Trichoderma: Population Structure and Genetic Diversity of Species with High Potential for Biocontrol and Biofertilizer Applications

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Article

Trichoderma: Population Structure and Genetic Diversity of Species with High Potential for Biocontrol and Biofertilizer Applications

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Abstract: Certain *Trichoderma* isolates provide biofertilizer, biocontrol, and other plant-beneficial activities while inhabiting the soil or internal plant issues; and their use in agricultural systems could contribute to sustainable food production. It is thought that this colonization of soil and internal plant tissues is fundamental for biocontrol and biofertilizer applications. Our collective analyses of prior surveys, where the *tef1* sequence was almost exclusively used to identify *Trichoderma* species, showed that isolates from the Harzianum Complex Clade, the *T. asperellum*/*T. asperelloides* group, *T. virens*, *T. hamatum*, and *T. atroviride* were prevalent in soil and/or as endophytes. Population structure and genetic diversity of these species were investigated, and new lineages with geographic significance within *T. atroviride*, *T. asperellum*, *T. hamatum*, and *T. virens* populations were found. The nearest relatives of some species were also revealed. Choosing isolates from among more than 500 known *Trichoderma* species for use in non-targeted evaluation screens for biocontrol or biofertilizer applications is time-consuming and expensive. Preferentially selecting species from *T. atroviride*, *T. asperellum*/*T. asperelloides*, *T. hamatum*, species from the *T. harzianum* Complex Clade, *T. virens*, and possibly nearest relatives, may speed the identification of candidates for commercialization due to the demonstrated ability of these species to successfully inhabit the soil and endorhizosphere. To our knowledge, this is the first report where dominant soil- and endophyte-inhabiting *Trichoderma* species were identified from past survey data and population structure and genetic diversity analyses conducted.

Keywords: biocontrol agent; biofertilizer; sustainable agriculture; *Trichoderma* species; population structure and genetic diversity

1. Introduction

Alternatives to synthetic fertilizers and pesticides must be considered in agricultural production systems if global food demand is to be increased in a sustainable manner. *Trichoderma* spp. (Kingdom: *Fungi*, Division: *Ascomycota*; Family: *Hypocreaceae*) are ideal alternatives to these synthetic agricultural inputs due to their demonstrated commercial successes and desirable traits such as direct and/or indirect negative effects on many plant pathogens, nematodes, and insects; multiple capabilities for crop protection in a single product due to this broad-spectrum activity against plant pathogens and pests; protection of the plant against abiotic stressors; stimulation of plant growth; and improvement of soil nutrient availability to plants. Certain *Trichoderma* isolates are also dominant in soil and thus have a higher opportunity to establish endophytic relationships with plants; which are thought to be important for providing these plant-beneficial activities [1,2].

Unfortunately, the genus *Trichoderma* is taxonomically complex, containing more than 500 species, with its taxonomy evolving due to the use of molecular taxonomic approaches [2]. To aid in selecting *Trichoderma* isolates for commercial development as biocontrol agents or biofertilizers (BCBFs) it would be helpful to narrow the more than 500 species of *Trichoderma* to a few known to have commercially desired attributes [3,4]. Here we surveyed the *Trichoderma* literature to identify

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species that were prevalent soil inhabitants and/or plant endophytes. We then used the gene sequences for translation elongation factor 1α (*tef1*) to assess genetic diversity and population structure with each species or species groups by phylogenetic analyses for the purpose of revealing lineages that may have more BCBF activities in certain geographic regions than the rest of the population.

2. Materials and Methods

2.1. Trichoderma in soil and endophytes

Data from 23 publications on surveys of Trichoderma in soil from different regions of four continents were collected to determine the dominant species in soil (Table 1). Similarly, data from 13 publications on surveys of endophytic Trichoderma from five different continents were presented (Table 2). These investigations were chosen primarily because of the reliability of the methods used in species identification, specifically, using the tef1 sequence data. The only two exceptions where tef1 was not used are indicated in Table 2. Literature using tef1 sequence for species identification was used as it is a powerful means for identification of Trichoderma to the species level [5,6] and it is the most prevalent locus reported in the literature for Trichoderma species. Species or species groups were considered most prevalent in soil if they were detected in at least 50% of the soil surveys and represented at least 5% of all isolates collected from the surveys.

2.2. Harzianum Complex Clade species

Literature was compiled on Trichoderma species from the Harzianum Complex Clade [3,7] using GenBank hits as the metric for intensity of study for individual species. Species with 20 hits or higher in GenBank were tabulated in Table 3. It was assumed that each GenBank submission represented a different Harzianum Complex Clade strain.

2.3. Phylogenetic analysis

Phylogenetic analyses of population structure and genetic diversity of T. atroviride, T. asperelloides, T. asperellum, T. hamatum, and T. virens were conducted with tef1 sequence to find variants or lineages within each species with or without geographic restriction. First, tef1 sequence of the type or ex-type for T. atroviride (GenBank accession: AY376051), T. asperellum (GenBank accession: AY376058), T. asperelloides (GenBank accession: GU198294), T. hamatum (GenBank accession: AF456911), and T. virens (GenBank accession: AY750891) were obtained from GenBank. Each sequence was separately subjected to a Basic Local Alignment Tool Search (BLAST) at the NCBI website, and the first 100 hits were downloaded as an alignment file in FASTA format. Alignments were improved using the website Guidance server (http://guidance.tau.ac.il/) and the ends of the sequences were trimmed. The alignment file was subsequently used to reconstruct phylogenetic trees using two methods: 1) Maximum Likelihood in MEGA X with substitution model predetermined using MEGA X [8]. Support for the clades was assessed with 1000 bootstrap replicates. 2) Phylogenetic trees were also constructed using the Parsimony criterion in PAUP version 4.0a, available at http://phylosolutions.com/paup-test/. The most parsimonious tree was obtained with a heuristic search with starting trees obtained via random stepwise addition (100 replicates) and with TBR as the branch-swapping algorithm. Support for branches was assessed with 1000 bootstrap replicates. The trees were pruned to between 50 and 60 strains for the purpose of presentation. Trees generated by the two methods were essentially identical in topology, and thus, only one tree is presented in the respective Figures, and clade support by both methods is shown on the branches.

Phylogenetic analyses for the Harzianum Complex Clade were conducted as follows. Sequences of tef1 of two or three strains of the dominant species (Table 3) were obtained from GenBank, plus additional sequences from two investigations [9,10]. The sequences used from these two investigations were identified as Trichoderma sp. or species in the Harzianum Complex Clade. The phylogenetic tree was subsequently constructed using the parsimony method described above. Support for branches was assessed with 1000 bootstrap replicates. The tree was rooted to two strains

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of T. pleurotum and T. pleuroticola, both species are positioned outside the Harzianum Complex Clade [7].

Phylogenetic trees were also constructed by both methods described above to reveal the nearest relatives for T. atroviride, T. asperellum, T. asperelloides, and T. hamatum. The tef-1 sequence of each type species was aligned with the nearest relatives of the species. The sequences of relatives were chosen from GenBank based on BLAST searches and previous studies. Trees generated using both methods were rooted to the type species of T. evansii (accession number EU883566).

