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In honor of John Bissett: authoritative guidelines on molecular identification of *Trichoderma*

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Abstract

Modern taxonomy has developed towards the establishment of global authoritative lists of species that assume the standardized principles of species recognition, at least in a given taxonomic group. However, in fungi, species delimitation is frequently subjective because it depends on the choice of a species concept and the criteria selected by a taxonomist. Contrary to it, identification of fungal species is expected to be accurate and precise because it should predict the properties that are required for applications or that are relevant in pathology. The industrial and plant-beneficial fungi from the genus *Trichoderma* (Hypocreales) offer a suitable model to address this collision between species delimitation and species identification. A few decades ago, *Trichoderma* diversity was limited to a few dozen species. The introduction of molecular evolutionary methods resulted in the exponential expansion of *Trichoderma* taxonomy, with up to 50 new species recognized per year. Here, we have reviewed the genus-wide taxonomy of *Trichoderma* and compiled a complete inventory of all *Trichoderma* species and DNA barcoding material deposited in public databases (the inventory is available at the website of the International Subcommission on Taxonomy of *Trichoderma* www.trichoderma.info). Among the 375 species with valid names as of July 2020, 361 (96%) have been cultivated in vitro and DNA barcoded. Thus, we have developed a protocol for molecular identification of *Trichoderma* that requires analysis of the three DNA barcodes (ITS, *tef1*, and *rpb2*), and it is supported by online tools that are available on www.trichokey.info. We then used all the whole-genome sequenced (WGS) *Trichoderma* strains that are available in public databases to provide versatile practical examples of molecular identification, reveal shortcomings, and discuss possible ambiguities. Based on the *Trichoderma* example, this study shows why the identification of a fungal species is an intricate and laborious task that requires a background in mycology, molecular biological skills, training in molecular evolutionary analysis, and knowledge of taxonomic literature. We provide an in-depth discussion of species concepts that are applied in *Trichoderma* taxonomy, and conclude that these fungi are particularly suitable for the implementation of a polyphasic approach that was first introduced in *Trichoderma* taxonomy by John Bissett (1948–2020), whose work inspired the current study. We also propose a regulatory and unifying role of international commissions on the taxonomy of particular fungal groups. An important outcome of this work is the demonstration of an urgent need for cooperation between *Trichoderma* researchers to get prepared to the efficient use of the upcoming wave of *Trichoderma* genomic data.

Keywords Diversity · DNA barcoding · Hypocreales · GCPSR · Species concept · Taxonomy · Whole-genome sequencing

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Introduction into the predicament of *Trichoderma* identification

Fungi are ubiquitous. They penetrate their environment and impact multiple facets of human life, ranging from biotechnology, phytopathology, and medicine to biodiversity conservation (Hyde et al. 2019). Precise identification of fungi is required for all mycological investigations and applications. It allows us to predict beneficial or pathogenic properties of individual fungal strains, monitor their distribution, and establish safety measures. The recent introduction of DNA Barcoding in fungal identification has significantly improved species identification and reduced the associated labor (Schoch et al. 2012; Vu et al. 2019). However, the precision of fungal identification is frequently impeded by development of the underlying taxonomy (Lücking et al. 2020).

Taxonomy, which is naming, classifying, and describing living organisms based on the similarity of their characteristics and evolutionary history, is not an exact science (Garnett et al. 2020; Lücking et al. 2020; Schoch et al. 2020). Different groups of organisms are classified based on their specific characteristics and their role in the ecosystem (*see below*). These differences can apply even to related organisms that have unique lifestyles (such as obligate biotrophs or saprotrophs) that are considered in species delimitation. Fungal species can be frequently delimitated by expert taxonomists, other fungal researchers, and amateurs. Although they all will provide sufficient material for the formal taxonomic descriptions, the taxonomic approaches will not be the same (Fontaine et al. 2012; Garnett et al. 2020). Expert taxonomists can represent different schools and generations, and thus, they will use unequal approaches and methodologies. Therefore, no nomenclatural codes can specify the criteria that were used to recognize taxa. Zoologists have recently proposed the establishment of global species lists that should be based on universal principles of science, transparency, and political compliance (Garnett et al. 2020). They specified the key role of taxonomic communities in consolidation of such a list and taxa approval/rejection. The implementation of such high-level taxonomic regulations supported by stakeholders (taxonomy users) can consolidate expert groups.

In fungi, which comprise one of the most diverse group of eukaryotes with the predicted diversity of several million species (Choi and Kim 2017; Hawksworth and Lücking 2017), the unification of taxonomic criteria is impeded by the scarcity of fossils, irregular lifecycles, and relative morphological simplicity. Species delimitation is hindered by the difficulties of defining boundaries of individual fungal organisms or populations, diminutive bodies that develop inside of a substrate, and exceptional metabolic and ecological plasticity for which observation may be hampered. Therefore, DNA-based techniques allowed a virtual restart of fungal taxonomy based on the new level of precision (Lücking et al. 2020), and unprecedented success with unification and standardization was achieved (Taylor 2011; May et al. 2019). Molecular techniques also led to discovery of the hidden fungal diversity and fueled the ongoing debate on the classification and naming rules for the fungal "dark taxa" that are only known from their DNA sequences and have attracted great attention of fungal taxonomists (Nilsson et al. 2019). The main consequence of the new methodology is probably not the taxonomic criteria unification but the sharp increase in the number of taxa (of all ranks) among known fungal groups (Taylor et al. 2000; Hawksworth and Lücking 2017). Numerous genera of common and industrially or agriculturally important fungi such as *Penicillium* and *Aspergillus* (Houbraken and Samson 2011; Sklenar et al. 2017; Steenwyk et al. 2019; Houbraken et al. 2020) have been recently taxonomically revised, and ample species combinations were proposed within previous species complexes or clades. Recognition of more species is considered to be a useful practice because it leads to the accurate and precise diagnosis of potential pathogens, prediction of beneficial properties, and an improved overall understanding of fungal diversity and ecology (Hyde et al. 2019; Bajpai et al. 2019). However, because the identifiability of new taxa (Box 1) is not always evaluated, even well-studied groups of fungi can rapidly move from the rear of fungal taxonomy to its frontline.

Trichoderma as a suitable model for integrative fungal taxonomy

Ubiquitous mycotrophic and phytosaprotrophic fungi from the genus *Trichoderma* (syn. *Hypocrea*, Hypocreales) have been known to mycologists from the beginning of the formal taxonomic records for fungi from the late 18th century (see Persoon 1794). For 200 years, investigation of *Trichoderma* (and *Hypocrea*) developed with the pace of all mycology, and it was mainly based on investigation of its teleomorphic stage *Hypocrea* [the name is not in use, (Taylor 2011; Rossman et al. 2013)] that is tractable in the scientific literature (reviewed elsewhere, for example in Rossman et al. 2013; Jaklitsch and Voglmayr 2013). In the mid-20th century, only a few species (or "species aggregates") of *Trichoderma* were proposed (Rifai 1969). However, similar to other common fungi, the last two decades sharply transformed *Trichoderma* to the species-rich genus (Druzhinina et al. 2006; Kubicek et al. 2008; Jaklitsch 2009, 2011; Atanasova et al. 2013; Bissett et al. 2015) that made it comparable to such fungi as *Fusarium* (Hypocreales), *Aspergillus*, or *Penicillium* (Eurotiales) and left all sister hypocrealean or even the model genus for fungal biology *Neurospora* (Sordariales) far behind

Box 1 Terms and definitions

TERMINOLOGY

The *Code,* CN International Code of Nomenclature for algae, fungi, and plants

The *International Code of Nomenclature for algae, fungi, and plants* is the set of rules and recommendations that govern the scientific naming of all organisms that are traditionally treated as algae, fungi, or plants, whether they are fossil or non-fossil, including blue-green algae (*Cyanobacteria*), chytrids, oomycetes, slime molds, and photosynthetic protists with their taxonomically related non-photosynthetic groups (but excluding *Microsporidia*). It is available at https://www.iapt-taxon.org/nomen/main.php.

Chapter F, San Juan Chapter F

Provisions of the *Code* relating solely to names of fungi are presented in its *San Juan Chapter F* that was revised based on decisions that were approved on 21 July 2018 by the closing plenary session of the 11th IMC, which was held in San Juan, Puerto Rico, and published as of (May et al. 2019).

DNA Barcoding

- =molecular identification; is the practice of using the sequences of specific DNA fragments for the identification of organisms. This can result in the detection of new species.
- determination of diagnostic regions that can be used to identify an organism.

DNA barcoding locus (primary)

A specific DNA fragment that is used for primary taxonomic identification. In fungi, the complete sequence of the internal transcribed spacers 1 and 2 of rRNA (ITS), including the sequence of the gene encoding 5.8 S rRNA, is considered to be a primary DNA barcode (Schoch et al. 2012).

DNA barcoding locus (secondary)

A DNA fragment that is accepted by most of the community members as the useful supplementary marker for the identification of a particular group of organisms. For *Trichoderma* and other hypocrealean fungi, such loci as partial fragments of the translation elongation factor 1 alpha (*tef1*) gene (Druzhinina and Kubicek 2005), and the RNA polymerase B subunit II (*rpb2*) gene (Liu et al. 1999; Druzhinina et al. 2006; Atanasova et al. 2013) were generally accepted as the secondary DNA barcodes. This study shows their role as primary DNA barcodes along with ITS. Sequences of a 42 kDa endochitinase gene [*chi18-5 = ech42*, (Lieckfeldt et al. 2000)], calmodulin 1 [*cal1,* (Carbone and Kohn 1999)], actin [*act*, (Carbone and Kohn 1999)], ATP citrate lyase large subunit [*acl1*, (Grafenhan et al. 2011)], nuclear small subunit rRNA (SSU = 18S rRNA), nuclear large subunit rRNA [LSU = 28S rRNA, (White et al. 1990)] and other genes remain secondary.

DNA barcoding locus ITS

The complete sequence of the internal transcribed spacers 1 and 2 (ITS1 and 2, ITS), including the sequence of the gene encoding 5.8 S rRNA, are a primary DNA barcode locus for fungi (White et al. 1990; Schoch et al. 2012). Depending on the technology, metabarcoding environmental studies usually use ITS1 and/or ITS2 fragments, and rarely the complete sequences.

DNA barcoding locus *rpb2*

The partial sequence of the *rpb2* gene encoding RNA polymerase II, the 2nd largest subunit was proposed for the tree of life (TOL) (Lutzoni et al. 2004), and it is frequently used for fungi (Liu et al. 1999; Druzhinina and Kubicek 2005; Schoch et al. 2009). Note, the *rpb2* DNA barcoding fragment is not equal to the whole gene sequence.

DNA barcoding locus *tef1*

The partial sequence of the *tef1* gene encoding translation elongation factor 1 α is frequently used for molecular evolutionary analyses of hypocrealean fungi, including *Trichoderma* spp. (Druzhinina and Kubicek 2005). The fragment corresponding to *tef1* DNA barcoding locus must include the long (forth) intron sequence (Kopchinskiy et al. 2005). Note, the *tef1* DNA barcoding fragment is not equal to the whole gene sequence.

Identification (molecular)

=DNA Barcoding; identification based on the similarity of given DNA fragments. DNA profiling techniques based on PCR and sequences of other biological macromolecules such as proteins, metabolome spectrum, or RNA can also be used (not considered in this study).

Identifiability

The property of a taxonomic group that allows a query organism to be assigned to it. Some species of *Trichoderma* can be unambiguously identified (i.e., high identifiability), while species borders of some other species remain ambiguous (i.e., low identifiability).

Identification accuracy

The quality of identification reflecting its correctness. Incorrect identifications (i.e., assignment to a wrong taxon) correspond to low accuracy. Highly accurate identification can be ambiguous on a low taxonomic level but unambiguous on a higher level.

Identification ambiguous

No taxon assigned (refers to a particular taxonomic level). Frequently, the relation to a sister taxon can be proposed using "affinis" (aff. [closely related species]) or a "confer" (cf. [one of the closely related species]) can be used to point to the neighboring clade.

Identification precision

The quality of the identification reflecting its taxonomic resolution. The highest precision is reached at the lowest taxonomic level. Low precision in identification (i.e., assignment to a higher taxonomic level) can be accurate.

Identification protocol

The list of sequential steps that are required for identification.

Identification tool

Software that is designed to aid the identification protocol.

Identification unambiguous

Box 1 Continued

A taxon name is assigned (refers to a particular taxonomic level). Unambiguous identification can have different levels of accuracy and precision, respectively.

Identification verification (Verification of identification)

Comparing the biological and ecological records for the query organisms and published features for the identified species.

Identification validation

A critical assessment of identification methodology and quality, and completeness of reference materials. Usually refers to the quality of reference materials.

Pairwise sequence similarity

In this study, the value from 0 to 100 reflects the percent of identical sites (i.e., identities) that are in the pairwise alignment of every two biological sequences (DNA or proteins); 100% similarity corresponds to identity.

Phylogenetic marker or locus

A specific DNA fragment that is used for DNA Barcoding or molecular evolutionary analysis. Usually, this is a partial gene fragment; see *DNA barcoding locus*

Reference material

(for DNA Barcoding) an organism or a biological sequence with formally confirmed (published) assignment to a valid taxon.

Reference strain

A strain that was deposited into an authorized public collection as etalon material for a given taxon. This has been published.

Reference sequence

A biological sequence (usually, DNA) that was deposited into an authorized public database as an etalon material for a given taxon. This has been published.

Sequence similarity search

The Basic Local Alignment Search Tool (BLAST) is a computer algorithm that compares biological sequences and uses a given method to calculate scores that describe the similarity and reliability of the result. It is used to find similar regions and estimate the significance of the obtained similarity (Ye et al. 2006).

Species

The taxonomic rank and the basic taxonomic unit that is assigned to a group of similar and evolutionary-related organisms using a given set of criteria (i.e., a species concept, *see below*). In fungi, species concepts are subjected to changes depending on the methodologies that are used or the role of the fungus in the environment or for humankind. Detection of fungal species boundaries is frequently impeded by the lack of morphological characters, pleomorphic lifestyle, asexual reproduction, or diminutive or unknown bodies (for fungal “dark taxa”).

Species anamorphic, agamospecies

Species of fungi for which sexual reproduction is either unknown or molecular evolutionary analysis points to its low probability.

Species complex, metaspecies

A monophyletic group of cryptic sister species that are usually delineated based on molecular evolutionary analysis or ecophysiological traits such as hosts or habitats.

Species cryptic

Species that are morphologically identical to one or several species that may be closely related.

Species holomorphic

Fungal species, for which sexual and asexual stages of the lifecycle were observed, which is pleomorphic in *Trichoderma*.

Species hypothesis

- A proposal to assign a species rank to a group of organisms, which is usually based on the molecular evolutionary analysis.
- The negative result of DNA Barcoding (no species name assigned) combined with the unambiguous, precise, and accurate identification.
- A proposal for species identification, which is usually based on a single DNA barcode, and it requires verification (Nilsson et al. 2019).

Species morphological

In fungi, it is a group of organisms that share similar micromorphological features and appearances of the culture *in vitro* and macromorphology of fruiting bodies (if available).

Species phylogenetic

A group of organisms sharing the same evolutionary history that is revealed by the molecular phylogenetic analysis of a gene or several loci.

Species concept

A set of criteria that should be met to assign a species to a group of organisms or (rarely) to a single isolate.

Species concept, biological

Two organisms belong to one species if they can mate and produce fertile progeny (e.g., De Queiroz 2007).

Species concept, ecological

Sister species can be distinguished based on the different interactions with other organisms or different responses to environmental conditions (Alves et al. 2008; Cai et al. 2020).

Species concept, Genealogical Concordance Phylogenetic Species Recognition (GCPSR)

Box 1 Continued

A species rank can be assigned to a group of organisms if their unique evolutionary history is confirmed by concordant topologies of at least two unlinked DNA loci and they are not contradicted by the others (Taylor et al. 2000). This requires consideration of reference materials (strains or sequences) and the use of at least three unlinked polymorphic loci. This is currently the most widely claimed species concept for fungi, including *Trichoderma.*

Species concept, morphological

A species can be distinguished based on the morphological dissimilarity to reference materials of the related species. This does not recognize cryptic species (Struck et al. 2018), and it was abandoned in *Trichoderma* after the introduction of DNA-based techniques and GCPSR (Samuels et al. 2010; Chaverri et al. 2015).

Species concept, phylogenetic

A species rank can be assigned to a group of closely related organisms if their unique evolutionary history is confirmed by the topology of a phylogram based on a single or multiple DNA loci. In contrast to GCPSR, the concordance of individual loci is not considered.

Species concept, polyphasic

- An assignment of a species rank to a group of organisms based on the integrative application of GCPSR concept and unique characteristics obtained based on multiple ecophysiological, phenotypic, and biogeographic assessments. It can require the development of (semi)quantitative measures.
- A cumulative species concept includes biological, ecological, morphological, and phylogenetic concepts. This is the most recommended approach for fungi like *Trichoderma*

Taxonomy providers

All those researchers who commit taxonomic acts, i.e., define and describe new taxa (all ranks but most frequently, species).

Taxonomy users

All those who use existing taxonomy and identification procedures to assign taxonomic names to query organisms. This can be researchers, industry and medical workers, and amateurs.

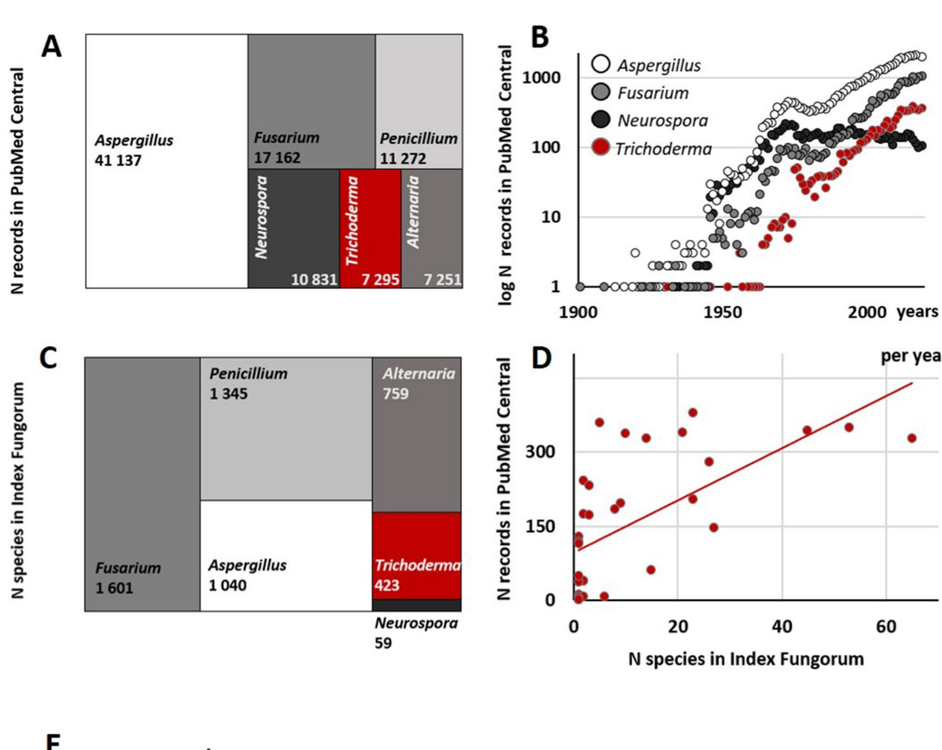
***TRICHODERMA* GENE NOMENCLATURE**

There is currently no agreement on the use of gene name nomenclature for fungi. Historically, the human gene nomenclature (Wain et al. 2002), the gene nomenclature for yeasts from Saccharomycotina (Kohli 1987), and the gene nomenclature for plant pathogenic fungi (Yoder et al. 1986) appeared to be the most advanced and developed. However, because *Trichoderma* is an industrial fungus that is gaining its significance as the model organism in fungal biology, we use the gene nomenclature that was proposed for *Neurospora* (Perkins 1999) and which is also widely used for *Aspergillus* (e.g., Yu et al. 2016), as follows:

- A gene name should consist of the three small letters and a number (all italicized); a protein encoded by this gene should be denoted using the same (most frequently) three letters and a number written in capital letters and all not italicized. E.g., *tef1* is the gene that encodes the translation elongation factor 1 α TEF1 protein (sometimes named as EF1 protein), or *rpb2* encodes the RNA polymerase B subunit II, RPB2 enzyme.
- The non-protein coding fragments of DNA, such as genes and intergenic spacers of the rRNA gene cluster, should be written in capital letters and not italicized. Although the full name of the rRNA locus used for the DNA Barcoding of fungi is the internal transcribed spacers 1 and 2 (ITS1 and 2), we use the truncated version (ITS), which is in agreement with the recent literature of fungal identification (Lücking et al. 2020).
- Strain or species names should not be incorporated into the names of genes or proteins because most genes are orthologous. Strain or species names can be indicated using subscripts before or after the name of a gene or a protein. For example, hydrophobin 4 (HFB4) is encoded by $_{Tg}$*hfb4* and $_{Th}$*hfb4* genes in *T. guizhouense* (Tg) and *T. harzianum* (Th), respectively (Cai et al. 2020).

An agreement on gene nomenclature that is suitable for *Trichoderma* research should be achieved by the community of *Trichoderma* scientists.

(Fig. 1). The increase in the total number of *Trichoderma* species was not strongly influenced by the general mycological movement "One fungus—one name" (Taylor 2011), as the connection with the single *Hypocrea* teleomorph (with only a few exceptions) has been established earlier and considered in the first species counts (Druzhinina et al. 2006; Atanasova et al. 2013). In addition to the unprecedented effort of *Trichoderma* taxonomists (*see below*), the drastic increase in *Trichoderma* species number has several explanations that are related to the technologies and applications. The first reason is the emerging importance of *Trichoderma* for humankind. Approximately 50 years ago, *T. reesei* was recognized as a highly efficient producer of plant biomass-degrading enzymes for biofuel and other industries. A couple of decades later, several other species (*T. atroviride*, *T. virens*, *T. harzianum*, and others) were proposed as potent bioeffectors for plant protection (*bio*fungicides) and plant growth promotion (*bio*fertilizers) (reviewed by Harman et al. 2004, Druzhinina et al. 2011 and others), and they are now widely used for biological control of fungal pests in sustainable agriculture (*bio*control). *Trichoderma* was also documented as the causative agent of the green mold disease on mushroom farms (Komoń-Zelazowska et al. 2007) and as an opportunistic pathogen in humans (Sandoval-Denis et al. 2014). This resulted in the rapid increase of scientific publications based on *Trichoderma* species (Fig. 1). The second reason that ultimately contributed to the sudden increase in the species number is the use of either phylogenetic (PSR, Box 1) or the



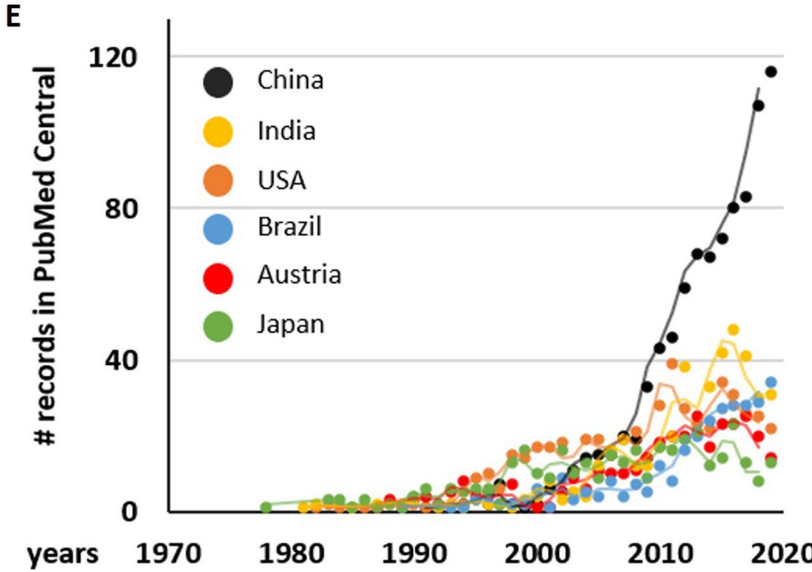


Fig. 1 Research interest to *Trichoderma* spp. as of July 2020. **a** The number of records in PubMed Central for the key word "*Trichoderma*" compared to other fungi with noticeable importance for humankind such as plant pathogens, industrial producers, and research model organisms. **b** Trends in research interest over last 100 years for *Trichoderma* compared to *Neurospora*, *Aspergillus,* and *Fusarium*. **c** The number of records in IndexFugorum. **d** The relationship between the number of species described per year and the number of *Trichoderma*-based research articles recorded in PubMed Central. **e** Research interest for *Trichoderma* in different countries, which is estimated as the number of publications and affiliations (including joint studies)

genealogical concordance phylogenetic species recognition (GCPSR, Box 1) concepts and DNA Barcoding techniques in *Trichoderma* taxonomy and the subsequent modification of the criteria for species delimitation. Before the introduction of DNA Barcoding, *Trichoderma* species were recognized based on their morphology and growth characteristics. However, the introduction of molecular methods and, in particular, the extensive use of GCPSR (Box 1) resulted in the recognition of several hundred *Trichoderma* species (reviewed in Druzhinina et al. 2006; Atanasova et al. 2013) many of which were delimitated within previously existing species complexes or clades. Although the applications are still restricted to a few species, the growth of species richness positively influences the *Trichoderma* science development as the number of *Trichoderma*-based publications grows proportionally to it (Fig. 1).

Another striking property of *Trichoderma* that makes it a useful model of taxonomic studies is the evident lack of hidden diversity or "dark *Trichoderma* species" (Migheli et al. 2009; Friedl and Druzhinina 2012; Hagn et al. 2007; Meincke et al. 2010; López-Quintero et al. 2013; Röhrich et al. 2014; Jaklitsch 2009, 2011; Jaklitsch and Voglmayr

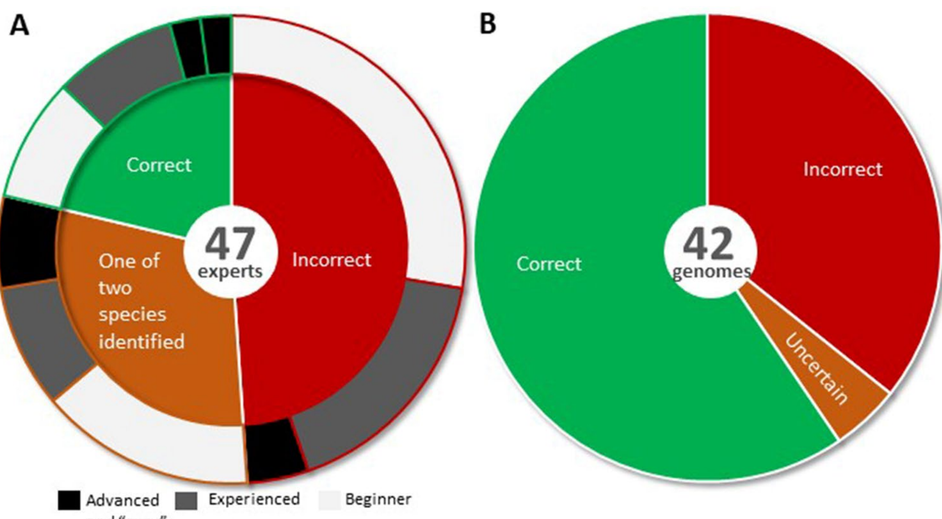


Fig. 2 Molecular identification of *Trichoderma* strains by experts. **a** The online survey results on the identification of the two unknown *Trichoderma* isolates based on the combination of primary (ITS) and secondary (*tef1* and *rpb2*) DNA barcodes. The survey was completed by 47 volunteers with experience in the area. The level of their expertise was provided by the respondents. **b** The correctness of species identification of 42 *Trichoderma* isolates, for which WGS are available in public databases in July 2020. "Uncertain" correspond to strains that were deposited as *Trichoderma* sp.

2015), meaning that most or all species can be successfully cultivated in vitro. Therefore, *Trichoderma* spp. can potentially be extensively phenotypically and physiologically characterized along with taxonomic or nomenclatural acts (Samuels et al. 2006, 2012; Druzhinina et al. 2010b; Chaverri et al. 2015; Bissett et al. 2015). The possibility of the extended ecophysiological profiling paves the way for the introduction of the integrative (polyphasic) taxonomy for species delimitation, i.e., the combination of genealogy (phylogeny), phenotype (including autecology), and reproductive biology (when feasible) (Lücking et al. 2020). The analysis of a relatively large number of whole-genome sequences (WGS) for *Trichoderma* spp. (*see below*) also provided insights into the evolutionary timeline of this genus (Druzhinina et al. 2018; Kubicek et al. 2019). Thus, *Trichoderma* can serve as a useful model for the observation of taxonomic development with an impact on the precision, accuracy, and ambiguity of species delimitation and subsequent identification.

The challenge and the aim: identification of *Trichoderma* species

To address the current state of *Trichoderma* identifiability at the species level, we invited researchers working with these fungi to perform an exercise on DNA Barcoding. The respondents were offered an anonymous online survey where they could insert their identification results along with the description of the identification procedure, their experience in the area, and comments. For this test, we picked two unpublished *Trichoderma* strains that had sequences of DNA barcoding loci that were similar but not identical to those that were available in public databases in May 2020. Each strain was represented by a set of the three sequences (ITS, partial sequences of *tef1*, and *rpb2* genes, respectively, see Box 1 and *below*) and a brief description of the habitat. No information on biogeography, morphology, or physiology was provided. As shown below, one strain belongs to *T. guizhouense* (TUCIM 10063, nick-named a "mycoparasite" in the survey), which is a sister species to *T. harzianum* (Li et al. 2013; Chaverri et al. 2015). Another strain (TUCIM 5640, nicknamed an "epiphyte") represents a putative new *Trichoderma* species (*T.* sp. TUCIM 5640), which is awaiting its formal description if additional material will become available.

The survey was completed by 47 respondents (Fig. 2). Among them, 82% described themselves as experienced *Trichoderma* researchers, including 15% who were also experienced in advanced DNA Barcoding of fungi (putative taxonomists). Ten (21%) replies diagnosed both strains correctly (*see below*), while 23 respondents (49%) failed to identify both sequences. *T. guizhouense* was identified correctly by 20 respondents, and the second strain was assigned to a putative new species by 14 respondents (*see below*). The accuracy of identification did not correlate with the experience because nearly one-half of the correct answers were given by beginners, while ten highly experienced *Trichoderma* scientists failed to identify both strains (Fig. 2). Similarly, time had no effect on the identification because the average time spent for the correct and incorrect answers was similar to the total average (55 min; ANOVA, $P > 0.05$).

Identification of the WGS strains provided an alternative measurement of *Trichoderma* species identifiability by the experts because genomes are usually deposited by researchers who specialize in this fungus. Therefore, we have assessed the identification of *Trichoderma* strains for which the WGSs have been available in public databases (Table 1). Among the 42 strains, two strains were deposited without species names (as *Trichoderma* sp. IMV 00454 and *Trichoderma* sp. TW21990_1), while the original identification of 15 strains (35%) was not accurate (Fig. 2, and *below*).

Table 1 *Trichoderma* strains with WGSs that were deposited in public databases before July 2020

Strain ID	Species		Identification accuracy	Genome ID	References
	This study	Initial			
QM6a[T]	*T. reesei*	*T. reesei*	√	GCA_002006585.1	Martinez et al. (2008)
CBS 999.97	*T. reesei*	*T. reesei*	√	GCA_001999515.1	Tisch et al. (2017)
CBS 125925[T]	*T. parareesei*	*T. parareesei*	√	GCA_001050175.1	Yang et al. (2015)
CBS 816.68[T]	*T. longibrachiatum*	*T. longibrachiatum*	√	GCA_003025155.1	Druzhinina et al. (2018)
MK1	*T. longibrachiatum*	*T. longibrachiatum*	√	JGI 1185339	–
SMF2	*T. longibrachiatum*	*T. longibrachiatum*	√	GCA_000332775.1	Xie et al. (2014)
JCM 1883	***T. longibrachiatum***	***T. koningii***	**O**	**GCA_001950475.1**	**Fanelli et al.** (2018)
TUCIM 6016	*T.* cf. *citrinoviride*	*T. citrinoviride*	√ O	GCA_003025115.1	Druzhinina et al. (2018)
CBS 226.95[T]	*T. harzianum*	*T. harzianum*	√	GCA_003025095.1	Druzhinina et al. (2018)
TR274	*T. harzianum*	*T. harzianum*	√	GCA_002838845.1	Kubicek et al. (2019)
B97	*T. harzianum*	*T. harzianum*	√	GCA_001990665.1	Compant et al. (2017)
T22	***T. afroharzianum***	***T. harzianum***	**O**	**JGI 1185335**	–
T6776	***T. afroharzianum***	***T. harzianum***	**O**	**GCA_000988865.1**	**Baroncelli et al.** (2015)
NJAU 4742	***T.* sp. NJAU 4742**	***T. guizhouense***	**O**	**GCA_002022785.1**	**Druzhinina et al.** (2018)
M10	***T.* sp. M10**	***T. harzianum***	**O**	**JGI 1185333**	–
IMV 00454	***T. simmonsii***	***T.* sp.**	**O**	**GCA_001931985.1**	**Fanelli et al.** (2018)
CFAM-422	***T.* cf. *endophyticum***	***T. lentiforme***	**O**	**GCA_011066345.1**	–
ITEM 908	*T.* cf. *atrobrunneum*	*T. atrobrunneum*	**√ O**	GCA_003439915.1	Fanelli et al. (2018)
TPhu1	***T.* sp. TPhu1**	***T. pleuroti***	**O**	**GCA_001721665.1**	**Fanelli et al.** (2018)
Tr1	***T. pleuroticola***	***T. harzianum***	**O**	**GCA_002894145.1**	–
Gv29-8[T]	*T. virens*	*T. virens*	√	GCA_000170995.2	Kubicek et al. (2011)
FT-333	*T. virens*	*T. virens*	√	GCA_000800515.1	Fanelli et al. (2018)
Tv-1511	***T. virens***	***T. viride***	**O**	**GCA_007896495.1**	–
IMI 304061	***T.* sp. aff. *neocrassum* IMI 304061**	***T. virens***	**O**	**GCA_001835465.1**	**Sherkhane et al.** (2017)
IMI 206040	*T. atroviride*	*T. atroviride*	√	GCA_000171015.2	Kubicek et al. (2011)
B10	*T. atroviride*	*T. atroviride*	√	JGI 1185343	–
JCM 9410	*T. atroviride*	*T. atroviride*	√	GCA_001599035.1	Fanelli et al. (2018)
F7	*T. atroviride*	*T. atroviride*	√	JGI 1185341	–
P1	*T. atroviride*	*T. atroviride*	√	JGI 1185337	–
XS2015	*T. atroviride*	*T. atroviride*	√	GCA_000963795.1	Shi-Kunne et al. (2015)
LY357	***T.* sp. LY357**	***T. atroviride***	**O**	**GCA_002916895.1**	–
T6085	*T. gamsii*	*T. gamsii*	√	GCA_001481775.2	Baroncelli et al. (2016)
A5MH	***T.* sp. aff. *gamsii* A5MH**	***T. gamsii***	**O**	**GCA_002894205.1**	–
POS7	***T.* sp. aff. *koningiopsis* POS7**	***T. koningiopsis***	**O**	**GCA_002246955.1**	**Castrillo et al.** (2017)
B05	*T.* cf. *asperellum*	*T. asperellum*	√	GCA_000733085.2	Fanelli et al. (2018)
CBS 433.97[T]	*T. asperellum*	*T. asperellum*	√	GCA_003025105.1	Druzhinina et al. (2018)
TR356	***T. asperelloides***	***T. asperellum***	**O**	**N.A.**	–
Ts93	***T. asperelloides***	***T. asperellum***	**O**	**GCA_004154885.1**	–
GD12	*T. hamatum*	*T. hamatum*	√	GCA_000331835.2	Studholme et al. (2013)
IBT 40837	*T. arundinaceum*	*T. arundinaceum*	√	GCA_003012105.1	Proctor et al. (2018)
IBT 40841	*T.* cf. *brevicompactum*	*T. brevicompactum*	**√ O**	GCA_003012085.1	Proctor et al. (2018)
TW21990_1[T]	*T. cyanodichotomus*	*T.* sp.	**O √**	GCA_010015515.1	Zhou et al. (2020)

[T], type strain; √, original identification was accurate; O, original identification was in correct. *T.* sp. [strain ID]—a putative new species of *Trichoderma* for which no sister species is known. *T.* sp. aff. [species name] [strain ID]—a putative new species of *Trichoderma* for which a sister species is detected; *T.* cf. [species name] and *T.* aff. [species name] cases where unambiguous identification is currently not achievable without a detailed taxonomic revision of the group. N.A., not available

Thus, these two tests demonstrate that the accurate molecular identification of *Trichoderma* species is a considerable challenge for experts who do research on this fungus. It is not easy even for specialists in fungal taxonomy. The difficulties related to identification are also reflected in the fact that more than 2000 *Trichoderma* records in the NCBI Taxonomy Browser were deposited as "*Trichoderma* sp." Identification of these 44 (2 + 42) strains also challenged our skills and triggered the study on how to identify a *Trichoderma* species, which is presented below.

