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# Species concepts and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species clusters?*

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**Abstract:** *Trichoderma*/*Hypocrea* is a genus of soil-borne or wood-decaying fungi containing members important to mankind as producers of industrial enzymes and biocontrol agents against plant pathogens, but also as opportunistic pathogens of immunocompromised humans. Species identification, while essential in view of the controversial properties of taxa of this genus, has been problematic by traditional methods. Here we will present a critical survey of the various identification methods in use. In addition, we will present an update on the taxonomy and phylogeny of the 88 taxa (which occur as 14 holomorphs, 49 teleomorphs and 25 anamorphs in nature) of *Trichoderma*/*Hypocrea* that have been confirmed by a combination of morphological, physiological and genetic approaches.

**Key words:** *Trichoderma*/*Hypocrea*, Genus, Molecular taxonomy-phylogeny
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## WHY IS *TRICHODERMA* IMPORTANT?

The anamorphic fungal genus *Trichoderma* (*Hypocreales*, *Ascomycota*) contains cosmopolitan soil-borne fungi frequently also found on decaying wood (Samuels, 1996; Klein and Eveleigh, 1998), of which some are economically important producers of industrial enzymes (*Trichoderma reesei=Hypocrea jecorina*) (Kubicek and Penttilä, 1998) and antibiotics (Sivasithamparam and Ghisalberti, 1998), or have application as biocontrol agents against plant pathogens (i.e. *T. harzianum=H. lixii*; *T. atroviride=H. atroviridis*; *T. asperellum*) (Hjeljord and Tronsmo, 1998). More recently, *T. longibrachiatum* has also become known as opportunistic pathogen of immunocompromised mammals including humans (Kredics *et al*., 2003). *Trichoderma* has been recognized to comprise a significant amount of fungal biomass in soil (Nelson, 1982; Widden and Abitbol, 1980) and is frequently present as an indoor contaminant (Thrane *et al*., 2001). These diverse implications of *Trichoderma*/*Hypocrea* with human society render an accurate species and strain identification to be an important issue. However, classical approaches based on the use of morphological criteria are, as in several other fungi, difficult to apply to *Trichoderma*, due to the plasticity of characters. As a consequence, we shall review the current state of perception of taxa and other taxonomic ranks in *Trichoderma* and *Hypocrea* in this paper.

## TOWARDS DEVELOPMENT OF A CONCEPT FOR THE GENUS *TRICHODERMA*

Although originally introduced by Persoon (1794), the taxonomy and identification of *Trichoderma* has remained problematic until relatively recently. Until 1969, nearly all strains of *Trichoderma* were identified in literature as "*T. viride*" (e.g. also all the cellulase-producing strains of *T. reesei*) owing to

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Bisby's (1939) concept that *Trichoderma* consists of a single species. This led to the erroneous statement even in textbooks that "*T. viride* is an industrial cellulose producer". As *T. viride sensu stricto* is a relatively rare species ([1]Kubicek, unpublished data; [2]Samuels, personal communications), most of the taxa sampled and determined before 1969 are probably misidentified. Rifai (1969) adopted the concept of 'aggregate' species, and distinguished nine 'species aggregates', and admitted that some of them (particularly *T. hamatum*) likely contain two or more morphologically indistinguishable species. Bissett (1984; 1991a; 1991b; 1991c; 1992; Gams and Bissett, 1998) revised Rifai's aggregate species by subtle recognition of continuities of morphological characters, expanded the morphological criteria to accommodate the wider range of morphological variation expressed by some anamorphs of *Hypocrea* and adopted some forms previously included in *Gliocladium*. While dissecting several of Rifai's species aggregates into several defined taxa, Bissett (1991a) also established a subdivision of the genus into five sections: *Longibrachiatum*, *Pachybasium*, *Trichoderma*, *Saturnisporum* and *Hypocreanum*.

The advent of molecular tools for investigations in fungal taxonomy prompted research in the mid-nineties to re-assess the morphology-based taxonomy in *Trichoderma*. The laboratories of G.J. Samuels (Beltsville, MD, USA), T. Börner (Berlin, FRG) and C.P. Kubicek (Vienna, Austria) collaboratively pioneered a revision of Bissett's section *Longibrachiatum*. They combined the use of molecular markers (ITS1 and ITS2 sequence analysis, RAPD), physiological (isoenzyme analysis) and phenetic characters, and also for the first time included an analysis of potential teleomorphs of the *Trichoderma* spp. from this section (Kuhls *et al*., 1996; 1997; Samuels, 1996; Samuels *et al*., 1998; Turner *et al*., 1997). As a result, section *Longibrachiatum* was recognized to be monophyletic and to contain ten taxa, within which four pairs displayed teleomorph-anamorph relationships: *H. schweinitzii*/*T. citrinoviride*; *H. pseudokoningii*/*T. pseudokoningii*; *H. jecorina*/*T. reesei* and *H. orientalis*/*T. longibrachiatum*, p/p. They further merged section *Saturnisporum*, which included only two species, *T. saturnisporum* and *T. ghanense* (Doi *et al*., 1987), with section *Longibrachiatum*, and recognized the synonymy of *T. ghanense* with *T. parceramosum*. Yet, as a whole, the concept of section *Longibrachiatum* as defined by Bissett (1984) was largely confirmed, suggesting a degree of correlation between morphological and molecular approaches to taxonomy in *Trichoderma*.

