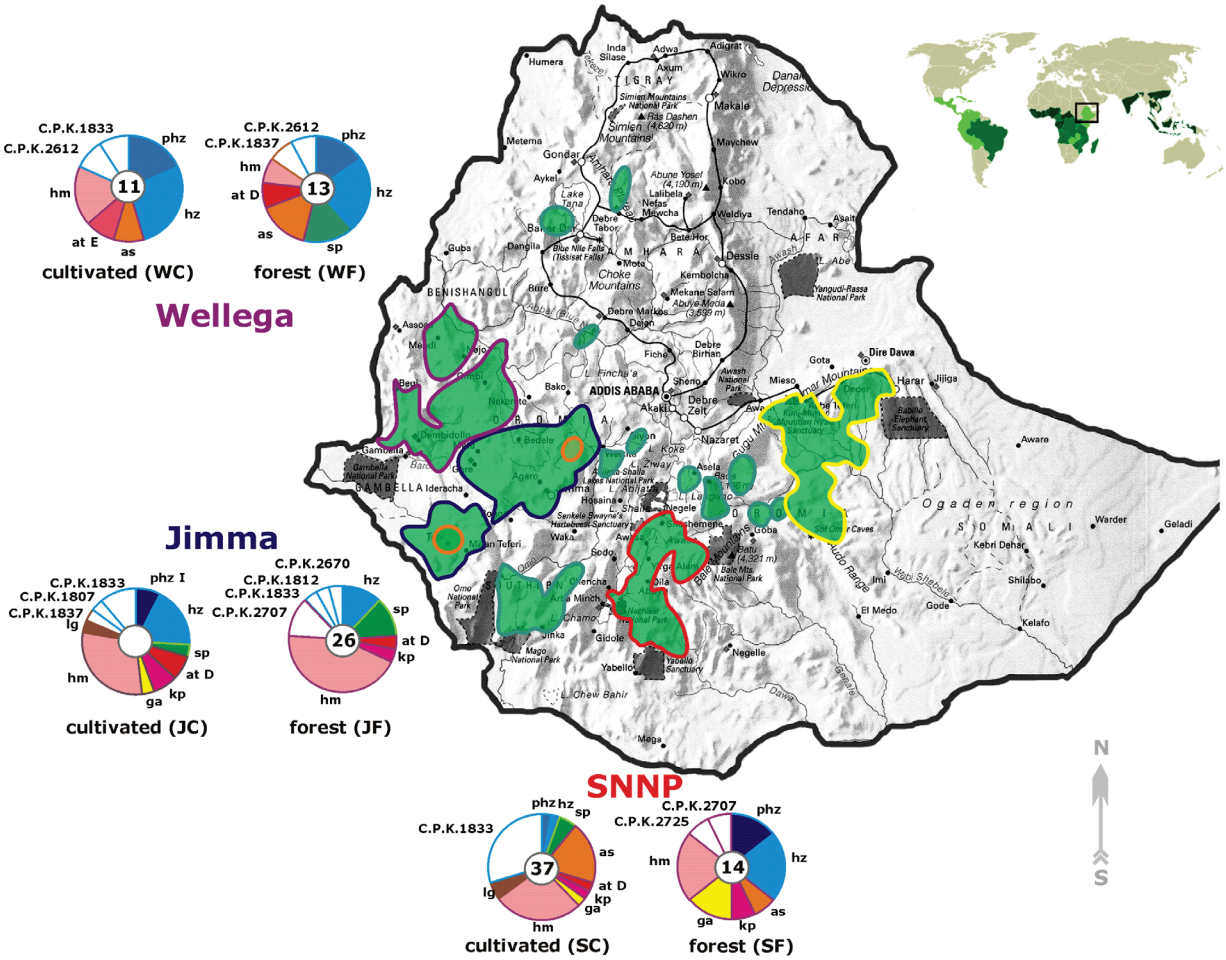
Table 1. Description of sampled areas and physical and chemical properties of soils.

Region	Ecosystem	Code	Alt. range (m)	Soil properties					No. of samples	No. of *Trichoderma* isolates
				Color	Texture	C%	N%	pH		
Jimma	native forest	JF	1320–2150	Reddish brown	Sandy loam	2.7	0.27	4.80	30	26
	semi-forest	JC	1370–2400	Reddish brown	Silt loam	2.7	0.6	4.72	29	27
Wellega	native forest	WF	1500–2300	Reddish brown	Silt loam	2.4	0.34	4.97	15	13
	semi-forest	WC	1570–2400	Reddish brown	Sandy loam	1.6	0.24	4.67	53	11
SNNP	native forest	SF	1650–2050	Reddish brown	Sandy loam	2.3	0.26	6.01	13	14
	semi-forest	SC	1670–2080	Reddish brown	Silt loam	2.3	0.32	4.81	12	37
Hararghe	native forest	HF	2150	Reddish brown	Sandy loam	2.5	0.23	6.53	2	2
	semi-forest	HC	1580–2350	Reddish brown	Sandy loam	2	0.19	4.01	6	4

2.2. Occurrence of Known Trichoderma Species

There were 134 isolates of *Trichoderma* recovered from the four major coffee growing regions of Ethiopia (Appendix 1; Figure 1). Among them, 54 came from the rhizosphere of coffee plants in forest soil, whereas 80 were obtained from *C. arabica* rhizosphere from semi-forests. Among these, 11 and 15 of the native (forest) and disturbed (semi-forest) soil samples respectively, were from plants infected with wilt disease (the presence of the disease was not visible at the time of sampling but later discovered in the laboratory).

Figure 1. Location of the main coffee growing areas (green areas) and the corresponding diversity of *Hypocrea/Trichoderma* in the rhizosphere of *C. arabica*. The purple line corresponds to Wellega, blue to Jimma, red for the SNNP region and yellow for the Hararghe region. The orange circles indicate the main commercial coffee plantation sites in Ethiopia (Jimma). The diversity of *Hypocrea/Trichoderma* is shown by separate pie plots (disturbed semi-forest and native forest, respectively) for all coffee growing areas except Hararghe, which was undersampled. The number in the center of each plot indicates the total number of *Trichoderma* strains isolated from each area. Species are abbreviated as *T. harzianum* sensu stricto (hz), 'pseudoharzianum matrix' (phz), *T. hamatum* (hm), *Hypocrea atroviridis* clade E (at E), *H. atroviridis* clade D (at D), *T. asperelloides* (as), *T. spirale* (sp), *T. longibrachiatum* (lg), *T. gamsii* (ga), and *H. koningiopsis* (kp), all potentially new species are numbered as "*T.* sp." followed by the corresponding strain number. The world map inset on the upper right side of the image shows the location of Ethiopia in the "coffee belt" where the light green indicates *C. arabica* production areas, and the dark green shows *C. robusta*.