Thus, this work addresses the problem of molecular identification of *Trichoderma* at the species level. We have selected the "white paper" format to provide a review of *Trichoderma* taxonomy and prepare the authoritative guidelines for the accurate unambiguous molecular identification of *Trichoderma* diversity that is recognized by the year 2020. For this, we first provided a complete inventory and a cumulative summary of *Trichoderma* nomenclature, and reviewed the current state of its molecular taxonomy. Second, we developed and explained the protocol for molecular identification of currently valid *Trichoderma* species. The comparison of ITS sequences for *Trichoderma* spp. and its neighboring genera allowed us to set up a similarity threshold to estimate a query strain for its possibility of being a member of the genus. We also used the variability of the two DNA barcoding markers (*rpb2* and *tef1*, Box 1) between the currently defined species and set the numerical standards of the similarity threshold at the level at which it is sufficient for species identification for most of the existing species. We then provided practical examples of DNA Barcoding showing how the identification results can be presented and gave examples on how a new species hypothesis can be proposed. Finally, we developed recommendations for *Trichoderma* taxonomy providers and taxonomy users on performing diversity studies. For this, we introduced the www.trichokey.com and the www.trichoderma.info web resources that dedicated to *Trichoderma* taxonomy and molecular identification. We concluded that the genus *Trichoderma* is highly suitable for the application of the integrative (polyphasic) taxonomy based on genealogy, ecophysiology, and biogeography, which was initially proposed by John Bissett for these and other fungi (Kubicek et al. 2003; Komoń-Zelazowska et al. 2007; Hoyos-Carvajal et al. 2009), and therefore, we dedicate this work to his memory. We also proposed a regulatory and unification role of International Commissions on Taxonomy of *Trichoderma* (ICTT) for the approval/rejection of new species proposals.

Assumptions made in this study

In this study, we assumed that the genus *Trichoderma* included species that were originally described as *Trichoderma* (basionym) or transferred to *Trichoderma* from other genera (*combinatio nova;* comb. nov.) such as *Hypocrea*, *Protocrea*, *Aphysiostroma*, or *Sarawakus,* according to Rossman et al. (2013). We also considered all *Hypocrea* and *Protocrea* records in the NCBI Taxonomy Browser that were transferred to *Trichoderma* because they were consistent with the aim of this study (molecular identification of *Trichoderma*). However, we did not consider all species names of *Hypocrea* that were deposited in the Index Fungorum and Mycobank that had not been formally transferred to *Trichoderma* because they may be members of other hypocrealean genera (e.g., *Hypomyces*, *Hypocrella*, *Moelleriella, Protocreopsis, Clintoniella, Atkinsonella, Stilbocrea, Battarrina, Podocrea, Nectriopsis, Myriogenospora, Ophiocordyceps, Arachnocrea, Dialhypocrea, Selinia, Nectria, Epichloe,* and others) or unrelated taxa (*Broomella, Amphisphaeria, Thuemenella, Hypoxylon, Penzigia,* or *Amplistroma* and *Plowrightia*).

Here, we focused on molecular identification using in silico methods and corresponding records in public databases. In some places, we indicated instances of incomplete reference material that were deposited into public databases or revealed identifications that could have increased accuracy, precision, and ambiguity. However, we assumed that the sequences and species descriptions were correct (i.e., we ignored incorrect sequences, not incorrect identifications).

We also assumed that all formally described species complied with the requirements of the *Code* (May et al. 2019; Box 1) irrespective of the species criteria applied, and that the material studied must be identifiable.

The importance of the *Trichoderma* taxonomic history, the scope of phenotypic assessments, morphology, biogeography, ecology, chemotaxonomy, reliability, and availability of reference specimens were highly appreciated but the detailed consideration of these aspects was beyond the scope of this survey.

For the sake of easier reading, we used the short taxonomic names, i.e. avoided listing authors' name(s) and the publication year of species names. For all species, this information is available in tables and in the accessory websites www.trichoerma.info and www.trichokey.com. Exceptions made for the case where these parts of the formal species name are discussed.

The state of *Trichoderma* nomenclature, taxonomy, and DNA Barcoding by the year 2020

To estimate the state of *Trichoderma* taxonomy, we first collected all *Trichoderma* names and the former *Hypocrea* names transferred to *Trichoderma* according to Rossman et al. (2013) that have been deposited in the three major taxonomic databases, which are Index Fungorum (http://

Table 2 The complete taxonomy of *Trichoderma* (July 2020)

		rpb2		TAXONOMY																			IDENTIFICATION				
PhyloOrder	Alphabetic order	Clade	In the tree	Genus	Species name	Author(s)	Year	Collection name	Collection code	NCBI Taxonomy	IndexFungorum	Mycobank	ITS1 and 2	*rpb2*	*tef1*	*chi18-5*	*cal*	*act*	*acl1*	18S rRNA	28S rRNA	others	# shared *rpb2*	Phylo Spec Hyp	Identifiability	Comments and warnings	Occurrence
Counts		8	340	3	371 (460)	179	226			336	422	436	293	310	322	87	113	103	140	43	63	52					
3570	315	6		*T.*	***protrudens***	Samuels & Chaverri	2008	CBS	121320																		
3560	31	6		*T.*	***arundinaceum***	Zafari, Gräfenhan & Samuels	2008	CBS	119575																		
3556	393	6		*H.*	***subcitrina***	Kalchbr. & Cooke	1880	*J.A.C.*	14420																	no GCPRS, no DNA Barcoding	
3550	60	6		*T.*	***brevicompactum***	Kraus, Kubicek & Gams	2004	CBS	109720																		
3545	110	6		*T.*	***cornu-damae***	(Pat.) Zhu & Zhuang	2014	*G.J.S.*	06-03																	*Podostroma*, no GCPRS, no DNA Barcoding	
3540	426	6		*T.*	***turrialbense***	Samuels, Degenkolb, Nielsen & Gräfenhan	2008	CBS	112445																		
3530	223	6		*T.*	***limonium***	Qin & Zhuang	2016	HMAS	248751																	not in NCBI, sequenced	
3520	173	6		*T.*	***grande***	Qin & Zhuang	2016	HMAS	248749																	not in NCBI, sequenced	
3510	339	6		*T.*	***rodmanii***	(Samuels & Chaverri) Jaklitsch & Voglmayr	2014	CBS	120895																		
3500	237	6		*T.*	***margaretense***	Jaklitsch	2011	CBS	120540																		
3491	41	6		*T.*	***auranteffusum***	Jaklitsch	2011	CBS	119284														-			*aurantioeffusum*	na
3490	42	6		*T.*	***aurantioeffusum***	Jaklitsch	2011	CBS	119284																		
3480	408	5		*T.*	***taxi***	Zhang, Lin & Kubicek	2007	CGMCC	1672																		
3470	345	5		*T.*	***rubi***	Jaklitsch & Voglmayr	2015	CBS	127380																	only secondary DNA Barcodes	
3460	194	5		*T.*	***hypoxylon***	Sun, Liu & Hyde	2016	CGMCC	3.1791																		
3440	304	5		*T.*	***placentula***	Jaklitsch	2011	CBS	120924																		
3430	156	5		*T.*	***foliicola***	(Jaklitsch & Voglmayr) Jaklitsch & Voglmayr	2014	CBS	130008																		
3420	52	5		*T.*	***bavaricum***	Jaklitsch	2011	WU	29196a																		
3410	35	5		*T.*	***atlanticum***	Jaklitsch	2011	CBS	120632																		
3400	142	5		*T.*	***europaeum***	Jaklitsch & Voglmayr	2015	CBS	121276																	only secondary DNA Barcodes	
3390	240	5		*T.*	***mediterraneum***	Jaklitsch & Voglmayr	2015	CBS	136469																	only secondary DNA Barcodes	
3380	249	5		*T.*	***minutisporum***	Bissett	1992	CBS	341.93																		
3370	210	5		*T.*	***lacuwombatense***	(Lu, Druzhin. & Samuels) Jaklitsch & Voglmayr	2014	CBS	122668																		
3360	21	5		*T.*	***alutaceum***	Jaklitsch	2011	CBS	120535																		
3350	276	5		*T.*	***pachypallidum***	Jaklitsch	2011	CBS	122126																		
3346	106	5		*H.*	***coprosmae***	Dingley	1952	PDD	10453																	only *tef1*	
3345	275	5		*H.*	***pachybasioides***	Doi	1972	CBS	820.68														-			*polysporum*	na
3344	17	5		*T.*	***album***	Preuss	1851																			*polysporum*; an outdated names assigned to sequences	
3343	116	5		*T.*	***croceum***	Bissett	1992																-			*polysporum*	na

www.indexfungorum.org/), Mycobank (http://www.mycobank.org/), and the NCBI Taxonomy Browser (https://www.ncbi.nlm.nih.gov/taxonomy). The cumulative list is presented in Table 2 (see the digital sortable version at https://trichokey.com/index.php/trichoderma-taxonomy-2020 and a printable version at https://trichoderma.info/trichoderma-taxonomy-2020/). It summarizes the results in which we screened *Trichoderma* for the names that are currently in use, names that are not in use, orthographic variants, and other synonyms. Then, for each species, we collected the records for the reference strain (holotype or ex-type specified with the original species description or its valid substitute) and recorded the distribution of DNA Barcoding markers and the total number of DNA Barcoding sequences archived per each species. The assessment of the accuracy of individual sequence attribution to a given species name was beyond the scope of this research (see "Assumptions" *above*), but this issue is partially addressed below.

Table 2 (continued)

3342	379	5	T.	*sporulosum*	(Link) Hughes	1958			-	*polysporum*	na
3341	380	5	T.	*stellatum*	(Lu, Druzhin. & Samuels) Jaklitsch & Voglmayr	2014			-	*polysporum*	na
3340	311	5	T.	*polysporum*	(Link) Rifai	1969	CBS	820.68			
3320	366	5	T.	*sinoluteum*	Zhu & Zhuang	2015	HMAS	252868			
3310	316	5	T.	*pruinosum*	Chen & Zhuang	2017	HMAS	247217		not in NCBI, sequenced	
3300	279	5	T.	*parapiluliferum*	(Lu, Druzhin. & Samuels) Jaklitsch & Voglmayr	2014	CBS	112771			
3290	301	5	T.	*piluliferum*	Webster & Rifai	1969	CBS	120927			
3281	232	5	T.	*luteffusum*	Jaklitsch	2011			-	*luteoeffusum*	
3280	234	5	T.	*luteoeffusum*	Jaklitsch	2011	CBS	120537		not in NCBI, sequenced	
3270	354	5	T.	*seppoi*	Jaklitsch	2008	CBS	122498			
3260	267	5	T.	*nybergianum*	(Ulvinen & Chamb.) Jaklitsch & Voglmayr	2014	CBS	122500			
3250	219	5	T.	*leucopus*	Jaklitsch	2011	CBS	122499			
3240	241	1	T.	*medusae*	Samuels	2012	CBS	125719	1	*rpb2* is not unique	
3230	69	1	T.	*caesareum*	Samuels	2012	CBS	124369	1	*rpb2* is not unique	
3220	389	1	T.	*stromaticum*	Samuels & Pardo-Schulth.	2000	CBS	101875			
3210	214	1	T.	*lanuginosum*	Samuels	2012	CBS	125718			
3200	343	1	T.	*rossicum*	Bissett, Kubicek & Szakács	2003	ATCC	MYA-483	3	*rpb2* is not unique	
3190	130	1	T.	*dimorphum*	Chen & Zhuang	2017	HMAS	247199	3	not in NCBI, sequenced, *rpb2* not unique	
3180	357	1	T.	*sichuanense*	Chen & Zhuang	2017	HMAS	248737	3	*rpb2* is not unique	
3170	432	1	T.	*verticillatum*	Chen & Zhuang	2017	HMAS	248740	3	*rpb2* is not unique	
3160	182	1	T.	*hebeiense*	Chen & Zhuang	2017	HMAS	248743			
3150	431	1	T.	*vermipilum*	Samuels	2012	CBS	127103			
3140	51	1	T.	*barbatum*	Samuels	2012	CBS	125733			
3130	200	1	T.	*ivoriense*	Samuels	2012	CBS	125734			
3120	155	1	T.	*floccosum*	Samuels	2011	CBS	124372			
3110	178	1	T.	*hainanense*	Chen & Zhuang	2017	HMAS	248837			
3100	358	1	T.	*silvae-virgineae*	Jaklitsch	2011	CBS	120922			
3091	90	1	T.	*chlamydosporum*	Chen & Zhuang	2017			-	*chlamydosporicum*	na
3090	89	1	T.	*chlamydosporicum*	Chen & Zhuang	2017	HMAS	248850	1	*rpb2* is not unique	
3070	416	1	T.	*tibetense*	Chen & Zhuang	2016	HMAS	245010			
3060	26	1	T.	*angustum*	Qin & Zhuang	2017	HMAS	273784		not in NCBI, sequenced	
3041	145	1	T.	*fasciculatum*	Bissett	1992			-	*strictipile*	na
3041	386	1	T.	*strictipilis*	Bissett	1992			-	*strictipile*	na
3040	385	1	T.	*strictipile*	Bissett	1992	CBS	347.93	1	*rpb2* is not unique	
3030	175	1	T.	*gregarium*	Chen & Zhuang	2017	HMAS	248887		not in NCBI, sequenced	
3020	356	1	T.	*shennongjianum*	Chen & Zhuang	2016	HMAS	245009			

Trichoderma nomenclature

The inventory of *Trichoderma* nomenclature resulted in a complete list of 464 nonredundant species epithets (Table 2). Among them, 90 names are not currently in use (Bissett et al. 2015), including 22 grammatically incorrect names (orthographic variants) that have been replaced by their corrected versions (Table 2). Several names are considered to be invalid because their description did not follow the requirements of the *Code* (May et al. 2019) or the deposition to public databases was not performed or was made incompletely (refer to *T. cyanodichotomous* nom. inval. at NCBI Taxonomy Browser as an example). The contemporary valid nomenclature of *Trichoderma* spp. consists of 375 species names.

The Latin names of *Trichoderma* spp. most commonly reflect macromorphology of the teleomorph and the culture appearance in vitro (e.g., *T. viride, T. citrinum, T.*

Table 2 (continued)

3013	230	1	T.	*longipilum*	Bissett	1992			-	*longipile*	na
3012	229	1	T.	*longipilis*	Bissett	1992			-	*longipile*	na
3011	119	1	T.	*cuenisporum*	Chaverri & Samuels	2003			-	*longipile*	na
3010	228	1	T.	*longipile*	Bissett	1991	CBS	120953		not in IndexFungorum	
3001	118	1	T.	*crystalligenum*	Qin & Zhuang	2017	HMAS	273783	-	Nom. illegit., sequenced	na
3000	422	1	T.	*tropicosinense*	(Liu) Zhu & Zhuang	2015	HMAS	252546			
2990	286	1	T.	*parestonicum*	Jaklitsch	2009	CBS	120636			
2980	83	1	T.	*ceramicum*	Chaverri & Samuels	2003	CBS	114576			
2970	140	1	T.	*estonicum*	Chaverri & Samuels	2003	CBS	111147			
2960	164	1	T.	*ganodermatis*	Chen & Zhuang	2017	HMAS	248856			
2955	129	1	H.	*dichromospora*	Doi	1968	CBS	337.69		no GCPRS, no DNA Barcoding	
2950	300	1	T.	*phyllostachydis*	Chaverri & Samuels	2003	CBS	114071			
2940	57	1	T.	*bomiense*	Zhang & Zhuang	2019	W.Z.	2018a		not in NCBI, sequenced	
2930	310	1	T.	*polypori*	Chen & Zhuang	2017	HMAS	248855			
2920	384	1	T.	*stramineum*	Chaverri & Samuels	2003	BPI	843667			
2910	227	1	T.	*longifialidicum*	Montoya, Meirelles, Chaverri & Rodrigues	2016	CBS	139785			
2900	430	1	T.	*velutinum*	Bissett, Kubicek & Szakács	2003	DAOM	230013			
2890	96	1	T.	*cinnamomeum*	Chaverri & Samuels	2003	G.J.S.	97-237			
2880	302	1	T.	*pinicola*	Oh, Park, & Lim	2019	KACC	48486		not in Mycobank	
2870	360	1	T.	*simplex*	Chen & Zhuang	2017	HMAS	248842			
2860	79	1	T.	*catoptron*	Chaverri & Samuels	2003	CBS	114232			
2851	320	1	T.	*pseudogelatinosa*	(Komatsu & Doi) Kim	2012			-	*pseudogelatinosum*	na
2850	321	1	T.	*pseudogelatinosum*	(Komatsu & Doi) Kim	2017	TUFC	60186			
2840	188	1	T.	*hirsutum*	Chen & Zhuang	2017	HMAS	248834			
2830	181	1	T.	*hausknechtii*	Jaklitsch & Voglmayr	2015	CBS	133493		only secondary DNA Barcodes	
2820	82	1	T.	*ceraceum*	Chaverri & Samuels	2003	BPI	843654			
2810	291	1	T.	*peberdyi*	Valadares-Inglis & Inglis	2020	CEN	1426		not in IndexFungorum	
2800	86	1	T.	*cerinum*	Bissett, Kubicek & Szakács	2003	DAOM	230012	1	*rpb2* is not unique	
2790	224	1	T.	*linzhiense*	Chen & Zhuang	2017	HMAS	248846	1	*rpb2* is not unique	
2780	451	1	T.	*viridulum*	Qin & Zhuang	2017	HMAS	273865	1	not in NCBI, sequenced, *rpb2* not unique	
2770	206	1	T.	*koreanum*	Oh, Park & Lim	2019	SFC	131005-S	1	*rpb2* is not unique	
2760	418	1	T.	*tomentosum*	Bissett	1992	CBS	349.93			
2750	390	1	T.	*rugulosum*	Park, Oh & Lim	2019	SFC	180301-0			
2740	196	1	T.	*inhamatum*	Veerkamp & Gams	1983	CBS	273.78			
2730	338	1	T.	*rifaii*	Rocha, Chaverri & Samuels	2015	CBS	130746			
2720	331	1	T.	*pyramidale*	Jaklitsch & Chaverri	2015	CBS	135574			

citrinoviride, T. pulvinatum) or the microscopic features of the species (e.g., *T. helicum, T. spirale, T. crystalligenum, T. compactum, T. oblongisporum, T. brevicompactum, T. longibrachiatum*). Some names indicate the species ecology (e.g., *T. psychrophilum, T. aggressivum, T. endophyticum*) or the substrates and hosts (e.g., *T. arenarium, T. bannaense, T. alni, T. parepimyces, T. epimyces, T. pleuroti, T. taxi*). The etymology of many *Trichoderma* species names corresponds to the names of continents or regions (e.g., *T. caribbaeum, T. sinense, T. americanum, T. sinoaustrale, T. europaeum, T. mediterraneum*), famous geographic hallmarks such as mountains or river basins (e.g., *T. shennongjianum, T. changbaiense, T. amazonicum, T. alpinum*), or they reflect political or historical–geographical names of the sampling locations (e.g., *T. aethiopicum, T. linzhiense, T. austriacum, T. britannicum, T. britdaniae, T. camerunense, T. costaricense, T. danicum, T. estonicum, T. guizhouense, T. hainanense, T. henanense, T. hispanicum, T. hongkongensis, T.*

Table 2 (continued)

2700	36	1	T.	*atrobrunneum*	Rocha, Chaverri & Jaklitsch	2015	CBS	548.92				
2680	109	1	T.	*corneum*	(Pat.) Jaklitsch & Voglmayr	2014	CBS	100541		only secondary DNA Barcodes		
2670	218	1	T.	*lentiforme*	(Rehm) Chaverri, Samuels & Rocha	2015	CBS	100542		only secondary DNA Barcodes		
2660	59	1	T.	*breve*	Chen & Zhuang	2017	CGMCC	3.184				
2650	50	1	T.	*bannaense*	Chen & Zhuang	2017	CGMCC	3.1839				
2640	460	1	T.	*zeloharzianum*	Yu & Du	2018	CGMCC	3.1908				
2620	8	1	T.	*afarasin*	Chaverri & Rocha	2015	CBS	130755	1	*rpb2* is not unique		
2610	137	1	T.	*endophyticum*	(Jaklitsch, Põldmaa & Samuels) Jaklitsch &	2015	CBS	130729	1	GCPSR, no DNA Barcoding		
2600	48	1	T.	*azevedoi*	Valadares-Inglis & Inglis	2020	CEN	1422		not in IndexFungorum		
2590	308	1	T.	*pollinicola*	Liu & Cai	2018	CGMCC	3.1878	1	not in NCBI, sequenced, *rpb2* not unique		
2580	225	1	T.	*lixii*	(Pat.) Chaverri	2015	CBS	110080	1	*rpb2* is not unique		
2570	176	1	T.	*guizhouense*	Li, McKenzie & Wang	2012	CBS	131803		not in Mycobank		
2565	72	1	T.	*camerunense*	Chaverri & Samuels	2015	CBS	138272		*identified by tef1*		
2560	359	1	T.	*simmonsii*	Chaverri, Rocha, Samuels, Degenkolb & Jaklitsch	2015	CBS	130431				
2555	12	1	T.	*albocorneum*	(Doi) Jaklitsch & Voglmayr	2014	G.J.S.	97-28		not in NCBI, sequenced		
2550	9	1	T.	*afroharzianum*	Chaverri, Rocha, Degenkolb & Druzhin.	2015	CBS	124620				
2545	158	1	T.	*fomitopsis*	(Liu & Doi) Liu, Zhu & Zhuang	2014	HKAS	26205		*name not in use*		
2540	180	1	T.	*harzianum*	Rifai	1969	CBS	226.95				
2530	409	1	T.	*tenue*	Qin & Zhuang	2017	HMAS	273785		not in NCBI, sequenced		
2520	347	1	T.	*rufobrunneum*	Zhu & Zhuang	2015	HMAS	252547				
2510	313	1	T.	*priscilae*	Jaklitsch & Voglmayr	2015	CBS	131487		only secondary DNA Barcodes		
2500	330	1	T.	*purpureum*	Qin & Zhuang	2017	HMAS	273787		not in NCBI, sequenced		
2490	195	1	T.	*ingratum*	Chen & Zhuang	2017	HMAS	248822				
2480	199	1	T.	*italicum*	Jaklitsch & Voglmayr	2015	CBS	132567		only secondary DNA Barcodes		
2470	285	1	T.	*parepimyces*	Jaklitsch	2009	CBS	122769				
2460	101	1	T.	*compactum*	Yu & Zhang	2007	CBS	121218				
2440	10	1	T.	*aggregatum*	Chen & Zhuang	2017	HMAS	248863				
2430	138	1	T.	*epimyces*	Jaklitsch	2008	CBS	120534				
2420	20	1	T.	*alpinum*	Chen & Zhuang	2017	HMAS	248821	3	*rpb2* is not unique		
2410	103	1	T.	*concentricum*	Chen & Zhuang	2017	HMAS	248833	3	*rpb2* is not unique		
2400	319	1	T.	*pseudodensum*	Chen & Zhuang	2017	HMAS	248828	5	*rpb2* is not unique		
2390	19	1	T.	*alni*	Jaklitsch	2008	CBS	120633	3	*rpb2* is not unique		
2380	391	1	T.	*subalni*	Zhang & Zhuang	2018	HMAS	247267	1	Nom. illegit., sequenced		
2370	459	1	T.	*zayuense*	Chen & Zhuang	2017	HMAS	248835	2	*rpb2* is not unique		
2361	92	1	T.	*christiani*	Jaklitsch & Voglmayr	2015	CBS	132572	1	GCPSR, rpb2 not uniqe, only secondary DNA Barcodes		
2360	93	1	T.	*christianii*	Jaklitsch & Voglmayr	2015			-	*christiani*	na	

hubeiense, T. istrianum, T. italicum, T. koreanum, T. moravicum, T. novae-zelandiae, T. sulawesense, T. taiwanense, T. thailandicum, T. tibetense, T. yunnanense). Naming after colleagues that contributed to *Trichoderma* research or the development of *Trichoderma*-based applications appears to be increasingly popular and appreciated, such as *T. beinartii, T. bissettii, T. chetii, T. christiani, T. dingleyae, T. eijii, T. evansii, T. gamsii, T. harzianum, T. lieckfeldtiae, T. parmastoi, T. petersenii, T. priscilae, T. reesei, T. rifaii, T. rogersonii, T. samuelsii, T. simmonsii, T. voglmayrii*, and others.

For cryptic species that are morphologically identical to previously described taxa, authors frequently compose Latin names by adding Greek or Latin affixes "*neo-*" (new) (e.g., *T. neocrassum, T. neokoningii, T. neorufoides, T. neorufum,*

Table 2 (continued)

2350	295	1	*T.*	***perviride***	Qin & Zhuang	2017	HMAS	273786	1	not in NCBI, sequenced, *rpb2* not unique	
2340	369	1	*T.*	***solum***	Chen & Zhuang	2017	HMAS	248848			
2330	186	1	*T.*	***hengshanicum***	Chen & Zhuang	2017	HMAS	248852			
2320	220	1	*T.*	***liberatum***	Chen & Zhuang	2017	HMAS	248831			
2310	170	1	*T.*	***globoides***	Qin & Zhuang	2017	HMAS	248747		not in NCBI, sequenced	
2300	11	1	*T.*	***aggressivum***	Samuels & Gams	2002	DAOM	222156			
2280	22	1	*T.*	***amazonicum***	Chaverri & Gazis	2011	CBS	126898			
2260	84	1	*T.*	***ceratophylli***	Yu	2019	YMF	1.0462	1	*rpb2* is not unique	
2250	104	1	*T.*	***confertum***	Chen & Zhuang	2017	HMAS	248896	1	not in NCBI, sequenced, *rpb2* not unique	
2241	307	1	*T.*	***pleurotum***	Yu & Park	2006			-	*pleuroti*	na
2240	305	1	*T.*	***pleuroti***	Yu & Park	2006	CBS	124387			
2230	306	1	*T.*	***pleuroticola***	Yu & Park	2006	CBS	124383			
2220	407	1	*T.*	***tawa***	Chaverri & Samuels	2008	CBS	114233			
2210	65	1	*T.*	***brunneoviride***	Jaklitsch	2008	CBS	121130			
2200	37	1	*T.*	***atrogelatinosum***	(Dingley) Jaklitsch & Voglmayr	2014	CBS	237.63			
2190	122	1	*T.*	***dacrymycellum***	Jaklitsch	2009	WU	29042a			
2170	255	1	*T.*	***neocrassum***	Samuels	2015	CBS	114230	1	*rpb2* is not unique	
2160	113	1	*T.*	***crassum***	Bissett	1992	CBS	336.93	1	*rpb2* is not unique	
2151	152	1	*T.*	***flavofuscum***	(Mill., Giddens & Foster) Bissett	1992			-	*virens*	na
2150	436	1	*T.*	***virens***	(Mill., Giddens & Foster) Arx	1987	CBS	249.59			
2140	121	1	*T.*	***cyanodichotomus***	Li & Chen	2018		*CK-2016a*		Nom. illegit., sequenced	
2130	183	1	*T.*	***helicolixii***	Jaklitsch & Voglmayr	2015	CBS	133499		only secondary DNA Barcodes	
2120	377	1	*T.*	***spirale***	Bissett	1992	CBS	346.93			
2110	192	1	*T.*	***hunanense***	Chen & Zhuang	2017	HMAS	248841			
2100	231	1	*T.*	***longisporum***	Chen & Zhuang	2017	HMAS	248843			
2090	165	1	*T.*	***gelatinosum***	Chaverri & Samuels	2008	CBS	114246			
2080	169	1	*T.*	***gliocladium***	Jaklitsch & Voglmayr	2015	CBS	130009			
2070	245	2	*T.*	***mienum***	Kim, Nakagiri & Maek.	2012	CBS	132690			
2060	87	2	*T.*	***changbaiense***	Chen & Zhuang	2017	HMAS	247198		not in NCBI, sequenced	
2050	146	2	*T.*	***fassatiae***	Nováková, Kubátová, Valinová, Hubka & Kolařík	2015	PRM	933821			
2040	268	2	*T.*	***oblongisporum***	Bissett	1992	CBS	343.93			
2030	147	2	*T.*	***fertile***	Bissett	1992	CBS	339.93			
2020	251	2	*T.*	***moravicum***	Jaklitsch	2011	CBS	120539			
2010	157	2	*T.*	***fomiticola***	Jaklitsch	2009	CBS	121136			
2000	193	2	*T.*	***hunua***	(Dingley) Jaklitsch & Voglmayr	2014	CBS	238.63			
1990	352	2	*T.*	***semiorbis***	(Berk.) Jaklitsch & Voglmayr	2014	CBS	130716			

T. neosinense, T. neotropicale), "*pseudo-*" (false) (e.g., *T. pseudobritdaniae, T. pseudocandidum, T. pseudodensum, T. pseudogelatinosum, T. pseudokoningii, T. pseudolacteum, T. pseudonigrovirens, T. pseudostramineum,*), "*para-*" (near) (e.g., *T. parareesei, T. pararogersonii, T. paratroviride, T. paraviridescens*), or "*-oides*" (likeness) (e.g., *T. asperelloides*). Prefixes such as "*eu-*" (true), "*sub-*" (under), "*mega-*", "*megalo-*" (big), "*proto*" (first), and "*zelo*" (zeal) are also used (e.g., *T. eucorticioides, T. euskadiense, T. subviride, T. subeffusum, T. megalocitrinum, T. melanomagnum, T. zeloharzianum).* There are no preferences for one naming strategy for *Trichoderma*. The etymology of each name is usually justified and explained along with the species description.

The common issue of *Trichoderma* nomenclature that is difficult to correct is the use of grammatically wrong formal scientific names (Table 2) (May et al. 2019). We calculated that nearly 80 *Trichoderma* spp. were first described using incorrect grammar. Even when corrected, such orthographic

Table 2 (continued)

			Genus	Epithet	Authority	Year	Collection	Number		Remarks	
1980	324	1	T.	*pseudonigrovirens*	Minnis, Samuels & Chaverri	2009	G.J.S.	99-64			
1970	66	1	T.	*byssinum*	Chen & Zhuang	2017	CGMCC	3.1839			
1960	184	1	T.	*helicum*	Bissett, Kubicek & Szakács	2003	DAOM	230022			
1951	428	1	T.	*undatipilosum*	Chen & Zhuang	2017			-	*undatipile*	na
1950	427	1	T.	*undatipile*	Chen & Zhuang	2017	HMAS	248854		*undatipilosum*	
1940	383	1	T.	*stipitatum*	Zhu & Zhuang	2015	HMAS	266613			
1930	449	1	T.	*viridicollare*	Zhang & Zhuang	2019	W.Z.	2018b		not in NCBI, sequenced	
1920	401	1	T.	*sulawesense*	(Doi) Jaklitsch & Voglmayr	2014	GJS	85-228			
1910	94	1	T.	*chromospermum*	Chaverri & Samuels	2003	CBS	114577			
1900	115	1	T.	*cremeum*	Chaverri & Samuels	2003	CBS	111146	2	*rpb2* is not unique	
1890	403	1	T.	*surrotundum*	Chaverri & Samuels	2003	BPI	843668		incomplete DNA Barcoding	
1880	114	1	T.	*cremeoides*	Jaklitsch & Voglmayr	2015		S112	2	rpb2 not uniqe, only secondary DNA Barcodes	
1870	61	1	T.	*brevicrassum*	Chen & Zhuang	2017	CGMCC	3.1841	2	*rpb2* is not unique, *tef1* is no unique	
1860	367	1	T.	*sinuosum*	Chaverri & Samuels	2003	CBS	114247		*tef1* is not unique	
1850	91	1	T.	*chlorosporum*	Chaverri & Samuels	2003	CBS	114231			
1840	412	1	T.	*thelephoricola*	Chaverri & Samuels	2003	CBS	114237			
1830	344	1	T.	*rosulatum*	Zhu & Zhuang	2015	HMAS	244906			
1820	461	1	T.	*zonatum*	Zhu, Zhuang & Li	2017	CGMCC	3.1876		only secondary DNA Barcodes	
1810	112	1	T.	*costaricense*	(Chaverri & Samuels) Chaverri, Jaklitsch &	2014	P.C.	21			
1800	411	1	T.	*thailandicum*	Chaverri & Samuels	2003	CBS	114234			
1790	437	1	T.	*virescentiflavum*	(Speg.) Jaklitsch & Voglmayr	2014	P.C.	278			
1780	160	1	T.	*fujianense*	Zhu, Zhuang & Li	2017	HMJAU	34830		only secondary DNA Barcodes	
1771	74	1	T.	*candidum*	Chaverri & Samuels	2003			-	*pseudocandidum*	na
1770	318	1	T.	*pseudocandidum*	Minnis, Samuels & Chaverri	2009	BPI	843652			
1760	43	1	T.	*aureoviride*	Rifai	1969	CBS	120536			
1750	15	8	T.	*albolutescens*	Jaklitsch	2011	CBS	119286			
1740	235	1	T.	*lycogaloides*	(Berk. & Broome) Jaklitsch, Lechat & Voglmayr	2014	CBS	123493		only secondary DNA Barcodes	
1730	376	1	T.	*spinulosum*	(Fuckel) Jaklitsch & Voglmayr	2014	CBS	311.5			
1720	123	1	T.	*danicum*	(Jaklitsch) Jaklitsch & Voglmayr	2014	CBS	121273			
1715	246	1	H.	*mikurajimensis*	Doi	2001	JCM	12018		no GCPRS, no DNA Barcoding	
1710	63	1	T.	*britannicum*	(Rifai & Webster) Jaklitsch & Voglmayr	2014	CBS	253.62			
1700	4	1	T.	*aerugineum*	Jaklitsch	2009	CBS	120541			
1690	189	5	T.	*hispanicum*	(Jaklitsch & Voglmayr) Jaklitsch & Voglmayr	2014	CBS	130540			
1680	423	5	T.	*samuelsii*	Jaklitsch & Voglmayr	2012	CBS	130537			
1670	434	5	T.	*vinosum*	Samuels	2006	CBS	119087			
1660	2	5	T.	*adaptatum*	Chen & Zhuang	2017	HMAS	248800		not in NCBI, sequenced	

variants remain recorded in public databases as synonyms. This ultimately affects the identifiability of the species and confuses the taxonomy users. For example, in MycoBank, the orthographic variant "*T. pleurotum* Yu & Park (2006)" [MB#504755] is recorded as synonym of grammatically correct *T. pleuroti* Yu & Park (2006) [MB#546965]. Although the details on the name status appear on the page with the detailed profile of the MycoBank record, the main page for the orthographic variant MB#504755 has no indications that the name should not be used (http://www.mycobank.org/BioloMICSDetails.aspx?Rec=440470). The NCBI Taxonomy Browser does not have the incorrect orthographic variant listed, but incorrect sequence information appeared largely in the NCBI Entrez search system. For example, "*Trichoderma pleurotum*" was used in the definitions of at least 14 nucleotide sequences and will appear in the results of the sequence similarity search (BLAST). Although on GenBank pages such as https://www.ncbi.nlm.nih.gov/nucco

Table 2 (continued)

1650	351	5	T.	*scalesiae*	Samuels & Evans	2006	CBS	120069			
1640	53	5	T.	*beijingense*	Chen & Zhuang	2017	HMAS	248804		not in NCBI, sequenced	
1630	441	5	T.	*valdunense*	Jaklitsch	2011	CBS	120923			
1620	238	5	T.	*martiale*	Samuels	2008	CBS	123052			
1610	282	5	T.	*paratroviride*	Jaklitsch & Voglmayr	2015	CBS	136489			
1590	163	5	T.	*gamsii*	Samuels & Druzhin.	2006	CBS	120075			
1585	260	5	T.	*neotropicale*	Chaverri & Rocha	2015	CBS	130633		*GCPSR*, no DNA Barcoding	
1580	256	5	T.	*neokoningii*	Samuels & Soberanis	2006	CBS	120070			
1575	264	5	T.	*nothescens*	Samuels & Jaklitsch	2013	CBS	134882			
1570	445	5	T.	*viridescens*	(Horne & Will.) Jaklitsch & Samuels	2006	CBS	433.34	8	*rpb2* is not unique	
1560	439	5	T.	*viridarium*	Jaklitsch, Samuels & Voglmayr	2013	CBS	132568	6	*rpb2* is not unique	
1550	448	5	T.	*viridialbum*	Jaklitsch, Samuels & Voglmayr	2013	CBS	133495	6	GCPSR, rpb2 not uniqe, only secondary DNA Barcodes	
1540	353	5	T.	*sempervirentis*	Jaklitsch & Voglmayr	2013	CBS	133498	7	GCPSR, rpb2 not uniqe, only secondary DNA Barcodes	
1535	6	5	T.	*aestuarinum*	Gonçalves & Alves	2019	MUM	H-19.05		no GCPRS, no DNA Barcoding	
1530	283	5	T.	*paraviridescens*	Jaklitsch, Samuels & Voglmayr	2013	CBS	119321	4	*rpb2* is not unique	
1520	421	5	T.	*trixiae*	Samuels & Jaklitsch	2013	CBS	134702	4	*rpb2* is not unique	
1510	27	5	T.	*appalachiense*	Samuels & Jaklitsch	2013	CBS	133558	5	*rpb2* is not unique	
1500	272	5	T.	*olivascens*	Jaklitsch, Samuels & Voglmayr	2013	CBS	132574	1	*rpb2* is not unique	
1490	259	5	T.	*neosinense*	Samuels & Jaklitsch	2013	CBS	134884	1	*rpb2* is not unique	
1480	102	5	T.	*composticola*	Samuels & Jaklitsch	2013	CBS	133497	1	*rpb2* is not unique	
1470	452	5	T.	*virilente*	Jaklitsch & Voglmayr	2013	CBS	132569			
1460	213	5	T.	*laevisporum*	Qin & Zhuang	2016	HMAS	273756		only secondary DNA Barcodes	
1451	39	5	T.	*atroviride*	Bissett	1984			-	*ghanense*	na
1450	38	5	T.	*atroviride*	Karst.	1892	IMI	206040			
1445	252	5	H.	*muroiana*	Hino & Katum.	1958	NBRC	31293			
1442	443	5	T.	*viride*	Schumach.	1803			-	*name not in use*	na
1441	442	5	T.	*viride*	Pers.	1794			-	*Trichoderma viride* Pers., Neues Mag. Bot. [Roemer's] 1: 92. 1794: Fries, Syst. Mycol. 3: 215. 1832	
1440	440	5	T.	*viride**	Pers.	1832	CBS	119325		*Trichoderma viride* Pers., Neues Mag. Bot. [Roemer's] 1: 92. 1794: Fries, Syst. Mycol. 3: 215. 1832	
1430	399	5	T.	*subviride*	Qin & Zhuang	2016	HMAS	273761		only secondary DNA Barcodes	
1420	202	5	T.	*junci*	Jaklitsch	2011	WU	29229a			
1410	374	5	T.	*speciosum*	Yu & Du	2018	CGMCC	3.1908			
1400	375	5	T.	*sphaerosporum*	Qin & Zhuang	2016	HMAS	273763		only secondary DNA Barcodes	
1390	382	5	T.	*stilbohypoxyli*	Samuels & Schroers	2006	CBS	992.97			

re/EU279975.1, the "Source" and "Organism", are correct (as *Trichoderma pleuroti*), unexperienced users may mistake the incorrect orthographic variant for species identification and, thus, unintentionally amplify the number of incorrect records. Therefore, it is highly recommended to carefully consider the grammar of the Latin language and ask the experts for the grammatic verification of a new name proposal. The San Juan Chapter F of the *Code* (Box 1) introduces the correctability for incorrectly cited identifiers of names and typifications (May et al. 2019).