Section *Longibrachiatum* is a comparably small section of *Trichoderma*, and phylogenetically most distant from the other sections. The relationship between morphological characters and molecular phylogeny became more complex, however, when larger sections of *Trichoderma* were investigated. Kindermann *et al*.(1998) attempted a first phylogenetic analysis of the whole genus. Using sequence analysis of the ITS1 region of rDNA they found that the largest section, section *Pachybasium*, is actually paraphyletic. Although the use of a single gene fragment alone is insufficient by today's standards, this finding has been confirmed by phylogenetic analysis of several other genes (Kullnig-Gradinger *et al*., 2002; Chaverri *et al*., 2003b). In this context it is noteworthy that the nomenclatural type strain of sect. *Pachybasium*, *Pachybasium hamatum* (Bonord.)=*T. hamatum*, is not a member of the major one of the two *Pachybasium* clades (clade B) (Kindermann *et al*., 1998), but clusters together with *T. pubescens* and *T. strigosum* in a clade otherwise containing almost all taxa (i.e. with the exception of *T. aureoviride*) from section *Trichoderma*. Because of the lack of a distinctive morphological hiatus between clades A and B of *Pachybasium* (Kindermann *et al*., 1998), researchers have so far maintained the name *Pachybasium* for both clades, but it is clear that this is an unsatisfactory situation from a taxonomic point of view. *Pachybasium* B contains all taxa attributed to this section by Bissett (1991b) with the exception of the three mentioned above. In addition, *Pachybasium* B also poses the problem of strong genetic variability of several of its species (e.g. *T. harzianum*). Since its species (both ana- as well as teleomorphs) account for the majority of taxa found in field investigations (Kullnig *et al*., 2000; Kubicek *et al*., 2003; Wuczskowski *et al*., 2003; Gherbawy *et al*., 2004; Druzhinina *et al*., 2004b), this raises the question of how to identify species in this large heterogenous group.

[1]Kubicek, C.P., 2003;
[2]Samuels, G.J. 2003

## WHAT IS A SPECIES IN *TRICHODERMA*?

Most of the taxa of *Trichoderma* have so far been defined on the basis of morphology, and gene sequence analysis was only used as a confirmative or distinctive complement. Thus, members of the genus are primarily defined by the application of the MSR (morphological species recognition) concept. Since strains of *Trichoderma* spp. apparently cannot be crossed, application of the BSR (biological species recognition) concept is impracticable. The genealogical concordance phylogenetic species recognition (GCPSR) (Taylor *et al*., 2000) is an attractive alternative or complement to the morphological species concept, but has not been widely applied to *Trichoderma*/*Hypocrea*. It requires the analysis of trees of several unlinked genes, and implies that the phylogenetic position of a true species will be concordant in at least some of them, and not be contradicted in the others. Kullnig-Gradinger *et al.*(2002) used four different loci (ITS1 and ITS2, mitssuDNA, short fifth *tef*1$\alpha$ intron, a fragment of *ech*42 large exon) to assess a global phylogeny of the genus. However, a stringent clade to clade concordance was not possible for most of the species because of insufficient phylogenetic resolution by the genes used. Chaverri *et al.*(2003a) analyzed ITS1 and ITS2, large *tef*1 intron, and short fragments of the actin (*act*1) and calmodulin (*cal*1) exon sequences in *H. lixii*/*T. harzianum*, and identified seven phylogenetic lineages which were concordant in most trees. However, since the isolates within the seven lineages could not be reliably distinguished morphologically, they were not given recognition as a species.

Taylor *et al.*(1999) proposed basing phylogenetic species concepts on the concordance between five or more gene trees. This requirement is not easily fulfilled in *Trichoderma*. In the past, most researchers made heavy use of ITS1 and/or ITS2 (Kuhls *et al*., 1997; Kindermann *et al*., 1998; Lieckfeldt *et al*., 1998; 2001; Dodd *et al*., 2000), because this gene cluster is present in >90 copies per genome and can thus be easily amplified. However, the use of ITS1 and ITS2 has meanwhile become discredited by the fact that some fungi, notably some sections in the closely related *Fusarium*, and plants have been shown to contain paralogous copies of the parts of the rDNA gene cluster (O'Donnell, 1992; Buckler *et al*., 1997; O'Donnell *et al*., 1998; Lieckfeldt and Seifert, 2000). Also, Chaverri *et al*.(2003b) reported unpublished data for the presence of divergent ITS1 and ITS2 sequences in *T. strictipile* and *T. crassum*. In contrast, we have so far not obtained evidence for the presence of paralogous ITS1 or ITS2 copies in most species of *Trichoderma*/*Hypocrea*, even though we specifically tested for it ([1]Mach *et al*., unpublished data). On the other hand, a serious drawback of the use of ITS1 and ITS2 is that it provides only poor phylogenetic resolution in some clades, particularly *Pachybasium* B (Kullnig-Gradinger *et al.*, 2002; Chaverri *et al*., 2003a). Among 11 tested loci/fragments (Table 1) the most promising loci seem to be different fragments of translation elongation factor 1-alpha (EF-1α=*tef*1) different fragments of which were sequenced by different research groups (Fig.1). The gene has been cloned from *H. jecorina* and exceeds 2 kb in length, and consisted of several relatively large and variable introns and exons. Also, the coding portions of endochitinase 42 (*ech*42) and RNA-polymerase subunit 2 (*rpb*2) have displayed significant intra- and interspecific variability while other genes, such as the D1 and D2 regions of the 28S rDNA, or the small subunit of the mitochondrial ribosomal DNA (ssu-mDNA) have been used with limited success. Fragments of the calmodulin- and the actin-encoding genes (*cal*1, *act*1) had also been used in *T. harzianum*/*H. lixii*, but exhibited less powerful resolution than the large exon of *tef*1 (Table 1) (Chaverri *et al*., 2003a). It is important to mention that two different unalignable intron fragments of *act*1 are available in GenBank (Table 1).

