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Molecular characterization of biocontrol isolates of *Trichoderma aureoviride*

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Abstract
Trichoderma has gained immense importance since last few decades due to its biological control ability against several plant pathogens. Twenty isolates of *T. aureoviride* were collected from different geographical locations of Chhattisgarh. Molecular characterization of isolates of *T. aureoviride* revealed homogeneity with ITS primer (internal transcribed spacer). In the present study the genetic relatedness among twenty isolates of *Trichoderma aureoviride* was analyzed by using sixteen gene specific markers. PCR amplification with ITS primers showed homogeneity of all *T. aureoviride* isolates indicating that they belong to the same species. Six different chitinase gene specific primers produced amplification of chitinase genes in almost all *T. aureoviride* isolates except one of the exochitinase encoding gene (*N*-acetyl- -glucosaminidase), *nag*70 based primer Nag70F-R and two endochitinase encoding gene specific primers Ech42-2F-R and Ech42-3F-R that could not amplify any of the isolates of *T. aureoviride*. PCR amplification of fragments of cellulase gene using CBH1 primer amplified a nucleotide fragment of the expected size from some isolates while PR808-PRegl4 primer pair amplified 340, 140 & 100 bp bands in all the isolates. Primer pair fRPB2-5F2- fRPB2-7cR2 generated major band above 1000 bp confirming the amplification of a 1.2-kb fragment of subunit 2 of the RNA polymerase B gene (RPB2) containing an ITS-like region. Almost all the isolates showed the presence of endochitinase and cellulose gene fragments which proves their ability to antagonize phytopathogens.

Keywords: *Trichoderma*, Molecular characterization, Internal transcribed spacer

Introduction
Trichoderma species are being shown to be the anamorphs of *Hypocrea* species (Kuhls *et al.* 1996, Samuels *et al.* 1998, Chaverri *et al.* 2001a). It's a cosmopolitan soil fungi, remarkable for their rapid growth, capability of utilizing diverse substrates, and resistant to noxious chemicals. They are often predominant components of the mycoflora in various soils, such as agriculture, prairie, forest salt marsh and desert soil in all climatic zones (Danielson, 1973; Klein and Eveleigh, 1998; Roiger, *et al*., 1991; Wardle, *et al*., 1993), where they are significant decomposers of woody and herbaceous material, and are also necrotrophic against the primary wood decomposers (Rossman, 1996). Some of the about 35 established species of *Trichoderma* (Gams and Bissett, 1998) are also of economic importance because of their production of enzymes and antibiotics, or use as biocontrol agents (Hjeljord and Tronsmo, 1998; Kubicek and Penttilä, 1998; Sivasithamparam and Ghisalberti, 1998).
Identification of the respective isolates at the species level has proved difficult, due to the degree of morphological similarities between them. As a solution, Rifai (1969) adopted the concept of 'aggregate' species, and distinguished nine aggregates, some of which comprised two or more morphologically indistinguishable species. In contrast, Bissett (1984, 1991a-c, 1992, Gams & Bissett, 1998) perceived non-continuous morphological characteristics of biological species. On this basis, he elevated Rifai's aggregate species to the sectional level, and expanded the morphological criteria to accommodate the wider range of morphological variation expressed by some anamorphs of *Hypocrea* and also to include some forms previously included in *Gliocladium.*
The phylogeny of *Trichoderma* and the phylogenetic relationships of its species were investigated by maximum parsimony analysis and distance analysis of DNA sequences from multiple genetic loci 18S rDNA sequence analysis suggests that the genus *Trichoderma* evolved at the same time as *Hypomyces* and *Fusarium* and thus about 110 M yr ago gene trees inferred from a combined analysis of the nuclear ribosomal internal transcribed spacer (ITS1 and 2), the Dl and D2 region of the 28S rDNA, the small subunit of the mitochondrial rDNA (mitSSU), the fifth and part of the sixth exon of translation elongation factor 2 *(tef1),* and a fragment of *ech42* provide strong statistical support for a phylogeny consistent with the