The ITS1 and 2 oligonucleotide barcode program *TrichO*Key [24] identified 104 of the 134 isolates at the species level. Eight known species were found: *T. harzianum* sensu lato and *T. hamatum* were most abundant (30 and 35 isolates respectively), followed by *T. asperelloides* (11 isolates), *T. spirale*

(eight isolates), *H. atroviridis*/*T. atroviride* (six isolates) and *H. koningiopsis/T. koningiopsis* (five isolates). *T. gamsii* and *T. longibrachiatum*, were represented by four and three specimens respectively. While ITS1 and 2 sequences are sufficient for distinguishing the majority of *Hypocrea/Trichoderma* species [24], they have only limited power to differentiate at the infraspecific level. As all identified species are very common and known to be cosmopolitan, it is interesting to learn whether the isolates from Ethiopia represent also common haplotypes of these species, which are already known from other geographic regions. In order to learn this, we sequenced the hypervariable 4th long intron of the elongation factor 1-alpha (*tef1)* of the corresponding strains. Comparison of the resulting sequences with those of the Database of Industrially Important Fungi of Vienna University of Technology and the NCBI GenBank databases showed that the *tef1* sequences of *T. longibrachiatum*, *H. koningiopsis/T. koningiopsis* and *T. gamsii* strains were identical to those of strains known from Europe or South America (data not shown). Isolates of *T. harzianum* sensu lato also covered a large part of the genetic diversity known for this complex taxon, although most strains were identical or highly similar to strains from Africa (Cameroon, Egypt). The majority of strains belonged to the network of recombining holomorphic strains known as the 'pseudoharzianum matrix' [25], while at least four isolates were attributed to the new putative agamospecies *T.* sp. 'afroharzianum' [25] and a single isolate C.P.K. 1818 resembled the *ex*-type strain of *T. harzianum* CBS 226.95 (*i.e.*, *T. harzianum* sensu stricto), which is usually found in a temperate climate [26].

Eleven isolates were initially identified as *T. asperellum*. However this species has been recently reconsidered as an aggregate of two morphological cryptic species *T. asperellum* s. s. and *T. asperelloides* [27]. Phylogenetic analysis of *tef1* intron and the detection of the diagnostic SNP of ITS2 of the rRNA gene cluster resulted in the attribution of Ethiopian isolates to *T. asperelloides* (Figure 2), which is frequent in Africa.

A different situation was encountered with the other three species: strains of *T. hamatum* exhibited three *tef1* alleles, which formed a clade separate from all other known *T. hamatum* strains in the phylogenetic analysis (data not shown). Most of the strains identified as *H. atroviridis/T. atroviride* clustered in *H. atroviridis* clade D [28], forming a terminal branch shared by an isolate from Nepal (C.P.K. 300). One isolate (C.P.K. 2622), although belonging to the *H. atroviridis/T. atroviride* clade, formed a basal single branch, which cannot be attributed to any clades specified Dodd *et al*. [28] (Figure 2).

A high, as yet unnoticed genetic diversity was found for *T. spirale*: a phylogenetic tree of *tef1* sequences resulted in the formation of several statistically supported clades with the *ex*-type strain CBS 346.93 forming a separate lineage (Figure 3). Six of the eight Ethiopian *T. spirale* isolates formed a so far unique terminal clade remote from the *ex*-type strain, whereas one (C.P.K. 2606) was located within a closely related subclade and one formed a single lineage which occupies an unresolved position on the *T. spirale* s. l. cladogram.

Figure 2. Identification phylogram of *Trichoderma* diversity from rhizosphere of *C. arabica* in a vicinity *of H. atroviridis/T. atroviride* and *T. asperellum* inferred from a Bayesian analysis of *tef1* intron alignments. The position of the potentially new species is shown in red, while hypothetically endemic populations of known species are marked by blue. Nodes with black circles indicate posterior probabilities >0.94. All reference strains are given by the accession number of the corresponding *tef1* sequence in GenBank and/or strain number. Infraspecific groups and species names are given above branches leading to the corresponding node.