Notwithstanding the fact that both regions exhibit an extremely weak phylogenetic signal it is important to reach an agreement between workers regarding which fragment of this gene should be sequenced. We have also attempted to use histon 3A and β-tubulin gene sequences, which have proven worthwhile for phylogenetic analysis in *Fusarium* and other genera, but while the variation in the histon 3A gene was not high, several *Trichoderma* spp. contained multiple heterologous copies of the *tub*1 gene, thus rendering both genes not applicable for this purpose ([2]Kullnig-Gradinger and Kubicek, unpublished data). Unfortunately, none of these genes is

[1]Mach, R.L., *et al.*, 1999

[2]Kullnig-Gradinger, C.M., Kubicek, C.P., 2004

**Table 1 Gene sequences used in molecular phylogeny of *Trichoderma*/*Hypocrea***

| Locus | Fragment | | Length (kb) | *N* | *Var.* (%)* | Reference |
|---|---|---|---|---|---|---|
| Internal transcribed spacer 1 and 2 | ITS1 and ITS2 | | 0.4 | 50 | 25 | Kullnig-Gradinger *et al.*, 2002 |
| RNA coding genes | | | | | | |
| Small subunit of the mitochondrial ribosomal DNA | ssu-mDNA | | 0.4 | 50 | 5 | Kullnig-Gradinger *et al.*, 2002 |
| 28S rDNA gene | 28S rDNA | | 0.5 | 51 | 8 | Kullnig-Gradinger *et al.*, 2002 |
| Protein coding genes | | | | | | |
| Calmodulin | *cal*1 | Intron | 0.45 | 35** | 15 | Chaverri *et al.*, 2003a |
| Actin | *act*1 | 1st intron | 0.35 | 35** | 5 | Chaverri *et al.*, 2003a |
| | | 2nd intron | 0.8 | 18** | 6 | [1]Samuels and Ismael, personal communication |
| RNA polymerase B subunit 2 | *rpb*2 | | 0.4 | 97 | 40 | Chaverri *et al.*, 2004 |
| Endochitinase 42 | *ech*42 | Last large exon | 0.6 | 44*** | 33 | Kullnig-Gradinger *et al*., 2002 |
| Translation elongation factor 1-alpha | *tef*1 | 5th (small) intron | 0.1 | 47 | 30 | Kullnig-Gradinger *et al.*, 2002 |
| | | 4th (large) intron | 0.35 | 125** | 29 | [2]Druzhinina, unpublished data |
| | | Last large exon | 0.7 | 84 | 29 | Chaverri *et al.*, 2004 |

* Portion of parsimoni informative sites from the length of fragment; ** Only tested for closely related taxa; *** Data for section *Longibrachiatum* are not available; *N* indicates number of species investigated

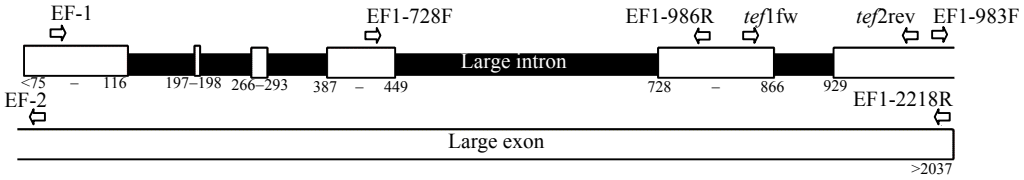


**Fig.1 A schematic structure of *tef*1 gene of *H. jecorina* (GeneBank accession number CAA80554), and location of primers used to amplify different parts of it for phylogenetic inferences**

optimal for phylogenetic resolution of the whole genus, or of large clades such as *Pachybasium* B: while the largest intron of *tef*1 provides excellent resolution and high clade support for closely related taxa such as the *T. harzianum*/*H. lixii* species clade (*H. lixii*, *T. harzianum*, *T. aggressivum*, *T. tomentosum*, *T. cerinum*, *T. velutinum*, *H. tawa*) or the group of *H. rufa* (*T. viride*, *T. atroviride*, *T. koningii*). However, the large introns in *tef*1 are less suited for resolving the phylogenetic elations of more distantly related species due to ambiguous alignments ([2]Druzhinina, unpublished data). On the other hand, the last large exon of *tef*1 contains only limited phylogenetic signals for analysis of diverse clades, and thus e.g. in a combined analysis of *Pachybasium* A and B resulted in lack of support for almost all basal branches, whereas the terminal branches had good support (Chaverri *et al*., 2003b; 2004). The same problem was even more apparent with *rpb*2. Therefore, the optimal combination of genes allowing the application of the GCPSR concept on the whole genus *Trichoderma* has not yet been applied. Detailed analysis of various loci available in GenBank (Table 1) may suggested that the simultaneous usage of (i) *tef*1 large intron and last large exon, (ii) *rpb*2 gene, (iii) *ech*42 last large exon and (iv) ITS1 and 2 as diagnostic regions may lead to the most reliable phylogeny. However a search for new phylogenetic markers is strongly recommended.

A further important point in the use of the GCPSR concept is the justified use of the correct phylogenetic approach, so far, most workers employed the maximum parsimony method to analyze sequence data, which does not employ any modelling

[1] Samuels, G.J., Ismael, E., 2004
[1] Druzhinina, I., 2004

of evolution and therefore poses several problems when dealing with either very closely or very distantly related taxa (Salemi and Vandamme, 2003). Maximum likelihood methods would be adequate, but suffer from the long computation time they usually require. For this purpose, the Bayesian approach to phylogenetic inferences represents the most recent advance in phylogenetic analysis (Rannala and Yang, 1996; Huelsenbeck and Roquist, 2001; Lutzoni *et al*., 2001). It shifts statistical interference away from an emphasis on hypothesis testing (*P*-values) and finding the single optimal tree, toward obtaining adequate estimates of uncertainty. Uncertainty is characterized through the use of the posterior distribution of a parameter, which is defined as the conditional probability of observing a particular parameter value given in the data. This involves the use of Markov chain Monte Carlo (MCMC) methods (Metropolis *et al*., 1953), which implements the simulation of a random walk through parameter space, which, if run long enough, will eventually converge on the stationary distribution of parameters (Lewis, 2001). Bayesian approaches have been introduced in the analysis of phylogenies of other genera, and had also been applied to *Trichoderma*/*Hypocrea* (Chaverri *et al*., 2003b; Druzhinina *et al*., 2004a). When combined with rigorous testing of evolutionary models, this approach yields excellent resolution even for difficult to resolve clades in *Trichoderma* ([1]Druzhinina *et al*., manuscript in preparation).