existence of four clades clade A comprises species of Bissett's (1991) sect *Trichoderma* but also *T. hamatum, T. pubescens, T. asperellum,* and *T. strigosum,* clade C comprises all the species contained m section *Longibrachiatum* as revised by Samuels *et al* (1998), and clade D contains only *T. aureoviride* which is genetically most distant to all other species Clade B, on the other hand, contains a large and taxonomically heterogeneous mixture of species (Kullnig-Gradinger et. al., 2002). Some *Trichoderma* species are morphologically similar to the anamorphs Hypocrea, and their internal transcribed spacer (ITS) sequences have revealed their taxonomic proximity (Monte, 2001; Hermosa *et al*., 2000). Molecular characterization and phylogenetic analysis have allowed strains of *T. aureoviride* originally identified as the same species to be assigned to different species clustered into distinct sections and groups.

Materials and Methods

Collection and isolation of the *Trichoderma aureoviride*

Twenty isolates of *T. aureoviride* isolated (Table 1) from the soil samples collected from different geographical location of Chhattisgarh. Different isolated were grown on Potato Dextrose Agar plates and incubated for 5 to 7 days at 25 ±2 ºC.

Table 1: Twenty isolates of *Trichoderma aureoviride* and pathogens obtained from soil samples collected from different geographical locations of Chhattisgarh.

S.No.	Isolate No.	Geographical Location
1	T5	Outside Fenugreek plantation, Horticulture Department IGKV, Raipur.
2	T29	Abhanpur
3	T37	Bhatagaon (Termitorium soil)
4	T50	Charama
5	T63	Chhati
6	T72	Dhamtari road, Raipur
7	T80	Jagtara
8	T82	Jagtara
9	T99	Jaisekara (rice field soil)
10	T107	Kanker (Forest 3 soil)
11	T114	Kodebor
12	T120	Kurdha
13	T131	Purur
14	T139	Purur (rice field soil)
15	T144	Rice field fallow land, Raipur
16	T149	Rice lathyrus field, Kanker road
17	T155	Tree soil Mana road Raipur
18	T159	Unknown location
19	T163	VIP road (Termitorium soil), Raipur
20	T175	VIP road rice field soil, Raipur

DNA extraction:

Fungal genomic DNA was extracted using the specific DNA extraction method. Isolates grown on potato dextrose broth (PDA) at 25˚C for 48 h will be used to harvest Mycelium Fungal tissue and DNA isolate using CTAB method modified with EBA and EBB. After incubation at 65°C, an RNase treatment was performed. DNA was precipitated with isopropanol, washed with ethanol (70%), and air-dried for 15 min. Store in TE for overnight an RNase treatment was performed on next day. DNA was rehydrated with 50 µl of DNA hydration solution which will further be quantified using Nanodrop Spectrophotometer (ND 1000). The acceptable absorbance ratio (A260/A280) for pure DNA is 1.8. Quantified DNA samples of the selected isolates will be subjected to PCR amplification using gene specific primers.

DNA amplification

PCR was performed in a total reaction volume of 20 µl containing 2µl of 10X buffer with $MgCl_2$, 2µl of dNTP, 1µl Taq DNA polymerase, 1µl each of primers (Forward and Reverse), 10 µl autoclaved distilled water and 3µl of genomic DNA (30 ng/ µl). The amplification program included an initial denaturation at 94ºC for 3 min, followed by 30 cycles of denaturation at 94ºC for 1 min, followed by 30 cycles of annealing at 52ºC for 1min, extension at 72ºC in a thermocycler. Following amplification, the PCR products were resolved on 5% non-denaturing PAGE.The list of 8 ITS markars, 6 Chytinolytic markars and 2 cellulolytic markars used in present investigation (**Table 2**).