3. Results

3.1. Trichoderma soil and endophyte survey compilation

Results from twenty-three Trichoderma-specific soil surveys conducted worldwide over the past 20 years were compiled where tef1 was used for identification of Trichoderma isolates to the species level (Table 1). Table 1 shows the dominant species of Trichoderma from these soil surveys and Supplementary Materials, Table S1 shows all the Trichoderma species identified in these soil surveys. Collectively there were 41 distinct species identified in this compilation, plus the T. asperellum/T. asperelloides group, the Harzianum Complex Clade, and Trichoderma isolates not identified to the species level. Isolates from the T. asperellum/T. asperelloides group and the Harzianum Complex Clade were not listed as separate species as isolates falling within these two species groupings are often misidentified and/or incorrectly deposited within GenBank (Ismaiel, unpublished). Collectively, there were 4,709 isolates when considering all species and all species groupings from these surveys. Species or species groups were considered most prevalent if they were detected in at least 55% of the soil surveys (at least thirteen of the twenty-three surveys) and represented at least 5% of all isolates collected from the twenty-three surveys (at least 235 isolates). The most prevalent species/species groups were the Harzianum Complex Clade species, T. asperellum/T. asperelloides group, T. virens, T. hamatum, and T. atroviride. Isolates from these species or species groups ranged from 42% to 100% of all isolates detected in each individual study in Table 1 and collectively 74% of all isolates detected from all studies listed in Table 1. Isolates from these species or species groupings were also found to be dominant in surveys of endophytic Trichoderma conducted worldwide on different plant species. Table 2 shows the dominant endophytic Trichoderma species and Supplementary Materials, Table S2 shows all the endophytic species identified in the studies. The total isolates of the dominant species were 254 out of 429, representing 59% of total strains isolated as endophytes. The species from Harzianum Complex Clade, and species from T. asperellum/T. asperelloides group represented the top two groups, respectively.

Table 1. Dominant *Trichoderma* species/groups from soil surveys from different geographic regions[a].

Region or Country	Total isolates identified[b]	*asperellum*/ *asperelloides*[c]	*harzianum*[d]	*virens*	*atroviride*	*hamatum*	Total dominant (% of all) isolates[e]	Reference[f]
Central and South America								
South America	183	60/0	49	8	3	2	122 (67%)	[5]
Colombia	21	10/0	3	0	4	0	17 (81%)	[11]
Brazil	54	2/13	23	0	0	2	40 (74%)	[12]
Central and South America	54	4/0	20	2	4	0	30 (56%)	[13]
Europe								
Poland	170	0	43	6	20	9	78 (46%)	[14]
Island of Sardinia	482	3/0	277	19	0	22	321 (67%)	[15]
Southern Italy	16	0	6	0	4	0	10 (63%)	[16
Africa								
Ethiopia	164	64/32	8	0	0	6	110 (67%)	[17]
Tunisia	53	0	15	0	7	14	36 (68%)	[18]
Algeria	9	0	4	0	0	0	4 (44%)	[19]
Egypt	20	0	14	0	0	0	14 (70%)	[20]
Asia								
Russia, Siberia, Himalaya	75	2/0	31	5	14	15	67 (89%)	[21]
Iran	159	0	87	19	0	0	106 (67%)	[22]
Nepal	41	18/19	4	0	0	0	41 (100%)	[23]
Malaysia	326	86/0	156	9	0	20	271 (83%)	[24]
South Korea	26	2/4	3	6	1	1	17 (65%)	[25]
China (East agri. Fields)	2078	425/0	429	340	73	397	1664 (80%)	[26]
China (Northwest)	312	20/0	108	0	3	1	132 (42%)	[27]
China (four regions)	64	4/0	26	2	13	0	45 (70%)	[28]
China (southeast sediments)	254	32/0	63	1	134	1	231 (91%)	[29]
China	13	12/0	1	0	0	0	13 (100)	[30]
China (Southwest)	57	0	49	0	0	0	49 (86%)	[31]
Southeast Asia	78	4/0	37	16	3	1	61 (78%)	[32]
Total[g]	**4,709**	**816**	**1456**	**417**	**283**	**491**	**3479 (74%)**	
Detection frequency among studies[h]	**N/A[i]**	**70%/17%**	**100%**	**52%**	**57%**	**57%**	**N/A**	

[a]Compilation of surveys published in the past 20 years that used *tef1* for *Trichoderma* species identification. Surveys were specific for *Trichoderma* isolates and all species listed in column headings are from the genus *Trichoderma*. [b]Total number of *Trichoderma* isolates (of all species) identified in this study. For complete information on species isolated see Supplementary Materials, Table S1. [c]Isolates from *T. asperellum* and *T. asperelloides* are grouped together because *T. asperelloides* is often misidentified as *T. asperellum* due to highly similar DNA sequences and identical morphology. Additionally, many strains of *T. asperelloides* are incorrectly deposited in GenBank as *T. asperellum*. [d]Harzianum Complex Clade species. Isolates from the different species in the Harzianum

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Complex Clade are not broken down into individual species as isolates are often misidentified as *T. harzianum* and deposited in GenBank as *T. harzianum*. [e]Total dominant (% of all) isolates. Total dominant isolates, sum of all isolates of the dominant species listed in this Table. (% of all), percent of all isolates in this study represented by isolates from these dominant species. [f]Reference for the information in this row. [g]Totals for information in each respective column. [h]Frequency of detection of this species in the different studies collectively. (Number of studies where this species was isolated)/(total number of studies) X 100.

Table 2. Isolation of endophytic species of *Trichoderma* from plants in different geographic regions[a].

Country	Host	Total isolates[b]	*asp*/*aspo*[c]	Harzianum Complex[d]	*virens*	*atroviride*	*hamatum*	Total and (%)	Reference[e]
North America									
Canada	Grapevines	29	0/4	8	0	7	0	19 (66%)	[33]
South America									
Brazil	Rubber trees	30	0	0	0	0	0	0	[34]
Brazil	Cerrado-Caatinga ecotone	19	0	0	0	0	0	0	[35]
Peru*	Wild rubber tree	39	0	31	0	0	0	31 (79%)	[36]
Europe									
United Kingdom	various garden trees	40	0	15	1	0	4	20 (50%)	[37]
Hungary	Grapevines	10	0	8	0	0	0	8 (80%)	[38]
Africa									
Ethiopia, Cameroon, Kenya	Coffee (cultivated and wild)	76	0	46	1	1	3	51 (67%)	[39]
Ethiopia	Coffee	48	0	14	0	0	20	14 (48%)	[9]
Asia									
Malaysia	35 plant families	93	13/22	27	22	0	0	84 (90%)	[10]
Indonesia	*Theobroma cacao*	21	19	0	2	0	0	21 (100%)	[40]
Thailand	Rubber trees	12	3/0	3	2	0	3	11 (92%)	[41]
Iran	*Vinca* sp.	7	1/0	0	0	0	0	1 (14%)	[42]
Iran*	Cuppressaceae family plants	5	0	0	0	4	0	4 (80%)	[43]
Total[g]	N/A[i]	429	62	152	28	12	27	281 (66%)	
Detection frequency among studies[h]	N/A	N/A	38%	61%	38%	23%	30%		

[a]Compilation of surveys published in the past 20 years that used tef1 for Trichoderma species identification. Surveys were specific for Trichoderma isolates and all species listed in column headings are from the genus Trichoderma. Complete isolate information from these surveys is in Supplementary Materials, Table S2. [b]Total number of Trichoderma isolates (of all species) identified in this study. [c]Isolates from T. asperellum and T. asperelloides are grouped together because T. asperelloides is often misidentified as T. asperellum due to highly similar DNA sequences and identical morphology. Additionally, many strains of T. asperelloides are incorrectly deposited in GenBank as T. asperellum. [d]Harzianum Complex Clade. Isolates from species within the Harzianum Complex Clade are not broken down into individual species as isolates are often misidentified as T. harzianum and deposited in GenBank as T. harzianum. [e]Reference for the information in this row. [f]T. sp., Trichoderma sp. [g]Totals for information in each respective column. [h]Frequency of detection of this species in the different studies collectively. (Number of studies where this species was isolated)/(total number of studies) X 100. [i]N/A, Not applicable. *Did not use tef1 for species identification.