## HOW CAN SPECIES OF *TRICHODERMA* BE IDENTIFIED?

While this review so far treated the concepts and advances in the recognition and definition of species of *Trichoderma*, nothing has been said about how an unknown isolate can be identified as one of these species. Morphological analysis is highly prone to error, and consequently roughly 50% of the *Trichoderma* spp. deposited in culture collections under names obtained by morphological analysis alone are wrong ([2]Kubicek, unpublished data)! Obviously, subjecting every new strain to a thorough GCPSR analysis would result in a safe identification, but most workers who study the occurrence of *Trichoderma* or *Hypocrea* in soil or other habitats will not invest such a massive use of money and time. In fact, several researchers are still mainly using morphological methods for identification of *Trichoderma*, although the use of ITS1 and ITS2 sequence analysis is becoming more and more popular. This is not in contradiction to the statement above that ITS1 and ITS2 do not provide sufficient phylogenetic resolution, because phylogeny and diagnosis underlie two different principles, i.e. even though the change in 2–3 nts may not provide sufficient phylogenetic information, it may be indicative of a given species if it is known that this species always contains these three nucleotides in the respective positions. Hence the intra- and interspecific variability must be fully known in order to confirm or refute species identity. Unfortunately, the preferred process used by some workers is to submit sequences to a BLAST, and then accept the best hit as species identity. This must be criticized for several reasons: first, the GenBank databases contain many sequences of *Trichoderma* isolates which had been incorrectly identified and thus occur under a false name (Table 2); second, many researchers without solid bioinformatic training are inexperienced with the principles of BLAST and take an *E*-value of 0.00 as identity without scrutinizing whether the sequence is identitical or only highly similar. Even if it is identical, it is imperative to know that no other species with the same sequence exists.

As a solution to this problem, we have recently developed a DNA-barcode system for quick identification on the basis of defined nucleotide sequence differences in the ITS1 and ITS2 region (Druzhinina *et al*., 2004b). The method relies on the discriminatory power of ITS1 and ITS2, which could be shown to be capable of identifying 70 of a total of 77 investigated species of *Trichoderma* and *Hypocrea*. Some taxa could not be distinguished because of ITS1 and ITS2 sequence identity: *T. crassum* and *T. longipile*, which however, once delimitated by the barcode, can then easily be distinguished on the basis of morphology (Bissett, 1991b; Chaverri *et al.*, 2003b); and *T. tomentosum* and *T. cerinum*. Although, the latter two are also difficult to distinguish morphologically,

[1]Druzhinina, I., Bissett, J., Kubicek, C.P., 2004. Bayesian inferences towards the phylogenetic species recognition of biocontrol fungi *Hypocrea lixii*/*Trichoderma* harzianum and closely related species. Manuscript in preparation

[2]Kubicek, C.P., 2000

**Table 2 Example of a BLAST search with an ITS1 and ITS2 sequence from *Trichoderma** which yielded ambiguous results**

| Sequences producing significant alignments: | | Score (bits) | *E*-value |
|---|---|---|---|
| gi\|19880152\|gb\|AF362109.1\| | *Trichoderma aureoviride* strain T... | 1132 | 0.0 |
| gi\|19880083\|gb\|AF359399.1\| | *Trichoderma aureoviride* strain T... | 1116 | 0.0 |
| gi\|32394933\|gb\|AY154947.1\| | *Trichoderma aggressivum* Ir. 560 ... | 1112 | 0.0 |
| gi\|19880067\|gb\|AF359267.1\| | *Trichoderma aureoviride* strain T... | 1108 | 0.0 |
| gi\|19032416\|gb\|AF345948.1\| | *Trichoderma harzianum* isolate GJ... | 1100 | 0.0 |
| gi\|19880081\|gb\|AF359397.1\| | *Trichoderma aureoviride* strain T... | 1100 | 0.0 |
| gi\|21239369\|gb\|AF501330.1\| | *Trichoderma aggressivum* f. europ... | 1096 | 0.0 |
| gi\|3095175\|gb\|AF055216.1\|AF055216 | *Trichoderma harzianum* str... | 1082 | 0.0 |
| gi\|27448757\|gb\|AF443912.1\| | *Trichoderma harzianum* G.J.S. 00−... | 1045 | 0.0 |
| gi\|9230639\|gb\|AF194019.1\|AF194019 | *Trichoderma aureoviride* s... | 1045 | 0.0 |
| gi\|9230631\|gb\|AF194011.1\|AF194011 | *Trichoderma harzianum* str... | 1045 | 0.0 |
| gi\|9230630\|gb\|AF194010.1\|AF194010 | *Trichoderma aureoviride* s... | 1045 | 0.0 |
| gi\|27448762\|gb\|AF443917.1\| | *Hypocrea lixii* G.J.S. 91-138 int... | 1041 | 0.0 |
| gi\|27448761\|gb\|AF443916.1\| | *Hypocrea lixii* G.J.S. 94-53 inte... | 1041 | 0.0 |
| gi\|27448758\|gb\|AF443913.1\| | *Trichoderma harzianum* G.J.S. 00−... | 1041 | 0.0 |
| gi\|27448771\|gb\|AF443926.1\| | *Hypocrea lixii* G.J.S. 90-254 int... | 1039 | 0.0 |
| gi\|27448769\|gb\|AF443924.1\| | *Hypocrea lixii* G.J.S. 92-110 int... | 1037 | 0.0 |
| gi\|27448764\|gb\|AF443919.1\| | *Hypocrea lixii* G.J.S. 92-100 int... | 1037 | 0.0 |
| gi\|27448760\|gb\|AF443915.1\| | *Hypocrea lixii* G.J.S. 90-22 inte... | 1037 | 0.0 |
| gi\|32394935\|gb\|AY154949.1\| | *Trichoderma harzianum* Ir. 112 C ... | 1033 | 0.0 |
| gi\|27448770\|gb\|AF443925.1\| | *Trichoderma harzianum* G.J.S. 92−... | 1033 | 0.0 |
| gi\|1813651\|gb\|U78881.1\|THU78881 | *Trichoderma harzianum* isola... | 1033 | 0.0 |
| gi\|32394941\|gb\|AY154955.1\| | *Trichoderma inhamatum* Ir. 286 18... | 1029 | 0.0 |