Table 2: List of DNA primers and their sequence

S.No.	Primer	Tm (ºC)	Primer Sequence (5'-3')
ITS, Calmodulin gene, Transcription Elongation factor based primers			
1	ITS 4	58.35	TCCTCCGCTTATTGATATGC
	ITS 5	56.5	GGAAGTAAAAGTCGTAACAAGG
2.	Hj ITS F	62.7	AGCGGAGGGATCATTACCGAGTTT
	ITSc R	62.7	TCGCATTTCGCTGCGTTCTTCATC
3.	SR6R(F)	53.2	AAGTAGAAGTCGTAACAAGG
	LR_1 (R)	47.9	GGTTGGTTTCTTTTCCT
4.	EF1 728f	56.5	CATCGAGAAGTTCGAGAAGG
	EF1-986 R	55.3	TACTTGAAGGAACCCTTACC
5.	EF1 728 F	56.5	CATCGAGAAGTTCGAGAAGG
	TEF1LLE rev	53.7	AACTTGCAGGCAATGTGG

6.	fRPB2-5F1	51.2	GATGATAGAGATCATTTTGG
	fRPB2-7cR1	55.3	CCCATAGCTTGTTTACCCAT
7.	fRPB2-5F2	61.4	GACGACCGTGATCACTTCGG
	fRPB2-7cR2	61.4	CCCATGGCTTGCTTGCCCAT
8.	HpCal F	62.7	AGAAAGTTGGAGGCGATTTCAGCG
	HpCal R	62.7	TGCCTAGCTCCTTGGTGGTAATCT
***Endochitinase* gene *ech42* primers**			
9.	Ech-42 -1 F	64.5	GCCAGTGGATACGCAAACGC
	Ech-42 -1 R	60.16	CTCTAGTTGAGACCGCTTC
10.	Ech-42-2F	63.08	CTTGTAGTCCCAAATAAAGTTCTCCA
	Ech-42-2 R	69.16	GGCGAAACGCCGTCTACTTCACCAACTGG
11.	Ech-42 -3 F	66.28	CACTTCACCATGTTGGGCTTCCTC
	Ech-42 -3 R	66.28	GATCTCTAGTTGAGACCGCTTCGG
12.	Ech-42 -4 F	66.47	GCGCTGCAGGCCACTCTCATT
	Ech-42 -4 R	66.47	AGTTGGGGTTGGACGGGTTGG
13.	Nag 1- F	66.4	GGCATTGACCGCGGTGTTGAGG
	Nag 1- R	67.98	CACGGGGCCAGATGATGTTGTCCA
14.	Nag 70 F	71.94	GCTCTAGACCGCCTCGGTCGTCCATCAT
	Nag 70 R	72.11	CGGGATCCTTATGCGAACAAGGTGCAAG CCGTA
***Cellulase* gene CBH Primers**			
15.	CBH1 F	65.7	CACCCGCCTCTGACATGGCAG
	CBH1 R	59.8	CAGGCATGAGAGTAGTAAGG
16.	PR808 F	52.8	ATCGCATTTCCTACCCC
	PR egl4 R	58.2	CCCACCCCAGACTCTGTA

Result and Discussion

Molecular characterization of *Trichoderma aureoviride*

In the present investigation molecular characterization of 20 isolates of *T. aureoviride* was carried out to amplify internal transcribed spacer region (ITS1, 5.8 S and ITS2), sections of the translation elongation factor (tef1), RNA polymerase II subunit (RPB2), calmodulin gene (*cal*), fragments of endochitinase gene *ech42* and fragments of cellulase gene *CBH* using 16 different primers. Out of the sixteen primer pairs three primer pairs (fRPB2-5f1-fRPB2-7cR1; Ech-42-2F-R and Ech-42-3F-R) failed to amplify any of the isolates.