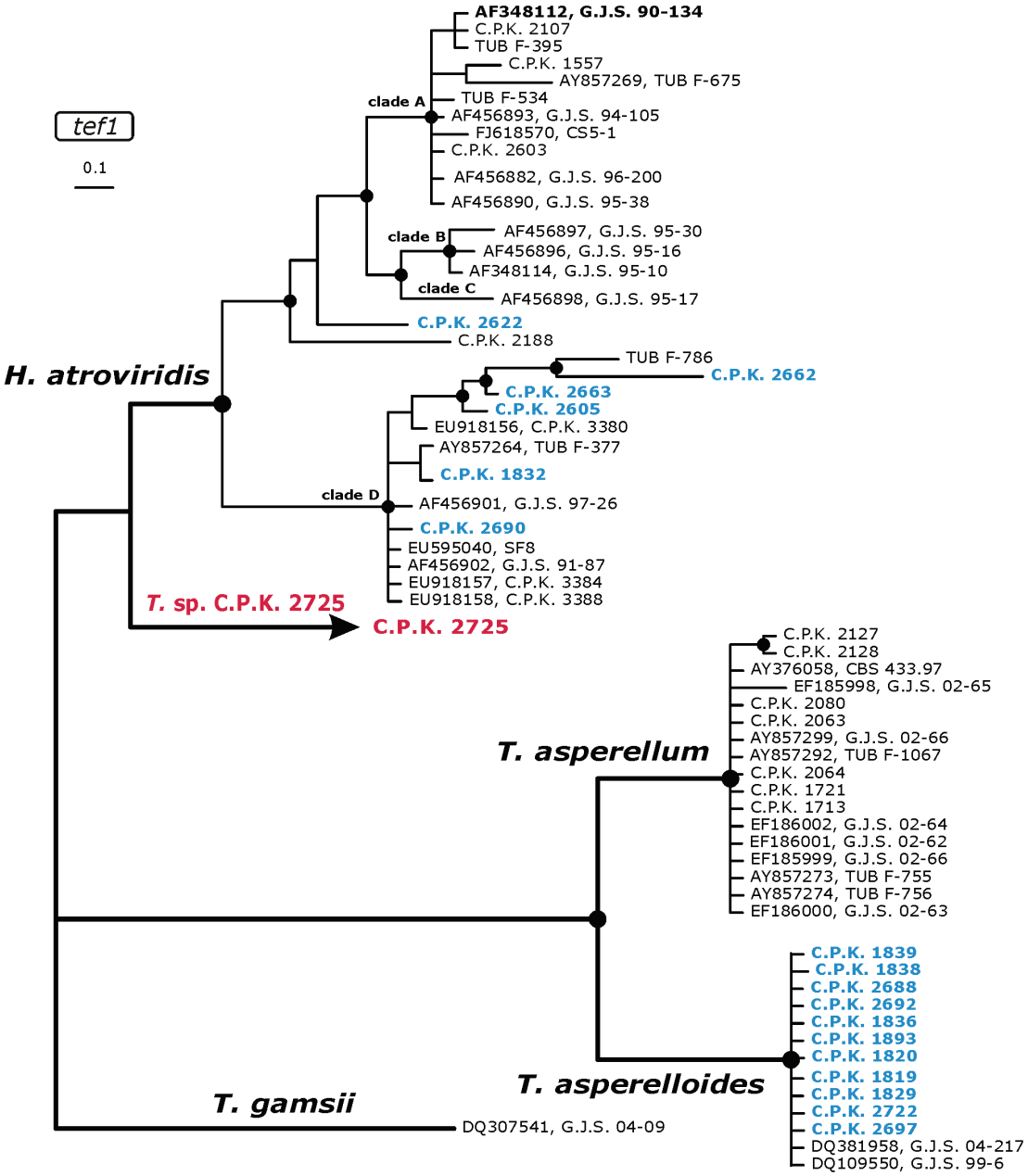
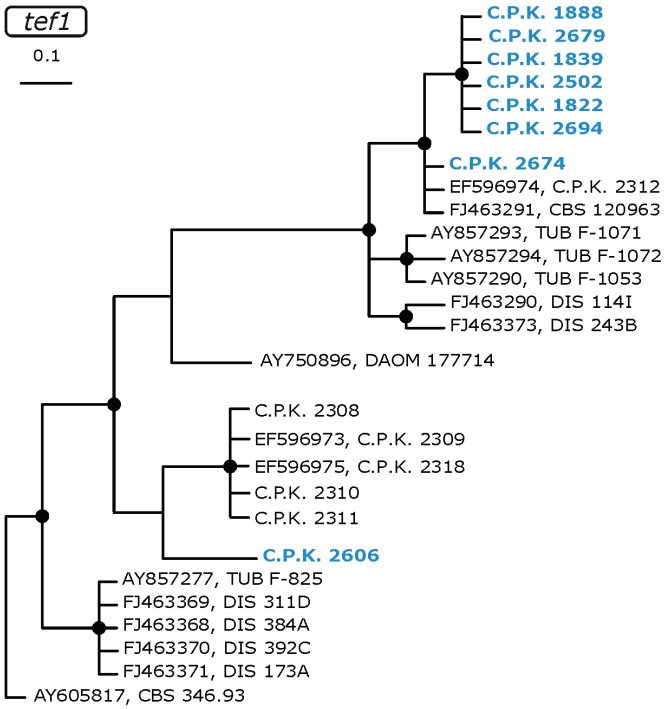


Figure 3. Identification phylogram of Ethiopian *tef1* alleles (in blue) of *T. spirale* based on a Bayesian analysis. Nodes marked by black circles indicate posterior probabilities >0.94. All reference strains are given by the accession number of the corresponding *tef1* sequence in GenBank and the strain number.

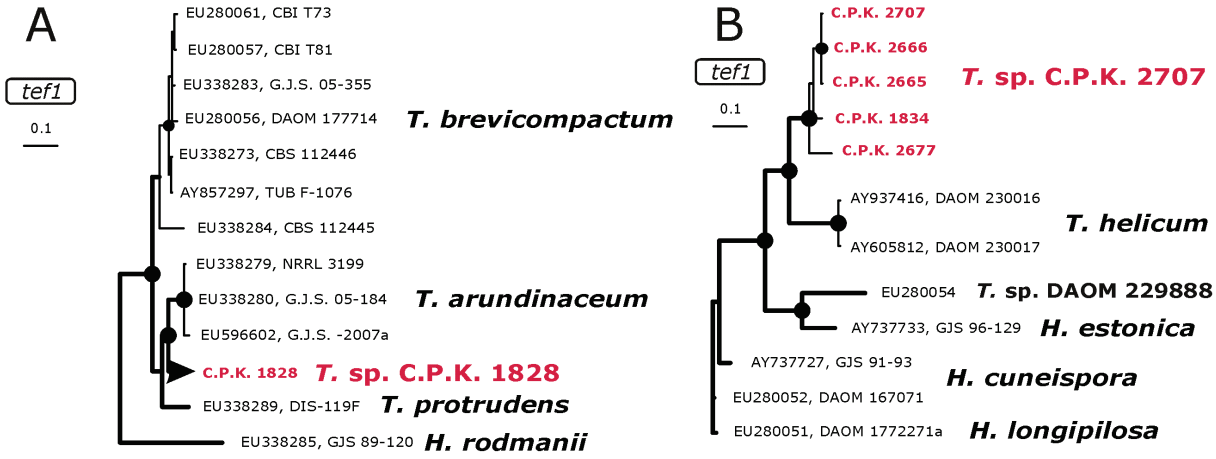


2.3. Occurrence of Potentially Endemic Trichoderma *Taxa*

Thirty (22.6%) of the isolates were identified as eight new alleles of ITS1 and 2 by *TrichO*Key and thus may constitute new, undescribed species. One of them (*T.* sp. C.P.K. 1833) accounted for the majority of isolates (16), whereas five of the others putative taxa were encountered only as single specimens. Five, three and two isolates were found for *T.* sp. C.P.K. 2707, *T.* sp. C.P.K. 1837 [29] and *T.* sp. C.P.K. 2612, respectively. *Trichoderma* sp. C.P.K. 1837 was recently shown to be a new phylogenetic species closely related to *H. orientalis* and *T. longibrachiatum* [29] of *Trichoderma* section *Longibrachiatum*. For the other putative new species hallmark sequences suggested that they are new members of *Trichoderma* section *Pachybasium* and section *Trichoderma*.