*Sequence AF 3362109 was used as query (*T. harzianum*, wrongly deposited as "*T. aureoviride*"), using BLASTN

phylogenetic analysis has shown that the latter two taxa are the result of a recent allopatric speciation, *T. cerinum* found only in Eurasia whereas *T. tomentosum* only in the Americas ([1]Druzhinina *et al.*, manscript in preparation). Therefore, knowledge of the geographic origin of an isolate with a *T. tomentosum*/*T. cerinum* sequence may be used to distinguish these two. Otherwise, the two must be distinguished by the aid of additional sequences or phenotype arrays (see below).

ITS1 and ITS2 sequence differences were also unable to consistently distinguish between three taxa from the "*H. rufa* species complex" namely *T. viride*, *T. koningii* and *T. atroviride*. This is not because no eventually appropriate nt-differences would be detected, but because the species concept of this clade is currently under revision and several additional new taxa will be defined among them ([2]Samuels, personal communication). Sequence analysis of the large *tef*1 intron has already been shown to distinguish groups within *T. viride* and *T. koningii*, and will probably be the method of choice to complement the ITS based DNA-barcode for identification of these taxa in future.

While we think that DNA-barcode will enable many researchers to reliably identify their *Trichoderma* strains, it is obvious that some researchers will not have access or financial capability to use DNA-based methods, and therefore need alternatives. Samuels and colleagues, advocating the morphologically and physiologically based methods, proposed an interactive key for strain identification in *Trichoderma*, which, besides subtile differences in morphology (http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm), makes use of differences in growth rates on PDA and SNA at 15, 20, 25, 30 and 35 °C (Chaverri *et al.*, 2003b). This method is inexpensive, but is time consuming and requires a sufficient number of repetitions ($n$>5) for each sample to be reliable, thus becoming laborious with more

[1]Druzhinina, I., Bissett, J., Kubicek, C.P., 2004. Bayesian inferences towards the phylogenetic species recognition of biocontrol fungi *Hypocrea lixii*/*Trichoderma harzianum* and closely related species. Manuscript in preparation

[2]Samuels, G.J., 2004

than 50 samples being considered as the lowest limit for any ecological investigation. [1]Bissett (unpublished) introduced a phenetic method, based on quantification of carbon assimilation patterns using BIOLOG MicroPlates™ (Biolog, Hayward, CA). Generally, phenotype arrays, commercialized by Biolog for identification of food and air-borne fungi, are not as suited for taxonomic purposes in *Trichoderma* because of the significant strain variation in some species (Kubicek *et al.*, 2003). However, we have recently included phenotype arrays into an 'integrated approach' when describing the new taxon *T. brevicompactum* (Kraus *et al.*, 2004). Phenotype array analysis of this new species and the phylogenetically closest neighbors method identified a number of carbon sources within the arrays, which were assimilated by *T. brevicompactum* at statistically different rates. This analysis is rapid and reliable, and even researchers for which phenotype arrays and/or the necessary reader are unavailable can use the published information to design simple agar plates with the respective carbon sources to verify an identification. We have recently used this system for distinguishing a number of species pairs which are morphologically very similar (e.g. *T. tomentosum* and *T. cerinum*; *T. harzianum* and *T. aggressivum*) ([2]Druzhinina and Bissett, unpublished data) and will continue to support by this method new species identified by GCPSR.

## WHAT DO THE CURRENT DATA TELL US ABOUT THE PHYLOGENETICS OF *TRICHODERMA*?

Notwithstanding the limitations and caveats outlined above, the use of molecular tools enabled the taxonomy of *Trichoderma* to advance strongly over the last years, and we will therefore attempt to summarize the outcome. So far, 88 taxa have been recently redefined by combination of molecular and phenetic tools (Table 3). Four taxa introduced by Chaverri *et al*.(2004) (Table 4) still lack phylogenetic analysis. Among the 88, 14 anamorph-teleomorph relationships have been demonstrated and thus represent holomorphs, 49 have been described in *Hypocrea*, while the remaining 25 were described as *Trichoderma*. In the latter, two cases, the other (sexual/asexual) form has not been found in nature. It is possible (but unlikely) that many of these *Hypocrea* species occur naturally only in their teleomorph state and that many of the hitherto described *Trichoderma* species may lack a teleomorph state due to clonal evolution.

Phylogenetic studies of these 88 species showed that *Trichoderma* and *Hypocrea* form a single holomorph genus, within which two major clades can be distinguished (Fig.2, Table 3): one, which contains all the taxa described in section *Longibrachiatum* (Samuels *et al*., 1998), *T. effusum* and *T. sinensis* (Bissett *et al.*, 2003), *Trichoderma* sp. MA 3239 (Wuczskowski *et al.*, 2003), *H. cerebriformis* and *H. poronoidea* ([3]Kubicek, unpublished data). *H. patella* forms a stable basal branch to the *Longibrachiatum* clade. At the moment it is unclear whether *H. peltata* should be included in section *Longibrachiatum* or forms a sister clade. For the sake of simplicity we include it in this section. The second clade forms several well supported subclades: one, leading to members of section *Trichoderma* including "*Pachybasium* A" (*T. viride*/*H. rufa*, *T. koningii*, *T. atroviride*, *T. ovalipsorum*, *H. neorufa*, *H. stilbohypoxyli*, *T. erinaceum*, *T. asperellum*, *T. hamatum*, *T. pubescens* and *T. strigosum*), and which also contains *H. pezizoides*, *H. avellanea* (Chaverri *et al*., 2003b), and *T. flavoconidia* (Druzhinina *et al.*, 2004a). The branch leading to this clade is frequently accompanied, but usually not strongly supported, by two sister clades: the *Pachybasioides* clade and the *Hypocreanum* clade. The former contains *H. pachybasioides*/*T. polysporum* (=*T. croceum*), *H. pilulifera*, *H. parapilulifera*, *H. stellata*, *H. minutispora*/*T. minutisporum* and *H. laciwombatensis* (Lu and Samuels, 2004), whereas the latter includes *H. citrina*, *H. lactea*, *H. sulphurea* and *H. pulvinata* (Chaverri *et al.*, 2004; [4]Overton, personal communication).