Timeline of *Trichoderma* taxonomy

The current taxonomy of *Trichoderma* was provided by 179 mycologists who researched the diversity of this genus for the last 236 years starting from the first proposed and

Table 2 (continued)

1380	139	5	T.	*erinaceum*	Bissett, Kubicek & Szakács	2003	DAOM	230018			
1370	370	5	T.	*songyi*	Park, Seung Oh & Lim	2014	CBS	138099			
1360	278	5	T.	*panacis*	Liu, Zhang, Yu & Zhang	2020	CGMCC	3.183		not in NCBI, sequenced	
1350	274	5	T.	*ovalisporum*	Samuels & Schroers	2004	CBS	113299			
1330	205	5	T.	*koningiopsis*	Samuels, Carm. Suárez & Evans	2006	CBS	119075			
1312	133	5	T.	*dorothopsis*	Tomah & Zhang	2020	HMAS	248251			
1311	31	5	T.	*arenarium*	Cai, Ding & Druzhin.	2020	CGMCC	19611			
1310	405	5	T.	*taiwanense*	Samuels & Wu	2006	CBS	119058			
1290	76	5	T.	*caribbaeum*	Samuels & Schroers	2006	CBS	119093	2	*rpb2* is not unique	
1280	198	5	T.	*istrianum*	Jaklitsch & Voglmayr	2015	CBS	130539	2	GCPSR, rpb2 not uniqe, only secondary DNA Barcodes	
1270	296	5	T.	*petersenii*	Samuels, Dodd & Schroers	2006	*G.J.S.*	91-99	1	*rpb2* is not unique	
1260	132	5	T.	*dorotheae*	Samuels & Dodd	2006	CBS	119089			
1250	410	5	T.	*texanum*	Montoya, Meirelles, Chaverri & Rodrigues	2016	CBS	139784			
1245	13	5	T.	*albofulvopsis*	Qin & Zhuang	2016	HMAS	273760		only secondary DNA Barcodes	
1240	269	5	T.	*ochroleucum*	(Berk. & Ravenel) Jaklitsch & Voglmayr	2014	CBS	119502			
1230	197	5	T.	*intricatum*	Samuels & Dodd	2006	CBS	119059			
1220	14	5	T.	*albofulvum*	(Berk. & Broome) Jaklitsch & Voglmayr	2014	CBS	114787		only secondary DNA Barcodes	
1210	131	5	T.	*dingleyae*	Samuels & Dodd	2006	CBS	119056			
1200	204	5	T.	*koningii*	Oudem.	1902	*G.J.S.*	96-117			
1190	406	5	T.	*tardum*	Chen & Zhuang	2017	HMAS	248798	1	not in NCBI, sequenced, *rpb2* not unique	
1180	55	5	T.	*bifurcatum*	Chen & Zhuang	2017	HMAS	248795	1	not in NCBI, sequenced, *rpb2* not unique	
1170	68	5	T.	*caerulescens*	(Jaklitsch & Voglmayr) Jaklitsch & Voglmayr	2014	CBS	130011			
1160	387	5	T.	*strigosellum*	López-Quint., Gams, Boekhout & Druzhin.	2013	CBS	102817			
1150	388	5	T.	*strigosum*	Bissett	1992	CBS	348.93			
1140	454	5	T.	*vulgatum*	Chen & Zhuang	2017	HMAS	248796		not in NCBI, sequenced	
1130	340	5	T.	*rogersonii*	Samuels	2006	*G.J.S.*	94-115			
1125	85	5	T.	*cerebriforme*	(Berk.) Samuels	2015	*G.J.S.*	85-245		no GCPRS, no DNA Barcoding	
1120	46	5	T.	*austrokoningii*	Samuels & Druzhin.	2006	CBS	119092		*GCPSR*, no DNA Barcoding	
1110	365	5	T.	*sinokoningii*	Qin & Zhuang	2016	HMAS	271397		only secondary DNA Barcodes	
1100	281	5	T.	*pararogersonii*	Jaklitsch & Voglmayr	2015	CBS	133496			
1090	236	5	T.	*mangshanicum*	Chen & Zhuang	2017	HMAS	248810		not in NCBI, sequenced	
1080	373	5	T.	*sparsum*	Qin & Zhuang	2016	HMAS	273759		only secondary DNA Barcodes	
1060	394	5	T.	*subeffusum*	Jaklitsch	2011	*W.M.J.*	2009-17			
1050	151	4	T.	*flavipes*	(Peck) Seifert, Jaklitsch & Voglmayr	2014	CBS	123070		only secondary DNA Barcodes	
1040	144	5	T.	*evansii*	Samuels	2009	CBS	123079			2
1030	328	5	T.	*pubescens*	Bissett	1992	CBS	345.93			

still-valid species name *T. viride* Pers. (1832) (Fig. 3) (*see below*). Before introducing molecular methods in fungal taxonomy, the most significant contributions were made by C. H. Persoon (11 species, http://www.indexfungorum.org/) and M. A. Rifai (six species) (Rifai 1969). J. Bissett with colleagues, worked on the edge of DNA Barcoding times (the 90's of the 20th century) and recognized 24 species, including some that have been DNA barcoded (Table 2). The most substantial contribution to *Trichoderma* taxonomy of all time was made by the groups of W. M. Jaklitsch [> 120 species, (for example, Atanasova et al. 2010; Jaklitsch 2009, 2011; Jaklitsch et al. 2005, 2006, 2008a, b, 2012, 2013, 2014; Jaklitsch and Voglmayr 2012, 2013, 2015)] and G. J. Samuels [> 70 species, (for example, Samuels et al. 2002, 2006, 2010, 2012; Chaverri et al. 2015; Chaverri and Samuels 2003; Lu et al. 2004; Chaverri et al. 2011)]

Table 2 (continued)

1020	355	5	T.	*shaoguanicum*	Chen & Zhuang	2017	HMAS	248809		not in NCBI, sequenced
1010	179	5	T.	*hamatum*	(Bonord.) Bainier	1906	CBS	102160		
1000	136	5	T.	*eijii*	Kim & Maek.	2013	CBS	133190		
990	1	5	T.	*acremonioides*	Zhang & Zhuang	2018	HMAS	279611		only secondary DNA Barcodes
980	290	5	T.	*paucisporum*	Samuels, Carm. Suárez & Solis	2006	CBS	118645		
970	413	5	T.	*theobromicola*	Samuels & Evans	2006	CBS	119120		
960	208	5	T.	*kunmingense*	Yu & Li	2018	YMF	1.0266	1	*rpb2* is not unique
955	312	5	T.	*poronioideum*	(Möller) Samuels	2015	CBS	139046		no GCPRS, no DNA Barcoding
950	33	5	T.	*asperellum*	Samuels, Lieckf. & Nirenberg	1999	CBS	433.97	2	*rpb2* is not unique
946	239	5	T.	*matsushimae*	(Webster) Yamag., Tsurumi, Chuaseehar. & Nakagiri	2012	IMI	266915		only *tef1*
945	168	5	T.	*glaucum*	Abbott	1927				*name not in use*
940	458	5	T.	*yunnanense*	Yu & Zhang	2007	CBS	121219	1	*rpb2* is not unique
930	32	5	T.	*asperelloides*	Samuels	2010	CBS	125938		
920	221	5	T.	*lieckfeldtiae*	Samuels	2009	CBS	123049		
910	3	5	T.	*aeroaquaticum*	Yamag., Tsurumi, Chuaseehar. & Nakagiri	2012	NBRC	108034		
900	127	5	T.	*densum*	Qin & Zhuang	2016	HMAS	273758	2	*no GCPSR*, no DNA Barcoding, rpb2 is not unique
890	258	5	T.	*neorufum*	(Samuels, Dodd & Lieckf.) Jaklitsch & Voglmayr	2014	CBS	111144	2	*rpb2* is not unique
880	257	5	T.	*neorufoides*	Jaklitsch	2011	CBS	119506	2	*rpb2* is not unique
875	336	5	T.	*restrictum*	du Plessis & Jacobs	2018	PPRI	19367		*GCPSR*, no DNA Barcoding
870	297	5	T.	*pezizoides*	(Berk. & Broome) Samuels, Jaklitsch & Voglmayr	2014	G.J.S.	01-257		
860	453	5	T.	*voglmayrii*	Jaklitsch	2006	CBS	117711		
850	40	1	T.	*attinorum*	Montoya, Meirelles, Chaverri & Rodrigues	2016	CBS	139783		
841	97	5	T.	*citrinella*	(Ellis) Zhuang & Zeng	2017			-	not in NCBI, sequenced
840	190	5	T.	*hongkongensis*	(Zhu & Zhuang) Zeng & Zhuang	2017	HMAS	75530		
830	271	7	T.	*oligosporum*	Zhu & Zhuang	2015	HMAS	252870		
820	71	7	T.	*calamagrostidis*	Jaklitsch	2011	WU	29198a		
810	117	7	T.	*crystalligenum*	Jaklitsch	2006	CBS	118980		
800	49	7	T.	*balearicum*	Jaklitsch & Voglmayr	2015	CBS	133222		GCPSR, only secondary DNA Barcodes
790	337	7	T.	*rhododendri*	(Jaklitsch) Jaklitsch & Voglmayr	2014	CBS	119288		
786	378	7	H.	*splendens*	Phillips & Plowr.	1885	CBS	336.69		not in NCBI, sequenced
780	327	7	T.	*psychrophilum*	Jaklitsch	2011	CBS	119129		
770	242	7	T.	*megalocitrinum*	(Doi) Jaklitsch & Voglmayr	2014	B.E.O.	00-09		
770	80	7	T.	*ceciliae*	Jaklitsch & Voglmayr	2015	CBS	130010		
760	191	5	T.	*hubeiense*	Qin & Zhuang	2016	HMAS	252888		only secondary DNA Barcodes
750	457	5	T.	*yui*	Zhu & Zhuang	2015	HMAS	266633		
740	243	6	T.	*melanomagnum*	Chaverri & Samuels	2003	G.J.S.	99-153	1	*rpb2* is not unique

that worked alone or collaborated with each other and such researchers as C. P. Kubicek, E. Lieckfeldt, H. Voglmayr, and P. Chaverri (Fig. 2). Most of the above-listed taxonomists except P. Chaverri have completed their research in *Trichoderma* diversity. Current active taxonomy providers for *Trichoderma* are W. Y. Zhuang and her colleagues who have named > 85 species in the last five years (for example, Chen and Zhuang 2016; Qin and Zhuang 2016a, c; Chen and Zhuang 2017a, b, c, d; Qin and Zhuang 2016b, 2017). However, the most recent species that appeared in 2020 were also described by scientists who are new to *Trichoderma* taxonomy (Tomah et al. 2020; Ding et al. 2020) (Table 2). Contact details for the current experts in *Trichoderma* taxonomy are available on the International Committee on Taxonomy of *Trichoderma* (ICTT) website (www.trichoderm

Table 2 (continued)

730	126	6	*T.*	*deliquescens*	(Sopp) Jaklitsch	2011	CBS	121131	1	*rpb2* is not unique	
720	233	6	*T.*	*luteocrystallinum*	Jaklitsch	2011	CBS	123828			
710	433	7	*T.*	*victoriense*	(Overton) Jaklitsch & Voglmayr	2014	CBS	140064	1	*rpb2* is not unique	
700	45	7	*T.*	*austriacum*	Jaklitsch	2011	CBS	122494	1	*rpb2* is not unique	
690	402	7	*T.*	*sulphureum*	(Schwein.) Jaklitsch & Voglmayr	2014	CBS	119929			
680	397	7	*T.*	*subsulphureum*	(Syd. & Syd.) Jaklitsch & Voglmayr	2014		M-141		*name not in use*	
670	244	7	*T.*	*microcitrinum*	(Doi) Jaklitsch & Voglmayr	2014	G.J.S.	91-61			
660	299	7	*T.*	*phellinicola*	Jaklitsch	2011	CBS	119283			
650	329	7	*T.*	*pulvinatum*	(Fuckel) Jaklitsch & Voglmayr	2014	CBS	121279			
640	23	7	*T.*	*americanum*	(Canham) Jaklitsch & Voglmayr	2014	CBS	976.69			
630	314	7	*T.*	*protopulvinatum*	(Doi) Jaklitsch & Voglmayr	2014	CBS	739.83			
621	209	7	*T.*	*lacteum*	Bissett	1992			-	*citrinum*	na
620	99	7	*T.*	*citrinum*	(Pers.) Jaklitsch, Gams & Voglmayr	2014	CBS	894.85			
610	381	7	*T.*	*stercorarium*	(Barrasa, Martínez & Moreno) Jaklitsch & Voglmayr	2015	CBS	148.85		Former *Aphysiostroma stercorarium* Barrasa, A.T. Martínez & G. Moreno, Can. J. Bot. 63(12): 2441 (1986) [1985]	
600	124	7	*T.*	*decipiens*	(Jaklitsch, Põldmaa & Samuels) Jaklitsch &	2014	G.J.S.	97-207			
591	325	7	*T.*	*pseudostraminea*	(Doi) Kim	2012			-	*pseudostramineum*	na
590	326	7	*T.*	*pseudostramineum*	(Doi) Kim	2012	TUFC	60104	1	*rpb2* is not unique	
580	105	7	*T.*	*confluens*	Qin & Zhuang	2016	HMAS	244993	1	only secondary DNA Barcodes, rpb2 not unique	
560	28	7	*T.*	*applanatum*	Zhu & Zhuang	2015	HMAS	245081			
550	415	7	*T.*	*tiantangzhaiense*	Zhu & Zhuang	2015	HMAS	252872			
540	141	7	*T.*	*eucorticioides*	(Overton) Jaklitsch & Voglmayr	2014	G.J.S.	99-61			
530	323	7	*T.*	*pseudolacteum*	Kim & Maek.	2013	CBS	133191			
520	217	3	*T.*	*leguminosarum*	Jaklitsch & Voglmayr	2015	CBS	130014	1	GCPSR, rpb2 not uniqe, only secondary DNA Barcodes	
510	34	3	*T.*	*asterineum*	Qin & Zhuang	2016	HMAS	271353	1	not in NCBI, sequenced, *rpb2* not unique	
500	440	8	*T.*	*undulatum*	du Plessis & Jacobs	2018	PPRI	19365			
490	125	4	*T.*	*delicatulum*	Jaklitsch	2011	CBS	120631			
480	47	4	*T.*	*avellaneum*	(Rogerson & Carey) Jaklitsch & Voglmayr	2014	CBS	121667			
475	24	3	*H.*	*ampulliformis*	Doi & Yamat.	1989	JCM	11982		no GCPRS, no DNA Barcoding	
470	64	3	*T.*	*britdaniae*	(Jaklitsch & Voglmayr) Jaklitsch & Voglmayr	2014	WU	31610			
460	317	3	*T.*	*pseudobritdaniae*	Qin & Zhuang	2016	HMAS	271355		not in NCBI, sequenced	
450	185	3	*T.*	*henanense*	Qin & Zhuang	2016	HMAS	252891		not in NCBI, sequenced	
440	270	3	*T.*	*odoratum*	Qin & Zhuang	2016	HMAS	271354		not in NCBI, sequenced	
430	395	3	*T.*	*sambuci*	(Jaklitsch & Voglmayr) Jaklitsch & Voglmayr	2014	WU	29467			

a.info), which replaces the currently unsupported www.isth.info (*see below*).

Beginning in the late 18th century and for the first 200 years, cumulative taxonomy for *Trichoderma* and *Hypocrea* developed at a steady rate, accumulating one or two new species every two years (Fig. 2). This mainly includes the teleomorphic species that were originally described as *Hypocrea* spp. and were recently transferred to *Trichoderma* according to the contribution of Rossman et al. (2013). In the 1990s, shortly before introducing DNA Barcoding in fungal diversity research, there were almost 100 *Hypocrea*/*Trichoderma* names deposited for this genus. However, with the introduction of DNA-based techniques, molecular phylogeny, and the GCPSR concept, the number of *Trichoderma* basionyms started to increase exponentially, resulting in a "hockey stick" shape of the plot showing the

Table 2 (continued)

					Species	Authority	Year	Coll.	No.		Remarks	
420	420	3		T.	*tremelloides*	Jaklitsch	2011	CBS	121140			
410	335	3		T.	*reesei*	Simmons	1977	CBS	383.78			
400	280	3		T.	*parareesei*	Atan., Jaklitsch, Komoń-Zel., Kubicek & Druzhin.	2010	CBS	125925			
390	348	3		T.	*rugosum*	Zhang & Zhuang	2018	HMAS	254548		Nom. illegit., sequenced	
380	414	3		T.	*thermophilum*	Qin & Zhuang	2016	HMAS	252912		not in NCBI, sequenced	
370	54	3		T.	*beinartii*	du Plessis, Druzhin., Atan., Yarden & Jacobs	2018	PPRI	19281		GCPSR, no DNA Barcoding	
360	303	3		T.	*pinnatum*	Samuels	2012	CBS	131292	1	GCPSR, rpb2 not uniqe, only secondary DNA Barcodes	
350	456	3		T.	*xanthum*	Chen & Zhuang	2017	HMAS	247202	1	not in NCBI, sequenced, *rpb2* not unique	
340	226	3		T.	*longibrachiatum*	Rifai	1969	CBS	816.68	1	*rpb2* is not unique	
330	56	3		T.	*bissettii*	Sand.-Den. & Guarro	2014	CBS	137447	1	*rpb2* is not unique	
325	67	3		T.	*caeruleimontis*	du Plessis & Jacobs	2018	PPRI	23903		GCPSR, no DNA Barcoding	
320	273	3		T.	*orientale*	(Samuels & Petrini) Jaklitsch & Samuels	2014	CBS	130428	1	*rpb2* is not unique	
310	7	3		T.	*aethiopicum*	Mulaw, Kubicek & Samuels	2012	CBS	130628	1	*rpb2* is not unique	
300	368	3		T.	*solani*	Samuels	2012	CBS	130506		GCPSR, only secondary DNA Barcodes	
290	172	3		T.	*gracile*	Samuels & Szakács	2012	CBS	130714		GCPSR, only secondary DNA Barcodes	
280	75	3		T.	*capillare*	Samuels & Kubicek	2012	CBS	130629			
270	81	3		T.	*centrosinicum*	Qin & Zhuang	2016	HMAS	252910		not in NCBI, sequenced	
260	150	5		T.	*flaviconidium*	(Chaverri, Druzhin. & Samuels) Jaklitsch &	2014	CBS	130688			
255	438	3		T.	*virgatum*	Cserjesi & Johnson	1972	ATCC	24961		*citrinoviride*	
250	98	3		T.	*citrinoviride*	Bissett	1984	CBS	258.85			
245	149	3		T.	*flavescens*	Zhu, Zhuang & Li	2017	HMJAU	34730		only secondary DNA Barcodes	
240	207	3		T.	*kunigamense*	Yabuki & Okuda	2014	TNS-F	38436		not in Mycobank	
230	424	3		T.	*tsugarense*	Yabuki & Okuda	2014	NBRC	109641		not in Mycobank	
220	322	3		T.	*pseudokoningii*	Rifai	1969	CBS	408.91	1	*rpb2* is not unique	
210	148	3		T.	*flagellatum*	Mulaw, Kubicek & Samuels	2012	CBS	130626	1	*rpb2* is not unique	
202	417	3		T.	*todica*	Sokoloff & Toda	1967			-	*ghanense*	na
202	277	3		T.	*paraceramosum*	Bissett	1992			-	*ghanense*	na
201	284	3		T.	*parceramosum*	Bissett	1992			-	*ghanense*	na
200	166	3		T.	*ghanense*	Doi, Abe & Sugiy.	1987	ATCC	208858			
190	350	3		T.	*saturnisporum*	Hammill	1970	CBS	330.7			
180	135	3		T.	*effusum*	Bissett, Kubicek & Szakács	2003	DAOM	230007			
170	143	3		T.	*euskadiense*	Jaklitsch & Voglmayr	2015	CBS	130013			
165	88	3		T.	*chetii*	du Plessis, Druzhin., Atan., Yarden & Jacobs	2018	PPRI	19363		GCPSR, no DNA Barcoding	
160	25	3		T.	*andinense*	(Samuels & Petrini) Samuels, Jaklitsch &	2014	CBS	345.97			
150	349	3		T.	*saturnisporopsis*	Samuels & Jaklitsch	2012	CBS	128829			
140	265	3		T.	*novae-zelandiae*	(Samuels & Petrini) Jaklitsch & Voglmayr	2014	CBS	639.92			

species number against time (Fig. 3). In 2006, the first 100 species were characterized using molecular data (Druzhinina et al. 2006), which was predicted to be accounted for one-half of the total diversity at that time, while in 2013, the number of DNA barcoded species doubled to about 200 (Atanasova et al. 2013). The maximum productivity of taxonomy providers was reached between 2014 and 2017 when > 50 molecularly characterized *Trichoderma* species were added per year (Fig. 3). The recent growth of molecular diversity in this genus has slightly declined, but it still leads to the addition of roughly a dozen new molecularly defined *Trichoderma* basionyms each year.

By the year 2020, most *Trichoderma* species have been characterized using DNA-based techniques. We have counted only 14 currently valid names that have not been characterized molecularly because DNA extraction from

Table 2 (continued)

				Species	Authority	Year	Collection	No.		Comment	
130	16	3	T.	*alboviride*	Chen & Zhuang	2017	HMAS	247224		not in NCBI, sequenced	
125	289	3	T.	*patellotropicum*	Samuels	2015	CBS	110084		no GCPRS, no DNA Barcoding	
120	288	3	T.	*patella*	(Cooke & Peck) Jaklitsch & Voglmayr	2014	CBS	110081			
100	203	3	T.	*konilangbra*	Samuels, Petrini & Kubicek	1998	CBS	100808			
91	362	3	T.	*sinensis*	Bissett, Kubicek & Szakács	2003			-	*sinense*	na
90	361	3	T.	*sinense*	Bissett, Kubicek & Szakács	2003	DAOM	230004			
80	167	3	T.	*gillesii*	Samuels	2012	CBS	130435		GCPSR, only secondary DNA Barcodes	
70	396	3	T.	*subiculoides*	Zeng & Zhuang	2019	HMAS	254600		Nom. illegit., sequenced	
60	392	3	T.	*subalpinum*	Jaklitsch	2011	CBS	119128			
50	293	4	T.	*peltatum*	(Berk.) Samuels, Jaklitsch & Voglmayr	2014	*G.J.S.*	08-207			
40	287	4	T.	*parmastoi*	(Overton) Jaklitsch & Voglmayr	2014	TFC	97-143			
30	309	4	T.	*polyalthiae*	Nuankaew & Boonlue	2018	TBRC	8737			
10	18	4	T.	*alcalifuscescens*	(Overton) Jaklitsch & Voglmayr	2014	CBS	122303			
0	70		T.	*caesium*	Pers.	1794			-	*name not in use*	na
0	261		T.	*nigrescens*	Pers.	1794			-	*name not in use*	na
0	342		T.	*roseum*	Pers.	1794			-	*name not in use*	na
0	425		T.	*tuberculatum*	Pers.	1795			-	*name not in use*	na
0	44		T.	*aureum*	Pers.	1796			-	*name not in use*	na
0	211		T.	*laeve*	Pers.	1796			-	*name not in use*	na
0	133		T.	*dubium*	Pers.	1801			-	*name not in use*	na
0	161		T.	*fuliginoides*	Pers.	1801			-	*name not in use*	na
0	332		T.	*pyrenium*	Pers.	1801			-	*name not in use*	na
0	58		T.	*brassicae*	Schumach.	1803			-	*name not in use*	na
0	78		T.	*carneum*	Schumach.	1803	DAOM	230012	-	*name not in use*	na
0	162		T.	*fuscum*	Schumach.	1803			-	*name not in use*	na
0	212		T.	*laeve*	Schumach.	1803			-	*name not in use*	na
0	292		T.	*pedunculatum*	Schumach.	1803			-	*name not in use*	na
0	333		T.	*pyrenium*	Schumach.	1803			-	*name not in use*	na
0	73		T.	*candidum*	Alb. & Schwein.	1805			-	*name not in use*	na
0	134		T.	*dubium*	Alb. & Schwein.	1805			-	*name not in use*	na
0	177		T.	*guttatum*	Alb. & Schwein.	1805			-	*name not in use*	na
0	5		T.	*aeruginosum*	Link	1816			-	*name not in use*	na
0	429		T.	*varium*	Ehrenb.	1818			-	*name not in use*	na
0	171		T.	*globosum*	Schwein.	1822			-	*name not in use*	na
0	253		T.	*mycophilum*	(Pers.) Schwein.	1822			-	*name not in use*	na
0	372		T.	*spadiceum*	Schwein.	1822			-	*name not in use*	na

old specimens failed (e.g., *T. latizonatum*, *T. sino-australe*, and *T. viridiflavum*) or samples were not available for this analysis (Bissett et al. 2015; Zhu and Zhuang 2014). The following eight unsequenced members of *Trichoderma* were transferred to this genus from *Sarawakus* (Hypocreales): *T. hexasporum*, *T. fragile*, *T. izawae*, *T. rosellum*, *T. sordidum*, *T. subtrachycarpum*, *T. succisum*, and *T. trachycarpum* by Jaklitsch et al. (2014) and *T. brevipes* from *Cordyceps* (Hypocreales) (Bissett et al. 2015) (Table 2). None of these species are available for DNA barcoding.

The remaining 361 *Trichoderma* species (96%) have been sequenced for at least one DNA Barcoding locus. In the following section, we provide an overview of the taxonomy and molecular identifiability of these species.

Table 2 (continued)

0	153	T.	*flavum*	Sommerf.	1826			-	*name not in use*	na
0	346	T.	***rubropallens***	Schwein.	1832			-	not sequenced	
0	95	T.	*cinnabarinum*	Wallr.	1833			-	*name not in use*	na
0	294	T.	*penicillatum*	Wallr.	1833			-	*name not in use*	na
0	298	T.	*pezizoideum*	Wallr.	1833			-	*name not in use*	na
0	174	T.	*granulosum*	Fuckel	1870			-	*name not in use*	na
0	222	T.	*lignorum*	(Tode) Harz	1872			-	*name not in use*	na
0	455	T.	*vulpinum*	Fuckel	1874			-	*name not in use*	na
0	215	T.	*lateritio-roseum*	Lib. ex Cooke	1880			-	*name not in use*	na
0	100	T.	*coliae*	(Schwein.) Sacc.	1886			-	*name not in use*	na
0	334	T.	*racemosum*	McAlpine	1902			-	*name not in use*	na
0	435	T.	*violaceum*	Oudem.	1904			-	*name not in use*	na
0	250	T.	*minutum*	Bainier	1906			-	*name not in use*	na
0	108	T.	*corfecianum*	Sacc.	1911			-	*name not in use*	na
0	446	T.	*varians*	Sartory & Bainier	1912			-	*name not in use*	na
0	128	T.	***desrochii***	Sartory & Bainier	1913			-	not sequenced	
0	262	T.	*nigrovirens*	Goddard	1913			-	*name not in use*	na
0	107	T.	*cordobense*	Speg.	1926			-	*name not in use*	na
0	254	T.	*narcissi*	(Tochinai & Shimada) Tochinai & Shimada	1931			-	*name not in use*	na
0	266	T.	*nunbergii*	Svilv.	1932			-	*name not in use*	na
0	404	T.	*sympodianum*	Kulik	1960			-	*name not in use*	na
0	247	T.	*minima*	(Speg.) Gunth. Müll.	1965			-	*name not in use*	na
0	248	T.	*minimum*	(Speg.) Gunth. Müll.	1965			-	*name not in use*	na
0	29	T.	*arachnoidea*	Kuritzina & Sizova	1967			-	*name not in use*	na
0	30	T.	*arachnoideum*	Kuritzina & Sizova	1967			-	*name not in use*	na
0	264	T.	*nigrovirens*	Chaverri & Samuels	2001	*G.J.S.*	99-64	-	*name not in use*	na
0	120	T.	*cuneisporum*	Chaverri & Samuels	2003			-	*name not in use*	na
0	263	T.	*nigrovirens*	Chaverri & Samuels	2003			-	*name not in use*	na
0	159	T.	***fragile***	(Doi) Jaklitsch & Voglmayr	2014			-	not sequenced	
0	187	T.	***hexasporum***	(Boedijn) Jaklitsch & Voglmayr	2014			-	not sequenced, *Sarawakus*	
0	201	T.	***izawae***	(Doi) Jaklitsch & Voglmayr	2014			-	not sequenced, *Sarawakus*	
0	341	T.	***rosellum***	Jaklitsch & Voglmayr	2014			-	not sequenced, *Sarawakus*	
0	364	T.	***sinoaustrale***	Zhu & Zhuang	2014	HMAS	23403	-	not sequenced	
0	371	T.	***sordidum***	(Doi) Jaklitsch & Voglmayr	2014			-	not sequenced	
0	398	T.	***subtrachycarpum***	(Doi) Jaklitsch & Voglmayr	2014			-	not sequenced, *Sarawakus*	
0	400	T.	***succisum***	(Rifai) Jaklitsch & Voglmayr	2014			-	not sequenced, *Sarawakus*	
0	419	T.	***trachycarpum***	(Syd.) Jaklitsch & Voglmayr	2014			-	not sequenced, *Sarawakus*	
0	450	T.	***viridiflavum***	Zhu & Zhuang	2014	HMAS	252549	-	not sequenced	
0	111	T.	*corrugatum*	(Doi, Liu & Tamura) Liu, Zhu & Zhuang	2014			-	*name not in use*	na
0	62	T.	***brevipes****	(Mont.) Samuels	2015	CBS	139044	-	not sequenced, Cordyceps brevipes, Podostroma brevipes	
0	216	T.	***latizonatum***	(Peck) Samuels	2015			-	not sequenced	

An online (updatable) version of this table is available at www.trichokey.com

PhyloOrder—order on a whole-genus *rpb2* phylogram (Fig. 6). This category determines neighboring species. **Species name**—names that are not in use are shaded gray. **Phylo Spec Hyp**—phylogenetic species hypothesis. Strong—the species has been recognized based on the genealogical concordance phylogenetic species recognition (GCPSR) concept applied to several strains and multiple loci; valid—GCPSR was applied, but the number of strains or loci was limited; weak—the strict sense of GCPSR was compromised by either insufficient number of loci or/and low polymorphism of *rpb2*; NO—GCPSR concept was not applied; na—the application of GCPSR concept is not possible. **Identifiability**—describes the possibility of the precise and accurate molecular identification of this species. NO—the name is not in use; OK—the identification is possible; warning—the identification is compromised, see comments. **Occurrence** is a conventional parameter that reflects the frequency of species sequences deposition in NCBI GenBank. This parameter should be critically considered as it may be influenced by incorrect sequence identification in public databases. Comments contain either currently correct names or explanations for identifiability warnings. See https://trichokey.com/index.php/trichoderma-taxonomy-2020

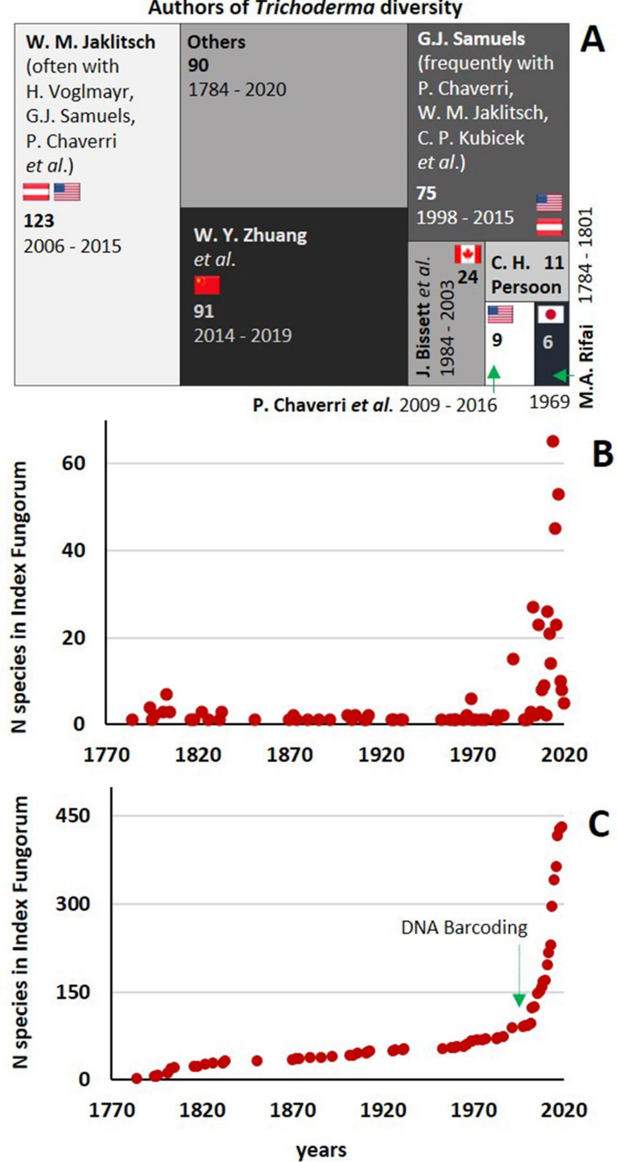


Fig. 3 Development of *Trichoderma* taxonomy over the last 236 years. **a** Groups of the most significant providers of *Trichoderma* taxonomy. **b** The number of *Trichoderma* species introduced to IndexFungorum per year. **c** The total number of *Trichoderma* species recorded in IndexFungorum

Reference materials that are available for molecular identification of *Trichoderma*

We first reviewed the *Trichoderma* species names that were deposited into the three main mycological taxonomic databases by May 2020. The largest number (all/valid) were recorded in Mycobank (436/361) and Index Fungorum (422/359) (See "Assumptions" *above*). The NCBI Taxonomy browser contained 336 names, among which 12 are not in use (Table 2), as follows: *T. album, T. glaucum*, *H. pachybasioides*, *T. luteffusum*, *T. fomitopsis*, *T. subsulphureum, T. undatipile*, *T. cyanodichotomus*, *T. subalni*, *T. rugosum*, *T. acremonioides*, and *T. subiculoides*. The four currently abandoned names—*T. album*, *T. glaucum*, *T. fomitopsis*, and *T. subsulphureum*,—were retrievable as valid from all three databases.