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3.2. Population structure and genetic diversity of T. atroviride

The tef1 sequence of T. atroviride type strain CBS 142.95 was used for a BLAST search, and a total of 100 sequences of T. atroviride strains were obtained including 11 from Italy, 11 from Canada, 9 from the US, and 9 from Poland. Very low numbers of sequences were from strains isolated in South America, India, and Indonesia (data not shown). The phylogenetic tree in Figure 1 shows a tree pruned to 54 taxa, including one outgroup. Many isolates (21/54) in Figure 1 including the type-strain CBS 142.45, clustered together as an unresolved group. The group was cosmopolitan and included isolates from the Southern Hemisphere (Brazil, Colombia, New Zealand). Within the overall group represented in Figure 1, there were four subclades (Lineages L1 – L4) moderately supported by bootstrap. L1 with 17 isolates had no isolates from the Southern Hemisphere. L2 had biogeographic restrictions as the six isolates were all from North and South America. L3 had biogeographic restrictions as well, with three isolates from the Mediterranean region. L4 had six isolates, all from Europe and the Mediterranean region except one from the United States.

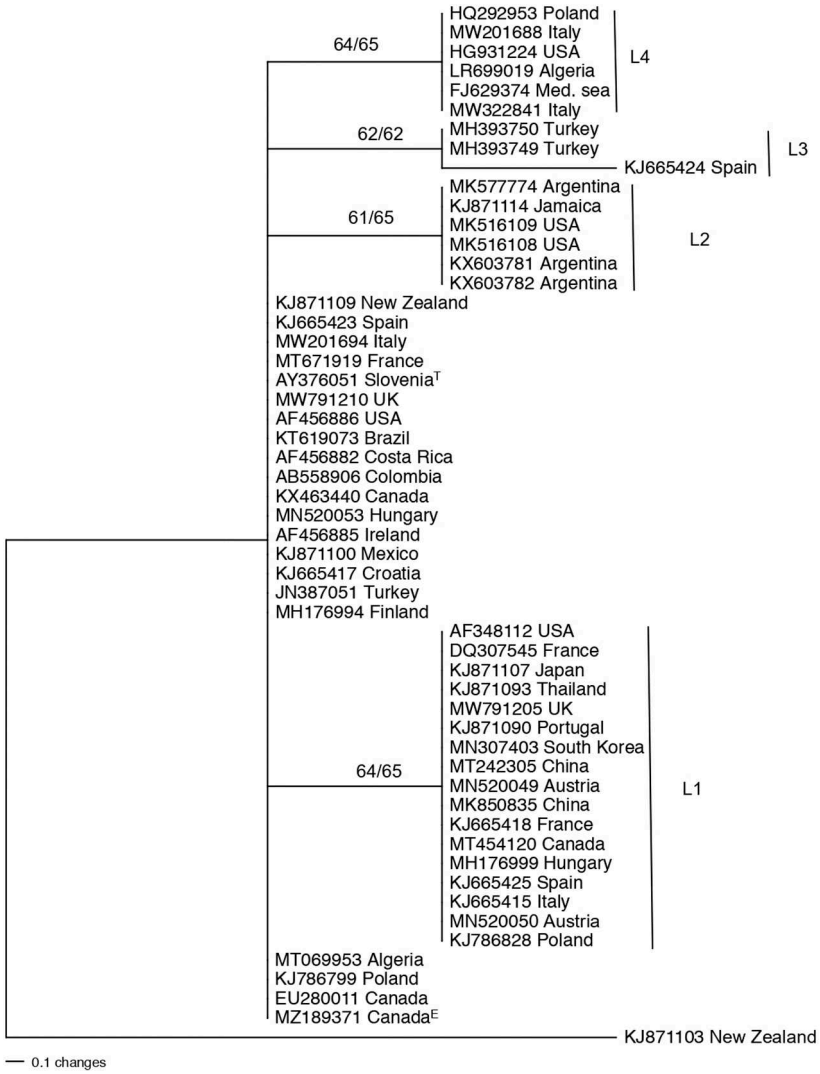

Figure 1. Phylogenetic tree revealing the genetic diversity of the T. atroviride population. The tree was produced using parsimony in PAUP. The numbers above the branches are bootstrap values obtained with 1000 bootstraps in parsimony with PAUP / Maximum likelihood in MEGA. Sequences are identified by GenBank accession number followed by the country of isolation; [T] at the end of leaf refers to type species; [E] indicates that the strain was isolated as an endophyte. L1-L4 refers to lineages with bootstrap support or geographic significance. The tree is rooted to a Trichoderma species from New Zealand. The scale bar represents the number of steps.

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3.3. Population structure and genetic diversity of the T. asperellum/asperelloides species group

The tef1 sequence of T. asperellum type-strain CBS 433.97 was used for a BLAST search, and a total of 100 sequences were obtained: most isolates being from Asia and South America. Countries represented by the most isolates were India (170), China (14), Malaysia (13), and Brazil (12) (data not shown). A substantial number of these tef1 sequences (48/100) were identical including the ex-type strain. The phylogeny of the analyzed T. asperellum population was pruned to 54 taxa for further analysis with the type species of T. asperelloides as the outgroup (Figure 2). This tree contained a major unresolved cluster that included the type-strain CBS 433.97. There was a highly supported subclade (L2) with six isolates all from Central and South America, except one isolate from Morocco. Sequences in this subclade differ from the major cluster by two nucleotides within tef1. There was another highly supported subclade (L4) with two isolates, one from Cameroon and the other from Colombia. Isolates in these two subclades (L2, L4) may qualify as new species based on future multi-locus phylogeny studies. There were two more subclades with moderate bootstrap support, one with biogeographic significance (L3) and one without biogeographic significance (L1).