In order to test the hypothesis that these are new phylogenetic species, we also sequenced the *tef1* fragment from these 30 isolates and used them for an analysis together with sequences of the most closely related reference species. Figure 4A shows that *T.* sp. C.P.K. 1828 is a member of the Brevicompactum clade [30], forming a separate branch between *T. arundinaceum* and *H. rodmanii*, and the four isolates of *T.* sp. C.P.K. 2707 form a sister branch to *T. helicum* (Figure 4B).

Figure 4. Identification phylogram of a potentially new species within the Brevicompactum clade (a) and a potentially new species related to *T. helicum* (b) based on the Bayesian analysis of *tef1* intron. Nodes marked by black circles indicate posterior probabilities > 0.94. All reference strains are given by the accession number of the corresponding *tef1* sequence in GenBank and strain number.



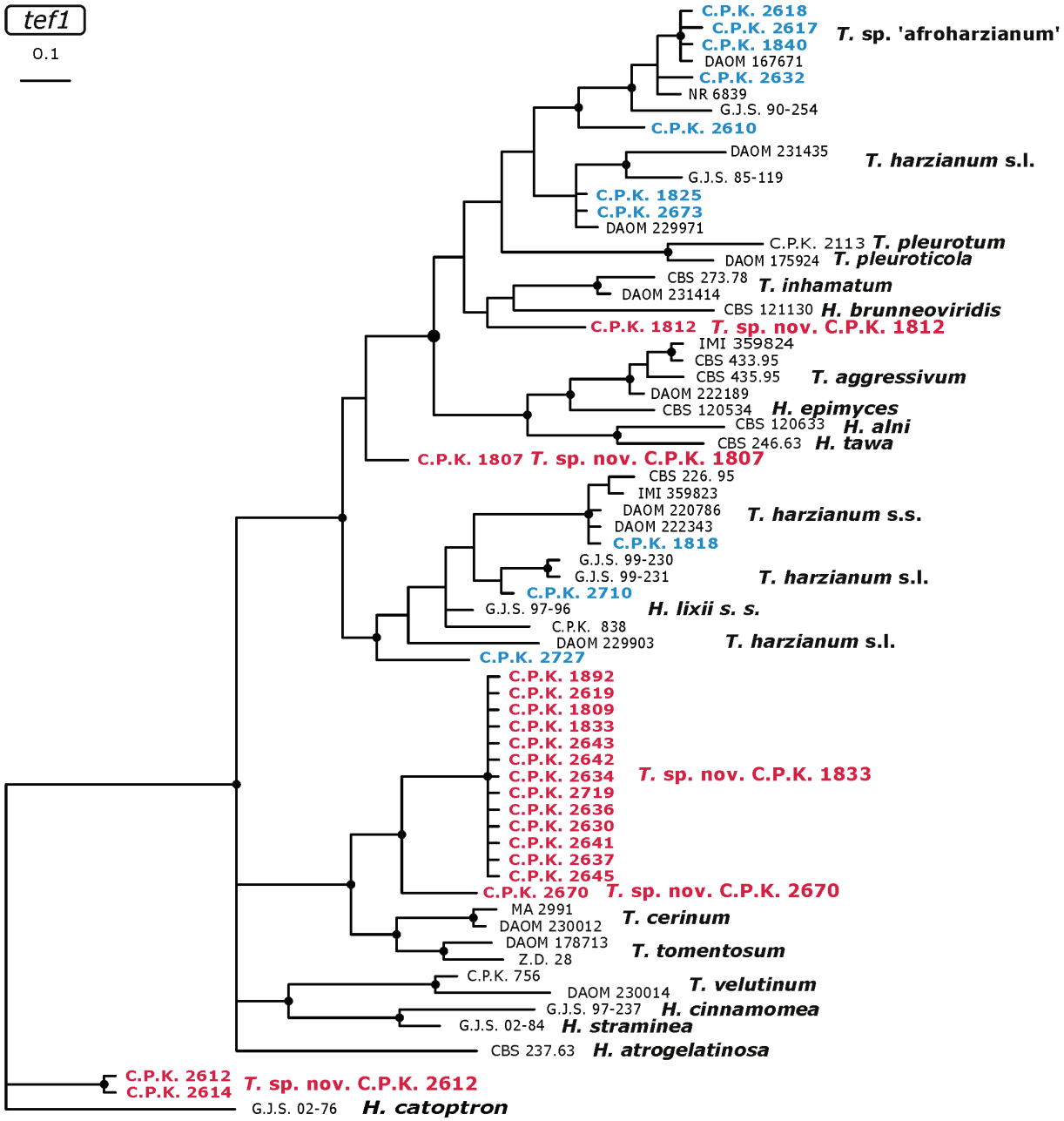
The remaining five putative new species were located within the Harzianum clade (Figure 5): one branch leading to the most abundant *T.* sp. C.P.K. 1833 and the single lineage corresponding to *T.* sp. C.P.K. 2670 formed a sister clade to the *T. cerinum–T. tomentosum* species pair. The two isolates of *T.* sp. C.P.K. 2612 formed an isolated branch basal to all other species. And the remaining two hypothetical new species—*T.* sp. C.P.K. 1812 and C.P.K. 1807 were located on basal branches to the clades containing e.g., *Trichoderma* species causing green mould disease of *Pleurotus* (*T. pleuroticola* and *T. pleurotum)* and *Agaricus* (*T. aggressivum)* respectively (Figure 5). The single putative new species of section *Trichoderma* (*T.* sp. C.P.K. 2725) formed a basal branch to *H. atroviridis* as shown in Figure 2.

2.4. Habitat Preference of Trichoderma

In order to assess the richness of *Trichoderma* in the rhizosphere of *C. arabica*, we calculated Shannon's biodiversity index H and evenness E, as well as the Simpson's biodiversity index (Table 2). Simpson's index and the evenness index were close to 1, indicating very high diversity and lack of dominance of any species. When the indexes are calculated separately for cultivated and uncultivated areas it becomes apparent that the diversity and evenness is equal in both ecosystems.

The high diversity was also reflected in the distribution of *Trichoderma* species. With the exception of species which were only obtained as single isolates, all other species were encountered from more than one area indicating that they may be evenly distributed within the coffee rhizosphere. Yet some remarkable exceptions could be seen: *T.* sp. C.P.K. 1833 was mostly isolated from semi-forest soils and *T.* sp. C.P.K. 2707 was only observed in the native forest. We also note that *T. hamatum* was particularly abundant in Jimma province, both in semi-forest as well as native forest rhizosphere (Figure 1).