PCR amplification of ITS region of 5.8s rRNA gene with primer ITS4-ITS5 yielded ITS fragment 700 bp length in all the isolates of *T. aureoviride*. No distinct inter or intra specific ITS length diversity was detected. However, its digestion with restriction endonuclease *MseI* and *Sau3AI* revealed monomorphism generating monomorphic bands of 290, 220 and 130bp with *MspI* and 190, 170 and 150bp with *Sau3AI*. Overall homogeneity was observed between different isolates of *T. aureoviride* suggesting its identity to be a single species. Parts of the conserved regions of the small-subunit rDNA (primer SR6R) and the large-subunit rDNA (primer LR1) flanking the ITSs were used as primers which amplified fragment of 700 bp length in all the isolates. Similarly, designed primer HjITSF-R generated 500, 470 and 290 bp band in all the 20 isolates of *T. aureoviride.* Calmodulin (CaM) (an abbreviation for CALcium-MODULated proteIN) is a calcium-binding messenger protein expressed in all eukaryotic cells. Using primer HpCalF-R 700 and 550 bp bands were observed in all isolates except T29, T63, T114, T149, T155 and T159.Similar digestion of species *Longibrachiatum* generated monomorphic fragments (gel data not shown) of 300, 140 and 110 bp. This reveals generation of inter specific variability after *MseI* digestion, suggesting its usefulness to discriminate species of *Trichoderma*. The resulting digestion patterns using this enzyme facilitate grouping of the respective isolates into the corresponding sections or species. Size differences of the intact ITS1 fragments can be used for distinguishing the strains into groups that correspond to sections and clades (Bulat *et al*., 1998; Kindermann *et al*., 1998; Lord *et al*., 2002).

The primers for translation elongation factor (tef1) includes EF1-728 EF1-986; EF1-728 - TEF1LLErev. Out of them EF1-728-EF1-986 primer generated 350bp band in all isolates of *T. aureoviride.* Isolate T139 has two bands at 350bp and 330bp (**Fig 1 a**). Primer EF1-728-TEF1LLErev (**Fig 1 a**) primer generated 1250bp; which is used for amplification of fragments of the large fourth intron of *tef1*.A 0.3kb fragment of *tef1*, containing the large intron, was amplified by the primer pair EF1-728F and EF1-986R as described by Druzhinina *et al*., (2004).(Chaverri *et al*., 2003a). This fragment (~1.3kb) included the fourth and fifth introns and a significant portion of the last large exon (Chaverri *et al*., 2003b, Druzhinina and Kopchinskiy 2006). Bayesian analysis of sequences of the large intron of *tef1* both confirmed species identification by the barcode, and also provided support for the unique nature of those isolates which could not be identified by the barcode (Druzhinina *et al*., 2005).

Similarly six different chitinase gene specific primers (Ech-42-1F-R; Ech-42-2F-R; Ech-42-3-F-R; Ech-42-4F-R; Nag1; Nag70) were used to analyse amplification of chitinase genes in *T. aureoviride*. PCR amplification of the 42-kDa endochitinase gene with primer Ech-42-1F-R resulted in a product of the expected size (900bp) for all investigated isolates except T50, T63, T72, T107, T114, T149, T155, and T159. Strikingly, there was an additional reproducible fragment (420 bp) in these isolates. PCR amplification with primer Ech42-4F-R amplified 754 bp in all isolates except T80. Similarly, primer Nag1 gave amplification of ~900 bp in all isolates.One of the exochitinase-encoding gene (*N*-acetyl- -glucosaminidase), *nag*70 based primer Nag70F-R and two endochitinase-encoding gene specific primers Ech42-2F-R and Ech42-3F-R could not amplify any of the isolates of *T. aureoviride.* The major band of chitinase seen in the electropherograms is likely due to c*hi18-5* (previously e*ch42* or c*hit42*), because it accounts for the major extracellular chitinase activity in *Hypocrea/Trichoderma* (Garcia *et al*., 1994, Hayes *et al*., 1994). According to phylogenetic analysis, the c*hi18-5* gene is a housekeeping gene rather than a tool

developed for mycoparasitism. It is likely that some of the other chitinase isoenzymes, which are expressed only poorly (such as that encoded by c*hi18*-10 (Seidl *et al*., 2005), have more subtle relevance to this process (Nagy *et al*., 2007).