The other, large clade contains one weakly supported branch, but otherwise remains essentially unresolved by all genes or gene fragments used so far (Kullnig-Gradinger *et al.*, 2002; Chaverri *et al*., 2003a; 2003b; 2004). This weakly supported branch

[1]Bissett, J., 2004
[2]Druzhinina, I., Bissett, J., 2004
[3]Kubicek, C.P., 2003
[4]Overton, B., 2004

**Table 3 Current status of *Trichoderma* and *Hypocrea* taxa, and their attribution to phylogenetic sections and clades***

| Section | Clade[1] | Anamorph[2] | Teleomorph[2] | Reference |
|---|---|---|---|---|
| *Longibrachiatum* | | | | |
| | | *T. longibrachiatum* | *H. orientalis* | Samuels *et al*., 1998 |
| | | *T. citrinoviride* | *H. schweinitzii* | Samuels *et al*., 1998 |
| | | *T. reesei* | *H. jecorina* | Samuels *et al*., 1998 |
| | | *T. ghanense* | | Samuels *et al*., 1998 |
| | | *T. pseudokoningii* | *H. pseudokoningii* | Samuels *et al*., 1998 |
| | | *T. saturnisporum* | | Samuels *et al*., 1998 |
| | | *T. konilangbra* | | Samuels *et al*., 1998 |
| | | *T. effusum* | | Bissett *et al*., 2003 |
| | | *T. sinensis* | | Bissett *et al*., 2003 |
| | | *T.* sp. MA | | Wuczskowski *et al*., 2003 |
| | | | *H. andinensis* | Samuels *et al*., 1998 |
| | | | *H. novazelandia* | Samuels *et al*., 1998 |
| | | | *H. cerebriformis* | [3]Kubicek, unpublished data |
| | | | *H. poronoidea* | [3]Kubicek, unpublished data |
| | | | *H. peltata* | Dodd *et al*., 2002 |
| *Trichoderma* | | | | |
| | | | *H. pezizoides* | Chaverri *et al*., 2004 |
| | | | *H. avellanea* | Chaverri *et al*., 2004 |
| | *Rufa* | *T. viride* | *H. rufa* | Lieckfeldt *et al*., 1999 |
| | | *T. atroviride* | *H. atroviridis* | Dodd *et al*., 2003 |
| | | *T. koningii* | *H. koningii* | Lieckfeldt *et al*., 1998 |
| | | *T. strigosum* | | Kullnig-Gradinger *et al*., 2002 |
| | | *T. ovalisporum* | | Samuels, 2004 |
| | | *T. erinaceum* | | Bissett *et al*., 2003 |
| | | | *H. stilbohypoxyli* | Lu and Samuels, 2004 |
| | *Pachybasium* A | *T. hamatum* | | Kullnig-Gradinger *et al*., 2002 |
| | | *T. pubescens* | | Kullnig-Gradinger *et al*., 2002 |
| | | *T. asperellum* | | Kullnig-Gradinger *et al*., 2002 |
| | | | *H. neorufa* | Dodd *et al*., 2002 |
| | | | *H. flavoconidia* | Druzhinina *et al*., 2004a |
| *Pachybasium* B | | | | |
| | *Pachybasioides* | *T. polysporum* | *H. pachybasioides* | Lu *et al*., 2004 |
| | | *T. minutisporum* | *H. minutispora* | Lu *et al*., 2004 |
| | | *T. piluliferum* | *H. pilulifera* | Lu *et al*., 2004 |
| | | | *H. parapilulifera* | Lu *et al*., 2004 |
| | | | *H. stellata* | Lu *et al*., 2004 |
| | | | *H. laciwombatensis* | Lu *et al*., 2004 |
| | *Hypocreanum* | | *H. citrina* | Chaverri *et al*., 2004 |
| | | | *H. lactea* | Chaverri *et al*., 2004 |
| | | | *H. sulphurea* | Chaverri *et al*., 2004 |
| | | | *H. pulvinata* | Chaverri *et al*., 2004 |
| | *Chlorospora* | | *H. aureoviridis* | Chaverri *et al*., 2004 |
| | | | *H. candida* | Chaverri *et al*., 2004 |
| | | | *H. cremea* | Chaverri *et al*., 2004 |
| | | | *H. surrotunda* | Chaverri *et al*., 2004 |
| | | | *H. sinuosa* | Chaverri *et al*., 2004 |

(Continued in the next page)