Although all three depositories are powered with an option to distinguish between currently legitimate names, synonyms, and names that are not in use, these records showed frequent disagreements. In Mycobank, *T. album* is correctly synonymized with *T. polysporum*, while in IndexFungorum, *T. citrinum* is listed as the currently correct name. The NCBI Taxonomy browser has no notes on the current status of *T. album* while 17 DNA sequences are attributed to this outdated taxonomic name, which appears in similarity search results (BLAST). Thus, none of the three depositories contain all 375 taxonomically valid names of *Trichoderma* spp. Therefore, none of the databases can be considered to be the only sufficient reference for currently valid *Trichoderma* nomenclature. Only 309 (82%) currently accepted names were deposited into all three databases (Table 2).

The description of the new fungal species requires deposition of the name into MycoBank (Seifert and Rossman 2010; May et al. 2019). Upon acceptance of the publication, a taxonomy provider (the author of the species name) is expected to manually release the name in this database for consideration by the curators. The name will be automatically copied to Index Fungorum without any manual update (Redhead and Norvell 2012), and therefore, these two databases will have concordant records. However, at least for *Trichoderma*, the validity of all names should still be cautiously considered, irrespective of the entry date.

The deposition of the name into the NCBI Taxonomy Browser is only possible along with the submission of DNA barcode sequences. Thus, this database does not contain currently used taxonomic names of the species for which DNA barcode sequences are not available.

Alternatively, deposition into the NCBI GenBank (and the Taxonomy Browser, respectively) without the deposition into Mycobank/Index Fungorum leads to an invalid species description (May et al. 2019). Some names have been abandoned by Mycobank/Index Fungorum because of the application of the "One fungus–one name" concept (Taylor 2011), which is specified for the order Hypocreales in Rossman et al. (2013), but it is still being recorded in the NCBI Taxonomy Browser. In *Trichoderma*, it refers to the name of the teleomorphic stage *Hypocrea*, or species of such sister genera as *Protocrea* (Jaklitsch 2009) or *Sarawakus* (Jaklitsch et al. 2014), which have been transferred to *Trichoderma*. For example, the NCBI Taxonomy Browser links the currently unused name "*Hypocrea pachybasioides* Doi 1972" to the correct name *T. polysporum*, but the "Definition" of the numerous individual sequences of *T. polysporum* remains

"*Hypocrea pachybasioides*". This disagreement should be considered when the results of the sequence similarity search (BLAST) against the NCBI GenBank are evaluated (*see below*). Five recently introduced species names were present in NCBI Taxonomy Browser but not deposited in Mycobank/Index Fungorum (Table 2). We assigned them as invalid for now based on the *Code* (May et al. 2019). Among them, *T. cyanodichotomus* is noted in the NCBI Taxonomy Browser as "*Trichoderma cyanodichotomus* J.S. Li & K. Chen, 2018, nom. inval." with the note "Nom. inval. (i.e., *nomen invalidum*, or invalid name) refers to a name that is not published in accordance with rules that were enumerated in the ICN", while *T. subalni*, *T. rugosum*, *T. acremonioides*, and *T. subiculoides* are not noted as such. *T. dorothopsis* (Tomah et al. 2020) has been deposited into MycoBank but not yet released. Therefore, we consider this species name to be valid. Thus, the status of each species name should be verified using multiple sources. Table 2 is designed to aid this search.

The name of the generic type species (*Trichoderma viride*) is presented differently in the three databases. The NCBI Taxonomy Browser contains *T. viride* Pers. 1832, while MycoBank and Index Fungorum refers to *T. viride* Pers. 1794, which is absent in the NCBI Taxonomy Browser. Jaklitsch et al. (2006) outlined the history of this species description in the 18th to 19th centuries, which allowed them to conclude that the correct taxonomic name should refer to both publications and be presented as *Trichoderma viride* Pers., Neues Mag. Bot. ([Roemer's] 1: 92. 1794: Fries, Syst. Mycol. 3: 215. 1832) (Jaklitsch et al. 2006). However, none of the databases accepts the double records for the authors, publications, and years, and only one of them should be chosen (Table 2).

To review the material that is available for molecular identification of *Trichoderma* species, we manually recorded the distribution of DNA barcodes that were deposited in the NCBI GenBank per each *Trichoderma* species that were recorded in NCBI Taxonomy Browser (Table 2). This analysis aimed to reveal gaps in the deposition of DNA barcoding markers, but could not allow verification of the correctness of available materials (see "Assumptions"). It showed that 224 (66%) *Trichoderma* species were characterized by four or more loci, 80 (22%) species were characterized by three loci, and 35 (10%) remain characterized by one or two loci. The most commonly deposited DNA barcode loci were *tef1* (322) and *rpb2* (310), followed by ITS (293). For 270 species (76% from the molecularly characterized and 72% from all taxa), these three DNA barcodes were available, and *tef1* and *rpb2* were available for 307 species (85% and 82%, respectively). ITS was missing for 73 (20%), *rpb2* was missing for 56 (16%), and *tef1* was missing for 43 (12%) species. The other phylogenetic markers were deposited for considerably fewer species, as follows: *acl1* for 140 (39%), *cal1* for 113 (32%), *act* for 103 (29%), and *chi18-5* for 87 (24%). Genes encoding LSU and SSU rRNA loci were sequenced for the small number of species (Table 2).

This analysis shows that the providers of molecular taxonomy of *Trichoderma* agreed on the use of the three DNA barcode loci (ITS, *rpb2*, and *tef1*) and deposited them for most of the molecularly characterized species. Consequently, independent of their properties and suitability for the purpose, only ITS, *rpb2*, and *tef1* can be used for molecular identification of contemporary diversity of *Trichoderma*. The community of *Trichoderma* taxonomy providers currently has no agreement on the suitability of other loci. Therefore, all other markers have incomparably smaller collections of reference sequences and cannot be considered for the comparison unless reference strains are available for sequencing. Below, we will also show that this lack of agreement and the resulting incomplete databases for phylogenetic loci and their distribution along the infrageneric clades considerably and adversely influenced the process of species delimitation by the taxonomists.

Properties of ITS, *rpb2*, and *tef1* DNA barcoding markers for *Trichoderma* spp.

In this study, we aimed to expand upon the protocol for accurate and unambiguous molecular identification of existing *Trichoderma* spp. based on the available DNA barcodes. In the following section, we estimate the genus-wide differences and similarities between the three DNA barcoding loci that are available for most molecularly defined species.

ITS is required to identify the genus *Trichoderma*

The theory suggests that accurate and precise molecular identification of such common and large fungal genera as *Trichoderma*, *Fusarium*, *Aspergillus*, and the others relies of the combined use of primary and secondary DNA barcodes (Stielow et al. 2015; Bissett et al. 2015; O'Donnell et al. 2015; Sklenar et al. 2017). The complete ITS region or more precisely, the internal transcribed spacers 1 and 2 of the rRNA gene cluster (See Box 1 and the discussion on the structure of ITS DNA barcoding locus below, Fig. 9), has been assigned as the primary DNA barcode marker for all fungi (Schoch et al. 2012). Although this locus can have insufficient polymorphism at a species level and numerous fungal sister species cannot be distinguished by the comparison of ITS sequences (e.g., Atanasova et al. 2013; Stielow et al. 2015; O'Donnell et al. 2015; Sklenar et al. 2017), it has the advantages of easy amplification and of the largest reference database (Nilsson et al. 2019; Schoch et al. 2020). The latter makes it more suitable for metabarcoding of fungal communities (Tedersoo et al. 2014; Abdelfattah et al. 2015) and thereby leads to the rapid growth of the number of

records on the environmental ITS sequences (usually either ITS1 or ITS2) that are deposited in public databases [e.g., UNITE (Nilsson et al. 2019)].

ITS was the first locus that was introduced in DNA Barcoding of *Trichoderma* in late 1990s (Kuhls et al. 1996), while in 2005, we used it to develop the on-line oligonucleotide DNA Barcoding tool to identify all 88 *Trichoderma* species that have been molecularly characterized at that time (Druzhinina et al. 2005). Although most species were reliably identified by the unique combinations of oligonucleotide ITS hallmarks, sister species such as *T. longibrachiatum* - *T. orientale*, *T. koningii* - *T. ovalisporum*, and others could not be distinguished at that time. Since then, and particularly along with the recent boom of *Trichoderma* taxonomy in 2014–2017, ITS was repeatedly criticized for the high number of homoplasious sites that evolve due to the high mutation rate and saturation (Samuels et al. 2006; Druzhinina et al. 2005; Chaverri et al. 2015) and for its insufficient resolution at the species level (Atanasova et al. 2010; Druzhinina et al. 2012; Sandoval-Denis et al. 2014; Samuels et al. 2006). Therefore, this locus has even been abandoned in some large surveys of *Trichoderma* diversity (Jaklitsch 2009, 2011; Jaklitsch and Voglmayr 2015), resulting in the description of at least 73 species that were not characterized by ITS (Table 2). This essentially compromised the status of ITS as a primary DNA barcode locus, at least for *Trichoderma* spp. identification.

In this study, we analyzed the pairwise similarities between the full-length reference ITS sequences (including the 5.8S rRNA gene, see the exact length in the Supplementary Datasets) representing all infrageneric groups of *Trichoderma* and compared it to sequences of *Protocrea*, *Hypomyces*, *Escovopsis*, *Sepedonium*, *Cladobotryum*, *Sphaerostilbella*, *Hypocreopsis*, *Mycogone*, and *Beauveria* (all from Hypocreales). The polymorphism reached 300 mutations from the total length of 760 base pairs in the alignment (63% similarity) (Fig. 4). However, we noticed that the ITS sequences in *Trichoderma* were significantly more similar to each other compared to the related genera (Fig. 4). The heat map and the principal component analysis showed that the infrageneric similarity of ITS in *Trichoderma* spp. is between 71 and 100% while the similarity between *Trichoderma* spp. and the currently recognized neighboring genera is almost 76%, which indicates that if a query ITS sequence shares a similarity ≥ 76% to at least one of the known *Trichoderma* spp., it most likely belongs to *Trichoderma* genus, and vice versa. This calculation allowed us to compose an *ITS56 Dataset* that contains representative ITS sequences from the genus *Trichoderma.* The dataset can be used for the identification of a query sequence on the generic level if its similarity is ≥ 76% to at least one of the records in the dataset (Supplementary Datasets). We then verified the above assumption by particularly checking the sequences of “basal” species from the genus *Trichoderma* such as *T. albolutescens* (Jaklitsch 2011), *T. undulatum* (du Plessis et al. 2018), and *T. alcalifuscescens* (Overton et al. 2006; Jaklitsch and Voglmayr 2013) that were characterized by the relatively long genetic distance to the core species of the genus (Jaklitsch and Voglmayr 2013). Moreover, this threshold was not contradicted by the results that were generated from other loci (*see below*).

Similar to previous studies, we also revealed that many closely related *Trichoderma* species shared the same ITS phylotypes [Fig. 4, (Samuels et al. 2006; Druzhinina et al. 2006, 2012)]. Thus, this locus cannot be used for the identification at the species level. We also showed that although ITS sequences are highly conserved between some infrageneric groups of *Trichoderma* (Section *Trichoderma* or *Viride* Clade, Fig. 4), it is not suitable for the identification of currently proposed infrageneric groups, which is likely due to the high level of homoplasious sites (Druzhinina et al. 2005; Sandoval-Denis et al. 2014).

We conclude that because ITS is highly diagnostic at the genus level and provides essential information for the molecular identification of *Trichoderma* spp., it remains the primary locus that is required for DNA Barcoding.

***Trichoderma* species can be identified based on ≥ 99% and ≥ 97% pairwise similarities of *rpb2* and *tef1*, respectively**

We then analyzed pairwise interspecific similarity values for the two other DNA barcoding loci that are available for *Trichoderma*—the partial sequences of *rpb2* and *tef1* (Fig. 4) genes. The exact length of the used fragments is given in the Supplementary Datasets and discussed *below*, Fig. 9). For this reason, we collected reference strains for all DNA barcoded species (Table 2) and used NCBI Entrez to retrieve the respective sequences. The lists of accession numbers for DNA sequences in public databases are highly prone to errors and become rapidly outdated because of taxonomic revisions of individual fungal groups. Therefore, we provided the list of suggested reference strains. We would like to recommend that taxonomy users address the literature and retrieve the reference strains for species of interest and then search the databases for the corresponding DNA barcode sequences. In this study, the correctness of each sequence was verified using taxonomic literature and records in Index Fungorum, MycoBank, and/or NCBI Taxonomy Browser. The sequences were trimmed to the standard length of a phylogenetic marker that was established for *Trichoderma* [*see below*, Kopchinskiy et al. (2005) and “Materials and Methods”].

The results indicated that the genetic border of the genus was not apparent on *rpb2* or *tef1* similarity plots (data not

Fig. 4 Sequence pairwise similarities of the three main DNA barcoding loci of *Trichoderma*. **a** Heatmap of ITS pairwise similarity between *Trichoderma* and other Hypocreales and within *Trichoderma* genus. Representative ITS sequences from 56 type strains belonging *Trichoderma* spp. (see *ITS56 Dataset* in Supplementary Datasets) and 22 other Hypocreales were respectively collected. **b** Principal component analysis (PCA) of the ITS pairwise similarity matrix. **c** Heatmap of *rpb2* pairwise similarities within *Trichoderma* genus (355 species). **d** Heatmap of *tef1* pairwise similarity within *Trichoderma* genus (200 species that produced significant alignment)

shown). Therefore, these two loci cannot be used for identification at the generic level.

The sequences of *tef1* (Box 1) were highly polymorphic (Fig. 4) and showed > 50% of mismatches between individual fragments, and therefore, they frequently did not produce a statistically significant alignment for most of their length. Consequently, most individual species can be distinguished by the *tef1* DNA barcode (Fig. 4). The high level of *tef1* polymorphism has the drawback of a high level of infraspecific variability that can lead to ambiguity and false-positive species hypotheses. Thus, a single 28 bp indel in the *tef1* sequence was used to recognize a cryptic species *T. bissettii* within the common putative agamospecies *T. longibrachiatum* (Sandoval-Denis et al. 2014). However, the polyphasic approach, i.e. the application of the GCPSR concept integrated with the detailed ecophysiological profiling and analysis of biogeography did not support the existence of *T. bissettii* as a single taxon because no other differences were detected (Hatvani et al. 2019).

Reference strains of several currently valid species shared highly similar (> 99.5%) phylotypes of *tef1* (for example, *T. afarasin* and *T. endophyticum*). Moreover, the history of *tef1* application for DNA Barcoding consists of several periods when researchers used different fragments of this large gene for phylogenetic reconstructions (Druzhinina and Kubicek 2005). Thus, in the early 2000s, we used the short fifth intron

of this gene, and J. Bissett's group then tested the applicability of the first two introns at the 5′ end of the gene, while P. Chaverri and G. J. Samuels et al. proposed the large portion of the last (sixth) exon (Chaverri and Samuels 2003). Most resolution is provided by the fragment spanning over the fourth intron, fifth exon, and fifth intron (Kopchinskiy et al. 2005). Consequently, the NCBI GenBank contains all these frequently non-overlapping fragments of the *tef1* gene, which complicates its use and in particular affects the results of the sequence similarity search. Together, these findings make the *tef1* locus insufficient to be used as the only DNA barcode marker for *Trichoderma* identification at the species level as it was also proposed by Rahimi et al. (2020) for the identifiction of *T. reesei*. The limitations outlined above also reveal that the application of *tef1* together with ITS will not allow unambiguous identification of *Trichoderma* species.

The sequences of *rpb2* (Box 1) were most conserved because many *Trichoderma* spp. shared highly similar phylotypes. Figure 4c shows large clusters of highly similar species and even clades indicating that the single use of this DNA barcode was also not suitable for species identification.

Thus, currently none of the three DNA barcode loci can be used as a sole sufficient marker for the identification of the 361 *Trichoderma* species.

In this study, we aim to determine how to distinguish currently valid *Trichoderma* species using the DNA barcode sequences that have been provided. To assess the sequence similarity threshold in a manner that is sufficient to identify species, we screened the subclades of species that exhibited highly similar *rpb2* and *tef1* sequences (Fig. 5). In such groups, we ignored rare species that were available from a low number of isolates, and focused on the well-established and common species with recorded values for humankind. As a reference example, we selected (1: *reesei*) the main industrial cellulase producer *T. reesei* (e.g., Druzhinina et al. 2016) and two of its sibling species *T. parareesei* (Atanasova et al. 2010) and *T. thermophilum* (Qin and Zhuang 2016a). (2: *harzianum*) The most common environmental opportunistic species with high suitability for biocontrol, plant growth promotion, and enzyme production are as follows: *T. harzianum* (Chaverri et al. 2015), and the two sibling species, *T. afroharzianum* (Chaverri et al. 2015) and *T. guizhouense* (Li et al. 2013; Grujic et al. 2019); and (3: *asperellum*) another common species with multiple applications in agriculture, *T. asperellum* (Rivera-Méndez et al. 2020) and the two recently recognized sibling species, *T. asperelloides* (Samuels et al. 2010) and *T. yunnanense* (Yu et al. 2007).

ITS was polymorphic in the 2: *harzianum* group, but *T. reesei–T. parareesei* (the 1: *reesei* group) and *T. asperelloides–T. yunnanense* (the 3: *asperellum* group) shared the same ITS phylotypes. In all three groups, the *rpb2* sequences were different, with similarities that were 98.15–98.77% for the 1: *reesei* group, 94.93–95.82% for the 2: *harzianum* cluster, and 98.65–99.14% for 3: *asperellum*. Thus, if none of these species hypotheses to be rejected based on *rpb2*, *Trichoderma* species should be only by 1% different. It corresponds to the maximum level of infraspecific polymorphism of eight mutations (substitutions or indels) if the total length of the alignment is fixed to the diagnostic region of 820 base pairs (see Fig. 9 below and "Materials and Methods" for the details). Thus, assignment to an existing species is possible if the similarity of *rpb2* is ≥ 99%. However, in this case, the uniqueness of *T. yunnanense rpb2* appears to be compromised (Fig. 5, Table 2).

Similar consideration of the *tef1* polymorphism resulted in 82.63–96.10% similarities between the 1: *reesei* group, 80.29–86.85% for the 2: *harzianum* cluster, and 89.29–95.39% for the 3: *asperellum* group. Thus, these species can be distinguished based on *tef1* similarity < 97% or identified based on ≥ 97%. This assumes that different strains of the same species can have up to 27 mutations in the diagnostic area of the *tef1* DNA barcode, which agrees well with the species where large populations were studied (Druzhinina et al. 2012; Hatvani et al. 2019).

We, therefore, conclude that a query strain can be assigned to the existing *Trichoderma* species if it is ≥ 99% similar for *rpb2* and has ≥ 97% *tef1* similarities to that of the reference strains. The molecular identification can only be achieved if both loci point to the same result species.

The high level of infrageneric conservation of *rpb2* (Atanasova et al. 2013; Jaklitsch 2009, 2011; Jaklitsch and Voglmayr 2015) has the advantage that allows construction of the most complete phylogram for the genus *Trichoderma* (Fig. 6) and, thus, reveal the "phylogenetic order" ("PhyloOrder") of the species that is provided in Table 2. To achieve this for all DNA barcoded 361 species, the approximate position of the species for which *rpb2* is not available or for which it is available but not attributed to the species in the NCBI Taxonomy Browser was determined based on the similarities of other loci and respective taxonomic literature (Fig. 6). The phylogenetic analysis of the alignment of 356 *rpb2* sequences revealed at least eight statistically supported *rpb2*-based infrageneric clades that largely correspond to those presented in previous reviews of *Trichoderma* taxonomy (Atanasova et al. 2013). To avoid further confusion and discrepancies, we skipped naming the clades, but we numbered them and highlighted the most prominent species within each clade (Fig. 6, Table 2).

Sorting all molecularly defined *Trichoderma* species according to their approximate phylogenetic position in Table 2 ("PhyloOrder") revealed the distribution of other phylogenetic markers (*chi18-5* = *ech42*, *cal1*, *act*, *acl1*,18S rRNA = SSU, 28S rRNA = LSU) along the genus genealogy. This demonstrates that the usability of such loci is limited

Fig. 5 Sequence pairwise similarities of each DNA barcoding locus between sets of selected model species. The three closely related sibling species. *T. reesei, T. parareesei,* and *T. thermophium* represent the *Longibrachiatum* Clade; *T. harzianum*, *T. afroharzianum,* and *T. guizhouense* represent the *Harzianum* Clade; and *T. asperellum*, *T. asperelloides,* and *T. yunnanense* represent the Section *Trichoderma*. Sequences were collected from the type strains and consistently trimmed as described in the Materials and Methods and in Fig. 9

Clade	Species	Pairwise similarity, %									Max
		T. reesei	*T. parareesei*	*T. thermophilum*	*T. harzianum*	*T. afroharzianum*	*T. guizhouense*	*T. asperellum*	*T. asperelloides*	*T. yunnanense*	
ITS											**100**
Longi-brachiatum	*T. reesei*										100
	T. parareesei	100.00									
	T. thermophilum	-	-								
Harzianum	*T. harzianum*										100
	T. afroharzianum				97.14						
	T. guizhouense				96.95	99.81					
Trichoderma	*T. asperellum*										100
	T. asperelloides							99.61			
	T. yunnanense							99.61	100.00		
rpb2											**99**
Longi-brachiatum	*T. reesei*										99
	T. parareesei	98.77									
	T. thermophilum	98.40	98.15								
Harzianum	*T. harzianum*										96
	T. afroharzianum				95.82						
	T. guizhouense				94.98	94.93					
Trichoderma	*T. asperellum*										99
	T. asperelloides							98.65			
	T. yunnanense							99.14	98.28		
tef1											**97**
Longi-brachiatum	*T. reesei*										97
	T. parareesei	82.63									
	T. thermophilum	89.36	96.10								
Harzianum	*T. harzianum*										87
	T. afroharzianum				80.29						
	T. guizhouense				85.57	86.85					
Trichoderma	*T. asperellum*										95
	T. asperelloides							90.06			
	T. yunnanense							95.39	89.29		

because none of *Trichoderma* clades have a complete reference dataset for any of them. Therefore, they can only be used if the providers of *Trichoderma* taxonomy will complement missing sequences or if all *Trichoderma* reference strains will become available for the research community (see "Discussions and suggestions" *below*). Consequently, molecular identification of *Trichoderma* spp. is only possible based on ITS, *tef1* and *rpb2* that are available in public databases.

Accuracy, precision, and ambiguity in DNA Barcoding of *Trichoderma*

With all the molecularly defined *Trichoderma* spp. ordered based on their approximate phylogenetic relation, we can estimate the potential identifiability of individual species and list warnings that should be considered by the users of *Trichoderma* taxonomy (Table 2).

Our analysis suggests that for at least 216 *Trichoderma* species (60%), molecular characteristics are sufficient for

1
aerugineum, afarasin, afroharzianum, aggregatum, aggressivum, albocorneum, alni, alpinum, amazonicum, angustum, atrobrunneum, atrogelatinosum, attinorum, aureoviride, azevedoi, bannaense, barbatum, bomiense, breve, brevicrassum, britannicum, brunneoviride, byssinum, caesareum, camerunense, catoptron, ceraceum, ceramicum, ceratophylli, cerinum, chlamydosporicum, chlorosporum, christiani, chromospermum, cinnamomeum, compactum, concentricum, confertum, corneum, costaricense, crassum, cremeoides, cremeum, dacrymycellum, danicum, dichromospora, dimorphum, endophyticum, epimyces, estonicum, floccosum, fujianense, ganodermatis, gelatinosum, gliocladium, globoides, gregarium, guizhouense, hainanense, harzianum, hausknechtii, hebeiense, helicolixii, helicum, hengshanicum, hirsutum, hunanense, ingratum, inhamatum, italicum, ivoriense, koreanum, lanuginosum, lentiforme, liberatum, linzhiense, lixii, longifialidicum, longipile, longisporum, lycogaloides, medusae, mikurajimensis, neocrassum, parepimyces, parestonicum, peberdyi , perviride, phyllostachydis, pinicola, pleuroti, pleuroticola, pollinicola, polypori, priscilae, pseudocandidum, pseudodensum, pseudogelatinosum, pseudonigrovirens, purpureum, pyramidale, rifaii, rossicum, rosulatum, rufobrunneum, rugulosum, shennongjianum, sichuanense, silvae-virgineae, simmonsii, simplex, sinuosum, solum, spinulosum, spirale, stipitatum, stramineum, strictipile, stromaticum, sulawesense, surrotundum, tawa, tenue, thailandicum, thelephoricola, tibetense, tomentosum, tropicosinense, velutinum, vermipilum, verticillatum, virens, virescentiflavum, viridicollare, viridulum, zayuense, zeloharzianum, zonatum

2
changbaiense, fassatiae, fertile, fomiticola, hunua, mienum, moravicum, oblongisporum, semiorbis

3
aethiopicum, alboviride, ampulliformis, andinense, asterineum, beinartii, bissettii, britdaniae, caeruleimontis, capillare, centrosinicum, chetii, citrinoviride, effusum, euskadiense, flagellatum, flavescens, ghanense, gillesii, gracile, henanense, konilangbra, kunigamense, leguminosarum, longibrachiatum, novae-zelandiae, odoratum, orientale, parareesei, patella, patellotropicum, pinnatum, pseudobritdaniae, pseudokoningii, reesei, sambuci, saturnisporopsis, saturnisporum, sinense, solani, subalpinum, thermophilum, tremelloides, tsugarense, virgineum, xanthum

4
alcalifuscescens, avellaneum, delicatulum, flavipes, parmastoi, peltatum, polyalthiae

5
adaptatum, aeroaquaticum, aestuarinum, albofulvopsis, albofulvum, alutaceum, appalachiense, arenarium, asperelloides, asperellum, atlanticum, atroviride, austrokoningii, bavaricum, beijingense, bifurcatum, caerulescens, caribbaeum, cerebriforme, citrinella, compostícola, coprosmae, densum, dingleyae, dorotheae, dorothopsis, eijii, erinaceum, europaeum, evansii, flaviconidium, foliicola, gamsii, hamatum, hispanicum, hongkongensis, hubeiense, hypoxylon, intricatum, istrianum, junci, koningii, koningiopsis, kunmingense, lacuwombatense, leucopus, lieckfeldtiae, luteoeffusum, mangshanicum, martiale, matsushimae, mediterraneum, medogense, minutisporum, muroiana, neokoningii, neorufoides, neorufum, neosinense, neotropicale, nothescens, nybergianum, ochroleucum, olivascens, ovalisporum, pachypallidum, panacis, palidulum, parapiluliferum, pararogersonii, paratroviride, paraviridescens, paucisporum, petersenii, pezizoides, piluliferum, placentula, polysporum, poronioideum, pruinosum, pubescens, restrictum, rogersonii, rubi, samuelsii, scalesiae, sempervirentis, seppoi, shaoguanicum, sinokoningii, sinoluteum, songyi, sparsum, speciosum, sphaerosporum, stilbohypoxyli, strigosellum, strigosum, subeffusum, subviride, taiwanense, tardum, taxi, texanum, theobromicola, trixiae, valdunense, vinosum, viridarium, viride, viridescens, viridialbum, virilente, voglmayrii, vulgatum, yui, yunnanense

6
arundinaceum, aurantioeffusum, brevicompactum, cornu-damae, deliquescens, grande, limonium, luteocrystallinum, margaretense, melanomagnum, protrudens, rodmanii, subcitrina, turrialbense

7
americanum, applanatum, austriacum, balearicum, calamagrostidis, ceciliae, citrinum, confluens, crystalligenum, decipiens, eucorticioides, fruticola, megalocitrinum, microcitrinum, oligosporum, phellinicola, protopulvinatum, pseudolacteum, pseudostramineum, psychrophilum, pulvinatum, rhododendri, splendens, stercorarium, sulphureum, tiantangzhaiense, victoriense

albolutescens
8
undulatum
Hypomyces austrlasiaticus
Sphaerostilbella aureonitens
Arachnocrea stipata

Trichoderma

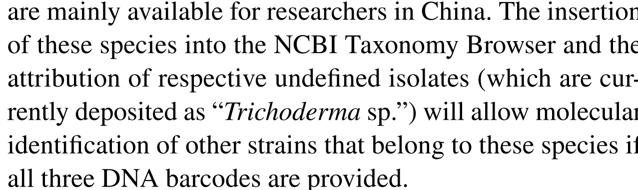
rpb2
0.06

Fig. 6 The list of all DNA barcoded *Trichoderma* spp. (361) sorted based on the phylogenetic position (PhyloOrder in Table 2). The core topology of the phylogram is based on the maximum-likelihood (ML) phylogeny of the currently *rpb2*-barcoded *Trichoderma* species. Eight main clades were collapsed and numerically named (see "Clade" in Table 2). Species names are sorted alphabetically within each clade. Well-known species are highlighted in red font for convenience purpose. The attribution of species that have no *rpb2* sequence available was approximately determined based on the other available loci. The nucleotide substitution model of TIM3 + F + R6 was chosen based on the Bayesian Information Criterion (BIC). Circles at the nodes indicate ultrafast bootstrap values > 80 given by IQ-TREE. The sequences of *rpb2* from *Arachnocrea stipata*, *Hypomyces austrlasiaticus,* and *Sphaerostilbella aureonitens* were used as the outgroups. The inset (top left) shows the complete topology of the *rpb2* phylogram

accurate and precise species identification based on three DNA barcodes (ITS, *tef1,* and *rpb2*) assuming that the deposited data are correct (Table 2) (See "Assumptions"). This group includes the most common species such as *T. harzianum* (= *T. harzianum* sensu stricto), *T. virens*, *T. gamsii*, *T. atroviride*, *T. koningiopsis*, *T. hamatum*, and *T. citrinoviride*, *T. reesei*, and around 100 rare species that are only known from a few or even one isolate (Table 2). Although these species have mostly complete records in all databases, some minor deviations should be considered. For example, *T. longipile* is deposited in IndexFungorum as *T. longipilis* (orthographic variant). *T. undatipile* Chen & Zhuang 2017 was molecularly characterized and deposited in MycoBank under its correct name, but it was deposited in IndexFungorum as *T. undatipilosum*. Four species, *T. pinicola*, *T. guizhouense*, *T. kunigamense*, and *T. tsugarense* are absent in MycoBank, which jeopardizes the validity of these taxa (Table 2).

Molecular identifiability of 141 *Trichoderma* species (40%) is compromised either by the lack of DNA barcodes or by the high similarity of *tef1* and/or *rpb2* sequences to their sister species. Among 73 species that lack ITS, 34 have *tef1* and *rpb2* and, therefore, can be potentially identified if their attribution to the genus is not in question. This group includes the very common or even dominant European species *T. europaeum* and *T. mediterraneum*, while many others are rare or very rare. Ten species, including *Hypocrea subcitrina*, *T. cornu-damae*, *H. dichromospora*, *T. aestuarinum*, *T. cerebriforme*, *T. poronioideum*, *T. densum*, *H. ampulliformis*, *T. surrotundum*, and *T. patellotropicum*, have ITS but lack either *tef1* or *rpb2* sequences and, therefore, cannot be accurately identified. It also suggests that these species were described without considering the GCPSR concept (see "Discussions and suggestions" *below*). *H. mikurajimensis* is only characterized using 28S rRNA sequence, and therefore, its molecular identification is not possible.

The following 37 species has been molecularly and phylogenetically characterized, but their taxonomic status was not updated in the NCBI Taxonomy Browser, and they are not available for sequence similarity search (Table 2): *T. limonium*, *T. grande*, *T. pruinosum*, *T. dimorphum*, *T. angustum*, *T. gregarium*, *T. bomiense*, *T. viridulum*, *T. pollinicola*, *T. tenue*, *T. purpureum*, *T. perviride*, *T. globoides*, *T. confertum*, *T. changbaiense*, *T. viridicollare*, *T. adaptatum*, *T. beijingense*, *T. panacis*, *T. tardum*, *T. bifurcatum*, *T. vulgatum*, *T. mangshanicum*, *T. shaoguanicum*, *T. citrinella*, *T. asterineum*, *T. pseudobritdaniae*, *T. henanense*, *T. odoratum*, *T. thermophilum*, *T. xanthum*, *T. centrosinicum, T. virgineum, T. fruticola, T. medogense, T. palidulum,* and *T. alboviride*. The reference cultures for these species were mainly deposited into the Fungarium (also as HMAS, Herbarium Mycologicum Academiae Sinicae) at the Institute of Microbiology, Chinese Academy of Sciences, and therefore, they



are mainly available for researchers in China. The insertion of these species into the NCBI Taxonomy Browser and the attribution of respective undefined isolates (which are currently deposited as "*Trichoderma* sp.") will allow molecular identification of other strains that belong to these species if all three DNA barcodes are provided.

For 49 *Trichoderma* spp., the *rpb2* sequences of reference strains showed high similarity to neighboring species (Fig. 7). Each of these species is marked by a respective warning in Table 2. Most of these species have *rpb2* similarity > 99% with only one other species, but *T. viridescens*, *T. viridarium*, *T. paraviridescens*, *T. trixiae*, *T. appalachiense*, *T. rossicum*, *T. sichuanense*, *T. verticillatum*, *T. alpinum*, *T. concentricum*, *T. alni*, and *T. pseudodensum* have from three to eight species that each shares a highly similar *rpb2* phylotype (> 99%). *T. cremeoides* also has no deposited ITS sequence, and thus, its molecular identification can only be putative. Our analysis also shows that *tef1* of *T. cremeoides* is > 97% similar to *T. sinuosum* and *T. brevicrassum* and accurate molecular identification of these three species is also not possible. The type strain of *T. asperellum* shares highly similar phylotypes of *rpb2* with *T. yunnanense* and *T. kunmingense* (Table 2, Fig. 4). Warnings related to the identification of all DNA barcoded *Trichoderma* spp. that are available to date are listed in Table 2.

Thus, accurate DNA Barcoding of a large portion (40%) of *Trichoderma* species is not possible based on the provided molecular characters, and further sampling and an integrated analysis of molecular, ecophysiological, and biogeographic features are required.

Validation of DNA barcoding results

Although DNA Barcoding is presented as a tool that provides the final level of precision in microbial identification (Valentini et al. 2009), studies on other fungi (Lücking et al. 2020) and this work indicate that verification is required. It appears to be reasonable to conclude that in silico analysis may result in a putative identification or a formulation of the species hypothesis (including the new species hypothesis), while final identification can be achieved after the verification step. Following the principle of scientific falsification, verification should consist of critical considerations of the putative identification result. Verifying of the molecular identification should include the consideration of biological features such as concordant phenotypes, growth profile, lifecycle, and habitat. However, before this, the correctness of the molecular identification can also be considered critically (i.e., it has been validated) because it depends on the correctness of the deposited reference materials.