Figure 5. Distribution of Ethiopian *Trichoderma* strains within Harzianum clade as inferred from a Bayesian analysis of *tef1* intron showing the position of potentially new species (red) and hypothetically endemic populations (blue). Nodes marked by black circles indicate posterior probabilities >0.94. GenBank accession numbers of the corresponding *tef1* sequence of the reference strains are given in [25].



In order to learn whether there would be any ecological preference of individual species, we performed a correlation analysis of the occurrence of strains with habitat specific properties such as altitude, pH, and the carbon and nitrogen content of the soils. Undisturbed forest soils in SNNP and Hararghe areas had a higher pH values (6.0–6.5) compared to other locations. Nevertheless, results from these analyses showed that there was no correlation between occurrence of any *Trichoderma* species and any of factors monitored in this study. Only exception was the observation that

cosmopolitan species which were also known from other geographic regions and continents appeared as better adapted to grow on soils poor in nitrogen and carbon. For example, occurrence of *T. asperelloides* was significantly higher on such soils (ANOVA, $P < 0.05$).

Table 2. Diversity of *Trichoderma* in major Ethiopian coffee growing regions.

			Ecosystem		Sampled regions			
Index		Average	Forest	Semi-forest	Wellega	Jimma	SNNP	Hararghe
Shannon diversity	H	2.47	2.34	2.29	2.14	2.19	2.15	1.56
Shannon evenness	E_H'	0.81	0.83	0.83	0.93	0.79	0.84	0.97
Simpson	D	0.88	0.87	0.87	0.9	0.84	0.86	0.93

2.5. Antagonistic Activity of Trichoderma Isolates against Gibberella xylarioides

In order to test whether the isolates of this study would be applicable as agents of biological control against plant pathogenic fungi (biocontrol agents), we tested a subset of randomly selected strains against an indigenous strain of *G. xylarioides* causing the coffee wilt in Ethiopia. Table 3 shows that all 10 isolates of *Trichoderma* tested were in fact able to inhibit the growth of *G. xylarioides in vitro* (PIRG between 55% and 76%). The hypothetically new taxon *T* sp. C.P.K. 2612 showed the highest antagonistic activity in this test. Interestingly, three strains (*T.* sp. C.P.K. 1817, *H. atroviridis/T. atroviride* C.P.K. 2622, and *T.* sp. C.P.K. 1834) also produced a zone of inhibition indicative of the formation of secondary metabolite(s) inhibiting *G. xylarioides*.

3. Discussion

Rhizosphere competence—*i.e.*, the ability to competitively colonize plant roots—has often been emphasized as a prerequisite for strains of genus *Trichoderma* to act as biocontrol agents (cf. [14]). However, to the best of our knowledge, the issue whether *Trichoderma* spp. prefer the rhizosphere as an ecological niche in soil has never been systematically addressed. In this study, we show that the rhizosphere of a *C. arabica* exhibits a notably high diversity of *Trichoderma* taxa. In fact, a correlation analysis failed to detect any other factor which appeared to influence species richness and distribution, and we thus conclude that the ecological-physiological constraint put by *C. arabica* ecological niche is the main parameter determining the presence of *Trichoderma*.

The major coffee growing regions of Ethiopia constitute one of the 34 biodiversity hotspot areas of the world [31]. In total they comprise 80% of the landmass of the Afromontane biodiversity hotspot that is inhabited by more than 700 plant species. The rhizosphere of *C. arabica* exhibited one of the highest richness of *Trichoderma* species detected so far (cf. [32-34]). The only study which may be comparable in sampling size relative to the studied area was performed in neotropical forests of South America, mainly in Colombia [34]. In that study the exclusively high diversity of *Trichoderma* (29 species detected among 183 isolates) was also accompanied by a high fraction of putatively new species (11 species corresponding to 6% of the sample). However the study of Hoyos-Carvajal *et al*. [34] included strains isolated from a great diversity of ecological niches and substrata and therefore the observed species richness likely reflects the overall microbial biodiversity

potential for neotropical regions. The principal difference of our findings is that we studied a limited and well defined microecological niche of rhizosphere of *C. arabica*. In another investigation of *Trichoderma* diversity based on a cultivation-dependent approach on Sardinia [32], around 500 *Trichoderma* strains have been isolated from different non-rhizosphere soil samples. The Shannon index calculated for all samples from the rhizosphere of *C. arabica* in Ethiopia is nearly two times higher compared to the same statistics calculated for soils in Sardinia (2.47 *versus* 1.59 respectively), although that sample was three times larger as compared to the present study. As Sardinian ecosystems were found to be seriously disturbed by various human activities and while in Ethiopia we sampled the largely undisturbed areas, our current results demonstrate that *Trichoderma* diversity may be used as an ecological indicator of 'soil health'.

Table 3. Ability of selected *Trichoderma* strains isolated from rhizosphere of *C. arabica* to inhibit *G. xylarioides.*

Isolate No.	Species	PIRG[1]	Clear Zone
C.P.K. 2612	*T.* sp. C.P.K.2612	76a	-
C.P.K. 2614	*T.* sp. C.P.K.2612	72b	-
C.P.K. 1808	*T.* sp. C.P.K.1833	64c	-
C.P.K. 2619	*T.* sp. C.P.K.1833	62c	-
C.P.K. 1817	*T.* sp. C.P.K.1837	62c	1.5
C.P.K. 2698	*T. hamatum*	60c	-
C.P.K. 2622	*H. atroviride*	56d	2
C.P.K. 1819	*T. asperelloides*	56d	-
C.P.K. 1888	*T. spirale*	55d	-
C.P.K. 1834	*T.* sp. C.P.K.2707	55d	1.5
F. xylarioides (Control)		0	-

[1] See the explanations in the Experimental Section.

The samples isolated from the rhizosphere of coffee plants also contain more of putatively new taxa than any other previous study (28% of all isolates; eight known species *versus* nine potentially new taxa; compare with Migheli *et al*. [32], Zachow *et al*. [33] Hoyos-Carvajala [34] or Kullnig *et al*. [35]). No new species have been detected among 500 isolates from soils in Sardinia. Although our identification of new taxa was based only on ITS1 and 2 sequence analysis and *tef1* phylogeny, and this claim still needs to be verified by analysis of additional loci, we consider this procedure reliable. In all our previous studies, prediction of new taxa by a combination of ITS1 and 2 and *tef1* polymorphisms has later on always been confirmed by multiloci phylogeny (e.g., [35,36]).