| | | | |
|---|---|---|---|
| | | *H. chlorospora* | Chaverri *et al*., 2004 |
| | | *H. thelephoricola* | Chaverri *et al*., 2004 |
| | | *H. costaricensis* | Chaverri *et al*., 2004 |
| | | *H. thailandica* | Chaverri *et al*., 2004 |
| | | *H. virecentiflava* | Chaverri *et al*., 2004 |
| *Lixii/catoptron* | *T. harzianum* | *H. lixii* | Chaverri *et al*., 2004 |
| | *T. aggressivum* | | Samuels *et al*., 2002 |
| | *T. tomentosum* | | Chaverri *et al*., 2004 |
| | *T. cerinum* | | Bissett *et al*., 2003 |
| | *T. velutinum* | | Bissett *et al*., 2003 |
| | *T.* sp. DAOM 175928 | | [4]Druzhinina, unpublished data |
| | | *H. tawa* | [4]Druzhinina, unpublished data |
| | | *H. atrogelatinosa* | Chaverri *et al*., 2003b |
| | | *H. ceracea* | Chaverri *et al*., 2004 |
| | | *H. cinnamomea* | Chaverri *et al*., 2004 |
| | | *H. straminea* | Chaverri *et al*., 2004 |
| | | *H. catoptron* | Chaverri *et al*., 2004 |
| *Virens* | *T. virens* | *H. virens* | Kullnig-Gradinger *et al*., 2002 |
| | *T. crassum* | *H. crassa* | Chaverri *et al*., 2004 |
| *Semiorbis* | | *H. semiorbis* | Chaverri *et al*., 2004 |
| | | *H. hunua* | Kullnig-Gradinger *et al*., 2002 |
| | *T. fertile* | | Kullnig-Gradinger *et al*., 2002 |
| | *T. oblongisporum* | | Kullnig-Gradinger *et al*., 2002 |
| *Strictipilis* | *T. strictipilis* | *H. strictipilosa* | Chaverri *et al*., 2004 |
| | *T. longipile* | | Chaverri *et al*., 2004 |
| | | *H. cuneispora* | Chaverri *et al*., 2004 |
| | | *H. aureoviridis* var. *macrospora* | [4]Druzhinina, unpublished data |
| *Stromatica* | *T. stromaticum* | | Chaverri *et al*., 2004 |
| | *T. rossicum* | | Bissett *et al*., 2003 |
| | *T.* sp. PPRI 3559 | | Kullnig-Gradinger *et al*., 2002 |
| *Ceramica* | | *H. ceramica* | Chaverri *et al*., 2004 |
| | | *H. estonica* | Chaverri *et al*., 2004 |
| *Lutea* | | *H. lutea* | Chaverri *et al*., 2004 |
| | | *H. megalomagna* | Chaverri *et al*., 2004 |
| | *T. brevicompactum* | | Kraus *et al*., 2004 |
| *Psychrophila* | | *H. psychrophila* | Chaverri *et al*., 2004 |
| | | *H. megacitrina* | Chaverri *et al*. 2004 |
| "*Lone lineages*" | *T. spirale* | | Kullnig-Gradinger *et al*., 2002 |
| | *T. helicum* | | Bissett *et al*., 2003 |
| | | *H. gelatinosa* | Chaverri *et al*., 2004 |
| | | *H. chromosperma* | Chaverri *et al*., 2004 |
| | | *H. sulawensis* | Chaverri *et al*., 2004 |
| | | *H. nigrovirens* | Chaverri *et al*., 2004 |
| | | *H. phyllostachidis* | Chaverri *et al*., 2004 |

[*]Only taxa, which have been verified by molecular analyses are included in the table. The following species were not included, as they have recently been abandoned: *T. inhamatum* (=*T. harzianum*); *T. fasciculatum* (=*T. strictipilis*), *T. flavofuscum* (=*T. virens*) and *T. croceum* (=*T. polysporum*); [1]Clades follow the nomenclature of Chaverri *et al*.(2004) and define phylogenetic groups of species, which received high statistic support in all investigations performed so far; [2]Species names listed in the same line indicate anamorph-teleomorph relationships, and are only given for cases where both forms have been found in nature; [3]Kubicek, C.P., 2003; [4]Druzhinina, I., 2004

**Table 4 *Hypocrea* spp. with *Trichoderma* anamorphs, whose phylogenetic position is unknown**

| Taxon | Reference |
|---|---|
| *Hypocrea albocornea* | Doi, 1972 |
| *Hypocrea centristerilis* | Doi, 1972 |
| *Hypocrea clusiae* | Chaverri *et al*., 2004 |
| *Hypocrea cornea* | Chaverri *et al*., 2004 |

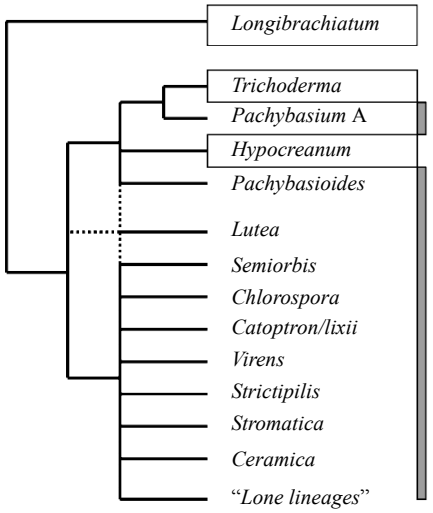


**Fig.2 Schematic representation of the phylogenetic relationships of the currently recognized sections and clades in the genus *Trichoderma*. Clades representing sections as defined by Bissett (1991a) are boxed. The two clades of *Pachybasium* (A and B) are given by a vertical grey bar. The dotted line indicates the ambiguous phylogenetic placement of the *H. lutea* clade. Note that this is not a phylogenetic tree, but only a scheme based on several different phylogenetic trees published by Kullnig-Gradinger *et al*.(2002) and Chaverri *et al*.(2004). Branches are only given if they were strongly supported in all trees obtained until now**

leads to *H. aureoviridis*/*T. aureoviride* and a group of *Hypocrea* spp. described recently (*H. aureoviridis* var. *macrospora*, *H. candida*, *H. cremea*, *H. surrotunda*, *H. sinuosa*, *H. chlorospora*, *H. thelephoricola*, *H. costaricensis*, *H. thailandica* and *H. virecentiflava*) with very similar morphological and genetic characters. Apart from this clade, several terminal subclades can be identifed which contain closely related species, but whose phylogenetic relationship remains unclear: *H. lixii*/*catoptron* (containing *H. lixii*/*T. harzianum*, *T. aggressivum*, *T. tomentosum*, *T. cerinum*, *T. velutinum*, *T.* sp. DAOM 172928, *H. tawa*, *H. atrogelatinosa*, *H. ceracea*, *H. cinnamomea*, *H. straminea* and *H. catoptron*); *H. virens* (*H. virens*/*T. virens*, *T. flavofuscum*, *T. crassum*); *H. semiorbis* (*H. semiorbis*, *T. oblongisporum*, *T. fertile*); *H. strictipilosa* (*H. strictipilosa*/*T. strictipile*, *H. aureoviridis* var. *macrospora*, *H. cuneispora*, *T. longipile*, *T. fasciculatum* (synonymized with *T. strictipile*); *H. ceramica* (*H. ceramica*, *H. estonica*); and *H. stromatica* (*H. stromatica*/*T. stromaticum*, *T. rossicum*, *T.* sp. PPRI 3559).