The correctness of reference materials that are used to formulate the species hypothesis should be critically assessed. The curators of public sequence databases (NCBI

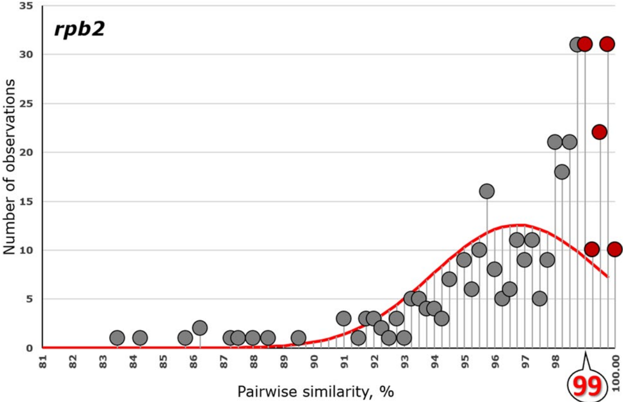


Fig. 7 Distribution of the pairwise similarities of *rpb2* between the 352 *Trichoderma* species and of each respective most closely-related species. The bars represent the number of observations at a certain similarity range. The delimitation of *rpb2* is set at 99%. Values < 99% are shown in grey and values ≥ 99% are in red

GenBank, EMBL, and DDBJ) take multiple measures to verify the quality of submitted materials (Lücking et al. 2020; Schoch et al. 2020). However, verification of species identification along with sequence submission is not a realistic task. Consequently, public databases contain a high proportion of sequences with incorrect species assignments. More than a decade ago, we estimated that 40% of such sequences were deposited into the NCBI GenBank for *Trichoderma* (Druzhinina et al. 2006; Atanasova et al. 2013). Molecular identification became essentially more complicated due to the rapid growth of species number, and we envision that the proportion of inaccurately identified sequence depositions will increase dramatically. Another source of incorrect species assignment for DNA barcode sequences is the common practice of taxonomic reclassifications that intends to improve the taxonomy of the group. However, such actions are not always reflect in the sequence annotations in public databases (see also *above*). Thus, hundreds of sequences that are available in the NCBI GenBank remain deposited under currently non-used "*Hypocrea lixii*", which has been maintained since the time when this combination was used for *Trichoderma harzianum* sensu lato (Chaverri and Samuels 2003; Druzhinina et al. 2010b). The latter species has been divided into a dozen sibling species including a rare *T. lixii*, which is known from a single isolate from Thailand (Chaverri et al. 2015). Thus, most sequences named "*Hypocrea lixii*" in the NCBI Taxonomy Browser should be considered to be inaccurately identified. Even *T. harzianum* name that has been assigned to the sequences of the most frequently deposited species is doubtful (irrespective of the DNA barcoding locus) because it may refer to the species concept that existed before the work of Chaverri et al. (2015), in which *T. harzianum* sensu lato was divided into several newly defined species form this complex including *T. harzianum* sensu stricto.

To show a quantitative example, we collected the 100 best hits from the sequence similarity search of the DNA barcode sequences for one of the strains (TUCIM 10063, *T. guizhouense*) that was used for the online survey earlier in this study (Supplementary Table S1). For ITS, at least 15 hits were incorrectly labeled as unrelated *T. atroviride* and *T. aureoviride* or as "*Hypocrea lixii*", and 31 were not identified. The *tef1* gene sequence can be submitted as it is (Supplementary Table S1, see "Materials and methods") or it can be trimmed for the length of the diagnostic fragment [see Kopchinskiy et al. (2005) or Fig. 9 *below*]. The respective lists of the best hits for untrimmed and trimmed *tef1* sequences contained at least 13 and 27 incorrect species names, and seven and 20 were not identified, respectively. We also detected *Trichoderma* sequences that were deposited as Dothideomycetes fungi such as *Neofusicoccum* spp. (KY024676.1 & KY024614.1) and *Lasiodiploidia* sp. (KY024673.1). It is likely that in these studies, *Trichoderma* parasitized these fungi [refer to the work of Druzhinina et al. (2018)], and its DNA was amplified instead of its hosts. These sequences were deposited under wrong names. Similarly, at least 27 *rpb2* sequences were also incorrectly named and six were not identified. This analysis revealed only the minimum number of incorrect records in the NCBI GenBank, but because the species borders in this group are difficult to establish (Druzhinina et al. 2010b), the actual number of incorrect records is likely to be higher.

The manually curated databases of sequences have fewer incorrect records, but they are usually outdated. The first multiloci database of reference *Trichoderma* sequences was powered by several on-line identification tools that were available at www.isth.info (Druzhinina et al. 2005; Kopchinskiy et al. 2005), and it is no longer supported (however we offer some updated tools *below*). The new tool, Multiloci Identification System for *Trichoderma* (MIST) is available at http://mmit.china-cctc.org/ (Dou et al. 2020), and it is based on the sequential sequence similarity search of ITS, *rpb2,* or *tef1* DNA barcode loci for a query strain against a MIST databases of reference and non-reference sequences. Although it provided correct identification of the query sequence in this case (*T. guizhouense*), for many other species it also exports numerous false-positive results (many species assigned at the identification step). When it was released in July 2020, it contained a database of *tef1* and *rpb2* sequences for 349 species (out of the current 361). Its usability will depend on the frequency of updates. If new species are not regularly added to the MIST database, it will lose its identification function but remain a useful support for searching for the approximate position of a query strain.

The use of the largest fungal database for sequence identification, UNITE https://unite.ut.ee/index.php#panel3, is not

suitable for *Trichoderma* species identification because it is only based on partial ITS (see *above*). Analysis of the test strain of *T. guizhouense* TUCIM 10063 in UNITE resulted in four species hypotheses, none of which were correct (*T. harzianum*, *T. tawa*, *T. lixii,* and *T. virens*). However, all these species are closely related to *T. guizhouense,* and therefore, this tool provides identification at the level of the *Harzianum* and *Virens* Clades. *Trichoderma* spp. are not yet included in the collection of MycoBank Polyphasic Identifications Databases (http://www.mycobank.org/DefaultInfo.aspx?Page=polyphasicID).

Thus, the molecular identification is solely dependent on sequences that are deposited into public databases (curated and non-curated). The current diversity of *Trichoderma* requires manual analysis of sequence similarities and phylogenetic analyses, but accurate automated identification of *Trichoderma* species is not available. However, several *Trichoderma*-dedicated tools provide useful supporting material (www.trichokey.com, www.trichoderma.info, and MIST http://mmit.china-cctc.org/).

The solution: molecular identification guideline for *Trichoderma* spp.

Synopsis of molecular taxonomic inventory for the genus *Trichoderma*

- The introduction of molecular evolutionary analyses resulted in exponential growth in the number of *Trichoderma* species, up to 50 new species that were described per each year.
- Among the 375 species with valid names as of July 2020, 361 (96%) are DNA barcoded.
- IndexFungorum and Mycobank do not contain complete lists of *Trichoderma* species. The NCBI Taxonomy Browser includes 90% of the species. Numerous species names that are not currently in use or not legitimate are listed in IndexFungorum and Mycobank. The NCBI Taxonomy Browser contains the fewest such names.
- As for July 2020, identification (DNA Barcoding) and evolutionary analyses of *Trichoderma* spp. are possible only based on three phylogenetic markers: ITS, *tef1*, and *rpb2*. Other DNA barcodes (*chi18-5* = *ech42*, *cal1*, *act*, *acl1*,18S rRNA = SSU, and 28S rRNA = LSU) are sequenced for less than one-half of the species, and therefore, they have limited or no suitability for molecular identification.
- *Trichoderma* spp. cannot be identified by phylogenetic analysis without considering the sequence similarity values.
- ITS can be used to identify *Trichoderma* at the generic level.
- For the accurate and precise molecular identification of *Trichoderma* isolates at the species level, sequencing of the three DNA barcodes (ITS, *tef1*, and *rpb2*) is required.
- Most closely related species of *Trichoderma* differ by 1% (approximately eight mutations) of *rpb2* and/or 3% (approximately 27 mutations including indels) of *tef1* sequences (if the specified region of each phylogenetic marker is considered, see Fig. 9 below). Some species and infrageneric groups share phylotypes of individual markers (ITS, *tef1*, or *rpb2*).
- Molecular identification can be achieved based on the analysis of sequence similarities between the query strain and the reference strains that are analyzed for *tef1* (≥ 97%) and *rpb2* (≥ 99%). If this condition is not met, the identification can be made based on sequence similarities and phylogenetic concordance, i.e., analysis of single loci tree topologies for *tef1* and *rpb2*.
- Molecular identification must be validated by the critical evaluation of non-biological aspects (quality and completeness of the reference taxonomic materials) and verified based on biological criteria (morphology, ecophysiology, biogeography, habitat, and occurrence).
- The inventory of DNA barcoding materials that were deposited in public databases revealed that only 60% of molecularly characterized *Trichoderma* species can potentially be unambiguously identified based on the reference sequences that were deposited by taxonomy providers.
- Identifiability of 40% of species is compromised by any of the following factors or their combinations: incomplete DNA barcoding, incomplete deposition of reference cultures or reference sequences, or insufficient polymorphism of one or several diagnostic sequences.
- *Trichoderma* spp. cannot be identified by the automated sequence similarity search (such as BLAST) irrespective of the reference database or DNA barcodes that are used as such results require in silico validation and biological verification.
- On-line tools for *Trichoderma* identification can provide a useful estimation of the taxonomic (phylogenetic) surroundings for a given strain. However, the tools that are currently available do not offer precise identification at the species level.
- Identification of *Trichoderma* species is an intricate and laborious task that requires a background in mycology, molecular biological skills, training in molecular evolution, and in-depth knowledge of taxonomic literature. For ambiguous cases, a consultation with *Trichoderma* taxonomy experts is recommended.

Molecular identification protocol for a single *Trichoderma* isolate

The following molecular identification protocol enables a user to do the following: (1) identify the genus *Trichoderma*, i.e., to exclude fungi other than *Trichoderma*; (2) identify *Trichoderma* species; and (3) verify the ambiguity of the identification. The protocol allows recognition of a putative new species as a particular case of species identification.

All steps proposed below refer to the taxonomic limitations that constrain the molecular diversity of the genus *Trichoderma* and recognized species that existed as of July 2020.

A *Trichoderma* species can be identified if its ITS sequence reaches at least one similarity value ≥ 76% compared to the sequences in the dataset that is attached to the protocol and the two other DNA barcoding markers are highly similar to the corresponding sequences of the reference strain from one species, with *rpb2* ≥ 99% and *tef1* ≥ 97%. These conditions can be shortened as shown in the following sequence similarity standard:

$\textbf{\textit{Trichoderma}}[\mathbf{ITS_{76}}] \sim \mathbf{sp}\exists!(\textbf{\textit{rpb2}}_{99} \cong \textbf{\textit{tef1}}_{97})$,

where "***Trichoderma***" means the genus *Trichoderma*, "**sp**" means a species, "**~**" indicates an agreement between ITS and other loci, "≅" refers to the concordance between "***rpb2***" and "***tef1***", and "∃!" indicates the uniqueness of the condition (only one species can be identified). Subscripts show the similarity per locus that is sufficient for the identification based on the assumptions of the protocol below. A flowchart of the protocol is presented in Fig. 8.

The result of molecular identification requires biological verification (Lücking et al. 2020) and consideration of the original taxonomic literature. The morphology and growth profile of the query strain should not contradict the published records for the identified species. It is recommended to compare the biogeography and occurrence records for the identified species with metadata for the query strain. The observed lifecycle, ecology (habitat and interactions with other organisms), and ecophysiology of the query strain should be in agreement with the description of the identified species. For ambiguous cases, it is useful to consult taxonomy experts.

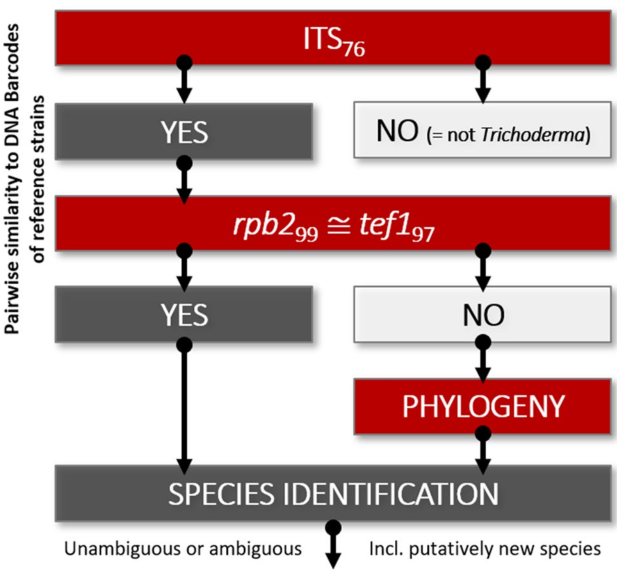


Fig. 8 The flowchart of the molecular identification protocol of *Trichoderma* based on three DNA barcode sequences. A species of *Trichoderma* can be identified if its ITS sequence reaches a similarity value ≥ 76% (ITS_{76}) compared to the sequences in the dataset that is attached to the protocol and the two other DNA barcoding markers are highly similar to the corresponding sequences of the reference strain of one species as *rpb2* ≥ 99% and *tef1* ≥ 97% ($\textbf{\textit{rpb2}}_{99} \cong \textbf{\textit{tef1}}_{97}$); "≅" refers to the concordance between *rpb2* and *tef1*

The check-list for materials, tools, and preparation steps.

- Isolate a single spore (asco- or conidiospore) culture from the putative *Trichoderma* sp. strain.

  Note: Although the fast growth on rich nutritional media, mycoparasitism, resistance to xenobiotics, and greenish conidiation are characteristic features for most of the *Trichoderma* cultures, some species have hyaline conidia or do not produce them in vitro (they appear white in culture), some are sensitive to fungicides, and some do not parasitize other fungi and/or have slow growth in vitro. Refer to the diversity of *Trichoderma* spp. morphotypes in monographs by Jaklitsch (2009, 2011) or elsewhere.
- Use PCR to amplify and sequence the three DNA barcode loci as follows: the complete fragment of ITS1 and 2 (including the 5.8S rRNA) of the rRNA gene clusters, and partial sequences of *rpb2* and *tef1* genes.

  Note: PCR protocols including the corresponding primer pairs are provided in Table 3, and the structure of the loci is shown in Fig. 9.

  Note: The quality of obtained sequences is crucially important for this protocol. No ambiguity in sequencing reaction is accepted. Ideally, sequences should be verified by sequencing from the 3′ and 5′ ends.
- Connect to the Internet.
- Trim the sequences. Use *Tricho*MARK 2020, which is available at www.trichokey.com, or use the reference *datasets* (Supplementary Datasets and www.trichoderma.info) and trim the length of the query sequences such that they correspond to the length of the reference DNA barcode loci, as shown in Fig. 9.

  Note: this step is required for the molecular identification protocol. If online tools are not available, the sequences can be trimmed manually using Aliview (Larsson 2014) or other sequence editors. The technical requirement to trim the sequences is also explained in Kopchinskiy et al. (2005).

Table 3 PCR conditions for the amplification of the three *Trichoderma* DNA barcodes

	***Trichoderma* DNA barcoding loci**					
	ITS		***rpb2***		***tef1***	
Primers (5′ – 3′)	ITS5		fRPB2-5f		EF1	
	GGAAGTAAAAGTCGTAACAAGG		GAYGAYMGWGATCAYTTYGG		ATGGGTAAGGARGACAAGAC	
	ITS4		fRPB2-7cr		EF2	
	TCCTCCGCTTATTGATATGC		CCCATRGCTTGTYYRCCCAT		GGARGTACCAGTSATCATGTT	
	(White et al. 1990)		(Liu et al. 1999)		(O'Donnell et al. 1998)	
PCR recipe (µL)						
template DNA, 100 ng	1		1		1	
2× Phanta Max Master Mix	12.5		12.5		12.5	
Forward primer, 100 µM	0.1		1		0.1	
Reverse primer, 100 µM	0.1		1		0.1	
ddH_2O	to 25		to 25		to 25	
PCR program	**T, °C**	**Time**	**T, °C**	**Time**	**T, °C**	**Time**
Pre-denaturation	95	3'	95	3'	95	3'
32 cycles: Denaturation	95	15''	95	15''	95	15''
32 cycles: Annealing	53	15''	58	15''	53	15''
32 cycles: Extension	72	1'	72	1'	72	1'
Final extension	72	5'	72	5'	72	5'

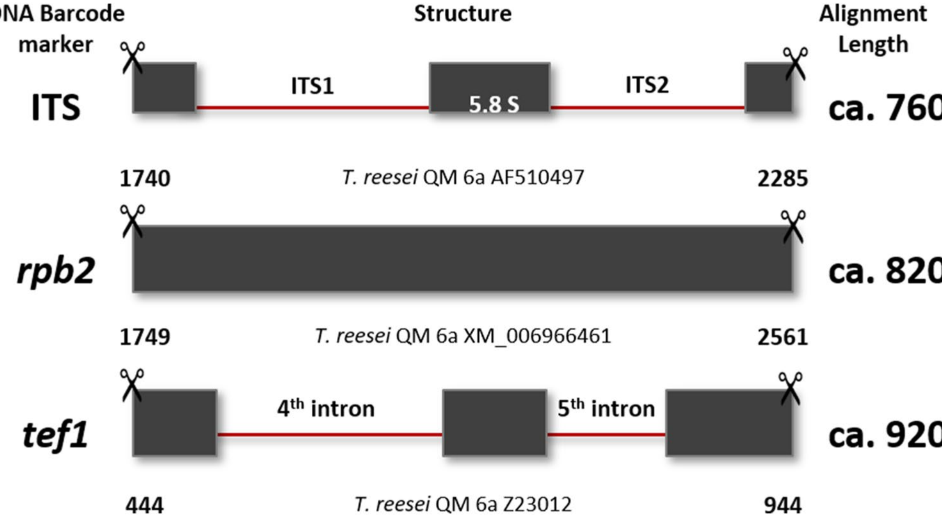


Fig. 9 Structure of DNA barcoding loci trimmed for molecular identification. Numbers below each locus show the 5′ and 3′ positions on the trimmed fragment that were suitable for molecular identification using reference loci from *T. reesei* QM 6a (Druzhinina et al. 2010a; Druzhinina et al. 2005; Chenthamara et al. 2020) as an example

- Use a text editor (e.g., Notepad) and put your trimmed query DNA barcode sequences into FASTA format and save the input files separately.
- Install Aliview, IQ-TREE (Nguyen et al. 2015b), and FigTree (http://tree.bio.ed.ac.uk/software/figtree/), or softwares with similar functions.

Step 1: ITS

Estimate the pairwise similarity between the ITS sequence of the query strain and the sequences that are given in the *ITS56 dataset* as described in **Comment 1** at the end of the protocol.

If the maximum similarity is ≥ 76%,

the query strain belongs to the genus *Trichoderma* spp. Continue to Step 2.

If the maximum similarity is < 76%,

the query strain belongs to a genus other than *Trichoderma*. Identification of *Trichoderma* spp. is not possible.

Step 2: *rpb2* and *tef1*

For each locus (*rpb2* and *tef1*), estimate the pairwise similarities between the query strain and the sequences of closely related reference strains, as described in **Comment 2**.

If the condition $\exists!(rpb2_{99} \cong tef1_{97})$ is met,

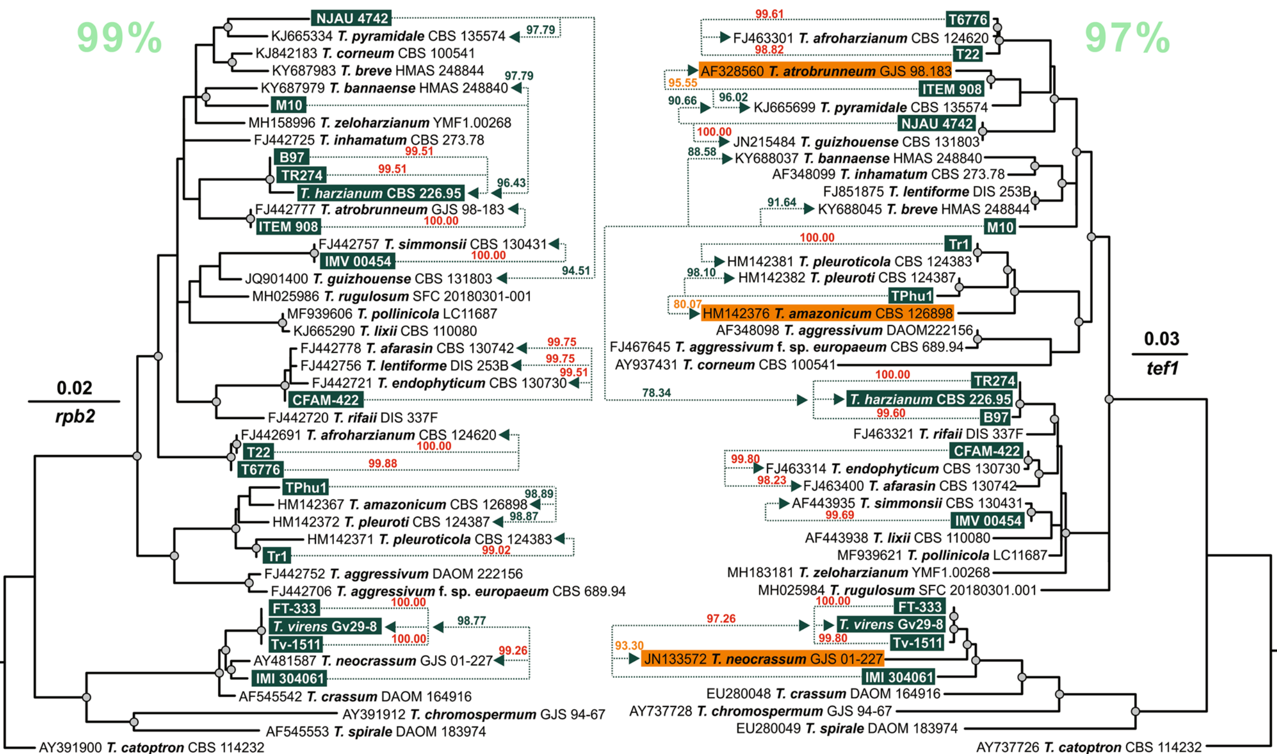


Fig. 10 Molecular identification of genome-sequenced strains from the *Harzianum* and *Virens* clades using maximum-likelihood (ML) phylogeny and pairwise sequence similarity calculation. The ML phylograms of *rpb2* and *tef1* were constructed in IQ-TREE 1.6.12 (bootstrap replicates = 1000) using the nucleotide substitution models of TNe + R3 and HKY + F+G4. Circles at the nodes indicate ultrafast bootstrap values > 80 given by IQ-TREE. Genome sequenced strains were shaded in green. The reference strains were provided with the GenBank accessions and the strain name, among which, strains with uncompleted reference information were shaded in orange. Results of the pairwise sequence similarity were illustrated on the dashed lines between the query strain and its closely related species (arrows point to the reference strains). The pairwise sequence similarity calculation was performed using the online tool of ClustalOMEGA (https://www.ebi.ac.uk/Tools/msa/clustalo/)

Record the identified species and continue to Step 4.

If the condition $\exists!(rpb2_{99} \cong tef1_{97})$ is not met,

Continue to Step 3.

Step 3: phylogenetic analyses of *rpb2* and *tef1*

- Open *rpb2* and *tef1* (separately) alignments that are produced in Step 2 (see **Comment 2**).
- Record cases of short or missing sequences for reference strains (if any).
- Run phylogenetic analyses separately for *rpb2* and *tef1* sequences. Given that the correct parameters were selected, the maximum likelihood or Bayesian methods are recommended; however maximal parsimony is also suitable. See details in the "Materials and methods".
- Visualize the tree files in Figtree and (optionally) export the data to a graphics software.
- On each tree, locate the query sequence and the most similar reference sequences; mark the pairwise similarities that were estimated in Step 2 (examples are shown on Figs. 10–13).
- Interpret the concordance of *rpb2* and *tef1* phylograms considering the similarity values that were estimated in Step 2.

Note: Consideration of single-loci phylograms for *tef1* and *rpb2* is required. The concatenated phylogram of the two loci is optional in addition to analysis of single-locus trees.

Note: For the interpretation of phylogenetic trees, refer to **Comment 3** and practical examples below.

Step 4: Validation of molecular identification

For the validation of the molecular identification and assignment of ambiguity status, the literature on *Trichoderma* taxonomy should be studied. Table 2 of this study provides supplementary information.

In some cases, results of phylogenetic analysis (Step 3) can be used to validate the identification results (**Comment 3**).

Validation of species identification

If all of the following criteria are met:

- The identified species is represented by the complete set of reference DNA barcodes (Table 2, taxonomic literature).
- The identifiability of the species is not compromised by insufficient polymorphism of *tef1* and *rpb2*, or other parameters (i.e., none of the warnings from Table 2 are present).
- The identified species was recognized based on the GCPSR concept using a polyphasic approach.

The identification is unambiguous, precise, and accurate.

If any of the following criteria are met:

- The identified species is represented by the incomplete set of reference DNA barcodes (see warnings in Table 2).
- The identifiability of the species is compromised by low *tef1* and *rpb2* polymorphism, or the quality of the reference sequences is not sufficient (usually, too short) (see warnings in Table 2).
- The identified species is recognized based on insufficient reference material or ambiguous species criteria.

The identification is ambiguous; the species name can be assigned as "confer" or "cf." (i.e., *compared to*) or as "affinis" or "aff." (i.e., *related to*) the most closely related species.

Note: In this case, the most closely related species can be revealed based on the results of phylogenetic analyses of *tef1* and *rpb2* (Step 3, **Comment 3**). ***Note***: Precise and accurate identification will usually require either taxonomic revision of reference materials, additional DNA sequencing, or/and sampling.

Note: If phylogenetic analyses of both loci point **to a single sister species** but it can't be identified because of incomplete reference materials, "aff." can be used to specify the related taxon: *T*. aff. [related species name]. If several sister species are proposed, the use of "cf." is more appropriate: *T*. cf. [one of the related species]. Here, it is suggested to point to the related species that is best studied or has similar features.

Validation of the new species hypothesis

If all of the following criteria are met:

- The query strain belongs to the genus *Trichoderma* (meets *Trichoderma*[ITS_{76}] standard).
- The query strain has unique sequences of *rpb2* or *tef1* (does not meet the $sp\exists!(rpb2_{99} \cong tef1_{97})$ standard for known species).
- The existing closely related species have complete sets of reference DNA barcodes.
- The new species hypothesis is supported by the topology of both phylograms (*rpb2* and *tef1*) and is not contradicted by other markers (GCPSR concept).

The new species hypothesis is unambiguous, precise, and accurate. Record the results as "*T*. sp. strain ID" before the formal name is given.

Note: the formal taxonomic description of a new fungal species requires the guidelines of Seifert and Rossman (2010) to be followed, including naming (see The *Code*), registration of the type (May et al. 2019), deposition of the reference materials into public databases, microbiological investigation, and imaging of microscopic features. It comprises the molecular evolutionary analysis (**Comment 3**) and comparison of morphological, eco-physiological, and biogeographical characteristics between the query strain(s) and closely related taxa.

If any of the following criteria met:

- Attribution of the query strain to the genus *Trichoderma* is ambiguous (does not meet the *Trichoderma*[ITS_{76}] standard, in particular if the similarity is $< 70\%$)
- Closely related species have incomplete sets of DNA barcodes, the quality of the reference sequences is not satisfactory, or related species were recognized based on insufficient DNA barcoding material.
- The position of a new species is not supported by the topology of both phylograms (*rpb2* and *tef1*) or is contradicted by other markers (GCPSR concept is not applicable).

The hypothesis of a new species remains ambiguous.

Note: In this case, the species name can be assigned as *T*. sp. with the addition of either "affinis" or "aff." [i.e., *related to*] (if there is only one sister species) or "confer" or "cf." [i.e., *compared to*] (if there is a group of related species) the most closely related species that can be revealed based on the results of phylogenetic analysis (Step 3, **Comment 3**). Precise and accurate identification of a new species will usually require either taxonomic revision of reference materials, additional sequencing, or/and sampling.

Step 5: Presentation of the identification result and data archiving

Record the identification results. An example is given in Table 4.

Archive your non-trimmed query DNA barcode sequences along with their identification (FASTA format is suggested).

Table 4 Molecular identification of *Trichoderma* strains that were used in an online survey and those that have their WGSs deposited in public databases before July 2020

Order	Figure	Strain	SIMILARITY STANDARD					Identified species	VERIFICATION				
			Genus		Species		Species		Completeness and quality of DNA Barcoding	Phylogenetic concordance of *tef1* and *rpb2*	Status of species identification	Species assigned	Taxonomy notes
			ITS ≥76%	~	*rpb2* ≥99%	≅	*tef1* ≥97%						
			to the sequences of reference strains (www.trichokey.com)										
Counts			Total number of isolates						Complete / Incomplete	Yes / No	Unambiguous / Ambiguous	Yes / No	Resolved / Requires revision / sp. nov.
			44						38 /6	38 /6	38 / 6	31 / 13	30 / 6 / 8
1	NA	**TUCIM 10063**	*Trichoderma*	~	*T. guizhouense*		*T. guizhouense*	***T. guizhouense***	Complete	Yes	Unambiguous	Yes	
2	NA	**TUCIM 5640**	*Trichoderma*	~	-		-	***T.* sp. TUCIM 5640**	Complete	**No**	Unambiguous	**No**	New species close to *T. compactum*
3	10	**NJAU 4742**	*Trichoderma*	~	-	≠	*T. guizhouense*	***T*. sp. NJAU 4742**	Complete	No, *rpb2* is more similar to *T. pyramidale* CBS 135574 (97.79%) than to *T. guizhouense* CBS 131803 (94.51%)	Unambiguous	**No**	New species close to *T. pyramidale*
4	10	**M10**	*Trichoderma*	~	-	≠	-	***T.* sp. M10**	Complete	**No**	Unambiguous	**No**	New species close to *T. bananense*
5	10	**CBS 226.95[T]**						***T. harzianum***	Complete	Yes	Unambiguous	Yes	-
6	10	**B97**	*Trichoderma*	~	*T. harzianum*		*T. harzianum*	***T. harzianum***	Complete	Yes	Unambiguous	Yes	-
7	10	**TR274**	*Trichoderma*	~	*T. harzianum*		*T. harzianum*	***T. harzianum***	Complete	Yes	Unambiguous	Yes	-
8	10	**T6776**	*Trichoderma*	~	*T. afroharzianum*		*T. afroharzianum*	***T. afroharzianum***	Complete	Yes	Unambiguous	Yes	-
9	10	**T22**	*Trichoderma*	~	*T. afroharzianum*		*T. afroharzianum*	***T. afroharzianum***	Complete	Yes	Unambiguous	Yes	-
10	10	**IMV 00454**	*Trichoderma*	~	*T. simmonsii*		*T. simmonsii*	***T. simmonsii***	Complete	Yes	Unambiguous	Yes	-
11	10	**CFAM-422**	*Trichoderma*	~	*T. afarasin* *T. lentiforme* *T. endophyticum*	≠	*T. afarasin* *T. endophyticum*	***T.* cf. *endophyticum***	Incomplete, *T. afarasin* shares *rpb2* phylotype with *T. endophyticum*	**No**	**Ambiguous**	**No**	The group requires taxonomic revision
12	10	**TPhu1**	*Trichoderma*	~	-	≠	*T. pleuroti*	***T.* sp. TPhu1**	Complete	**No**	Unambiguous	**No**	New species close to *T. pleuroti*
13	10	**Tr1**	*Trichoderma*	~	*T. pleuroticola*		*T. pleuroticola*	***T. pleuroticola***	Complete	Yes	Unambiguous	Yes	-
14	10	**ITEM 908**	*Trichoderma*	~	*T. atrobrunneum*	≠	-	***T.* cf. *atrobrunneum***	Incomplete, the reference seq. of *T. atrobrunneum* is short	Yes	**Ambiguous**	**No**	Phylogeny does not exclude *T. atrobrunneum; T. pzramidale* or a new species close to these two
15	10	**Gv29-8[T]**						***T. virens***	Complete	Yes	Unambiguous	Yes	-
16	10	**FT-333**	*Trichoderma*	~	*T. virens*		*T. virens*	***T. virens***	Complete	Yes	Unambiguous	Yes	-
17	10	**Tv-1511**	*Trichoderma*	~	*T. virens*		*T. virens*	***T. virens***	Complete	Yes	Unambiguous	Yes	-
18	10	**IMI 304061**	*Trichoderma*	~	*T. neocrassum*	≠	*T. virens*	***T.* sp. aff. *neocrassum* IMI 304061**	Incomplete, the reference seq. of *T. neocrassum* is short	**No**	**Ambiguous**	**No**	Phylogeny does not exclude a new species close to *T. neocrassum*
19	11	**QM6a[T]**						***T. reesei***	Complete	Yes	Unambiguous	Yes	-
20	11	**CBS 999.97**	*Trichoderma*	~	*T. reesei*		*T. reesei*	***T. reesei***	Complete	Yes	Unambiguous	Yes	-
21	11	**CBS 125925[T]**						***T. parareesei***	Complete	Yes	Unambiguous	Yes	-

Table 4 (continued)

22	11	**CBS 816.68[T]**						***T. longibrachiatum***	Complete	Yes	Unambiguous	Yes	-
23	11	**SMF2**	*Trichoderma*	~	*T. longibrachiatum*		*T. longibrachiatum*	***T. longibrachiatum***	Complete	Yes	Unambiguous	Yes	-
24	11	**MK1**	*Trichoderma*	~	*T. longibrachiatum*		*T. longibrachiatum*	***T. longibrachiatum***	Complete	Yes	Unambiguous	Yes	-
25	11	**JCM 1883**	*Trichoderma*	~	*T. longibrachiatum*		*T. longibrachiatum*	***T. longibrachiatum***	Complete	Yes	Unambiguous	Yes	-
26	11	**TUCIM 6016**	*Trichoderma*	~	-	≠	*T. citrinoviride*	***T.*** **cf.** ***citrinoviride***	Incomplete, the reference seq. of *T. citrinoviride* is short	Yes	**Ambiguous**	**No**	Phylogeny does not exclude *T. citrinoviride* or a new species close to it
27	12	**IMI 206040**	*Trichoderma*	~	*T. atroviride*		*T. atroviride*	***T. atroviride***	Complete	Yes	Unambiguous	Yes	-
28	12	**XS2015**	*Trichoderma*	~	*T. atroviride*		*T. atroviride*	***T. atroviride***	Complete	Yes	Unambiguous	Yes	-
29	12	**P1**	*Trichoderma*	~	*T. atroviride*		*T. atroviride*	***T. atroviride***	Complete	Yes	Unambiguous	Yes	-
30	12	**F7**	*Trichoderma*	~	*T. atroviride*		*T. atroviride*	***T. atroviride***	Complete	Yes	Unambiguous	Yes	-
31	12	**B10**	*Trichoderma*	~	*T. atroviride*		*T. atroviride*	***T. atroviride***	Complete	Yes	Unambiguous	Yes	-
32	12	**JCM 9410**	*Trichoderma*	~	*T. atroviride*		*T. atroviride*	***T. atroviride***	Complete	Yes	Unambiguous	Yes	-
33	12	**LY357**	*Trichoderma*	~	-	≠	-	***T.*** **sp. LY357**	Complete	Yes	Unambiguous	**No**	New species close to *T. paratroviride* and *T. atroviride*
34	12	**T6085**	*Trichoderma*	~	*T. gamsii*		*T. gamsii*	***T. gamsii***	Complete	Yes	Unambiguous	Yes	-
35	12	**A5MH**	*Trichoderma*	~	*T. gamsii*	≠	-	***T.*** **sp. aff.** ***gamsii*** **A5MH**	Complete	**No**	Unambiguous	**No**	New species close to *T. gamsii*
36	12	**POS7**	*Trichoderma*	~	-	≠	-	***T.*** **sp. aff.** ***koningiopsis*** **POS7**	Complete	Yes	Unambiguous	**No**	New species close to *T. koningiopsis*
37	12	**CBS 433.97[T]**						***T. asperellum***	Complete	Yes	Unambiguous	**No**	-
38	12	**B05**	*Trichoderma*	~	*T. asperellum* *T. kunmingense*	≅	*T. asperellum*	***T.*** **cf.** ***asperellum***	Incomplete, similarity to *tef1* of *T. kunmingense* is 91.92% because the reference *tef1* sequence is short	Yes	**Ambiguous**	Yes	The taxonomy of *T. kunmingense* requires revision
39	12	**TR356**	*Trichoderma*	~	*T. asperelloides*		*T. asperelloides*	***T. asperelloides***	Complete	Yes	Unambiguous	Yes	-
40	12	**Ts93**	*Trichoderma*	~	*T. asperelloides*		*T. asperelloides*	***T. asperelloides***	Complete	Yes	Unambiguous	Yes	-
41	12	**GD12**	*Trichoderma*	~	*T. hamatum*		*T. hamatum*	***T. hamatum***	Complete	Yes	Unambiguous	Yes	-
42	13	**IBT 40837**	*Trichoderma*	~	*T. arundinaceum*		*T. arundinaceum*	***T. arundinaceum***	Complete	Yes	Unambiguous	Yes	-
43	13	**IBT 40841**	*Trichoderma*	~	*T. brevicompactum*	≠	-	***T.*** **cf.** ***brevicompactum***	Incomplete, similarity to *tef1* of *T. brevicompactum* is 93.67% because the reference *tef1* sequence is short	Yes	**Ambiguous**	**No**	Phylogeny does not exclude *T. brevicompactum* or a new species close to it
44	NA	**TW21990_1**						***T. cyanodichotomus*** **nom. inval.**	Complete	Yes	Unambiguous	Yes	Species description is not valid. Requires taxonomic revision.