One of these potentially new species (*T.* sp. C.P.K. 1833) was remarkable as it was the third most frequent taxon sampled in this study (16 isolates), while at the same time mainly being recovered from *C. arabica* rhizosphere growing in semi-forests. Comparison with our database revealed that strains of this putative species have been isolated previously from soil in Siberia ([35], erroneously identified as *T. oblongisporum*), Guatemala [24], Slovakia and Kenya (unpublished data), and thus it is likely cosmopolitan. Phylogenetically, this species appears to be closely related to *T. tomentosum* and *T. cerinum*. The fact that it was so abundant in Ethiopia and that its phylogenetic clade was even

accompanied by a basal branch to a further potentially new taxon (*T*. sp. C.P.K. 2670) may suggest that *T*. sp. C.P.K. 1833 has an East African origin. Its high antagonistic ability renders it a potential candidate for biological control trials, because it has been isolated from disturbed forests.

The diversity of known species was dominated by *T. harzianum*, *T. hamatum*, *T. spirale* and *T. asperelloides*. All of these species are regarded as the most frequent representatives of the genus in soil and known to be cosmopolitan. They all have been also abundantly detected in Sardinian soils. We therefore consider them as ecological opportunists, which have very efficient abilities in conidial dispersal and germination. Their presence could thus be interpreted to be due to invasion. However, *T. spirale* is more frequently isolated from tropical environments [24,34-36], and also *T. asperelloides* has been reported to be abundant in South America and Cameroon [27,37]. In addition, most strains of *T. harzianum* sensu lato, for instance, *T.* sp. 'afroharzianum' [25] also belonged to *tef1* phylogenetic clades that were either rich in tropical isolates or being exclusively African.

In this study, we also tested the hypothesis that *Trichoderma* taxa hypothetically endemic to the rhizosphere of *C. arabica* would exhibit antagonistic activity against the coffee pathogen *G. xylarioides*. Although we randomly selected only a small subsample of our isolates for these tests and made only *in vitro* dual confrontation assays, the results performed verify this hypothesis. Moreover, *in planta* experiments, performed with different varieties of *C. arabica*, gave similar results (T. B. Mulaw, unpublished data). The fact, that the putative new taxa were particularly efficient antagonists, supports the above hypothesis for enrichment of these taxa in the coffee rhizosphere. Selecting biocontrol strains by looking for isolates associated with the respective plant may therefore be a method best suited for ecosystems with a largely preserved microbial flora.

4. Experimental Section

4.1. Soil Sampling and Characterization

At each sampling site, a visually healthy adult *C. arabica* plant was located and a sample was taken within its rhizosphere area. The top layer of a soil litter and the upper soil horizon (4–6 cm) was discarded, and 100 g of soil from approximately 10 cm depth was collected, placed in polyethylene bags and labeled. Afterwards, similar samples from one subarea (geographic region, type of growth of *C. arabica*, latitude and tracheomycosis state of the forest) were merged in several larger samples, which were subsequently sieved and dried on sterile paper for 2–3 days. Thereafter, they were stored at 4 °C until isolation of *Trichoderma* strains occurred.

The soil color was defined using a standard color scale for soil science (Munsell Soil Color Charts, U.S. Dept. of Agriculture). 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 after shaking for one hour pH was determined with a glass electrode. The total nitrogen content was determined according to the Kjeldahl method [38] on a Vapodest 30 (Gerhardt, Germany). The total organic carbon content was measured using the Liechtenfelder method [38], which oxidizes carbon with potassium dichromate, and quantifies the generated Cr^{3+} photometrically (DIN 19684).

4.2. Strain Isolation and Purification

10 g of soil samples (if necessary pulverized by means of a mortar and pestle, and passed through a 0.5 mm soil screen mesh to remove large debris and root fragments; [39]) were suspended in 90 mL sterile distilled water and thoroughly mixed. A 10 mL aliquot was then used to prepare a series of dilutions in the range of 10^{-1} to 10^{-3}, and inoculated on to potato dextrose agar (PDA), malt extract agar (MEA) and synthetic low nutrient agar (SNA) [40], each supplemented with streptomycin (50 mg/L) to prevent bacterial growth. Three replicates were done for each medium and soil sample. Plates were incubated at 25 °C for a period of 10 days, and examined daily for colony development. Putative *Trichoderma* colonies, indicated by their extensive fluffy white mycelium and distinctively green sporulation, were collected, and used to prepare single spore cultures. They were maintained in 50% (w/v) glycerol at −80 °C in the Collection of Industrially Important Microorganisms of Vienna University of Technology, Austria.

4.3. DNA Extraction, PCR Amplification and Sequencing

Genomic DNA was extracted from mycelia grown on MEA with the Plant DNeasy Minikit (QIAgen GmbH, Hilden, Germany) according to the manufacturer's instructions. A region of nuclear DNA containing the ITS1 and two regions of the rRNA gene cluster, was amplified by PCR with the primer combinations SR6R and LR1 [40], and an approximately 1-kb portion of the gene encoding translation elongation factor 1-alpha (*tef1*) was amplified and sequenced using the primers EF1 (5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and EF2 (GGA(G/A)GTACCA GT(G/C)ATCATGTT-3) as described by Jaklitsch *et al*. [40]. PCR products were purified with the QIAquick PCR Purification Kit (QIAgen) and were subjected to automated sequencing at MWG (Martinsried, Germany). The sequences obtained in this study have been deposited at GenBank; their accession numbers are given in Appendix 1.

4.4. Identification of Trichoderma

For species identification, ITS 1 and 2 sequences were subjected to analysis by *TrichO*Key (http://www.isth.info/tools/molkey/index.php; [24]). In ambiguous cases, the result was re-checked by sequence analysis of the large intron of *tef1* using a sequence similarity search against a database of type sequences implemented in *Tricho*BLAST (www.isth.info/tools/blast, [41]). For analysis of unusual ITS1 and 2 or *tef1* alleles, sequences were automatically aligned with ClustalX [42] and visually checked using Genedoc 2.6.002 [43]. Potentially unique alleles were then confirmed by BLAST analysis against NCBI GenBank, and the database of Collection of Industrially Important Microorganisms of Vienna University of Technology, Austria which currently contains more than 3500 *Hypocrea/Trichoderma* strains with more than 5800 sequences of phylogenetic marker loci.