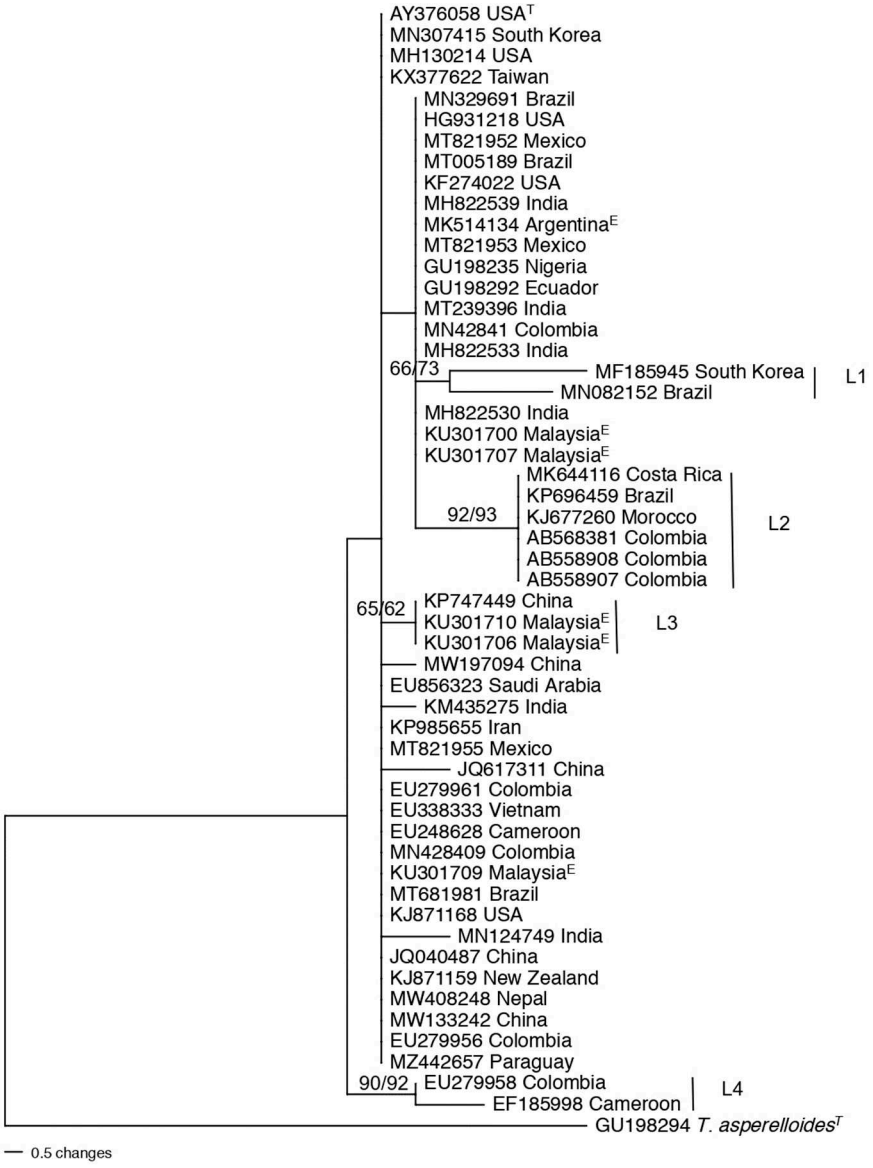

Figure 2. Phylogenetic tree revealing the diversity of the T. asperellum population. The tree was produced using parsimony in PAUP. The numbers above the branches are bootstrap values obtained with 1000 bootstraps in parsimony with PAUP / Maximum likelihood in MEGA. Sequences are identified by GenBank accession number followed by the country of isolation; ^{T} at the end of leaf

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names refers to type species; [E] indicates the strain was isolated as an endophyte. L1-L4 refers to lineages with bootstrap support or geographic significance. The tree is rooted to T. asperelloides from China. The scale bar represents the number of steps.

The tef1 sequence of the T. asperelloides type strain CBS 125398 was used for a BLAST search, and a total of 100 sequences were obtained. Of these, approximately 30% were deposited in GenBank under the wrong identity, mostly as T. asperellum with a few as T. pseudoasperelloides and T. yunnanense (Ismaiel, unpublished). The most prevalent countries of origin for the isolates of T. asperelloides were Malaysia (21), Brazil (21), China (11), and India (9) (data not shown). These were also the most prevalent countries for T. asperellum. The phylogenic tree for the analyzed T. asperelloides population was pruned to 53 taxa for further analysis with T. yunnanense as the outgroup (Figure 3). Most tef1 sequences within the pruned tree were homogenous, with 43 identical sequences, including the type strain from Vietnam. There were two subclades (L1, L2) that were moderately supported. The first subclade (L1) consisted of three isolates from Malaysia, and these strains had one nucleotide difference from the main cluster within tef1. The second subclade consisted of two isolates from India (L2). Isolates of this subclade also differed from the main cluster by one nucleotide.

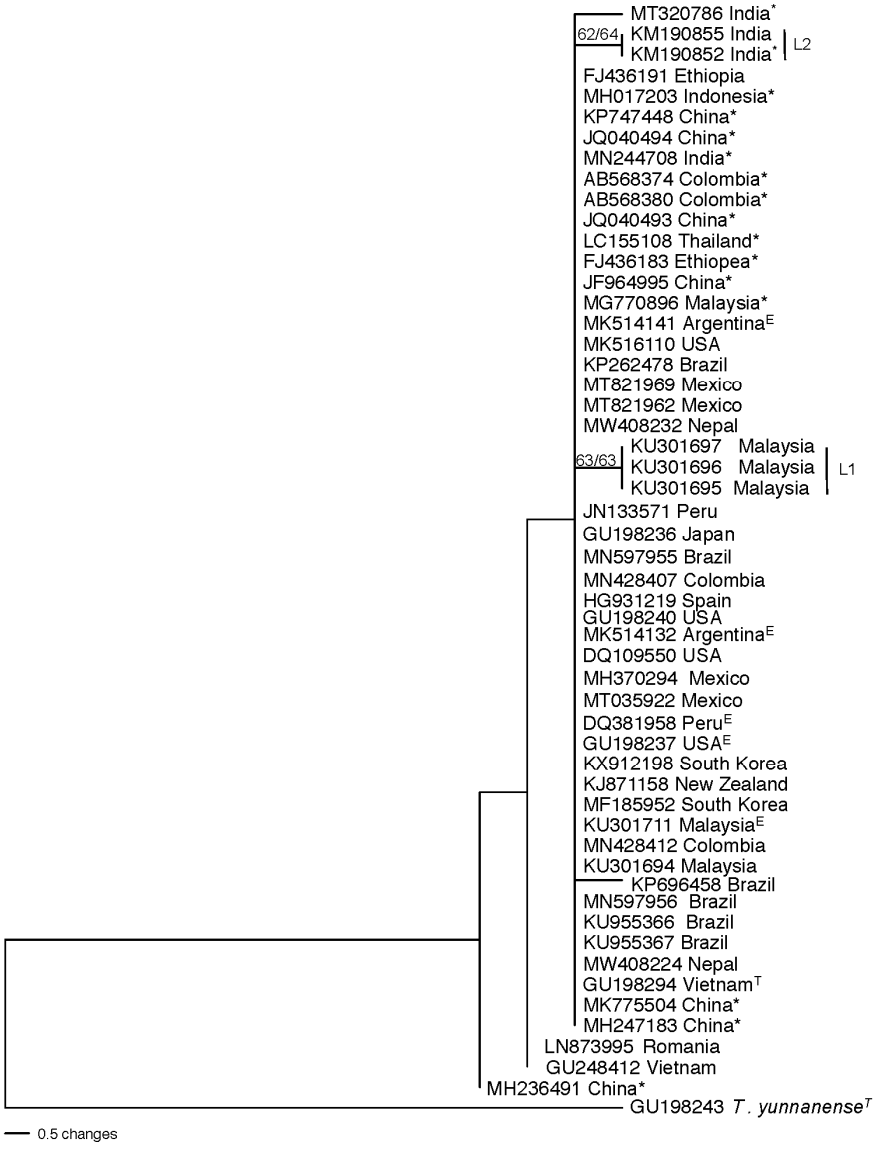

Figure 3. Phylogenetic tree revealing the genetic diversity of the T. asperelloides population. The tree was produced using parsimony in PAUP. The numbers above the branches are bootstrap values