T. *Trichoderma* genus; ~ in agreement; ≅ concordant; ≠ conflicting, *NA* not available. *T.* sp. [strain ID]—a putative new species of *Trichoderma* for which no sister species is given. *T.* sp. aff. [species name] [strain ID]—a putative new species of *Trichoderma* for which a sister species is given; *T.* cf. [species name] and *T.* aff. [species name] cases where unambiguous identification is currently not achievable without a detailed taxonomic revision of the group

Comments:

Comment 1. Calculation of pairwise similarities between the query and reference sequences using ITS:

- Download the sequence *ITS56 dataset* from Supplementary Datasets from this study or www.trichokey.com and open in the text editor. Add the query ITS sequence to the dataset.
- Insert the sequences in Aliview and use "Realign everything" option in "Align" menu.
- Check whether the length of the query sequence fits the *ITS56 dataset*. If not, the identification result will be ambiguous.
- Export the alignment as a .fasta file and save it.
- Upload the exported .fasta file or paste the sequences into the input box of the online ClustalOMEGA tool for pairwise similarity calculation (https://www.ebi.ac.uk/Tools/msa/clustalo/) or use other tools for pairwise sequence similarity calculation.
- Select the option of "DNA", setup your parameters ("ClustalW" is recommended), and click the "submit" button.
- Download the .pim file, which contains the results of the pairwise similarity calculation, from the "results summary" page.
- (Optional) A "guide tree" can also be obtained from the "results summary" page and visualized in FigTree for your interest.
- Open the .pim file using Microsoft Excel or a text editor, search for the maximum similarity value(s) between your query sequence and the references. Make sure you have excluded the value showing the similarity to the query sequence (100%).

Note: The *ITS56 dataset* contains 56 selected reference ITS sequences that represent intrageneric polymorphism of the *Trichoderma* genus.

Comment 2. Manual calculation of pairwise similarities between the query and reference sequences using tef1 *or* rpb2:

- Submit the trimmed *rpb2* sequence to *Tricho*BLAST (www.trichokey.com) and detect the most closely related species.
- Use the most updated data in Table 2 (i.e., the latest updated version is on www.trichokey.com) and taxonomic literature that was published after the release of this manual, and compose lists of the most closely related species, 6 < N < 10.
- Find the taxonomically confirmed reference strains (ex-type, type, vouchered; Table 2) for each species and retrieve *rpb2* and *tef1* sequences from public databases.
- Align and trim the sequences, and calculate the pairwise sequence similarities as described in **Comment 1**.

Comment 3. Application of phylogenetic analysis in molecular identification and its use for the validation of identification results.

Phylogenetic analysis can contribute to unambiguous or ambiguous identification of either a known species or a putative new species, as described below.

- If the sequence similarity standard (whether it is *rpb2* and/or *tef1*) indicates several species (e.g., *T.* cf. *endophyticum* CFAM-422, Tables 1 and 4), phylogenetic analysis of both loci will reveal the closest species and allow accurate but imprecise (ambiguous) identification as *Trichoderma* cf. [closest species]. Thus, this analysis will usually indicate a need for the taxonomic revision of the reference group. In this case, phylogeny is used as an identification step.
- If the two loci indicate different species (existing or putatively new), the phylogenetic analysis results can demonstrate that the loci are not concordant (e.g., *T.* sp. NJAU 4742, Tables 1 and 4). In this case, and considering that only two markers are currently available, phylogeny is used as a validation step. With the introduction of genomic techniques in fungal taxonomy, such cases may be resolved by the application of phylogenomic analyses (Galtier and Daubin 2008).
- If the reference sequences are not complete, the results of phylogenetic analysis will reveal the closest species and allow accurate but imprecise (ambiguous) identification as *Trichoderma* aff. [closest species] or *Trichoderma* cf. [closest species] (e.g., *T.* cf. *atrobrunneum* ITEM 908, Tables 1 and 4). In this case, phylogeny is used as a validation step.
- If a new species is found, phylogeny is a required as part of the new species recognition. In this case, the topologies of both phylograms are expected to be concordant and pairwise sequence similarities should support the unambiguous new species hypothesis.

Practical examples of *Trichoderma* identification

To verify the suitability of the molecular identification protocol and to demonstrate how the identification results can be presented, we list below the detailed identification

diagnoses for the two strains that were used for the on-line survey (see above) and the 42 WGS *Trichoderma* strains that were available in public databases as of July 2020.

Note: Sequences of all phylogenetic markers were trimmed before the analysis using *Tricho*MARK 2020, which is available at www.trichokey.com or the reference *datasets* (Supplementary Datasets and www.trichoderma.info), so that they correspond to the length of the reference DNA barcode loci, as shown in Fig. 9.

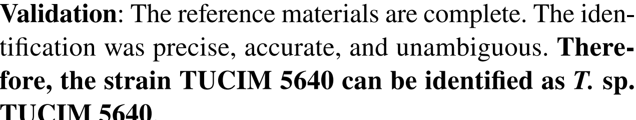
Identification of strains that were used in the on-line survey

(1) **TUCIM 10063** (called "mycoparasite" in the on-line survey)

Identification: The pairwise sequence similarity of ITS (MT792072) between strain TUCIM 10063 and the references that are given in the *ITS56 dataset* showed several values > 76%, which indicated that strain TUCIM 10063 belongs to the genus *Trichoderma*. The similarity of *rpb2* (MT802437) between strain TUCIM 10063 and the most closely related species *T. guizhouense* (strain CBS 131803) that is found at this locus was 100.00% (Table 2), and the similarity of *tef1* (MT802439) between strain TUCIM 10063 and the most closely related species *T. guizhouense* (strain CBS 131803) that is found at this locus was 100.00% (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).
Validation: The reference materials are complete, and identification was precise, accurate, and unambiguous. **Therefore, strain TUCIM 10063 can be identified as *T. guizhouense***.

(2) **TUCIM 5640** (called "epiphyte" in the on-line survey)

Identification: The pairwise sequence similarity of ITS (MT792073) between strain TUCIM 5640 and the references that is given in the *ITS56 dataset* showed several values > 76%, which indicated that strain TUCIM 5640 belongs to the genus *Trichoderma*. The similarity of *rpb2* (MT802438) between strain TUCIM 5640 and the most closely related species including *T. compactum* (strain CBS 121218) and *T. aggregatum* (strain HMAS 248863) that are found at this locus was 96.55% and 96.05% (Table 2), respectively, and the similarity of *tef1* (MT802440) between strain TUCIM 5640 and the most closely related species including *T. compactum* (strain CBS 121218) and *T. aggregatum* (strain HMAS 248863) that are found at this locus was 95.84% and 91.51% (Table 2), respectively, (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was not met). This indicates that strain TUCIM 5640 can be recognized as a putative new species (**Comment 3**).



Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain TUCIM 5640 can be identified as *T.* sp. TUCIM 5640**.

Identification of *Trichoderma* isolates for which WGSs have been deposited in public databases before July 2020

Corresponding sequences can be retrieved from public databases. Accession numbers, references for WGS, and the initial species identifications are listed in Table 1 and Fig. 2. The dataset includes several ex-type strains that do not require identification (i.e., they are reference strains). However, the sequence similarity analysis is also described for these strains.

We deliberately skipped the WGS mutants of *T. reesei* because the pedigree for the type strain QM 6a that leads to diverse industrial mutants is well documented in the literature (Druzhinina and Kubicek 2016). However, we included mutants of several other species that are used in agriculture and may be confused with the wild-type strains.

(3) **NJAU 4742** (Tables 1 and 4; Fig. 10)

Identification: Pairwise sequence similarity of ITS between strain NJAU 4742 and the references that are given in the *ITS56 dataset* showed several values > 76%, which indicated that strain NJAU 4742 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain NJAU 4742 and the most closely related species *T. pyramidale* (strain CBS 135574) that is found at this locus was 97.79% (Table 2; Fig. 10), while the similarity of *tef1* between strain NJAU 4742 and the most closely related species *T. guizhouense* (strain CBS 131803) that is found at this locus was 100.00% (Table 2; Fig. 10) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was not met). This indicates that strain NJAU 4742 can be recognized as a putative new species that has non-concordant phylogenies of *rpb2* and *tef1* (**Comment 3**).
Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain NJAU 4742 can be identified as a putative new species *T.* sp. NJAU 4742. Due to the value of this strain for the development of biofertilizers, we propose a provisional name to this species as *T. shenii* nom. prov**. The formal taxonomic description will be presented elsewhere upon additional sampling.

(4) **M10** (Tables 1 and 4; Fig. 10)

Identification: The pairwise sequence similarity of ITS between strain M10 and the references that are given in the *ITS56 dataset* showed several values > 76%, which indicated that strain M10 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain M10 and the most closely

related species *T. bannaense* (strain HMAS 248840) that is found at this locus was 97.79% (Table 2; Fig. 10), and the similarity of *tef1* between strain M10 and the most closely related species that are found at this locus were all < 97% (Table 2; Fig. 10) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was not met). This indicates that strain M10 can be recognized as a putative new species (**Comment 3**).

Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain M10 can be identified as *T.* sp. M10**.

(5) ***T. harzianum* CBS 226.95, type strain** (Tables 1 and 4; Fig. 10)

Identification: not required for the type strain

The pairwise sequence similarity of ITS between strain CBS 226.95 and the references that are given in the *ITS56 dataset* showed several values > 76%, which indicated that strain CBS 226.95 belongs to the genus *Trichoderma*. Strain CBS 226.95 is the ex-type strain of species *T. harzianum* sensu stricto. The similarity of *rpb2* and *tef1* between strain CBS 226.95 and the most closely related species *T. harzianum* (itself) that is found at this locus was 100.00% (Table 2; Fig. 10), and the similarity of *tef1* between strain CBS 226.95 and the most closely related species *T. harzianum* (itself) that is found at this locus was 100.00% (Table 2; Fig. 10) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).

Validation: The reference materials are complete.

(6) **B97** (Tables 1 and 4; Fig. 10)

Identification: The pairwise sequence similarity of ITS between strain B97 and the references that are given in the *ITS56 dataset* showed several values > 76%, which indicated that strain B97 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain B97 and the most closely related species *T. harzianum* (strain CBS 226.95) that is found at this locus was 99.51% (Table 2; Fig. 10), and the similarity of *tef1* between strain B97 and the most closely related species *T. harzianum* (strain CBS 226.95) that is found at this locus was 99.60% (Table 2; Fig. 10) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).

Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain B97 can be identified as *T. harzianum***.

(7) **TR274** (Tables 1 and 4; Fig. 10)

Identification: The pairwise sequence similarity of ITS between strain TR274 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain TR274 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain TR274 and the most closely related species *T. harzianum* (strain CBS 226.95) that is found at this locus was 99.51% (Table 2; Fig. 10), and the similarity of *tef1* between strain TR274 and the most closely related species *T. harzianum* (strain CBS 226.95) that was found at this locus was 100.00% (Table 2; Fig. 10) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).

Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain TR274 can be identified as *T. harzianum***.

(8) **T6776** (Tables 1 and 4; Fig. 10)

Identification: The pairwise sequence similarity of ITS between strain T6776 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain T6776 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain T6776 and the most closely related species *T. afroharzianum* (strain CBS 124620) that is found at this locus was 99.88% (Table 2; Fig. 10), and the similarity of *tef1* between strain T6776 and the most closely related species *T. afroharzianum* (strain CBS 124620) that is found at this locus was 99.61% (Table 2; Fig. 10) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).

Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain T6776 can be identified as *T. afroharzianum***. The same conclusion was obtained in Kubicek et al. (2019).

(9) **T22** (Tables 1 and 4; Fig. 10)

Identification: The pairwise sequence similarity of ITS between strain T22 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain T22 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain T22 and the most closely related species *T. afroharzianum* (strain CBS 124620) that is found at this locus was 100.00% (Table 2; Fig. 10), and the similarity of *tef1* between strain T22 and the most closely related species *T. afroharzianum* (strain CBS 124620) that was found at this locus was 98.82% (Table 2; Fig. 10) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).

Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain T22 can be identified as *T. afroharzianum***. Similar conclusion was obtained in Chaverri et al. (2015).

Note: This is a laboratory strain that was obtained in vitro as a UV treated protoplast fusion hybrid of the benomyl-resistant strain T-95 (ATCC 60850) and T12m (ATCC 20737) (Stasz et al. 1988).

(10) **IMV 00454** (Tables 1 and 4; Fig. 10)

Identification: The pairwise sequence similarity of ITS between strain IMV 00454 and the references that are given in the *ITS56 dataset* showed several values > 76%, which indicated that strain IMV 00454 belongs to the genus

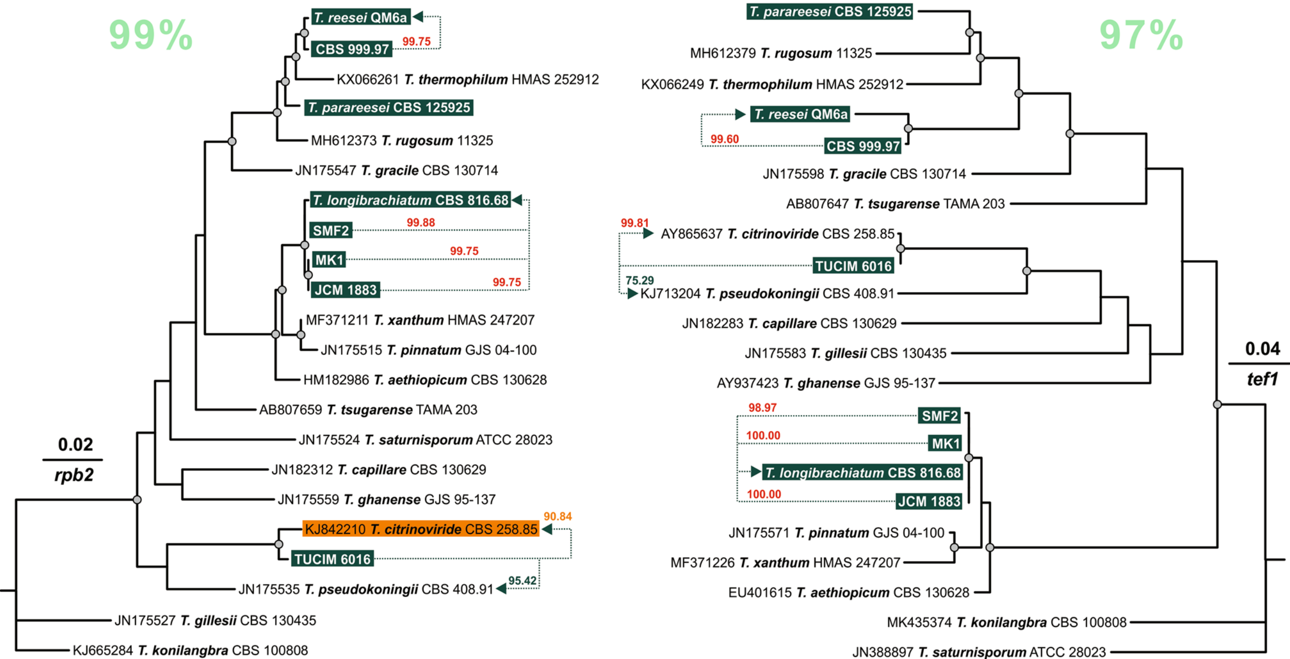


Fig. 11 Molecular identification of genome-sequenced strains from the Section *Longibrachiatum* using maximum-likelihood (ML) phylogeny and pairwise sequence similarity calculation. The ML phylograms of *rpb2* and *tef1* were constructed in IQ-TREE 1.6.12 (bootstrap replicates = 1000) using the nucleotide substitution models of TN + F + I + G4 and TN + F + R2. Circles at the nodes indicate ultrafast bootstrap values > 80 given by IQ-TREE. Genome sequenced strains were shaded in green. The reference strains were provided with the GenBank accessions and the strain name, among which the strains with uncompleted reference information were shaded in orange. Results of the pairwise sequence similarity were illustrated on the dashed lines between the query strain and its closely related species (arrows point to the reference strains). The pairwise sequence similarity calculation was performed using the online tool Clustal OMEGA (https://www.ebi.ac.uk/Tools/msa/clustalo/)

Trichoderma. The similarity of *rpb2* between strain IMV 00454 and the most closely related species *T. simmonsii* (strain CBS 130431) that is found at this locus was 100.00% (Table 2; Fig. 10), and the similarity of *tef1* between strain IMV 00454 and the most closely related species *T. simmonsii* (strain CBS 130431) that is found at this locus was 99.69% (Table 2; Fig. 10) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).

Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain IMV 00454 can be identified as *T. simmonsii***.

(11) **CFAM-422** (Tables 1 and 4; Fig. 10)

Identification: The pairwise sequence similarity of ITS between strain CFAM-422 and the references that are given in the *ITS56 dataset* showed several values > 76%, which indicated that strain CFAM-422 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain CFAM-422 and the most closely related species including *T. afarasin* (strain CBS 130742), *T. lentiforme* (strain DIS 253B), and *T. endophyticum* (strain CBS 130730) that are found at this locus was 99.75%, 99.75%, and 99.51%, respectively (Table 2; Fig. 10), while the similarity of *tef1* between strain CFAM-422 and the most closely related species including *T. afarasin* (strain CBS 130742) and *T. endophyticum* (strain CBS 130730) that are found at this locus was 98.23% and 99.80%, respectively (Table 2; Fig. 10) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was not met. This indicates that strain CFAM-422 can be recognized as *T. afarasin* or *T. endophyticum* (**Comment 3**).

Validation: The reference materials that were used in the molecular identification in this case were not complete due to the lack of sequences from the ex-type strains of several related species (the reference sequences used in this case were obtained from the published voucher materials, which may require taxonomic revision) (**Comment 3**). The identification was precise, but inaccurate, and ambiguous. **Therefore, the strain CFAM-422 can be identified as *T.* cf. *endophyticum***.

(12) **TPhu1** (Tables 1 and 4; Fig. 10)

Identification: The pairwise sequence similarity of ITS between strain TPhu1 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain TPhu1 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain TPhu1 and the most closely related species *T. amazonicum* (strain CBS

126898) and *T. pleuroti* (strain CBS 124387) that are found at this locus were 98.89% and 98.87%, respectively (Table 2; Fig. 10), and the similarity of the *tef1* between strain TPhu1 and the most closely related species *T. pleuroti* (strain CBS 124387) that was found at this locus was 98.10% (Table 2; Fig. 10) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was not met), indicating strain TPhu1 can be recognized as a putative new species (**Comment 3**).

Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain TPhu1 can be identified as *T.* sp. TPhu1**.

(13) **Tr1** (Tables 1 and 4; Fig. 10)

Identification: The pairwise sequence similarity of ITS between strain Tr1 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain Tr1 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain Tr1 and the most closely related species *T. pleuroticola* (strain CBS 124383) that is found at this locus was 99.02% (Table 2; Fig. 10), and the similarity of *tef1* between strain Tr1 and the most closely related species *T. pleuroticola* (strain CBS 124383) that is found at this locus was 100.00% (Table 2; Fig. 10) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).

Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain Tr1 can be identified as *T. pleuroticola***.

(14) **ITEM 908** (Tables 1 and 4; Fig. 10)

Identification: The pairwise sequence similarity of ITS between strain ITEM 908 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain ITEM 908 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain ITEM 908 and the most closely related species including *T. atrobrunneum* (strain G.J.S. 98-183) that is found at this locus was 100.00% (Table 2; Fig. 11), while the similarity of *tef1* between strain ITEM 908 and the most closely related species *T. atrobrunneum* (strain G.J.S. 98-183) that is found at this locus was 95.55% (Table 2; Fig. 11) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was not met).

Validation: The reference materials that were used in the molecular identification in this case were not complete due to the short sequence of *tef1* from the ex-type strain of *T. atrobrunneum* (**Comment 3**). The identification was precise, but inaccurate and ambiguous. **Therefore, the strain ITEM 908 can be identified as *T.* cf. *atrobrunneum***.

(15) ***T. virens*** Gv29-8, type strain (Tables 1 and 4; Fig. 10)

Identification: not required for the type strain

The pairwise sequence similarity of ITS between strain Gv29-8 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain Gv29-8 belongs to the genus *Trichoderma*. Strain Gv29-8 is the ex-type strain of species *T. virens*. The similarity of *rpb2* between strain Gv29-8 and the most closely related species *T. virens* (itself) that was found at this locus was 100.00% (Table 2; Fig. 10), and the similarity of *tef1* between strain Gv29-8 and the most closely related species *T. virens* (itself) that was found at this locus was 100.00% (Table 2; Fig. 10) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).

Validation: The reference materials are complete.

(16) **FT-333** (Tables 1 and 4; Fig. 10)

Identification: The pairwise sequence similarity of ITS between strain FT-333 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain FT-333 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain FT-333 and the most closely related species *T. virens* (strain Gv29-8) that is found at this locus was 100.00% (Table 2; Fig. 10), and the similarity of *tef1* between strain FT-333 and the most closely related species *T. virens* (strain Gv29-8) that is found at this locus was 100.00% (Table 2; Fig. 10) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).

Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain FT-333 can be identified as *T. virens***.

(17) **Tv-1511** (Tables 1 and 4; Fig. 10)

Identification: The pairwise sequence similarity of ITS between strain Tv-1511 and the references that were given in *ITS56 dataset* showed several values > 76%, which indicated that strain Tv-1511 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain Tv-1511 and the most closely related species *T. virens* (strain Gv29-8) that is found at this locus was 100.00% (Table 2; Fig. 10), and the similarity of *tef1* between strain Tv-1511 and the most closely related species *T. virens* (strain Gv29-8) that were found at this locus was 99.80% (Table 2; Fig. 10) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).

Validation: The reference materials are complete. The identification was precise, accurate and unambiguous. **Therefore, the strain Tv-1511 can be identified as *T. virens***.

(18) **IMI 304061** (Tables 1 and 4; Fig. 10)

Identification: The pairwise sequence similarity of ITS between strain IMI 304061 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain IMI 304061 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain IMI 304061 and the most closely related species *T. neocrassum*

(strain G.J.S. 01–227) that is found at this locus was 99.26% (Table 2; Fig. 10), while the similarity of *tef1* between strain IMI 304061 and the most closely related species *T. virens* (strain Gv29-8) that is found at this locus was 97.26% (Table 2; Fig. 10) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was not met). The similarity assessment and phylogeny of the *tef1* locus indicated that strain IMI 304061 can be recognized as a putative new species other than *T. virens* and *T. neocrassum* (**Comment 3**).

Validation: The reference materials used in the molecular identification in this case were not complete due to the short sequence of *tef1* from the ex-type strain of *T. neocrassum* (strain G.J.S. 01-227, **Comment 3**). The identification is precise and accurate but ambiguous. **Therefore, the strain IMI 304061 can be identified as *T.* sp. aff. *neocrassum* IMI 304061.**

(19) ***T. reesei* QM 6a, type strain** (Tables 1 and 4; Fig. 11)

Identification: not required for the type strain

The pairwise sequence similarity of ITS between strain QM 6a and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain QM 6a belongs to the genus *Trichoderma*. Strain QM 6a is the ex-type strain of species *T. reesei*. The similarity of *rpb2* between strain QM 6a and the most closely related species *T. reesei* (itself) that is found at this locus was 100.00% (Table 2; Fig. 11), and the similarity of *tef1* between strain QM 6a and the most closely related species *T. reesei* (itself) that is found at this locus was 100.00% (Table 2; Fig. 11) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).

Validation: The reference materials are complete.

(20) **CBS 999.97** (Tables 1 and 4; Fig. 11)

Identification: The pairwise sequence similarity of ITS between strain CBS 999.97 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain CBS 999.97 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain CBS 999.97 and the most closely related species *T. reesei* (strain QM 6a) that is found at this locus was 99.75% (Table 2; Fig. 11), and the similarity of *tef1* between strain CBS 999.97 and the most closely related species *T. reesei* (strain QM 6a) that is found at this locus was 99.60% (Table 2; Fig. 11) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).

Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain CBS 999.97 can be identified as *T. reesei*.**

(21) ***T. parareesei* CBS 125925, type strain** (Tables 1 and 4; Fig. 11)

Identification: not required for the type strain

The pairwise sequence similarity of ITS between strain CBS 125925 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain QM 6a belongs to the genus *Trichoderma*. Strain CBS 125925 is the ex-type strain of species *T. parareesei*. The similarity of *rpb2* between strain CBS 125925 and the most closely related species *T. parareesei* (itself) that is found at this locus was 100.00% (Table 2; Fig. 11), and the similarity of *tef1* between strain CBS 125925 and the most closely related species *T. parareesei* (itself) that is found at this locus was 100.00% (Table 2; Fig. 11) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).

Validati on: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain CBS 125925 can be identified as *T. parareesei*.**

(22) ***T. longibrachiatum* CBS 816.68, type stain** (Tables 1 and 4; Fig. 11)

Identification: not required for the type strain

The pairwise sequence similarity of ITS between strain CBS 816.68 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain CBS 816.68 belongs to the genus *Trichoderma*. Strain CBS 816.68 is the ex-type strain of species *T. longibrachiatum*. The similarity of *rpb2* between strain CBS 816.68 and the most closely related species *T. longibrachiatum* (itself) that is found at this locus was 100.00% (Table 2; Fig. 11), and the similarity of *tef1* between strain CBS 816.68 and the most closely related species *T. longibrachiatum* (itself) that is found at this locus was 100.00% (Table 2; Fig. 11) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).

Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain CBS 816.68 can be identified as *T. longibrachiatum*.**

(23) **SMF2** (Tables 1 and 4; Fig. 11)

Identification: The pairwise sequence similarity of ITS between strain SMF2 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain SMF2 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain SMF2 and the most closely related species *T. longibrachiatum* (strain CBS 816.68) that is found at this locus was 99.88% (Table 2; Fig. 11), and the similarity of *tef1* between strain SMF2 and the most closely related species *T. longibrachiatum* (strain CBS 816.68) that is found at this locus was 98.97% (Table 2; Fig. 11) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).

Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous.

Therefore, the strain SMF2 can be identified as *T. longibrachiatum*.

(24) **MK1** (Tables 1 and 4; Fig. 11)

Identification: The pairwise sequence similarity of ITS between strain MK1 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain MK1 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain MK1 and the most closely related species *T. longibrachiatum* (strain CBS 816.68) that is found at this locus was 99.75% (Table 2; Fig. 11), and the similarity of *tef1* between strain MK1 and the most closely related species *T. longibrachiatum* (strain CBS 816.68) that is found at this locus was 100.00% (Table 2; Fig. 11) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).
Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain MK1 can be identified as *T. longibrachiatum*.**

(25) **JCM 1883** (Tables 1 and 4; Fig. 11)

Identification: The pairwise sequence similarity of ITS between strain JCM 1883 and the references that are given in the *ITS56 dataset* showed several values > 76%, which indicated that strain JCM 1883 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain MK1 and the most closely related species *T. longibrachiatum* (strain CBS 816.68) that is found at this locus was 99.75% (Table 2; Fig. 11), and the similarity of *tef1* between strain JCM 1883 and the most closely related species *T. longibrachiatum* (strain CBS 816.68) that is found at this locus was 100.00% (Table 2; Fig. 11) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).
Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain JCM 1883 can be identified as *T. longibrachiatum*.**

(26) **TUCIM 6016** (Tables 1 and 4; Fig. 11)

Identification: The pairwise sequence similarity of ITS between strain TUCIM 6016 and the references that are given in the *ITS56 dataset* showed several values > 76%, which indicated that strain TUCIM 6016 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain TUCIM 6016 and the most closely related species including *T. citrinoviride* (strain CBS 258.85) that is found at this locus was 90.84% (Table 2; Fig. 11), while the similarity of *tef1* between strain TUCIM 6016 and the most closely related species *T. citrinoviride* (strain CBS 258.85) that is found at this locus was 99.81% (Table 2; Fig. 11) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was not met).
Validation: The reference materials used in the molecular identification in this case were not complete due to the short sequence of *rpb2* from the ex-type strain (**Comment 3**). The identification was precise, but inaccurate and ambiguous. **Therefore, the strain TUCIM 6016 can be identified as *T.* cf. *citrinoviride*.**

(27) ***T. atroviride* IMI 206040**, (Tables 1 and 4; Fig. 12)

The pairwise sequence similarity of ITS between strain IMI 206040 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain IMI 206040 belongs to the genus *Trichoderma*. Strain IMI 206040 is not the ex-type strain of species *T. atroviride* but is considered as a reference strain (Kubicek et al. 2011, 2019). The similarity of *rpb2* between strain IMI 206040 and the most closely related species *T. atroviride* (itself) that is found at this locus was 100.00% (Table 2; Fig. 12), and the similarity of *tef1* between strain IMI 206040 and the most closely related species *T. atroviride* (itself) that is found at this locus was 100.00% (Table 2; Fig. 12) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).
Validation: The reference materials were complete. The identification was precise, accurate and ambiguous. **Therefore, the strain IMI 206040 can be identified as *T.* atro*viride*.**

(28) **P1,** (Tables 1 and 4; Fig. 12)

Identification: The pairwise sequence similarity of ITS between strain P1 and the references that were given in *ITS56 dataset* showed several values > 76%, which indicated that strain P1 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain P1 and the most closely related species *T. atroviride* (strain IMI 206040) that is found at this locus was 100.00% (Table 2; Fig. 12), and the similarity of *tef1* between strain P1 and the most closely related species *T. atroviride* (strain IMI 206040) that is found at this locus was 99.43% (Table 2; Fig. 12) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).
Validation: The reference materials are complete.

Note: this is a laboratory strain that was obtained in vitro from "*T. harzianum* 107" (DNA Barcoding was not available) by screening towards increased resistance to xenobiotics (Tronsmo, 1991). The strain is frequently treated as a wild-type isolate in research related to plant protection and growth promotion (biocontrol).

(29) **XS2015** (Tables 1 and 4; Fig. 12)

Identification: The pairwise sequence similarity of ITS between strain XS2015 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain XS2015 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain XS2015 and the most closely related species *T. atroviride* (strain IMI 206040) that is found at this locus was 100.00% (Table 2; Fig. 12), and the similarity of *tef1* between strain XS2015 and the most

closely related species *T. atroviride* (strain IMI 206040) that is found at this locus was 100.00% (Table 2; Fig. 12) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).
Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain XS2015 can be identified as *T. atroviride*.**

(30) **F7** (Tables 1 and 4; Fig. 12)

Identification: The pairwise sequence similarity of ITS between strain F7 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain F7 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain F7 and the most closely related species *T. atroviride* (strain IMI 206040) that is found at this locus was 100.00% (Table 2; Fig. 12), and the similarity of *tef1* between strain F7 and the most closely related species *T. atroviride* (strain IMI 206040) that is found at this locus was 99.43% (Table 2; Fig. 12) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).
Validation: The reference materials are complete. The identification is precise, accurate, and unambiguous. **Therefore, the strain F7 can be identified as *T. atroviride*.**

(31) **B10** (Tables 1 and 4; Fig. 12)

Identification: The pairwise sequence similarity of ITS between strain B10 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain B10 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain B10 and the most closely related species *T. atroviride* (strain IMI 206040) that is found at this locus was 100.00% (Table 2; Fig. 12), and the similarity of *tef1* between strain B10 and the most closely related species *T. atroviride* (strain IMI 206040) that is found at this locus was 99.43% (Table 2; Fig. 12) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).
Validation: The reference materials are complete. The identification was precise, accurate and unambiguous. **Therefore, the strain B10 can be identified as *T. atroviride*.**

(32) **JCM 9410** (Tables 1 and 4; Fig. 12)

Identification: The pairwise sequence similarity of ITS between strain JCM 9410 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain JCM 9410 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain JCM 9410 and the most closely related species *T. atroviride* (strain IMI 206040) that is found at this locus was 99.02% (Table 2; Fig. 12), and the similarity of *tef1* between strain JCM 9410 and the most closely related species *T. atroviride* (strain IMI 206040) that is found at this locus was 100.00% (Table 2; Fig. 12) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).
Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain JCM 9410 can be identified as *T. atroviride*.**

(33) **LY357** (Tables 1 and 4; Fig. 12)

Identification: The pairwise sequence similarity of ITS between strain LY357 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain LY357 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain LY357 and the most closely related species including *T. paratroviride* (strain CBS 136489) and *T. atroviride* (strain IMI 206040) that are found at this locus were 98.65% and 97.79%, respectively (Table 2; Fig. 12), and the similarity of *tef1* between strain LY357 and the most closely related species including *T. paratroviride* (strain CBS 136489) and *T. atroviride* (strain IMI 206040) that are found at this locus were 83.37% and 91.29%, respectively (Table 2; Fig. 12) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was not met). This indicates that strain LY357 can be recognized as a putative new species (**Comment 3.4**).
Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain LY357 can be identified as *T.* sp. LY357.**

(34) **T6085** (Tables 1 and 4; Fig. 12)

Identification: The pairwise sequence similarity of ITS between strain T6085 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain T6085 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain T6085 and the most closely related species *T. gamsii* (strain G.J.S. 04-09) that is found at this locus was 99.38% (Table 2; Fig. 12), and the similarity of *tef1* between strain T6085 and the most closely related species *T. gamsii* (strain G.J.S. 04-09) that is found at this locus was 97.31% (Table 2; Fig. 12) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).
Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain T6085 can be identified as *T. gamsii*.**

(35) **A5MH** (Tables 1 and 4; Fig. 12)

Identification: The pairwise sequence similarity of ITS between strain A5MH and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain A5MH belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain A5MH and the most closely related species *T. gamsii* (strain G.J.S. 04-09) that is found at this locus was 99.63% (Table 2; Fig. 12), and the similarity of *tef1* between strain A5MH and the most closely related species *T. gamsii* (strain G.J.S. 04-09) that is found at this locus was 95.98% (Table 2; Fig. 12) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was not met).Thus, A5MH is

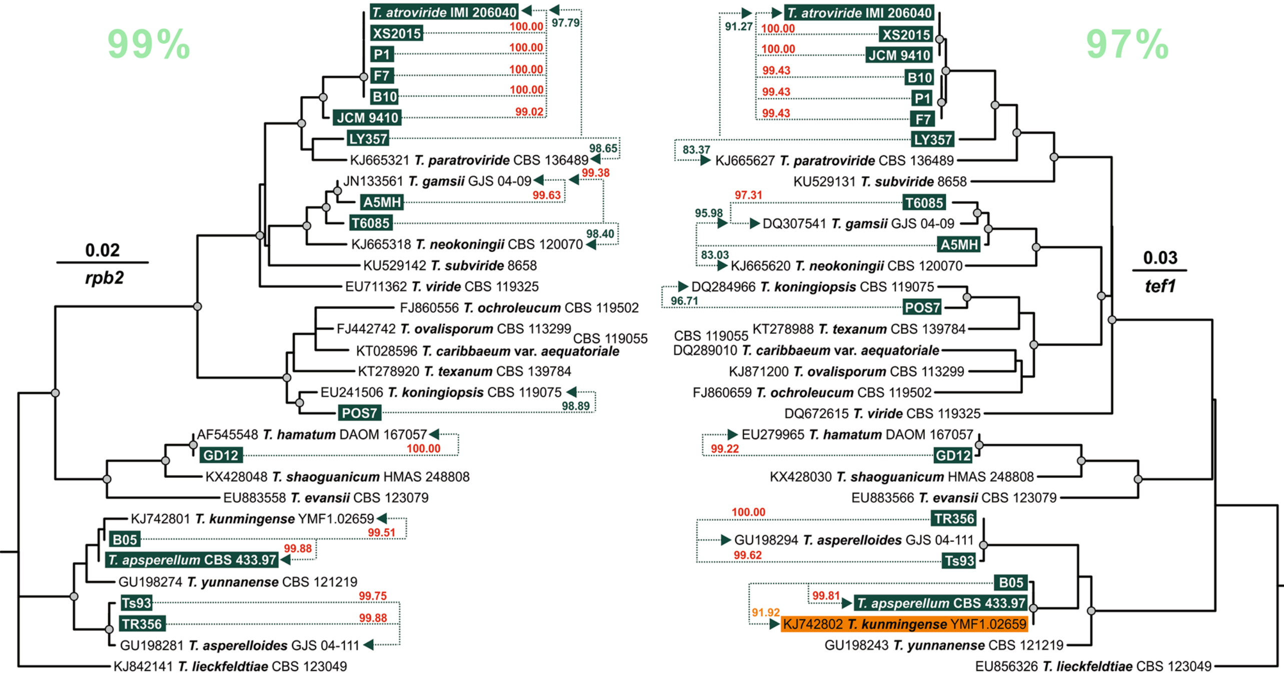


Fig. 12 Molecular identification of genome-sequenced strains from the Section *Trichoderma* using maximum-likelihood (ML) phylogeny and pairwise sequence similarity calculation. The ML phylograms of *rpb2* and *tef1* were constructed in IQ-TREE 1.6.12 (bootstrap replicates = 1000) using the nucleotide substitution models of TNe + G4 and HKY + F + G4. Circles at the nodes indicate ultrafast bootstrap values > 80 given by IQ-TREE. Genome sequenced strains were shaded in green. The reference strains were provided with the GenBank accessions and the strain name, among which the strains with uncompleted reference information were shaded in orange. Results of the pairwise sequence similarity were illustrated on the dashed lines between the query strain and its closely related species (arrows point to the reference strains). The pairwise sequence similarity calculation was performed using the online tool ClustalOMEGA (https://www.ebi.ac.uk/Tools/msa/clustalo/)

a putative new species that is closely related to *T. gamsii* (**Comment 3**).

Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain A5MH can be identified as *T.* sp. aff. *gamsii* A5MH.**

(36) **POS7** (Tables 1 and 4; Fig. 12)

Identification: The pairwise sequence similarity of ITS between strain POS7 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain POS7 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain POS7 and the most closely related species *T. koningiopsis* (strain CBS 119075) that is found at this locus was 98.89% (Table 2; Fig. 12), and the similarity of *tef1* between strain POS7 and the most closely related species *T. koningiopsis* (strain CBS 119075) that is found at this locus was 96.71% (Table 2; Fig. 12) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was not met). This indicates that strain POS7 can be recognized as a putative new species closely related to *T. koningiopsis* (**Comment 3**).

Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain POS7 can be identified as *T.* sp. aff. *koningiopsis* POS7.**

(37) ***T. asperellum* CBS 433.95, type strain** (Tables 1 and 4; Fig. 12)

Identification: not required for the type strain

The pairwise sequence similarity of ITS between strain CBS 433.95 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain CBS 433.95 belongs to the genus *Trichoderma*. Strain CBS 433.95 is the ex-type strain of species *T. atroviride*. The similarity of *rpb2* between strain CBS 433.95 and the most closely related species *T. asperellum* (itself) that is found at this locus was 100.00% (Table 2; Fig. 12), and the similarity of *tef1* between strain CBS 433.95 and the most closely related species *T. asperellum* (itself) that is found at this locus was 100.00% (Table 2; Fig. 12) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).

Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain CBS 433.95 can be identified as *T. asperellum*.**

(38) **B05** (Tables 1 and 4; Fig. 12)

Identification: The pairwise sequence similarity of ITS between strain B05 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain B05 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain B05 and the most closely related species including *T. kunmingense* (strain YMF1.02659) and *T. asperellum* (strain CBS 433.97) that are found at this locus were 99.88% and 99.51%, respectively (Table 2; Fig. 12), and the similarity of *tef1* between strain B05 and the most closely related species including *T. kunmingense* (strain YMF1.02659) and *T. asperellum* (strain CBS 433.97) that are found at this locus were 91.92% and 99.81%, respectively (Table 2; Fig. 12) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was not met).
Validation: The reference materials that were used in the molecular identification in this case were not complete due to the short sequences of *tef1* from the ex-type strain of *T. kunmingense* (strain YMF1.02659) (**Comment 3**), indicating that strain B05 can be recognized as *T. asperellum* without excluding its possibility of being *T. kunmingense* (**Comment 3**). Thus, the group of *T. asperellum* and the species closely related to it may need a critical taxonomic revision. The identification was precise, but inaccurate and ambiguous. **Therefore, the strain B05 can be identified as *T.* cf. *asperellum***.

(39) **TR356** (Tables 1 and 4; Fig. 12)

Identification: The pairwise sequence similarity of ITS between strain TR356 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain TR356 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain TR356 and the most closely related species *T. asperelloides* (strain G.J.S. 04-111) that is found at this locus was 99.88% (Table 2; Fig. 12), and the similarity of *tef1* between strain TR356 and the most closely related species *T. asperelloides* (strain G.J.S. 04-111) that is found at this locus was 100.00% (Table 2; Fig. 12) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).
Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain TR356 can be identified as *T. asperelloides***.

(40) **Ts93** (Tables 1 and 4; Fig. 12)

Identification: The pairwise sequence similarity of ITS between strain Ts93 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain Ts93 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain Ts93 and the most closely related species *T. asperelloides* (strain G.J.S. 04-111) that is found at this locus was 99.75% (Table 2; Fig. 12), and the similarity of *tef1* between strain Ts93 and the most closely related species *T. asperelloides* (strain G.J.S. 04-111) that is found at this locus was 99.62% (Table 2; Fig. 12) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).
Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain Ts93 can be identified as *T. asperelloides***.

(41) **GD12** (Tables 1 and 4; Fig. 12)

Identification: The pairwise sequence similarity of ITS between strain GD12 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain GD12 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain GD12 and the most closely related species *T. hamatum* (strain DAOM 167057) that is found at this locus was 100.00% (Table 2; Fig. 12), and the similarity of *tef1* between strain GD12 and the most closely related species *T. hamatum* (strain DAOM 167057) that is found at this locus was 99.22% (Table 2; Fig. 12) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).
Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain GD12 can be identified as *T. hamatum***.

(42) **IBT 40837** (Tables 1 and 4; Fig. 13)

Identification: The pairwise sequence similarity of ITS between strain IBT 40837 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain IBT 40837 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain IBT 40837 and the most closely related species *T. arundinaceum* (strain CBS 119575) that is found at this locus was 100.00% (Table 2; Fig. 13), and the similarity of *tef1* between strain IBT 40837 and the most closely related species *T. arundinaceum* (strain CBS 119575) that is found at this locus was 100.00% (Table 2; Fig. 13) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).
Validation: The reference materials are complete. The identification was precise, accurate, and unambiguous. **Therefore, the strain IBT 40837 can be identified as *T. arundinaceum***.

(43) **IBT 40841** (Tables 1 and 4; Fig. 13)

Identification: The pairwise sequence similarity of ITS between strain IBT 40841 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain IBT 40841 belongs to the genus *Trichoderma*. The similarity of *rpb2* between strain IBT 40841 and the most closely related species *T. brevicompactum* (strain CBS 109720) that is found at this locus was 100.00% (Table 2; Fig. 13), and the similarity of *tef1* between strain IBT 40841 and the most closely related species including *T. brevicompactum* (strain CBS 109720) that is found at this locus was 93.67% (Table 2; Fig. 13) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was not met).

Validation: The reference materials that were used in the molecular identification in this case were not complete due to the short sequences of *tef1* from the ex-type strain of *T. brevicompactum* (strain CBS 109720) **(Comment 3). The identification was** precise and accurate but ambiguous. **Therefore, the strain IBT 40841 can be identified as *T.* cf. *brevicompactum.***

(44) **TW21990_1** (Tables 1 and 4)

Identification: The pairwise sequence similarity of ITS between strain TW21990_1 and the references that were given in the *ITS56 dataset* showed several values > 76%, which indicated that strain CBS 433.95 belongs to the genus *Trichoderma*. Strain TW21990_1 is the ex-type strain of species *T. cyanodichotomus*. The similarity of *rpb2* between strain TW21990_1 and the most closely related species *T. cyanodichotomus* (itself) that is found at this locus was 100.00% (Table 2), and the similarity of *tef1* between strain TW21990_1 and the most closely related species *T. cyanodichotomus* (itself) that is found at this locus was 100.00% (Table 2) (i.e., the condition $\exists!(rpb2_{99} \cong tef1_{97})$ was met).
Validation: The reference materials are complete. The identification is precise, accurate and unambiguous. **Therefore, the strain TW21990_1 can be identified as *T. cyanodichotomus*.**

Thus, the molecular identification protocol that was applied to 44 *Trichoderma* strains resulted in unambiguous identification of 38 (86%) strains and allowed assignment of 31 species names (including seven ex-type strains) (70%) and the proposal of eight new species (18%). Six (14%) identifications remained ambiguous because of either incomplete reference material or ambiguous taxonomy of the related species. Together, this result indicates the urgent need to achieve an agreement on the genus-wide criteria that are suitable to allow recognition of the species in *Trichoderma* and the requirement to complete the reference materials based on these criteria. Furthermore, the recognition of a considerable amount of putative new species indicates further rapid growth of *Trichoderma* diversity in the near future.

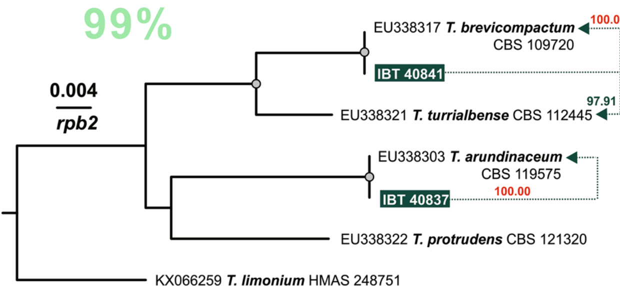
Discussion and suggestions

This study shows that identification of species is challenging for us and for most experts. As shown by the survey, *Trichoderma* researchers spent an average of one hour identifying the two strains based on three DNA barcodes for each, and achieved 50% accuracy. The rate of new species descriptions in the genus of *Trichoderma* was as high as approximately 50 per year, and this number is expected to increase faster in the future. Therefore, we selected a white paper format to present a detailed review on *Trichoderma* taxonomy, exploring the problem of molecular identification and proposing a possible solution in a form of an authoritative guideline.

We aimed to develop a protocol for the molecular identification of *Trichoderma* that should reflect the contemporary taxonomy of the genus. This means that where possible, we avoided an option of a taxonomic revision for a particular group or the entire genus (see for example, Houbraken et al. 2020). Instead, we considered *Trichoderma* to be a genus in its privileged taxonomic position because most of its species have been delimitated after the introduction of DNA-based methods. *Trichoderma* has received much attention from fungal taxonomists, which has resulted in the ample new species descriptions based on the newest (DNA-based) technologies and concepts (Seifert and Rossman 2010) that were mainly published over the last decade (Fig. 2). The "recently taxonomically resolved" state for *Trichoderma* taxonomy (that we believe is a correct assessment) was also considered to be an argument in support of the initiation of the whole-genus genomics project for *Trichoderma* (https://genome.jgi.doe.gov/portal/Genwidrichoderma/Genwidrichoderma.info.html) as taxonomy underlines all biological studies. Thus, our intention was to "measure" genetic similarities and dissimilarities that have already been used by the *Trichoderma* taxonomy providers and incorporate them into the DNA Barcoding protocol. In this manner, we hoped to balance the contradiction between the ultimate subjectivity in the species recognition and the need for the exact species identification that is crucial for applications, patenting, and research purposes. The availability of such a protocol should facilitate the accurate, precise, and unambiguous identification of *Trichoderma* species and beneficially contribute to the development of applications and research on these fungi.

We previously proposed an automated oligonucleotide DNA Barcoding tool for *Trichoderma* (Druzhinina et al. 2005; Kopchinskiy et al. 2005) that was based on ITS for approximately 100 species and was widely appreciated by the researchers for its unambiguous results and simplicity. Due to the insufficient variability of ITS between *Trichoderma* species (*see above*), this tool is no longer functional. The current study reveals the following features and their combination that impeded the simplicity of the molecular identification protocol that is presented here:

1. Most *Trichoderma* species cannot be identified by a sequence similarity search or by the multiloci phylogenetic analysis if it is applied alone.
2. The identification procedure requires three DNA barcoding loci, and sequences have to be prepared (trimmed) for the analysis.
3. The retrieval of reference materials and the calculations of the pairwise similarities are tedious and they frequently need to be performed manually.
4. In *silico* results require validation against the availability of reference materials (Figs. 10–13).



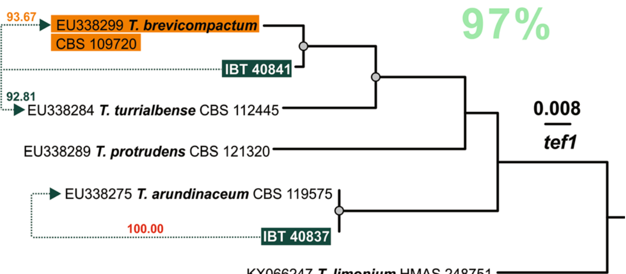


Fig. 13 Molecular identification of genome-sequenced strains from the *Brevicompactum* clades using maximum-likelihood (ML) phylogeny and pairwise sequence similarity calculation. The ML phylograms of *rpb2* and *tef1* were constructed in IQ-TREE 1.6.12 (bootstrap replicates = 1000) using the nucleotide substitution models of TIM2e and HKY + F + I. Circles at the nodes indicate ultrafast bootstrap values > 80 given by IQ-TREE. Genome sequenced strains were shaded in green. The reference strains were provided with the GenBank accessions and the strain name, among which, strains with uncompleted reference information were shaded in orange. Results of the pairwise sequence similarity were illustrated on the dashed lines between the query strain and its closely related species (arrows point to the reference strains). The pairwise sequence similarity calculation was performed using the online tool of ClustalOMEGA (https://www.ebi.ac.uk/Tools/msa/clustalo/)

It is now evident for all DNA barcoded fungi that any molecular identification requires its biological verification as a necessary step (Lücking et al. 2020). The combination of several in silico methods was already appreciated by *Trichoderma* experts because 27 of the 47 respondents who completed our online survey did not rely on any of the methods alone, and instead, they used all the available tools. Therefore, the users of *Trichoderma* taxonomy are expected to have skills not only in mycology, fungal taxonomy, basic DNA techniques, but also sequence analysis.

To date, the sufficient training of taxonomy users is essential because there is no clear distinction between taxonomy users and taxonomy providers. The diversity of *Trichoderma* is such that the initial taxonomy users frequently detect potentially novel species and start their descriptions, i.e., become taxonomy providers. Conversely, taxonomy providers are usually the most dedicated users of existing taxonomy, but the work of taxonomy providers is essentially more laborious and is associated with more responsibility because the outcome (the taxonomic and nomenclatural acts, e.g., the formally described taxonomic entities) influences the development of taxonomic standards that are applied to a particular group of organisms. This study demonstrates how the results that were obtained by a few *Trichoderma* taxonomy providers in the last decade strongly impact the ambiguity of *Trichoderma* species identification and the application of species recognition criteria.

The transformations of the genealogical concordance species concept in *Trichoderma* taxonomy

Regardless of the species recognition criteria that are used, fungal taxonomy allows room for subjectivity in the assessment of species borders. In *Trichoderma,* this can be exemplified by many cases when taxonomists observed a considerable genetic, ecological, and phenotypic polymorphism within a particular group, but they did not find it sufficient for the species delimitations [see *T. harzianum* sensu Chaverri and Samuels (2003) or *T. guizhouense* sensu Chaverri et al. (2015)]. It is also possible that the same researchers change their assessment of species borders as more materials are studied [see the revision of the *Harzianum* Clade by Chaverri et al. (2015)]. However, numerous morphologically identical and genetically highly similar species have been named and formally described as cryptic taxa based on subtle genetic distance [e.g., *T. bissettii* was delimited from *T. longibrachiatum* (Sandoval-Denis et al. 2014) and *T. kunmingense* was separated from *T. asperellum* (Qiao et al. 2018)].

The ambiguity of taxonomy reflects the diversity of species recognition criteria that are applicable for fungi [recently reviewed by Lücking et al. (2020)]. However, only a few could be potentially suitable for the genus *Trichoderma* (Druzhinina and Kubicek 2005). Among them, the morphological species concept is no longer suitable for this genus because even the largest infrageneric groups, sections, are not always morphologically distinguishable [for example, see the transfer of the famous biocontrol strain P1 from *T. harzianum* (Tronsmo 1991) to *T. atroviride* (Mach et al. 1999)]. The high ambiguity of morphological identification of *Trichoderma* is no longer discussed. The biological species concept that is verifiable through in vitro mating is restricted to a single species *T. reesei* (Seidl et al. 2009) because none of the other species that have been found to date could repeatedly produce fruiting bodies in vitro. Therefore, the genealogical concordance phylogenetic species recognition (GCPSR) concept (Taylor et al. 2000) is the most widely claimed approach in this genus (*see* references *below*). After detecting many cryptic species, GCPSR

became the only suitable option for species delimitation in *Trichoderma*. Although this concept was shown to be a powerful tool for species delimitation (Druzhinina and Kubicek 2005; Druzhinina et al. 2005; Jaklitsch 2009, 2011; Jaklitsch et al. 2013; Jaklitsch and Voglmayr 2015; Chen and Zhuang 2017a, b, d; Qin and Zhuang 2017), the two decades of its application, at least in *Trichoderma*, revealed several shortcomings. GCPSR requires the concordance of phylogram topologies from at least two unlinked loci that are not contradicted by the other loci (Taylor et al. 2000) (Fig. 14). In practice, the application of GCPSR assumes (i) the consideration of individual trees and (ii) sets of several strains per each species, which ultimately include reference materials for all species in questions. For example, Druzhinina et al. (2008) studied the evolutionary relationships between such species as *T. longibrachiatum*, *T. orientale*, and several related strains (Fig. 14a). They constructed single locus phylograms for *tef1*, *chi18-5* (*ech42*), and *cal1* (Box 1). The topologies and statistical supports for HTUs (hypothetical taxonomic units, internal nodes on phylograms) for *tef1* and *chi18-5* were highly concordant and revealed four monophyletic phylogenetic species (PS I–PS IV, Fig. 14a), which were supported by statistically significant posterior probabilities. The topology of *cal1* did not contradict this conclusion. However, the resolution in *cal1* phylogram was low. Nevertheless, this analysis allowed the application of GCPSR and the conclusion that individual PSs corresponded to four phylogenetic species (Druzhinina et al. 2008) that were then taxonomically described (Druzhinina et al. 2012; Samuels et al. 2012). Conversely, a similar analysis that was performed in the *Harzianum* Clade [a "demon" of *Trichoderma* taxonomy, Druzhinina et al. (2010b)], which revealed that GCPSR could not be applied to this group (Fig. 14b) because all strains "jumped" from clade to clade in single loci phylograms (Fig. 14b). Thus, no clades seen on a combined phylogram (based on the concatenated three loci) were apparent on single-gene phylograms. Based on the species delimitation proposal (Taylor et al. 2000), the whole clade represented a single species [that was provisionally named as "pseudoharzianum matrix," (Druzhinina et al. 2010b)] because the phylograms of tested loci contradicted each other at this level. To explain the cases of concordant phylogenies for the analysis of Section *Longibrachiatum* and non-concordant for the *Harzianum* Clade, the authors of each study performed analyses of genetic recombination. This showed that *T. longibrachiatum* s. s. is likely a (clonal) agamospecies, while *T. orientale* is most likely holomorphic (Druzhinina et al. 2008). However, the evidence of intensive sexual recombination was obtained for most of the strains in the *Harzianum* Clade, except the *T. harzianum* s. s. subclade (Druzhinina et al. 2010b) explaining "jumping" positions of individual strains on single-loci phylograms. This result prevented the authors from delimiting the *Harzianum* Clade in several species because the GCPSR concept was not applicable. There were no other species recognition criteria available, therefore no taxonomic acts were performed. Thus, these examples illustrated one frequent shortcoming when applying for GCPSR in *Trichoderma*. The analysis of single loci phylograms is a critical and compulsory step in the application of GCPSR. Additionally, ambiguous cases can be verified by the in silico tests for sexual recombination (Rossman et al. 2016) or other analyses.

Unfortunately, in a rapidly increasing number of studies, the new *Trichoderma* species are delineated and described based on the analysis of a combined phylogram that was obtained from a concatenated alignment of several loci (Chaverri et al. 2011, 2015; Chen and Zhuang 2017a, b, d; Qin and Zhuang 2016c; Jaklitsch 2009, 2011; Jaklitsch and Voglmayr, 2015) without consideration of the single locus trees. Such studies do not rely of genealogical concordance. Although GCPSR is usually cited and claimed, species are delimitated based on the topology of a single tree, i.e. based on the phylogenetic species concept (Box 1). The authors use such parameters as the branch length, and statistical support for individual HTU to assign a species rank to a group of strains, or even frequently to a single strain (*see below*). One example is the delimitation of the *Harzianum* Clade (mentioned above) in a dozen new species based on the combined phylogram of *act*, *tef1*, *cal1*, and ITS (Chaverri et al. 2015). Our evaluation of the sequences provided by the authors showed that the taxonomic act was largely completed based on the phylogenetic signal mainly obtained from polymorphism of an approximately 250 bp-long fragments of the *tef1* gene. This is because the three other loci (ITS, *act*, and *cal1*) were sampled for roughly 60% of isolates, and *act* and ITS were highly conserved. Because individual phylograms were not assessed, the strict sense GCPSR was not applied in that study. Moreover, the monographs of Jaklitsch (2009, 2011) on European species of *Hypocrea* and the work on *Trichoderma* diversity in Southern Europe (Jaklitsch and Voglmayr 2015) also do not contain single loci trees, but species were delimitated mainly (not only) based on the strict consensus phylogram of *tef1* and *rpb2*. Moreover, W. Jaklitsch used not the hypervariable fragment of *tef1* considered above, but the sixth exon from the *tef1* gene, although he noted that it "shows less variability among species than *rpb2*" (Jaklitsch 2009). [Refer to Fig. 4c and respective text *above* describes that *rpb2* is already highly conserved and species are delimited based on minor (< 1%) dissimilarity. It means that the polymorphism of *tef1* exon fragment is neglectable.] This algorithm based on the concatenated phylograms of the two conserved loci (*tef1* and *rpb2*) was then adopted in more recent taxonomic studies on *Trichoderma* that assigned > 90 new species (Chen and Zhuang 2016, 2017a, b, c; d; Qin and Zhuang 2016a, b, c, 2017). The drawback of this approach is the lack of the third marker that is strictly required for

Genealogical concordance of *tef1* and *chi18-5* is not contradicted by *cal1*

from Druzhinina et al., 2008

A

tef1
chi18-5
cal1
0.1
PS I
PS II
PS III
PS IV

No genealogical concordance

from Druzhinina et al., 2010

B

tef1
cal1
chi18-5
0.1

◂**Fig. 14** Examples of genealogical concordance in the genus *Trichoderma*. **a** Phylogenetic trees taken from Druzhinina et al. (2008) that describe phylogenetic concordance of the three loci (*tef1, chi18-5,* and *cal1*) in the Section *Longibrachiatum*. PS I–PS IV correspond to phylogenetic species. Colors indicate statistically supported clades of the concatenated phylogram of the three loci. See Druzhinina et al. (2008) for details. **b** Phylogenetic trees taken from Druzhinina et al. (2010b) describing the lack of phylogenetic concordance of the three loci (*tef1*, *chi18-5* and *cal1*) in the *Harzianum* Clade. Colors indicate clades seen on the concatenated phylogram of the three loci. See Druzhinina et al. (2008) for details

GCPSR. Moreover, the use of combined phylogeny does not allow evaluation of the concordance between the two loci and does not reveal their polymorphism. If one of the combined markers is not sufficiently variable or conserved, it does not contribute to the structure of the combined tree. The recombination and incongruences between tree topologies have become neglected. Thus, despite claims in the publications, species resolved based on the combined phylograms of the two or sometimes even several loci were not recognized based on the strict sense of the GCPSR concept, although a phylogenetic species recognition (PSR) concept was applied (See Definitions in Box 1). If we consider that these are at least 200 species described by the groups of W.M. Jaklitsch and W.Y. Zhuang, we can conclude that GCPSR, the most powerful and widely accepted species concept for fungi, have not been applied for the delimitation of the majority of *Trichoderma* species. Because the choice of a species criteria and concepts are not determined in the *Code*, we refrained from any evaluation of the rationale for some of species delimitations. Instead, we used this example to show how the work of taxonomy providers influences the applicability of species recognition criteria.

For about the first 10 years since its introduction by Taylor et al. (2000), the GCPSR concept in *Trichoderma* was implemented in its strictest sense (Atanasova and Druzhinina 2010; Druzhinina et al. 2008, 2010b; Komoń-Zelazowska et al. 2007; Jaklitsch et al. 2008a, b; Chaverri and Samuels 2003; Lu et al. 2004; Samuels et al. 2000, 2010; Degenkolb et al. 2008), which resulted in the deposition of DNA barcoding sequences for additional loci such as *chi18-5* (*ech42*), *acl1*, *cal1*, *act,* and some others (Table 2). However, the shift to the two loci that was initiated during the last decade and the massive introduction of new species without consideration for the supplementary barcodes and frequently also without ITS (Table 2), reduced the usability of these supplementary DNA barcodes almost to zero.

The second drawback that comes from the non-strict application of GCPSR appears when species are recognized based on a few or even a single isolate. In this case, it is not possible to distinguish between species and populations. Therefore, multiple *Trichoderma* species that were described based on a single available isolate are ambiguous unless the unique ecophysiological or morphological features were detected.

The factual retreat in *Trichoderma* taxonomy from the application of GCPSR to the less powerful PSR has practical and theoretical explanations and consequences for precision and accuracy of taxonomy. First, the genus-wide taxonomic revisions [such as that performed by (Jaklitsch 2009, 2011)] require the simultaneous analysis of several hundred isolates and sequences of several hundred reference strains. Ideally, GCPSR could be applied if *tef1* and *rpb2* phylograms could be confronted. However, because the intron-containing *tef1* DNA barcode locus is highly polymorphic, respective sequences cannot be aligned across the genus. The analysis will require the construction of numerous smaller separate phylogenetic trees for individual sections (such as those shown in Figs. 10–13). The conserved exon-containing *tef1* fragment that was selected by Jaklitsch (2009, 2011) allowed the avoidance of multiple phylograms because it was suitable for alignment across the genus. However, the poor resolution of resulting trees was shown before (Chaverri and Samuels 2003) and also mentioned by this author. We would like to warn the researchers who are aiming at identification of the large collections of *Trichoderma* strains that the correct application of GCPSR will require the construction and analysis of numerous phylograms.

Second reason why the GCPSR concept was replaced by the PSR, is theoretical. GCPSR alone does not allow a decision to be made on the rank of concordant clades. For cryptic species, even the strict application of GCPSR cannot distinguish between taxa of different ranks (such as populations, species, or genera). For this reason, *T. aggressivum* and *T. caribbaeum* consist of ambiguously defined varieties (Samuels et al. 2002, 2006). Thus, we can conclude that although GCPSR is considered to be the most powerful concept (Nguyen et al. 2015a), it did not yet find its broad application in *Trichoderma* taxonomy.

As it has been already explained above, the revision of the distribution of DNA barcoding loci revealed that the currently available material for species identification within the genus *Trichoderma* (Table 2) makes DNA Barcoding limited to the three loci analysis among which, the concordance *rpb2* ≅ *tef1* should not be contradicted by ITS. Unexpectedly, it further raised the taxonomic value of ITS. In fungi, ITS fragments have numerous features that limit its taxonomic applicability [reviewed by Lücking et al. (2020)], but most of them are not known for *Trichoderma*. To the best of our knowledge, there were no reports on intragenomic polymorphism of this locus. However, a high number of homoplasious sites was demonstrated (Druzhinina et al. 2005) and there was insufficient polymorphism between many related species (Druzhinina and Kubicek 2005). Therefore, we do not recommend using ITS for phylogenetic analysis, but

we suggest the similarity analysis for this locus that can be applied for assigning the genus delimitation.

The search for the best phylogenetic markers by *Trichoderma* taxonomy providers resulted in the mosaic and incomplete distribution of DNA barcoding loci and the genus phylogram (Table 2). These gaps can be filled if taxonomists worldwide have easy access to the reference strains' cultures for additional sequencing. However, the practice shows that in some countries where fungal taxonomy develops very fast (such as China), the acquisition of reference strains from culture collections abroad is overly burdensome and costly such that it cannot be accomplished by most researchers. Conversely, shipment of reference strains, even from the authorized collections in China to other countries, is also complicated, expensive, and time-consuming. These non-scientific obstacles result in a bottleneck for the development of *Trichoderma* taxonomy and lead to the emergence of ambiguous species descriptions and increase the incomplete distribution of phylogenetic markers.

The only solution that we can propose is cooperation within the community of *Trichoderma* taxonomists. For example, a colleague "A" who is working on the taxonomic description of a species "X" that is related to species "Y", which was described by colleague "B," can request the latter person to provide sequences of additional DNA barcoding loci (Table 2) for species "Y". For example, for *T. changbaiense* in a group of species that are related to *T. fertile*, providing either four missing *chi18-5* (*ech42*) or three missing *acl1* sequences could allow the application of the GCPSR concept and unambiguous species recognition. The current state of *T. changbaiense* species is ambiguous because its description does not correspond to the recommendations for the new fungal species description (Seifert and Rossman 2010). It has been described based on a single strain and the concatenated analysis of the two loci (Chen and Zhuang 2017a). Moreover, the morphology of *T. changbaiense* did not correspond to the related morphospecies, which also suggests the need for further sampling. Thus, the cooperation between taxonomists can aid in the in silico analysis. However, the exchange of sequence data will not replace the need to perform the comparative analysis of phenotypes and ecophysiological features will require consideration of the reference cultures in vitro, not only in silico.

In summary, sequencing of ITS, *tef1*, and *rpb2* is currently the minimum sufficient set of phylogenetic markers that is required for the application of the GCPSR concept. In those cases, when these markers are not concordant (see examples *above*), consideration of other loci is required. The WGSs can provide enough material to resolve evolutionary positions of species with non-concordant phylogenies of *rpb2* and *tef1* and shared (identical) ITS sequences. However, because only 10% for *Trichoderma* species have been whole genome sequenced to date, the phylogenomic analysis for *Trichoderma* will not be available in the near future. We anticipate many new species that will be described based only on a few phylogenetic markers.

Testing the identifiability of every new species using the currently available materials for related strains is essential for species recognition. Comparative analysis of ecophysiological traits along with multiparametric phenotypes of a putative new species and the closely related taxa along with the application of the GCPSR concept will result in the most reliable species delimitation practice, a polyphasic approach (Lücking et al. 2020).

Comparative ecology aids identification of *Trichoderma* species

The reliability of species recognition in *Trichoderma* can be further aided by the analysis of DNA barcodes that are deposited for environmental samples and corresponding metadata that are recorded in public databases. In almost all cases, it will include the analysis of ITS. In fortunate cases where there are unique ITS sequences [e.g., *T. asperelloides* delimitated from *T. asperellum*, Samuels et al. (2010)], the sequences of new species can be searched in public databases for their occurrence in various habitats and ecosystems worldwide. The sequence similarity search in public databases that is performed with *tef1* and *rpb2* can also reveal other strains of a given new taxon among the pool of nearly 2000 taxonomically undefined records that were deposited as "*Trichoderma* sp." in public databases (July 2020). The metadata for such records of the respective sequences can also serve as a useful supporting material for species description. Because most *Trichoderma* DNA barcodes were deposited in public databases within the last two decades, the authors of most sequences can be contacted, and a collaboration can frequently be established. For example, in our earlier study of *Trichoderma* diversity in Mediterranean sponges that was performed in collaboration with Oded Yarden's group (Israel), we identified several potentially new species of *Trichoderma* (Gal-Hemed et al. 2011). The sequence similarity search in the NCBI GenBank revealed that strains with identical or highly similar DNA barcodes were already deposited by Karin Jacobs' group (South Africa). These findings essentially supported our new species hypotheses because highly similar strains were found on the other continent. We contacted Professor Jacobs' group and the cooperation between the three groups and the active exchange of materials between Austria, Israel, and South Africa resulted in the joint description of five new species (du Plessis et al. 2018). This cooperation arose from the analysis of sequences and respective metadata for strains deposited in a public database.

Suggestions for *Trichoderma* diversity studies

The popularity of the large-scale biodiversity surveys among mycologists worldwide and the relative ease of *Trichoderma* sampling and isolation attract many new researchers in this area. Based on our personal communications, at least several groups throughout the world, in particular, but not only, in China, are possessing collections consisting of several hundred or even thousands of *Trichoderma* isolates pending their taxonomic evaluations. As described above, the GCPSR (Taylor et al. 2000) and concept of cryptic fungal species (Struck et al. 2018) together with the broad availability of basic DNA techniques (PCR amplification and Sanger sequencing) result in the relative simplicity of the new species delimitation in this genus. Our assessments allow foreseeing the description of a considerable number of new species in the near future and urge us to propose genus-wide standards to discuss at the upcoming nomenclatural and taxonomic meetings. The most active providers of *Trichoderma* systematics are a few groups of highly experienced fungal taxonomists (Fig. 2, Table 2) who are invited to share their skills and knowledge with the beginners [see also fungi-wide recommendations in Lücking et al. (2020)]. The International Commission on *Trichoderma* Taxonomy (ICTT, www.trichoderma.info) or regular meetings such as the International Workshop on *Trichoderma* and *Gliocladium* or the *Trichoderma* Workshop that satellites the European Conference on Fungal Genetics (ECFG) offer opportunities for such exchanges. In Box 2 and below, we summarize practical recommendations that arose from this study and that can be useful for *Trichoderma* scientists that shift their research interest towards a taxonomy and hold collections of unidentified isolates.

We also propose that genus-wide standardization of species criteria that can be achieved if every new species hypothesis is to be first submitted to the ICTT board for the review and approval before committing to a taxonomic and nomenclatural act. In this way, the researchers can effectively communicate, exchange their *Trichoderma* experience and methods, and also compose the UpToDate global list of *Trichoderma* species names that is started in this study. The regulations and principles of such approvals can be discussed at the upcoming international meeting in consultation with the members of the parental International Commission on Taxonomy of Fungi (ICTF) (www.fungaltaxonomy.org), and the conclusions can be recorded in ICTT statues.

The responsible curation of deposited material upon the taxonomic and nomenclatural acts is another essential recommendation that should be given to the providers of *Trichoderma* taxonomy. This practice will result in reduced ambiguity in *Trichoderma* taxonomy. It is strongly suggested to revise species identifications for all DNA barcoding materials upon the release of species names. As shown above, the names of several dozen *Trichoderma* species have not been updated in the NCBI Taxonomy Browser (Table 2). Therefore, they are not visible in a sequence similarity search and may be easily overlooked by the beginner users of *Trichoderma* taxonomy.

Another (repeated) recommendation is the ultimate provision of ITS sequences for all *Trichoderma* species, including those that have already been described. Although species can be recognized based on the use of other phylogenetic markers in some cases, ITS should be provided to record this taxon in metagenomic studies. Even if the ITS phylotype of a given species is not unique, it is essential to associate all possible taxonomic names with each phylotype of ITS. Because the resolution of metabarcoding is expected to improve with the integration of new technologies and longer reads (Feng et al. 2015; Rhoads and Au 2015), ITS sequences will gain further value in the diversity research of all fungi, including *Trichoderma*. Furthermore, ITS can serve as the third locus, complementing the strict GCPSR that is applied for *tef1* and *rpb2* (*see above*).