4.5. Molecular Phylogenetic Analysis

A multiple sequence alignment file was formatted with PAUP*4.0b10, and manually adapted for the MrBayes v 3.0B4 program [44-46]. The model of evolution and prior settings for individual loci were used as estimated for different taxa of *Hypocrea/Trichoderma* [47]. Metropolis-coupled Markov

chain Monte Carlo (MCMCMC) sampling was performed with four incrementally heated chains that were simultaneously run for 1,000,000 and 3,000,000 generations. To check for potentially poor mixing of MCMCMC, each analysis was repeated several times. The convergence of MCMCMC was monitored by examining the value of the marginal likelihood through generations. Convergence of substitution rate and rate heterogeneity model parameters were also checked. Bayesian posterior probabilities (PP) were obtained from the 50% majority rule consensus of trees sampled every 100 generations after removing the 500 first trees.

4.6. Antagonism of Trichoderma Isolates against Gibberella xylarioides

Ten different isolates of *Trichoderma* were individually tested for their antagonistic property against *G. xylarioides* using the dual culture technique. Agar pieces of 6 mm diameter of *G. xylarioides* and *Trichoderma* isolates were taken from pure cultures 7 days old, and placed on plates of potato dextrose agar (3%, Merck, Germany) in a distance 6 cm between *G. xylarioides* and the *Trichoderma strain*. Plates were incubated at room temperature for 5 days. Five plates were prepared for each isolate. Plates inoculated with *G. xylarioides* alone served as control. Clear zone of inhibition (CZI) was also determined by measuring the clearance between the colony margins of the *G. xylarioides* and *Trichoderma*. Radial growth of both *G. xylarioides* and *Trichoderma* were measured after 5 days after inoculation.

The percentage of inhibition of radial growth (PIRG) was calculated as ([R1-R2]x100)/R1, where R1 is the colony diameter of the pathogen in the control and R2 is the diameter of the pathogen during antagonistic interaction. Antagonism was assessed in semi-quantitative means [48]: >75 PIRG indicating very high antagonistic activity, 61–75 PIRG indicating high antagonistic activity, 51–61 PIRG, indicating moderate antagonistic activity, <50 PIRG, indicating low antagonistic activity, and 0 indicating no activity.

The data were analyzed using SPSS version 17.0 software.

4.7. Statistical Analysis

The Shannon biodiversity index H was used to evaluate the species diversity, which appears as the product of evenness E and the number of species [49]. It measures the likelihood that the next individual will be the same species as the previous sample. Given a sample size with many (more than 5) species, a value near 0 would indicate that every species in the sample is the same, whereas a value near or 4.6 would indicate that the number of individuals is evenly distributed between the 5 species. Dominance of individual species was also measured by Simpson's diversity index [50], using formula $D= 1 - \sum_i n_i (n_i - 1)_i / N(N - 1)$, where n_i represents specimens of a species and N represents the total number of species. This index reflects the probability that two individuals randomly selected from a sample will belong to different species.

5. Conclusions

Our data strongly support the speculation that the *C. arabica* rhizosphere in Ethiopia is a hotspot for speciation of several *Trichoderma* spp. The putative Ethiopian endemic nature of several of the new taxa and populations encountered in this study would be consistent with an allopatric speciation

scenario. Yet this usually occurs by geographic isolation [51], and although it is possible that the East African highland could present a barrier causing such isolation, the cosmopolitan nature of many of the taxa found in this study argues against this possibility. Therefore, we rather suppose that the association with rhizosphere of *C. arabica*, which is native for the region, considerably contributed to this speciation. *C. arabica* is known to display a high genetic diversity in Ethiopia [52] and Tanzania [53]. We consider it possible that this genetic diversity has given rise to new *Trichoderma* populations and taxa capable of establishing themselves in the rhizosphere of a genetically variable plant. It will thus be interesting to look for *Trichoderma* populations outside the rhizosphere of *C. arabica* or/and to compare rhizosphere samples from Tanzania, as well as in areas growing other coffee species (for instance, *C. robusta)* and/or genetically less variable cultivars of Arabica coffee such as Indonesia or Central America [54].

Given that this investigation is the first of its kind in coffee growing areas of Ethiopia, and that studies on the wild *C. arabica*-associated microbial community are generally lacking, there is high demand for further research in this field. It is also recommended that further studies be conducted to determine microbial communities using both culture-dependent and culture-independent techniques to reveal the true picture of *Trichoderma* diversity in such specific microbial communities.

Acknowledgements

This work was partly supported by the Austrian Science Fund grants FWF P-17859 to I.S.D and by OeAD 894/07 North-South Dialogue Program, doctorate scholarship to T.B. Mulaw. The authors are grateful to Monika Komon-Zelazowska (Vienna University of Technology, Austria) for her laboratory assistance and to Vera Terekhova (Moscow State University, Soil Science Faculty, Russia) for her help with the soil analyses.

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Appendix 1. Strains of *Trichoderma* isolated in this study and NCBI GenBank accession numbers of their sequences.

Region	Ecosystem	Alt. (m)	Species	No. of Isolates	Strain No. C.P.K.	GenBank accession No. ITS 1 and 2	*tef1*
Wellega	disturbed semi-forest	2500	*T.* sp. 'afroharzianum'	2	2618	FJ412028	FJ436158
		2400			2617	FJ412059	FJ436157
		2250	*T. harzianum* sensu lato	3	2620	FJ412029	
		2500			2616	FJ412026	
		2800			2615	FJ412025	
		1900	*T. asperelloides*	1	1839	FJ412074	FJ436141
		2800	*H. atroviridis/T. atroviride* clade E	1	2622	FJ412094	FJ436160
		1570	*T. hamatum*	2	1826	FJ411979	FJ436130
		2400			2613	FJ411986	
		2500	***T.* sp. C.P.K.2612**	1	2614	FJ412013	FJ436156
		2400	***T.* sp. C.P.K.1833**	1	2619	FJ412060	FJ436159
	native forest	1750	*T. harzianum* sensu lato	5	2610	FJ412024	FJ436154
		1900			1825	FJ412017	FJ436129
		1750		3	2611	FJ412027	
		1750			2608	FJ412023	
		1750			2609	FJ412032	
		1750	*T. spirale*	2	2606	FJ412078	FJ436153
		1750			2607	FJ412079	
		1560	*T. asperelloides*	2	1893	FJ412049	FJ436148
		1500			1838	FJ412047	FJ436140
		1950	*H. atroviridis/T. atroviride* clade D	1	2605	FJ412093	FJ436152
		2300	*T. hamatum*	1	1827	FJ411980	
		1750	***T.* sp. C.P.K.2612**	1	2612	FJ412011	FJ436155
		1740	***T.* sp. C.P.K. 1837**	1	1837	FJ412090	FJ436139
Hararghe	disturbed semi-forest	2300	*T.* sp. 'afroharzianum'	1	1840	FJ412018	FJ436142
		2100	*T. asperelloides*	1	1829	FJ412048	FJ436132
		2080	***T.* sp. C.P.K.1828**	1	1828	FJ412085	FJ436131