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obtained with 1000 bootstraps in parsimony with PAUP / Maximum likelihood in MEGA. Sequences are identified by GenBank accession numbers followed by country of isolation; [T] at the end of leaf names refers to type species; *, refers to strain deposited under the wrong name; and [E] indicates that the strain was isolated as an endophyte. L1-L2 refers to lineages with bootstrap support or geographic significance. The tree was rooted to T. yunnanense from China. The scale bar represents the number of steps.

3.4. Population structure and genetic diversity of T. hamatum

The tef1 sequence of T. hamatum type-strain DAOM 167057 (CBS 102160) was used for a BLAST search, and a total of 100 sequences were obtained. Three of the sequences were deposited under the incorrect species name (Accession numbers FJ763170, FJ763171, and FJ763161). The first 100 sequences were from T. hamatum strains originating on five continents (i.e., Africa, North and South America, Asia, Europe), as well as Oceana (New Zealand) showing the cosmopolitan nature of this species. The countries, Ethiopia, Italy, and Brazil were highly represented. An initial tree was constructed to observe clades or lineages within the population (data not shown). The tree was pruned to 52 taxa, plus one outgroup and three nearest relatives (Figure 4). There were 7 clades with high bootstrap values. None of the clades had biogeographical restrictions except Clade 3 where the isolates originated from the Far East, particularly China and South Korea. The T. hamatum population appeared to be highly diverse compared to other species studied here.

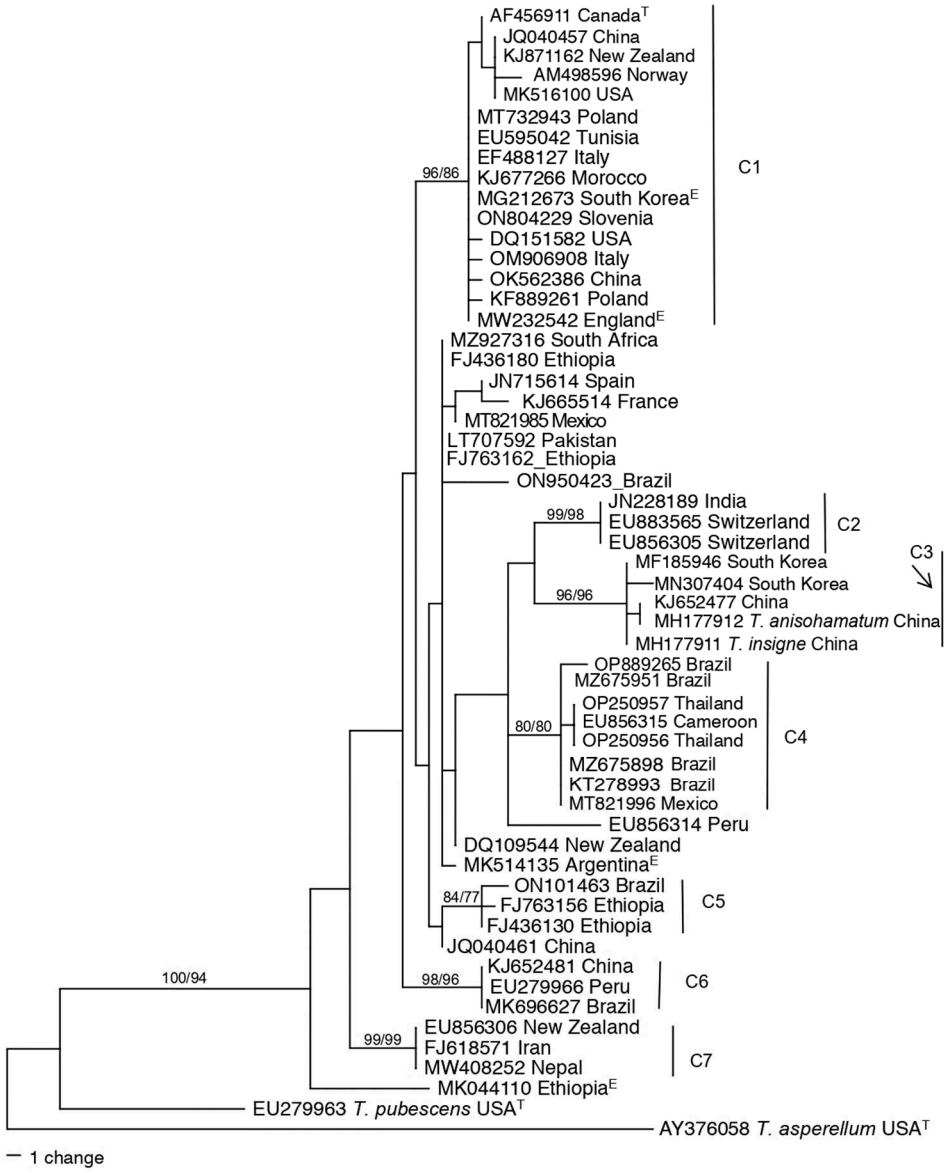

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Figure 4. Phylogenetic tree revealing the genetic diversity of the T. hamatum population. The tree was produced using parsimony in PAUP. The numbers above the branches are bootstrap values obtained with 1000 bootstraps in parsimony with PAUP/ Maximum likelihood in MEGA, respectively. Sequences are identified by GenBank accession number followed by the country of isolation; [T] at the end of leaf names refers to type species; [E] indicates that the strain was isolated as an endophyte. The tree is rooted to T. asperellum. C1-C7 marked with vertical lines refers to lineages with bootstrap support of 70 and greater. Reference species in the tree are identified by species name.

3.5. Genetic diversity of Harzianum Complex Clade species

Table 3 lists the most studied species from the Harzianum Complex Clade using GenBank hits as the metric when searching with species names in the clade. Table 3 also lists geographic regions with references. Species with less than 20 GenBank hits were not included. Using this metric T. afroharzianum, T. lentiforme, T. atrobrunneum, and T. guizhouense were the most studied species, each with over 200 GenBank hits. From Table 3 it is evident that there are some species with worldwide distribution (T. guizhouense, T. afroharzianum) and others that have not been detected in some regions. For example, T. camerunense, T. botryosum, T. pseudopyramidale, and T. afarasin were only detected in Africa.