The presence of species of section *Hypocreanum* as a subclade in clade "*Pachybasium* B" deserves some comments: Samuels (1996) hypothesized that the anamorphs of species of *Hypocrea* with effused stromata having colourless conidia on irregularly verticillate conidiophores, which Bissett (1991a) placed in *Trichoderma* sect. *Hypocreanum* may be synanamorphs or spermatial states, and therefore inappropriately placed in *Trichoderma*. Because the anamorphs had not been found in nature, Gams and Bisset (1998) omitted sect. *Hypocreanum* from their treatment of *Trichoderma*. However, phylogenetic data proved that the respective taxa are clearly members of the genus. Kullnig-Gradinger *et al.*(2002) postulated that the ability to form the *Trichoderma*-like anamorph may have been lost during evolution of these species. A phylogeny based on 28S-rDNA sequence analysis showed that genera having *Verticillium*-like anamorphs (*Aphysiostroma*, *Podocrea*, *Arachnocrea*) occur in a stable basal position to the genus *Trichoderma* implying that *Trichoderma* evolved from them. We thus favour the interpretation that the ability to form the *Trichoderma*-like anamorph morphology, which may have supported the switch from fungicolous to saprophytic habitats, was subsequently lost in some evolutionary lines. Other *Hypocrea* species with *Verticillium*-anamorph morphology such as *H. tawa* or *H. hunua* occur nested in other clades (Table 1) (Kullnig-Gradinger *et al*., 2002), indicating that the loss of genes (or their expression) required form the *Trichoderma*-like anamorph occured several times during the evolution of the genus.

Phylogenetic analysis revealed that three other species (*H. lutea*/*H. melanomagna*/*T. brevicompactum*) also cluster together, but their association with any of the large clades is unclear and dependent on the genes and species used ([1]Kubicek, unpublished data).

[1]Kubicek, C.P., 2003

Besides these, there are still a number of species for which no close neighbor is known, and for which the phylogenetic position within *Pachybasium* B could so far not be identified: *H. psychrophila*/*H. megacitrina*, which are found in the vicinity of the *Hypocreanum* clade; *T. spirale*, which has phylogenetic affinity to *T. virens* and *T. harzianum*; *T. helcum*, which has a slight affinity to *T. spirale*; and *H. gelatinosa*, *H. chromosperma*, *H. sulawensis*, *H. nigrovirens* and *H. phyllostachidis*, which all occur as *Lone Lineages* of unresolved *Pachybasium* B.

## WHAT NEXT?

The current information, as summarized above, places *Trichoderma* among the fungal genera most thoroughly investigated taxonomically today, in view of the fact that at least two but on average more gene sequences are known for every recognized species. While the 88 species recently characterized by molecular methods are phylogenetically well supported, their evolution and phylogenetic relationship has so far been more difficult to resolve. This may either reflect the lack of known ancestors (as with most other fungi), or the occurrence of high selection pressure during the evolution of these clades, consequently leading to dichotomous trees. However, we must emphasize that these 88 species are still probably only a minor fraction of the existing number of taxa. The CABI online database of fungal names (http://www.indexfungorum.org) lists 401 *Hypocrea* spp., and while several of them may be redundant (i.e. synonyms of other described species) or not correctly placed in the genus (e.g. *H. pallida*), this may be compensated by others yet to be described. One should bear in mind that many of the currently known *Hypocrea* spp. had been isolated and described by Yoshimichi Doi (see Samuels, 1996) from sampling in Japan, the Western Pacific and South America. In our lab, Walter Jaklitsch has recently initiated a study on the biodiversity of *Hypocrea* spp. in Central Europe, and his preliminary data indicate the presence of at least 10–15 new undescribed taxa in the samples from the first 18 months. Given the comparatively small area investigated, and the fact that huge areas like Africa or Central Asia have not been studied at all, we expect that 400 will be even too low.

The number of *Trichoderma* spp. may remain lower, but also here a further rise can be anticipated. From our own study on the biodiversity of *Trichoderma*, at least 10 putative new 'phylogenetic spp.' are currently in the pipeline, and will be described in the near future. In addition, screening of so far neglected geographic areas will likely show new taxa (Kullnig *et al.*, 2000; Kubicek *et al.*, 2003; Wuczskowski *et al.*, 2003), and several such investigations have recently been completed or are being undertaken (e.g. New Zealand, S.L. Dodd and coworkers; Iran, D. Zafari; Sardinia, Q. Migheli and I. Druzhinina; China, T. Xu; Rwanda, J. Bissett and I. Druzhinina). Still, areas like most of the African continent and the Pacific have not been investigated.

As emphasized above, most of the phylogenetic analyses are incomplete, due to the limited suitability of the gene sequences used so far (Table 1). To improve this situation, novel gene sequences are needed. Researchers need to be aware that there is no single "universal, all-purpose" gene for phylogenetic analysis of this genus, and the suitability of a given gene should always be tested first before applying it to a given phylogenetic problem.

Finally, a safe species concept may also aid in identification and safe comparison of biochemical and physiological properties of *Trichoderma* strains used in biocontrol. While these strains have been uniformly been called "*T. harzianum*" in the past, leading to the situation that the name *T. harzianum* is synonymized with biocontrol agent, there is now increasing evidence that actually several, genetically diverse species are used in biocontrol (Hermosa *et al*., 2000; 2004; Kullnig *et al*., 2001). The species identification tools now in hand will help to answer the question whether particular taxa are to be preferred on particular hosts or plants.

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