Appendix 1. *Cont.*

		1580	***T.* sp. C.P.K. 1837**	1	1841		FJ436143
	native forest	2150	*T. harzianum* sensu lato	2	2623	FJ412030	
		2150			2624	FJ412031	
Jimma	disturbed semi-forest	1950	*T. harzianum* sensu lato	1	2673	FJ412038	FJ436175
		2200	*T. harzianum* sensu stricto	1	1818	FJ412016	FJ436123
		1750	*T. harzianum* sensu lato	5	2664	FJ412034	
		1800			2506	FJ412020	
		1850			2507	FJ412021	
		1950			2672	FJ412037	
		1590			2684	FJ412036	
		1660	*T. spirale*	1	1888	FJ412075	FJ436144
		1650	*H. atroviridis/T. atroviride* clade D	2	2662	FJ412095	FJ436170
		1700			2663	FJ412096	FJ436171
		1360	*H. koningiopsis/T. koningiopsis*	2	1816	FJ412100	FJ436121
		1850	*T. gamsii*	1	2508	FJ412103	FJ436151
		1600	*T. hamatum*	8	2682	FJ411994	
		1500			2686	FJ411996	
		1500			2687	FJ411997	FJ436180
		2400			1810	FJ411977	FJ436116
		1770			1814	FJ411981	FJ436119
		1500			2685	FJ411995	FJ436179
		1680			1811	FJ411978	
		2000			2505	FJ411985	FJ436150
		1330	*H. koningiopsis/T. koningiopsis*	1	1831	FJ412091	FJ436133
		1950	*T. longibrachiatum*	1	1815	FJ412086	FJ436120
		2300	***T.* sp. C.P.K. 1837**	1	1817	FJ412089	FJ436122
		2150	***T.* sp. C.P.K.1807**	1	1807	FJ412014	FJ436113
		2250	***T.* sp. C.P.K.1833**	3	1808	FJ412055	FJ436114
		2300			1809	FJ412056	FJ436115

Appendix 1. *Cont.*

		1800			2671	FJ412069	
	native forest	2100	*T. harzianum* sensu lato	3	2510	FJ412022	
		1750			2504	FJ412019	
		1700			2668	FJ412035	
		1670	*T. spirale*	3	2674	FJ412071	FJ436176
		1880			2679	FJ412072	FJ436178
		1800			2502	FJ412076	FJ436149
		1800	*H. atroviridis/T. atroviride* clade D	1	1832	FJ412092	FJ436134
		1950	*H. koningiopsis/T. koningiopsis*	1	1813	FJ412099	FJ436118
		1750	*T. hamatum*	11	2499	FJ411982	
		1880			2678	FJ411991	
		1900			2680	FJ411992	
		1780			2675	FJ411989	
		1600			2500	FJ411983	
		1700			2501	FJ411984	
		1800			2667	FJ411987	
		1640			2669	FJ411988	
		1780			2676	FJ411990	
		1900			2681	FJ411993	
		1950			1835	FJ436137	
		1960	***T.* sp. C.P.K.1833**	1	1833	FJ412058	FJ436135
		2150	***T.* sp. C.P.K.1812**	1	1812	FJ412015	FJ436117
		1720	***T.* sp. C.P.K.2670**	1	2670	FJ412107	FJ436174
		1830	***T.* sp. C.P.K.2707**	3	2677	FJ412083	FJ436177
		1600			2666	FJ412082	FJ436173
		1800			1834	FJ412080	FJ436136
SNNP	disturbed semi-forest	2070	*T.* sp. 'afroharzianum'	1	2632	FJ412033	FJ436162
		2000	*T. harzianum* sensu lato	1	2718	FJ412039	
		1750	*T. spirale*	2	2694	FJ412077	FJ436184

Appendix 1. *Cont.*

1670			1822	FJ412073	FJ436127
1670	*T. asperelloides*	7	1836	FJ412046	FJ436138
1750			2692	FJ412051	FJ436183
2000			2722	FJ412053	FJ436191
1750			2697	FJ412052	FJ436185
1750			1819	FJ412044	FJ436124
1700			1820	FJ412045	FJ436125
1750			2688	FJ412050	FJ436181
1750	*H. atroviridis/T. atroviride* clade D	1	2690	FJ412097	FJ436182
1780	*H. koningiopsis/T. koningiopsis*	1	1823	FJ412102	FJ436128
2000	*T. gamsii*	1	2723	FJ412106	FJ436192
1750	*T. hamatum*	10	2689	FJ411998	
1750			2700	FJ412004	
1750			2703	FJ412007	
1750			2691	FJ411999	
1750			2695	FJ412000	
1750			2696	FJ412001	
1750			2698	FJ412002	
1750			2699	FJ412003	
1750			2702	FJ412005	
1750			2704	FJ412006	
1800	*T. longibrachiatum*	2	1889	FJ412087	FJ436145
1770			1890	FJ412088	FJ436146
1690	***T.* sp. C.P.K.1833**	11	2645	FJ412068	FJ436169
2000			2719	FJ412070	FJ436190
2200			2634	FJ412063	FJ436163
1750			1892	FJ412057	FJ436147
1850			2626	FJ412061	
1970			2630	FJ412062	FJ436161

Appendix 1. *Cont.*

	2200			2636	FJ412064	FJ436164
	2260			2637	FJ412065	FJ436165
	1790			2641	FJ412066	FJ436166
	1690			2642	FJ412067	FJ436167
	1700			2643		FJ436168
native forest	1850	*T. harzianum* sensu lato	5	2710	FJ412040	FJ436189
	1900			2727	FJ412012	FJ436194
	1850			2712	FJ412042	
	1850			2711	FJ412041	
	1900			2728	FJ412043	
	2000	*T. asperelloides*	1	*2724*	FJ412054	
	1750	*H. koningiopsis/T. koningiopsis*	1	1821	FJ412101	FJ436126
	1650	*T. gamsii*	2	2706	FJ412104	FJ436186
	2000			2709	FJ412105	FJ436188
	1650	*T. hamatum*	3	2705	FJ412008	
	2050			2715	FJ412009	
	2050			2717	FJ412010	
	2050	***T.* sp. C.P.K. 2725**	1	2725	FJ412098	FJ436193
	1950	***T.* sp. C.P.K.2707**	1	2707	FJ412084	FJ436187

Bold font indicates potentially new species.