The phylogeny of species within the Harzianum Complex Clade segregate into two highly supported clades (Figure 5). Clade I included T. camerunense, T. rifaii, T. harzianum, T. simmonsii, T. endophyticum, T. neotropicale, T. afarasin T. botryosum, and T. lixii. Clade II included species T. lentiforme, T. inhamatum, T. afroharzianum, T. atrobrunneum, T. pyramidale, T. pseudopyramidale, and T. guizhouense. The two main clades were not separated based on biogeographic separation. The tree had four lineages (L1 – L4) with no association with the identified species or with associations with long branches. All these lineages have endophytic isolates obtained from plants in Malaysia and Ethiopia. This identifies endophytic species as a source for possible new species within Harzianum Clade Complex.

Table 3. Most studied *Trichoderma* species from the Harzianum Complex Clade[a].

Species[b]	GenBank Hits[c]	Habitat	Geographic Region	Reference[d]
T. lentiforme	481	endophytes; few from soil	South America	[7]
T. inhamatum	117	Soil	South America	Mycobank# 104673
T. guizhouense	204	commonly in soil; endophytes in Africa	World-wide	[44]
T. afroharzianum	644	commonly in soil; few endophytic	World-wide	[7]
T. pyramidale	26	soil and decaying wood	Europe	[7]
T. atrobrunneum	234	commonly in soil	Europe	[7]
T. simmonsii	176	commonly in soil or decaying wood	North America, Europe	[7]
T. harzianum	N/A[e]	soil, endophytic	North America, Europe	[7,45]
T. camerunense	38	commonly in soil	Africa	[7]
T. botryosum	44	endophytic in coffee	Africa	[39]
T. pseudopyramidale	82	endophytic in coffee and mycoparasite	Africa	[39]
T. afarasin	27	mostly endophytic	Africa	[7]
T. neotropicale	37	endophytes of tropical trees	South America	[7]
T. endophyticum	72	endophytes of tropical trees	South America	[7]
T. rifaii	40	endophytes of tropical trees	South America	[7]
T. lixii	113	soil or decaying wood	Southeast Asia	[7]

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[a]Compilation of species from the Harzianum Complex Clade with 20 or more GenBank Hits when using the species names in the search. GenBank Hits was the metric used to indicate the degree to which a species has been studied. [b]All species listed are from the genus *Trichoderma*. [c]Number of sequence deposits from isolates of this species in GenBank. [d]Literature describing this species. [e]N/A, not applicable. For *T. harzianum*, number of Hits is not accurate as newly classified species from the Harzianum Complex Clade were previously deposited as *T. harzianum*.

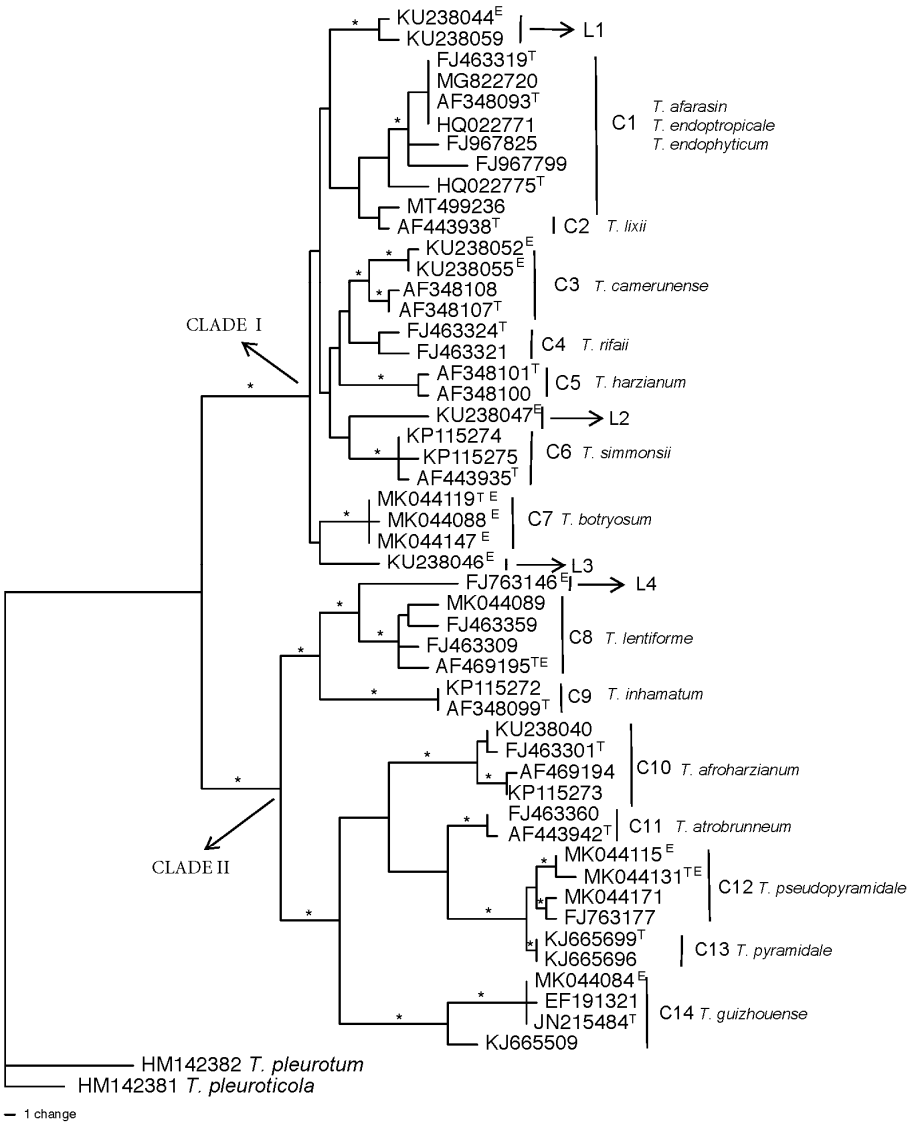

Figure 5. One of the most parsimonious trees obtained via PAUP based on sequences of tef1 resolving the relationship of Trichoderma species within the Harzianum Complex Clade. Tree leaves are labeled with Genbank accession numbers for Trichoderma species sequences. Stars above the branches indicate bootstrap support of 70 or greater. [E] at the end of the accession number indicates that the strain was isolated as an endophyte. [T] at the end of the accession number indicates a type species. Clades are marked with vertical lines and numbers 1-14 represent identified species. The scale bar represents the number of steps. Lineages marked with vertical lines (L1-L4) represent unidentified lineages. The tree was rooted to T. pleurotum and T. pleuroticola.

3.6. Population structure and genetic diversity of T. virens

The first 100 hits when doing a BLAST search in GenBank with the tef1 sequence from the type strain (GLI 39) for T. virens were sequences of isolates from China (27), Brazil (11), Malaysia (8), and Hungary (7) (data not shown). T. virens is truly cosmopolitan having been isolated from South America, North America, Europe, Africa, and Asia. Africa had the least number of GenBank hits with

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only two, one from Cameroon and the other from the Ivory Coast. A phylogenetic tree was constructed for T. virens pruned to 55 taxa, plus T. crassum as the outgroup (Figure 6). The population structure and genetic diversity of T. virens is highly variable compared to the other Trichoderma species analyzed in this study, containing seven lineages (L1 – L7) with strong bootstrap values. Three of the lineages, L1, L2, and L5 had biogeographic restrictions as they had strains from only China, Mexico, and Turkey, respectively. Within Clade L6 there were two isolates from Mexico (MT075514, MT075513), deposited in GenBank as T. neocrassum. The position of T. neocrassum between T. virens lineages L5 and L7 is possibly due to errors in identification.