Description of a new species that is based on a single strain is not recommended (Seifert and Rossman 2010). Exceptional cases require justification and a clear statement that genealogical concordance was not accessed (*see above*). The need for the nomenclatural act for a single isolate (assigning of a new name) can be considered to be convincing if the specimen was collected in a habitat that cannot be further sampled [as from clinical material (Druzhinina et al. 2008)], if the strain has some unique and clearly distinguishing ecophysiological properties [*T. cyanodichotomous,* (Li et al. 2018)], if it is particularly relevant for applications [*T. taxi*, (Zhang et al. 2007)], or if it has pathological significance. Single strains can be assigned as putative new species and communicated using their strain ID. Thus, in this study, we refrain from describing the strain that was used as an example, *T.* sp. TUCIM 5640, as a formal new species because it meets all but this criterion (*see above*). The formal taxonomic description should be completed when more samples become available. Unfortunately, a formal taxonomic description based on a single isolate is still common in *Trichoderma* taxonomy (Chen and Zhuang 2017a; Jaklitsch 2009, 2011; Jaklitsch and Voglmayr 2015), which frequently results in ambiguous species that can also not be unambiguously identified. It is recommended that measures should be taken to perform additional sampling and search public databases, strain collections, fungaria, and herbaria for the specimens and cultures with matching properties and/or DNA barcodes.

Besides the increasing number of the WGS strains in the *Trichoderma* spp., the applicability of WGS in taxonomy and DNA Barcoding did not reach its potential importance. Researchers repeatedly select strains that belong to the same species for WGS (Table 4). Thus, for now, three whole genomes of *T. harzianum* s. s., four genomes of *T.*

Box 2 Recommendations related to *Trichoderma* taxonomy

Irrespective on the intention and final goal (either taxonomy, biology or applications), every *Trichoderma* diversity research starts from the identification of sampled species, i.e., use of the existing taxonomy. Below, we list a few practical recommendations that aim to provide answers to the most frequent questions that were addressed to us in our practice of molecular identification of *Trichoderma* and also aid in the evaluation of *Trichoderma* biodiversity studies by reviewers, editors, and decision-makers in organizations financing such studies.

Key references on fungal taxonomy

Reading of the following literature is highly recommended before approaching *Trichoderma* taxonomy:

The latest edition of Chapter F by (May et al. 2019) in the *Code*, (https://www.iapt-taxon.org/nomen/pages/main/chapter_f.html) and the *Code* (https://www.iapt-taxon.org/nomen/main.php). Additionally, become familiar with the original requirements regarding the deposition of reference materials and types in public databases, naming, and imaging. It is also recommended to address the most recent fungal taxonomy and fungal DNA Barcoding guidelines (Lücking et al. 2020; Schoch et al. 2020; Vu et al. 2019; May et al. 2019) and the original publication on the new species description standard in fungi by Seifert and Rossman (2010). Independent of the publication date, taxonomic descriptions of all related species, taxonomic revisions of the related infrageneric groups, and non-taxonomic literature on the species that belong to the group of interest should be investigated.

Consulting with the experienced experts

Specialists in fungal taxonomy and nomenclature can be contacted through the International Committee of Taxonomy of Fungi (www.fungaltaxonomy.info), the Nomenclature Committee for Fungi (NCF) (https://www.ima-mycology.org/nomenclature/nomenclature-committee-fungi), the International Mycological Association (IMA) (https://www.ima-mycology.org/), or through the regional Member Mycological Organizations http://www.ima-mycology.org/society/member-mycological-organizations or also listed in Wikipedia (https://en.wikipedia.org/wiki/Category:Mycology_organizations).

Experts on *Trichoderma* taxonomy can be contacted through the International Commission of Taxonomy of *Trichoderma* (ICTT) (www.trichoderma.info) (Fig. 16).

***Trichoderma* diversity surveys and DNA Barcoding**

(1) Do not expect high diversity of *Trichoderma* in soil. It is not a soil fungus (Friedl and Druzhinina 2012; Kubicek et al. 2019).
(2) Do not add fungicides to the isolation medium. The growth of numerous rare species is reduced by such fungicides as Rose Bengal and others (I.S. Druzhinina, unpublished).
(3) Do not rely on phenotypical or morphological similarity for grouping the strains for DNA Barcoding. Many *Trichoderma* spp. are morphologically identical (cryptic) (Jaklitsch 2009, 2011; Jaklitsch and Voglmayr 2015; Chaverri et al. 2015).
(4) Do not rely on ITS for the preliminary grouping of isolates for the subsequent DNA Barcoding. Many sister species share the same ITS phylotype (Druzhinina et al. 2012; Sandoval-Denis et al. 2014; Druzhinina et al. 2005). The probability to isolate two or more of such species from the same habitat is considerable because several related *Trichoderma* species co-occur (Komoń-Zelazowska et al. 2007; Friedl and Druzhinina 2012) and therefore cannot be distinguished by ITS.
(5) Sequence of DNA barcoding fragments of ITS, *tef1,* and *rpb2* for all isolates. Consider selecting primer pairs of *tef1* that will guarantee the sequencing of the diagnostic region (see example in Table 3, Fig. 9, note other primer pairs listed in Rahimi et al. (2020).
(6) Use on-line tools and public databases for the preliminary analysis of the obtained DNA barcodes [such as MIST, (Dou et al. 2020) or NCBI BLAST, (Ye et al. 2006)]. These analyses will help to reveal genetically unique or common isolates. Consider the results that were obtained using automated tools as preliminary or putative molecular identification.
(7) Follow the molecular identification protocol for a single *Trichoderma* isolate including the validation step.
(8) Use original taxonomic literature and the metadata for the query strains (morphology, physiology, ecology, biogeography, occurrence) for the biological verification of the identification results. Assign ambiguous identification if the biological verification fails.
(9) While depositing sequences in public databases, taxonomic accuracy is more appreciated over precision. For ambiguous results *T*. sp. [strain ID] is preferred over the assignment of an ambiguously identified species name. Alternatively, use *T*. aff. [closest species] or *T*. cf. [one of several close species] strain ID format.
(10) If the molecular identification and subsequent biological verification suggest that a putative new species has been detected, consider the following requirements:
- Check the compliance with *the Code*.
- Verify Latin grammar for the new species name.
- Consider intraspecific polymorphism (more than one strain or specimen).
- Apply GCPSR concept (compulsory consideration of single locus trees).
- Aim to use the polyphasic approach that implies detailed comparative ecophysiological characterization of the putative new species and closely related taxa.
- Deposit the maximum number of DNA barcodes for each isolate and for more than a single isolate. Collect and provide the most explicit metadata.
- Test the identifiability of the strain.
- In ambiguous case, consult with experts.
(11) Obtain the most precise species identification before subjecting a *Trichoderma* strain for a WGS. Genomics is highly useful for the study of fungal biology, but its applicability in taxonomy and identification is still limited.
(12) Verify the use of *Trichoderma* gene nomenclature.

longibrachiatum, and seven genomes of *T. atroviride* are available in public databases (see references in Tables 1 and 4).

The diversity surveys of *Trichoderma* are now frequently based on large samples of several hundred or even thousands of isolates (Migheli et al. 2009; Ma et al. 2020). The development of the protocol for handling such datasets requires a bioinformatic approach that will be presented elsewhere. However, we would like to specify the need to perform biological verification of the identification results that were obtained in silico. For example, if the soil is not sampled, the most common species in the genus in Europe are *T. europaeum* and its sister species *T. mediterraneum* (Jaklitsch and Voglmayr 2015). However, isolation-based surveys and metagenomic diversity studies did not identify these species or the closely related *T. minutisporum* in bulk soil or rhizosphere (Friedl and Druzhinina 2012; Hagn et al. 2007; Meincke et al. 2010). This does not mean that isolation of these species from the soil is not expected, but that identification of one of these species that is isolated from bulk soil requires critical evaluation. Generally, most of the infrageneric diversity of the genus *Trichoderma* is found in habitats other than soil (Jaklitsch 2009, 2011; Jaklitsch and Voglmayr 2015; Qin and Zhuang 2016c) and only a limited number of highly environmentally opportunistic *Trichoderma* species can establish in this environment (Friedl and Druzhinina 2012; Hagn et al. 2007; Meincke et al. 2010).

Similarly, *T. reesei* is a common and cosmopolitan species with a distribution that is limited to 20° south and north of the equator (Druzhinina et al. 2010; Druzhinina and Kubicek. 2016). The abundant detection of this species in temperate soils in Austria reported by Hinterdobler[1] requires verification by repeated sampling and consideration of artifacts.

The aspects of the *Trichoderma* lifecycle can also be considered to verify the in silico identification. Thus, *T. longibrachiatum* s. s. is a common species with a cosmopolitan distribution. Its isolates are known from all continents, including Antarctica, and subjected to several molecular evolutionary investigations that revealed that this was most likely a clonal species (agamospecies) (Druzhinina et al. 2008). Consequently, molecular identification of a teleomorph-derived isolate as *T. longibrachiatum* should be questioned and verified.

Concluding remarks and outlooks: *Trichoderma* genomics and polyphasic approach

For two centuries, the identification of *Trichoderma* (and other common cultivable fungi) required microscopic preparations, scientific drawings, and growth observation on multiple nutritional media. It was a laborious practice that frequently resulted in ambiguous species assignments (Fig. 15). The introduction of DNA-based techniques first slightly complicated the process by the need to equip mycological labs with molecular biological devices, but then it resulted in a drastic decrease in the labor that was required for the identification (DNA Barcoding). In a few years, the commercial kits for DNA extraction, ready PCR mixes, well-optimized PCR components, and the broad availability of Sanger sequencing service made DNA Barcoding a widely accepted technique. Additionally, the public databases of DNA sequences became powered by automated sequence analysis tools such as BLAST (Ye et al. 2006). Some online identification tools also become available for individual genera and fungal groups [*Tricho*Key, (Druzhinina et al. 2005); MIST, (Dou et al. 2020); UNITE, (Nilsson et al. 2019)]. Together with the GCPSR and PSR concepts, this prepared a simple methodological framework for the relative ease of species delimitation and triggered the ongoing boost of *Trichoderma* taxonomy (Fig. 3). Within a short time, the labor that was subsequently required for species identification sharply increased (Fig. 15), and the rapid growth of newly described species also contributed to the increased ambiguity of species diagnosis. Based on our estimation, 40% of *Trichoderma* species can not be unambiguously identified because either the respective reference materials are incomplete or species criteria that were used for the species delimitation has become ambiguous. The standardization of species recognition criteria and an agreement between *Trichoderma* taxonomy providers will allow us to avoid reaching the level when unambiguous species diagnosis will become rare or impossible (Fig. 15).

The current diversity of *Trichoderma* species is mostly recognized based on *tef1* and *rpb2* polymorphisms and supported by ITS allowing the development of the molecular identification protocol that will result in the frequent proposal of putative new species. Thus, we anticipate the future rapid growth of *Trichoderma* species to 1000 in the next decade. We agree that the particular species delimitation allows the precise identification and prediction of useful properties. However, we also hope that advances in taxonomy will improve rather than hinder our understanding of fungal biology and evolution.

[1] The talk of Wolfgang Hinterdobler who presented W. Hinterdobler, J. Scholda, G. Li, S. Böhmdorfer, M. Schmoll "*Trichoderma* spp. impact mycotoxin production of the plant pathogen *Fusarium graminearum*" on the ECFG15 Satellite Workshop "*Trichoderma*, *Clonostachys* and other biocontrol fungi" (February, 2020, Rome, Italy). The abundant detection of *T. reesei* in a soil sample from Austria was also reported earlier by the same group, e.g. on the 15th International *Trichoderma* and *Gliocladium* Workshop (June, 2018, Salamanca, Spain). A respective publication is anticipated (W. Hinterdobler, personal communication).

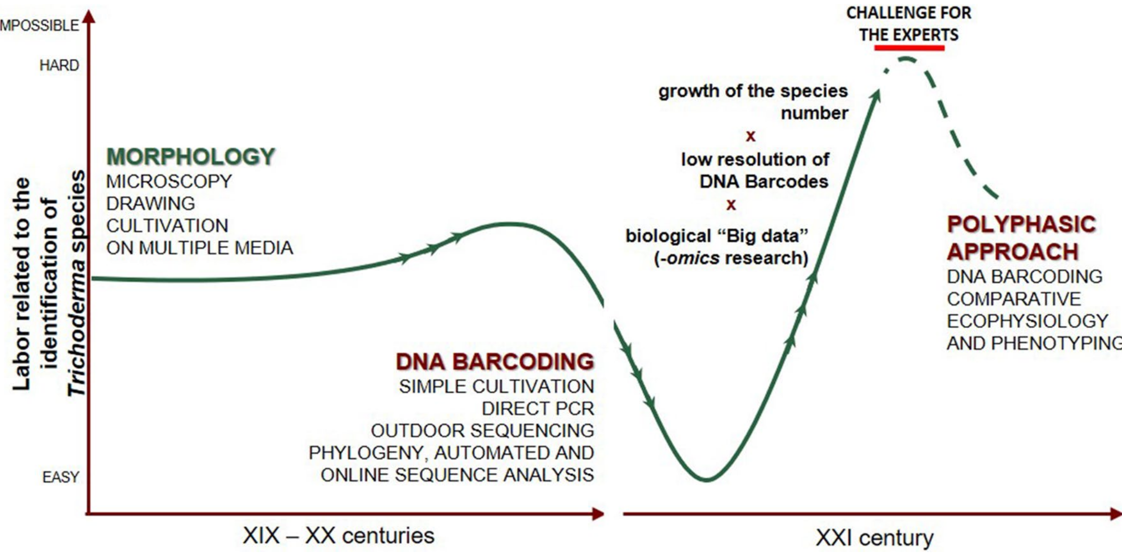


Fig. 15 A schematic diagram showing the changes of labor related to species identification in *Trichoderma* over > 230 years

Favorable opportunity and venture of the whole-genus genomics

Compared to some other ubiquitous fungi, the genus *Trichoderma* is relatively young. Its origin likely coincided with the Cretaceous–Paleogene extinction event, which was roughly 66 million years ago (mya) (Kubicek et al. 2019). It was approximately 15 million years after the putative origin of *Aspergillus* (81.7 mya) and about 10 million years after the formation of the ancestor of *Penicillium*. (73.6 mya) (Steenwyk et al. 2019). However, compared to the evolution of other groups, 66 million years are long. It includes the time passed from the end of the Cretaceous period and the entire Mesozoic Era, which was sufficient for the evolution of Hominidae (humans and other higher apes) from the placental mammalians similar to a rat-sized *Purgatorius* (O'Leary et al. 2013) that hardly had any features of modern primates. In contrast to mammals, fungal taxonomy is complicated by the lack of distinctive features (either phenotypic or DNA-barcodes) and fossils. However, the immense evolutionary time that has passed since the genus' origin is reflected in the diversity of *Trichoderma* genomes (Kubicek et al. 2011, 2019). In the first comparative genomic study, syntenic orthologs of *Trichoderma* spp. were evaluated to be only 70% (*T. reesei versus T. atroviride*) to 78% (*T. reesei versus T. virens*) similar, which is comparable to the similarity between species of other fungal genera [69% for *Aspergillus fumigatus* versus *A. niger* (Galagan et al. 2005)] and to those between fish and man (Nadeau and Taylor 1984; Fedorova et al. 2008). Our more recent genomic investigations of a dozen *Trichoderma* spp. showed that the formation of the three major infrageneric groups, Section *Longibrachiatum*, Section *Trichoderma* (sensu *Viride* Clade), and the *Harzianum*–*Virens* Clades started 20–30 mya. Thus, these lineages were already separated by millions of years of independent evolution. The divergence between sister species, such as *T. reesei* and *T. parareesei* (Section *Longibrachiatum*), cryptic species *T. harzianum, T. afroharzianum,* and *T. guizhouense* happened several mya (4 to 8 mya) (Kubicek et al. 2019). In that study, Kubicek et al. (2019) found this evolutionary distance to be a supportive argument for delimitation of respective lineages in separate species (Druzhinina et al. 2010a; Atanasova et al. 2010; Chaverri et al. 2015). However, this judgment remained subjective because no standards on genomic or genetic similarities or the length of evolutionary distance were proposed that were sufficient to recognize a species. The number of intraspecific genomic studies for *Trichoderma* spp. remains limited. In the same work, the divergence between the two strains of the putative agamospecies *T. harzianum* sensu stricto (Druzhinina et al. 2010b) (the ex-type strain from the UK and a strain isolated from Brazil) was calculated to have occurred approximately 460,000 years ago. By all taxonomic means described in this study, these strains are not distinguishable. However, probably the most taxonomically-relevant and remarkable finding of the comparative genomics is the detection of 1699 genes in the genome of the ex-type *T. harzianum* strain CBS 226.95 (12% of the entire genome) that were absent from TR274 strain, and 1419 genes that were present in the latter (10.1%) were absent from the type strain. Most of these genes encoded orphan proteins for the species, and a function could only be predicted for less than 200 of them (Kubicek et al. 2019). Notably, the lack or presence of > 1000 entire genes in an individual genome a more significant distinction that 1–3% dissimilarity between *rpb2* or *tef1* DNA barcoding markers,

which was used to identify species above. Thus, the level of taxonomic precision can be strongly influenced by the resolution of the method. Because the separation of species due to the long evolutionary history can be further powered by the high resolution of advanced -omics techniques, such as genomics, transcriptomics, epigenomics, metabolomics, or phenomics, the distinctions between any individual strains will appear deeper as more such tools become available for taxonomic studies, but the decision of the boundaries for particular fungal species may remain subjective.

The availability of the genomes opened an avenue for ecological genetics, which is the study of the role of individual genes and proteins in fungal fitness that was largely impeded in pre-genomic time. Cai et al. (2020) revealed that a single gene encoding the amphiphilic surface-active protein hydrophobin (HFB4) that covers *Trichoderma* conidia could drastically influence species-specific traits of *T. guizhouense* and *T. harzianum* that are related to spore dispersal and stress resistance. The results of that research pointed to another dimension that can be applied to distinguish between the two species that were previously considered to be cryptic and sympatric (Druzhinina et al. 2010b; Li et al. 2013; Chaverri et al. 2015). The ecophysiological profiling of HFB-deletion mutants suggested that *T. guizhouense* has features of anemophilous aero-aquatic fungi, while the *T. harzianum* has evolved towards pluviophilous dispersal (by rain droplets) and is adapted to habitats that are not flooded by water (soil or plant tissues) (Cai et al. 2020).

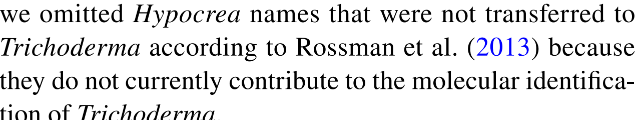
Thus, the application of the modern techniques will ultimately reveal more differences between individual fungal taxa (of all ranks) than similarities and, thus, improve cladistics (search of clades within clades) and phylogenetic resolution. Besides the differences, taxonomy also aims to reveal similarities between the organisms and, thus, improve our understanding of relationships and evolutionary history. Therefore, we anticipate that *Trichoderma* taxonomy and DNA Barcoding will be further challenged by choices between the biological accuracy and high precision of genetic delimitation of species and possibly subsequent identification. The results of the ongoing whole-genus genomic project for *Trichoderma* (https://genome.jgi.doe.gov/portal/Genwidrichoderma/Genwidrichoderma.info.html), which aims for whole-genome sequencing of all *Trichoderma* spp., will drastically increase the precision of strain recognition. However, it may result in the distinction on the level of populations and even individual isolates rather than species and, thus, severely jeopardize the identifiability of *Trichoderma* species and ecological studies that are crucial for understanding the genomes. The urgent task for the *Trichoderma* community is to achieve an agreement on the genus-wide criteria that are used to recognize species and, thus, prepare for the release of massive genomic data.

Polyphasic approach and the work of John Bissett

Lücking et al. (2020) wrote that "the lack of accuracy of fungal identifications cannot be excused by the lack of adequate tools, and so the availability of tools determines which fungi can be studied. However, lack of molecular tools can be partially balanced by expertise: talented and knowledgeable mycologists may provide more accurate species identifications through non-molecular approaches than unexperienced users do through DNA-based identifications."

We dedicate this work to the distinguished *Trichoderma* taxonomist John Bissett (1948–2020). Almost immediately after the introduction of DNA-based techniques in *Trichoderma* diversity studies, he proposed the integration of these tools with the advanced semiquantitative phenotypic characterization of individual strains and species. Today, the urgent need for the comprehensive implementation of such an approach—a polyphasic approach in species recognition, i.e. the combination of molecular phylogeny, phenotyping and ecology—is highly supported by fungal taxonomists including members of the ICTF [see Lücking et al. (2020)].

J. Bissett developed a fungal version of the microplate-based simultaneous characterization of fungi growth on 95 carbon sources and water (Phenotype MicroArrays). For *Trichoderma*, the system was first applied to the collection of South-East Asian isolates (Kubicek et al. 2003), and then this concept was used for the taxonomic description and characterization of numerous species (Atanasova et al. 2010; Ding et al. 2020; Druzhinina et al. 2006, 2008, 2010a, b; López-Quintero et al. 2013), strain collections (Komoń-Zelazowska et al. 2007; Gal-Hemed et al. 2011; Hatvani et al. 2019; Friedl and Druzhinina 2012; Cai et al. 2020), or individual mutants (Friedl et al. 2008; Seidl et al. 2006, 2008; Schuster and Schmoll 2010; Derntl et al. 2017, Wang and Zhuang 2020). The principle of semiquantitative phenotype profiling based on spectrophotometric or nephelometric measurements (Joubert et al. 2010) is becoming accepted in research on *Trichoderma* and other fungi [see Atanasova and Druzhinina (2010) for the review]. Cai et al. (2020) introduced REPAINT, which is the advanced version of Phenotype Microarrays that is powered by the artificial intelligence algorithm for the semiquantitative assessment of the reproductive potential such as production of aerial hyphae and conidiation. We propose that these or similar quantitative or semiquantitative tools for multiparametric automated phenotyping can rapidly find its applicability in the formal taxonomy of *Trichoderma* and of other fungi. It will allow the development of standardized phenotypic databases that are available for taxonomy and identification, and thus, prepare for the use of upcoming wave of *Trichoderma* "Big Data".



Materials and methods

Strains, cultivation conditions, PCR, and sequencing

In this study, the two *Trichoderma* isolates (TUCIM 5640 and TUCIM 10063) from our collection were used as test material for a DNA barcoding exercise. For DNA extraction, *Trichoderma* cultures were maintained on potato dextrose agar (PDA; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) plates at 25 °C in darkness. Fungal strains used for DNA Barcoding were cultivated for 48 h on PDA plates in darkness. Genomic DNA was extracted using a Phire Plant Direct PCR kit (Thermo Scientific, Waltham, Massachusetts, USA), according the manufacturer's instructions. PCR amplification of the phylogenetic markers corresponding to ITS 1 and 2 of the rRNA gene cluster (ITS, including the 5.8S rRNA), the fragments of RNA polymerase II subunit B gene (*rpb2*), and the translation elongation factor 1-α (*tef1*) were set as described in Table 3. Amplicons were sent for Sanger sequencing.

Online survey

To estimate the molecular identifiability of *Trichoderma* spp. by the experts, we performed an on-line survey (the detailed questions can be seen in https://www.surveymonkey.com/r/?sm=hgTrOEkKaUnBxAsJkS5pSw_3D_3D) that was titled "*Trichoderma* 20x20". The respondents were shown two sets of DNA barcoding markers (ITS, *rpb2*, and *tef1*) for two unknown isolates that had not been deposited into public databases. The questions concerned species identification or each strain, time spent, methods and loci used, and self-estimation of the respondent's experience in the area of *Trichoderma* research and fungal taxonomy. The survey could have been completed anonymously or the respondents could leave their name and comments. The link to the survey was sent to > 200 respondents using the mailing list from the regular International Workshop on *Trichoderma* and *Gliocladium*.

Retrieval of taxonomic data

The information regarding taxonomy of the genus *Trichoderma*, including species names, publication year, and author names were exported from Index Fungorum (http://www.indexfungorum.org/), Mycobank (http://www.mycobank.org/), and the National Center for Biotechnology Information (NCBI) Taxonomy Browser (https://www.ncbi.nlm.nih.gov/taxonomy/). The latter was manually screened for all loci that were deposited per each taxonomic name of *Trichoderma*. Sequences that were assigned to undefined species of *Trichoderma* were not sampled. In our survey, we omitted *Hypocrea* names that were not transferred to *Trichoderma* according to Rossman et al. (2013) because they do not currently contribute to the molecular identification of *Trichoderma*.

The reference sequences of each marker locus for each type strain was retrieved from the NCBI database, which is based on the information that was provided by the NCBI RefSeq Targeted Loci Project (Robbertse et al. 2017) or from related publications (Bissett et al. 2015). Overall, 42 *Trichoderma* genomes (listed in Table 1) that were publicly available from the NCBI and the Joint Genome Institute (JGI) databases were used as the sequence resources for strain identification with author's permissions for yet unpublished records. The respective sequences of each marker from *T. reesei* QM 6a, *T. harzianum* CBS 226.95, and *T. asperellum* CBS 433.97 were used in BLASTn when querying the genomes.

Online tools supporting *Trichoderma* taxonomy

The retrieved taxonomic data from the above three resources were manually confirmed and summarized in Table 2, which is also shown on the official website of the International Commission on *Trichoderma* Taxonomy (ICTT, https://www.Trichoderma.info (Fig. 16) as well as on https://www.trichokey.com (Fig. 17). The list of *Trichoderma* species contains species names that were valid as of July 2020, including those that are currently invalid species that lack DNA Barcoding information.

Due to the lack of consistency within the *Trichoderma* community as to which primers to use for amplifying and sequencing of marker loci, there is considerable variation in the length and fragment area of sequences that are deposited into public databases under the same locus name. Additionally, a partial, rather than the whole fragment, of the marker locus is informative for molecular identification (Druzhinina and Kubicek 2005; Druzhinina et al. 2005; Kopchinskiy et al. 2005). Thus, we released the updated on-line tool *Tricho*MARK 2020 (https://trichokey.com/index.php/trichomark), by which the diagnostic area of each phylogenetic marker (ITS, *rpb2*, and *tef1*) with no flanking fragments can be retrieved. As described in Kopchinskiy et al. (2005), *Tricho*MARK is a specifically script-written tool for detecting and retrieving phylogenetic markers in query sequences, and it is based on genus specific oligonucleotides both on 5′ and 3′ ends of the marker.

We also developed and updated another online tool *Tricho*BLAST 2020 (https://trichokey.com/index.php/trichoblast), which covers all 361 currently genetically characterized species of *Trichoderma* and contains almost complete sets of the diagnostic fragments of the *rpb2* locus from these 361 species and ITS sequences from the 56 type strains of each species that were representatively distributed in the whole

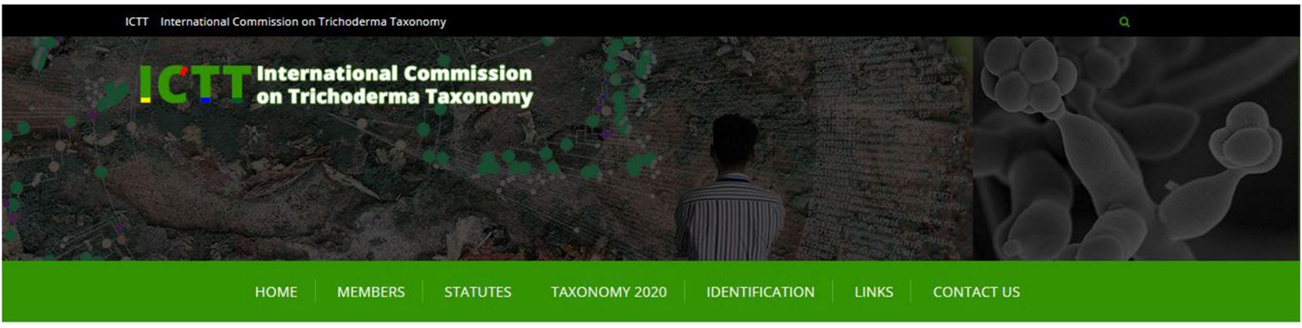


Fig. 16 www.Trichoderma.info. **A snapshot showing the design and content of the website of the International Subcommission of Taxonomy of** *Trichoderma* (ICTT)

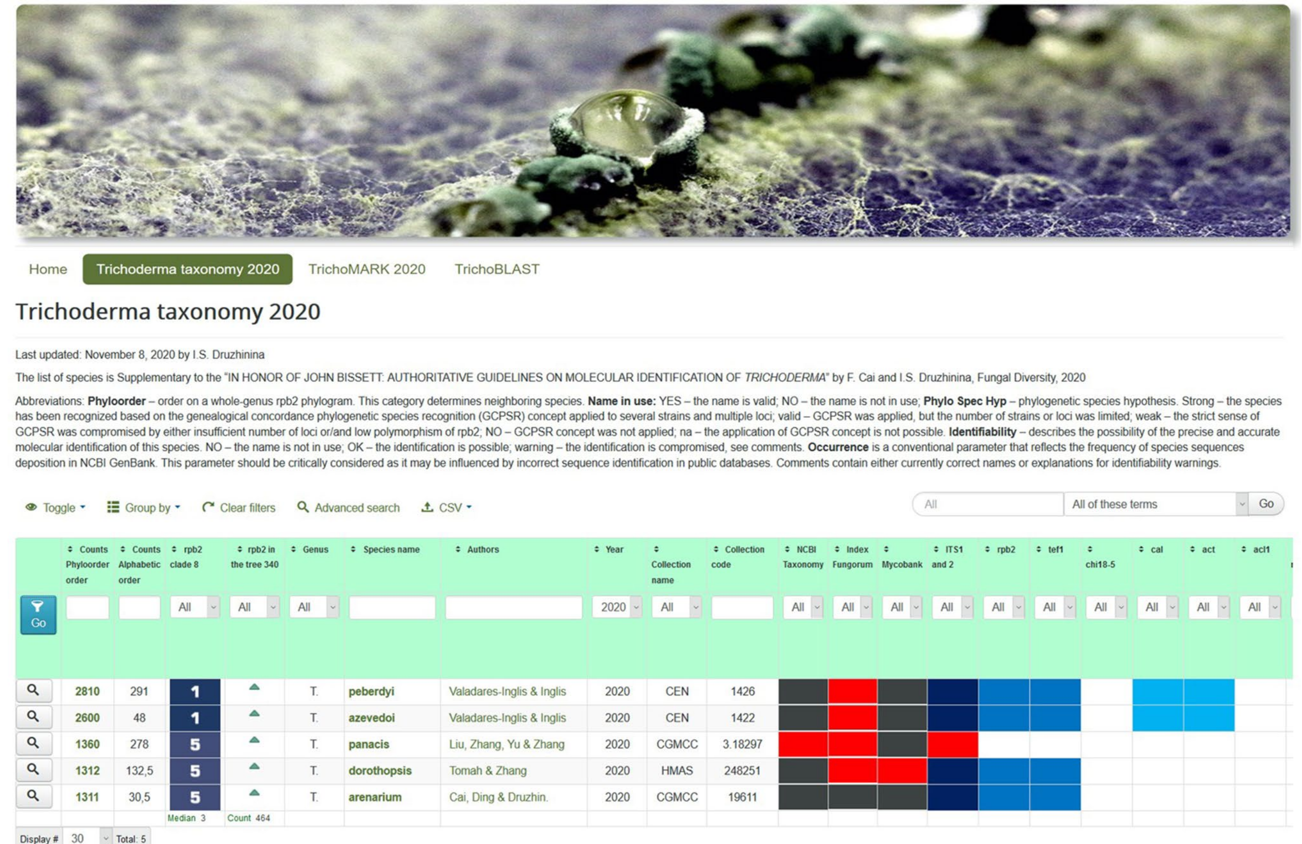
genus. *Tricho*BLAST is a publicly available database that supports the similarity search tool to find the "best hit" of the query strain (sequence) within the genus that is based on a single locus of *rpb2* or ITS. With respect to ITS as the marker locus harboring the largest dataset for fungal identification, *Tricho*BLAST, with 56 representative ITS sequences, allows estimation of whether a query strain belongs to the genus of *Trichoderma* (based on the current scope) if the subsequent calculation of the similarity between the query sequence (after trimmed by *Tricho*MARK) and the "best hit" is performed afterwards (*see below*).

Phylogenetic analysis

Sequences of each marker from the query strains and from the reference strains were consistently trimmed using *Tricho*MARK. The processed sequences were then aligned using Muscle 3.8.31 (Edgar 2004) available Aliview 1.23 (Larsson 2014). Maximum-likelihood (ML) phylogeny was performed using IQ-TREE 1.6.12 (Nguyen et al. 2015b). Statistical bootstrapping support was computed with 1000 replicates. The nucleotide substitution model was selected by ModelFinder (Kalyaanamoorthy et al. 2017) integrated in IQ-TREE, based on the Bayesian Information Criterion (BIC). Phylogenetic trees were visualized in FigTree v1.4.2 and annotated using CorelDraw 2017 (Corel, Ottawa, Ontario, Canada).

Pairwise similarity calculation

The multiple sequence alignment matrix of each locus was submitted to the online tool, Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), for pairwise similarity calculation between two sequences.



Home | Trichoderma taxonomy 2020 | TrichoMARK 2020 | TrichoBLAST

Trichoderma taxonomy 2020

Last updated: November 8, 2020 by I.S. Druzhinina

The list of species is Supplementary to the "IN HONOR OF JOHN BISSETT: AUTHORITATIVE GUIDELINES ON MOLECULAR IDENTIFICATION OF *TRICHODERMA*" by F. Cai and I.S. Druzhinina, Fungal Diversity, 2020

Abbreviations: **Phyloorder** – order on a whole-genus rpb2 phylogram. This category determines neighboring species. **Name in use:** YES – the name is valid; NO – the name is not in use; **Phylo Spec Hyp** – phylogenetic species hypothesis. Strong – the species has been recognized based on the genealogical concordance phylogenetic species recognition (GCPSR) concept applied to several strains and multiple loci; valid – GCPSR was applied, but the number of strains or loci was limited; weak – the strict sense of GCPSR was compromised by either insufficient number of loci or/and low polymorphism of rpb2; NO – GCPSR concept was not applied; na – the application of GCPSR concept is not possible. **Identifiability** – describes the possibility of the precise and accurate molecular identification of this species. NO – the name is not in use; OK – the identification is possible; warning – the identification is compromised, see comments. **Occurrence** is a conventional parameter that reflects the frequency of species sequences deposition in NCBI GenBank. This parameter should be critically considered as it may be influenced by incorrect sequence identification in public databases. Comments contain either currently correct names or explanations for identifiability warnings.

Toggle | Group by | Clear filters | Advanced search | CSV | All | All of these terms | Go

Counts Phyloorder order	Counts Alphabetic order	rpb2 clade 8	rpb2 in the tree 340	Genus	Species name	Authors	Year	Collection name	Collection code	NCBI Taxonomy	Index Fungorum	Mycobank	ITS1 and 2	rpb2	tef1	chi18-5	cal	act	acl1
		All	All	All			2020	All		All	All	All	All	All	All	All	All	All	All
2810	291	1		T.	peberdyi	Valadares-Inglis & Inglis	2020	CEN	1426										
2600	48	1		T.	azevedoi	Valadares-Inglis & Inglis	2020	CEN	1422										
1360	278	5		T.	panacis	Liu, Zhang, Yu & Zhang	2020	CGMCC	3.18297										
1312	132,5	5		T.	dorothopsis	Tomah & Zhang	2020	HMAS	248251										
1311	30,5	5		T.	arenarium	Cai, Ding & Druzhin.	2020	CGMCC	19611										
		Median 3	Count 464																

Display # 30 | Total: 5

Fig. 17 www.Trichokey.com**. A snapshot of the** *Trichoderma* taxonomy 2020 page containing the digitally searchable and sortable copy of Table 2 described in this study

Statistical analysis

The pairwise similarity data for each locus was illustrated using heatmaps that were generated by R (v3.6.1). The distribution of the data matrix was analyzed using STATISTICA 6 (StatSoft, Hamburg, Germany). One-way analysis of variance (ANOVA) and Tukey honest significance difference multiple comparison were set at the significance threshold $P \leq 0.05$.

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Data availability All data are available as supplementary materials and on accessory websites www.trichoderma.info and www.trichokey.com.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interests.

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