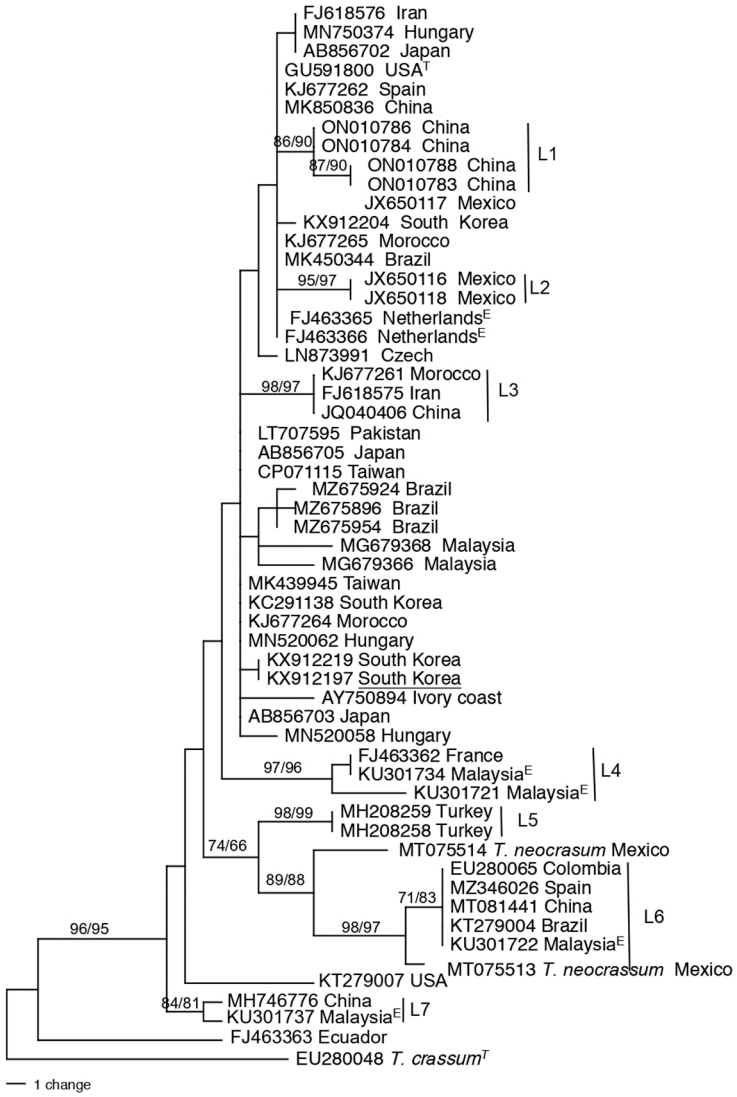

Figure 6. Phylogenetic tree revealing the genetic diversity of the T. virens population. The tree was produced using parsimony in PAUP. The numbers above the branches are bootstrap values obtained with 1000 bootstraps in parsimony with PAUP / Maximum likelihood in MEGA. Sequences are identified by GenBank accession numbers followed by country of origin; [T] at the end of leaf names refers to type species; [E] indicates that the strain was isolated as an endophyte. L1-L7 refer to lineages with bootstrap support or geographic significance. The tree is rooted to the T. crassum type species. Reference species in the tree are identified by species name.

3.7. Nearest relative analysis

The phylogenetic relatives of T. atroviride, T. asperellum, T. asperelloides, and T. hamatum are shown in Figure 7. In Clade C1 of the resulting tree, the T. atroviride type species clustered with T. atroviride B from New Zealand which was previously identified as T. atroviride [46]. The other

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species in Clade C1 was T. nordicum from China. These two species in Clade C1 were the nearest relatives to T. atroviride and together formed a highly supported clade. The next closest relatives to T. atroviride were the species in Clade C2, T. uncinatum, T. paratroviride, and T. obovatum. Clade C2 also contained isolates incorrectly identified in GenBank as T. atroviride (accession numbers KJ634780 and KJ634765) as they clustered with T. paratroviride and T. obovatum.

The nearest relative for T. asperellum was T. yunnanense (Figure 7, Clade C3), while the nearest relative for T. asperelloides was T. pseudoasperelloides. In fact, the type species of T. asperelloides (GU198294) had an identical tef1 sequence with the type species of T. parasperelloides (MK775504). Clade C3 also contained sequence of an isolate incorrectly identified in GenBank as T. yunnanense from India, as it did not cluster with the type species of T. yunannense. The nearest relatives of T. hamatum (C4) were the two species T. insigne and T. anisohamatum followed by T. pubescens. The four species formed a highly supported clade C4. However, when the two species of T. insigne and T. anisohamatum were included in the population structure and genetic diversity of T. hamatum, they clustered with three other isolates of T. hamatum from China and South Korea (C3, Figure 4). The discrepancy regarding whether these species are new species or T. hamatum deserves revisiting. The closest relative for T. virens was T. neocrassum (Figure 6, Clade L6). However, there was no sequence for the type species CBS 114230 (T. neocrassum) to know the exact relation of this species to T. virens. Nearest relative analysis was not conducted for the Harzianum Complex Clade since the Clade was previously divided.

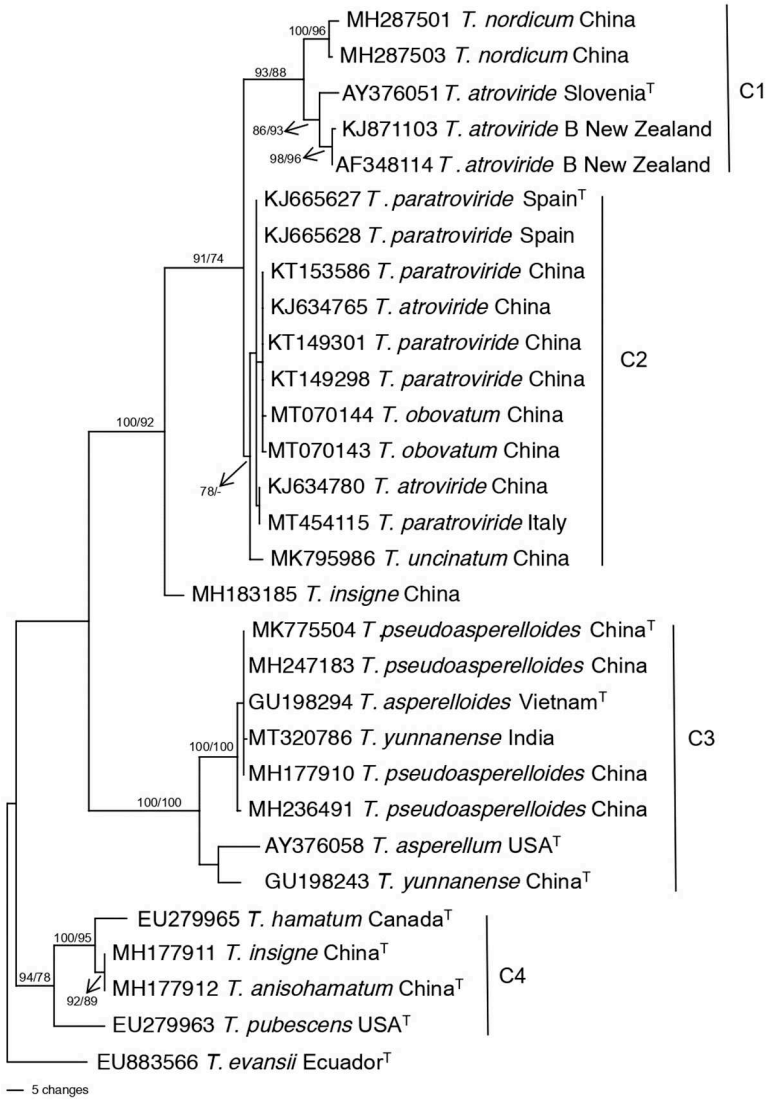

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Figure 7. One of the most parsimonious trees obtained via PAUP based on DNA sequence of tef1 showing the nearest relatives to Trichoderma species, T. atroviride, T. asperellum, T. asperelloides, and T. hamatum. Sequences are identified by GenBank accession number followed by species names and country of origin. Clades C1-C4 are marked by vertical lines. Numbers above the branches represent bootstrap support values of 70 and greater from 1000 replicates from Parsimony with PAUP/Maximum likelihood in MEGA, respectively. ^{T} at the end of leaf names refers to type species. The tree is rooted to T. evansii. The scale bar represents the number of steps.

4. Discussion

The genus *Trichoderma* was first recognized by Persoon in 1794 [47]. However, the taxonomy of the genus remained obscure until 1969, when Rifai [45] proposed nine species or species aggregates based mainly on morphological characteristics of conidiophores and phialides. Approximately 20 years later, Bissett revised Rifai's proposal and replaced the nine aggregate species by formally recognizing five sections comprising 27 species [48–51]. In the late 1990s, molecular identification based on DNA sequence data started and showed inaccuracies in the taxonomy based on morphological characters primarily due to the homoplasy and plasticity of the characters [52]. As a result, taxonomy based on molecular data was adopted and used for the classification and identification of *Trichoderma* spp. [3]. Although the Internal Transcribed Spacers Sequences (ITS) was informative at the genus level, it was found to be the least informative, whereas *tef1* and the gene for the second largest RNA polymerase subunit (*rpb2*) were the most informative loci for species level differentiation [6,7]. Amongst the latter two loci, however, *tef1* was the most utilized marker for *Trichoderma* phylogeny (Ismaiel, unpublished). Today, there are about 500 species of *Trichoderma* based on legitimate names available in Mycobank (https://www.mycobank.org).

A strategy to enhance the successful commercial development of *Trichoderma* and other microbes for agriculture is to preferentially seek isolates of species that have been demonstrated to have desired traits and/or are adapted to local crops, soils, and farming practices [47,53]. To use this strategy to aid in selecting *Trichoderma* isolates for development as biocontrol agents or biofertilizers (BCBFs) we reduced the more than 500 species of *Trichoderma* identified based on *tef1* sequence homology and/or phylogeny to a few that were prevalent soil inhabitants and/or endophytes as persistence in soil and within plant tissues is important for microbes to function as BCBFs [1,2]. For this, prior *Trichoderma*-specific soil surveys and endophyte surveys were analyzed. This analysis revealed that isolates from *T. atroviride, T. hamatum, T. virens*, the *T. asperellum/asperelloides* grouping, and the species in the Harzianum Complex Clade were prevalent soil inhabitants. Isolates from these species and species groupings were also often detected as endophytes in various plants worldwide. Supporting their preferred selection for this strategy, these *Trichoderma* species have demonstrated importance in commercial products used in several countries. In a prior analysis, 51 of 56 commercial products had at least one of these species as active ingredients [47]. Further, in a compilation of biocontrol investigations directed at combating various diseases of crops 21 of 28 isolates, or 75% of isolates, were from one of the above *Trichoderma* species or species groupings [54]. Each of these species or species groupings has been shown to induce systemic disease resistance in various crop plants, an important trait for biological control and biological fertilizer [2,55,56]. Various BCBF *Trichoderma* isolates also produce or induce plant growth hormones and volatile compounds and are involved in promoting the uptake of macro– and micro-nutrients by crops [57].

Population structure and genetic diversity was performed to further characterize *T. atroviride, T. hamatum, T. virens*, and the *T. asperellum/asperelloides* grouping and to demonstrate whether there were lineages that had potential to be a different species; as was done for the Harzianum Complex Clade. The analyses revealed new lineages for *T. atroviride* (L2, L3), *T. asperelloides* (L1), *T. asperellum* (L2), *T. hamatum* (L1, L2, L3, L4, L5, L6, L7), *T. virens* (L1, L2, L5), and the Harzianum Complex Clade (L1, L2, L3, L4). Some of these lineages may qualify as new species after multi-locus phylogeny is performed [3]. Further, we found that within the *T. hamatam* population, two new species have been described as *T. insigne* and *T. anisohamatum* (Figure 4 Clade 3). Several lineages or distinct clades reported here have distinct biogeographic restrictions. It is possible that populations of *T. atroviride* and the other

Trichoderma species reported here with biogeographic restrictions may have evolved separately due to distinct regional environmental conditions or restrictions due to the endophytic lifestyle. The coevolution of plants and *Trichoderma* species has been postulated just as has been demonstrated for plant-pathogen interactions [55–57]. These regional adaptations may make these isolates ideal candidates for commercial development in these regions of the world.

In summary, microbials are proving to be important in many applications in sustainable agriculture including their use as biocontrol agents and biofertilizers [2,53,57,58]. Unfortunately, there has been limited commercialization of microbial agricultural products relative to the volume of research on plant-beneficial microbes [53]. The strategy of preferential selection of isolates from species known to have beneficial properties that are also compatible with commercialization is more robust than prior, non-preferential approaches where hundreds to thousands of randomly selected isolates may need to be screened to identify a few strains with desired characteristics [60]. Consistent with this strategy we propose narrowing the search for biocontrol agents and biofertilizers within the increasingly complex genus *Trichoderma* to the subset of *Trichoderma* species: *T. asperellum*, *T. asperelloides*, *T. virens*, *T. atroviride*, *T. hamatum*, and some species in the Harzianum Complex Clade based on solid existence in the soil and as endophytes and prior commercialization. Although persistence in soil or as an endophyte does not guarantee effectiveness as a BCBF [2] use of these attributes in this approach should speed the selection of candidate isolates for downstream, in-depth screening. We also identified lineages within some of these species that are geographically restricted that may be highly effective BCBFs, particularly when used for crops grown in the region of isolation, due to region- or plant-specific environmental adaptations [46]. Finally, we identified nearest-neighbor *Trichoderma* species, which could be additional sources of isolates for commercialization.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1. Dominant *Trichoderma* species/groups from soil surveys from different geographic regions. Table S2. Isolation of endophytic species of *Trichoderma* from different geographic regions.

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