Along with ITS and chitinase gene primers two cellulase gene specific (CBH1F-R; PR808-PRegl4) primers also used. Among them CBH1 primer (**Fig 2a**) pair readily amplified a nucleotide fragment of the expected size (~1300bp) from some isolates (T29, T37, T72, T82, T120, T131, T155, T159). While PR808-PRegl4 primer (**Fig 2b**) pair amplified 340, 140 & 100 bp bands. This primer was used by Karlsson *et al*. (2001) for homologous expression and characterization of Cel61A (EG IV) of *Trichoderma reesei*. Using PCR, Kubicek *et al*., (1996) were able to amplify fragments of the gene encoding the major cellulose of *T.reesei*-cbh1 from a selection of isolates of other species of *Trichoderma* section *Longibrachiatum* with low and high cellulase production. This indicated that the differences in cellulase production were not due to the absence of one of these two genes. The low production in most strains must therefore be due to other reasons, such as differences in the 5'-non coding sequences or in the chromosomal location. Similar results are observed in the present investigation because isolates T29 [(L) 30.5 ± 2.12], T37 [(M) 37 ± 1.414], T72 [(H) 40.5 ± 2.121], T82 [(L) 30.5 ± 2.121], T120 [(M) 37 ± 4.243], T131 [(H) 40 ± 1.414], T155 [(L) 35 ± 2.828] and T159 [(L) 34.5 ± 2.121] which were amplified with the primer CBH1 showed different response for cellulose hydrolysis.

The RNA polymerase II subunit (RPB2) in isolates of *T. aureoviride* amplified using primer pair fRPB2-5F2- fRPB2-7cR2 generated major band above 1000 bp confirming the amplification of a 1.2-kb fragment of subunit 2 of the RNA polymerase B gene (RPB2) containing an ITS-like region. However, primer pair fRPB2-5F1- fRPB2-7cR1 could not amplify any of the isolates of *T. aureoviride.* The RNA polymerase II subunit (RPB2) is generally used to evaluated multiple phenotypic characters. James *et al*. (2006) evaluated the contribution of protein data (*RPB*1, *RPB*2, and *EF*1-α) and ribosomal data (nucSSU, nucLSU, and 5.8S) for basal relationships within fungi.Ribosomal RNA genes are the most commonly used loci in molecular systematic studies of fungi (Lutzoni *et al*., 2004). RPB2 encodes the second largest subunit of RNA polymerase II and has been established as a suitable alternative to the widely used 18S rRNA (ITS) system for molecular systematic of fungi (Liu *et al*., 1999).

Phylogenetic Analysis

The band appeared in Gene specific primers were scored in the form a matrix with '1' and '0', which indicate the presence and absence of bands in each isolate respectively.For phylogenetic analysis of the twenty isolates of *T. aureoviride*, a dendrogram was constructed through the method of UPGMA (Sneath and Sokal, 1973) using of NTSYS-pc software, version 2.02 (Rohlf, 1998) Considerable variation was observed among some of the isolates of *T. aureoviride*. The combined tree of the of chitinase gene specific primer produced for *T. aureoviride* strains split into two strongly supported cluster, A and B with the similarity coefficient ranging from 0.44 to 0.94 (Fig 2a). Major cluster A contained 14 isolates(T5, T82, T139, T29, T99, T131, T80, T120, T149, T107, T159, T144, T63and T72) .whereas minor cluster B containing only 6 isolates(T37, T50, T155, T114, T163 and T175) of *T. aureoviride.* Similarly cellulase gene specific primer also produced two clusters A and B with the similarity coefficient ranging from 0.31 to 1.00 ((Fig 2b) for *T. aureoviride* strains. In these case major cluster A represented 19 isolates(T5, T82, T139, T29, T99, T131, T80, T120, T149, T107, T159, T144, T63,T37, T50, T155, T114, T163 and T175)while minor cluster B represented only 1 isolate T72.

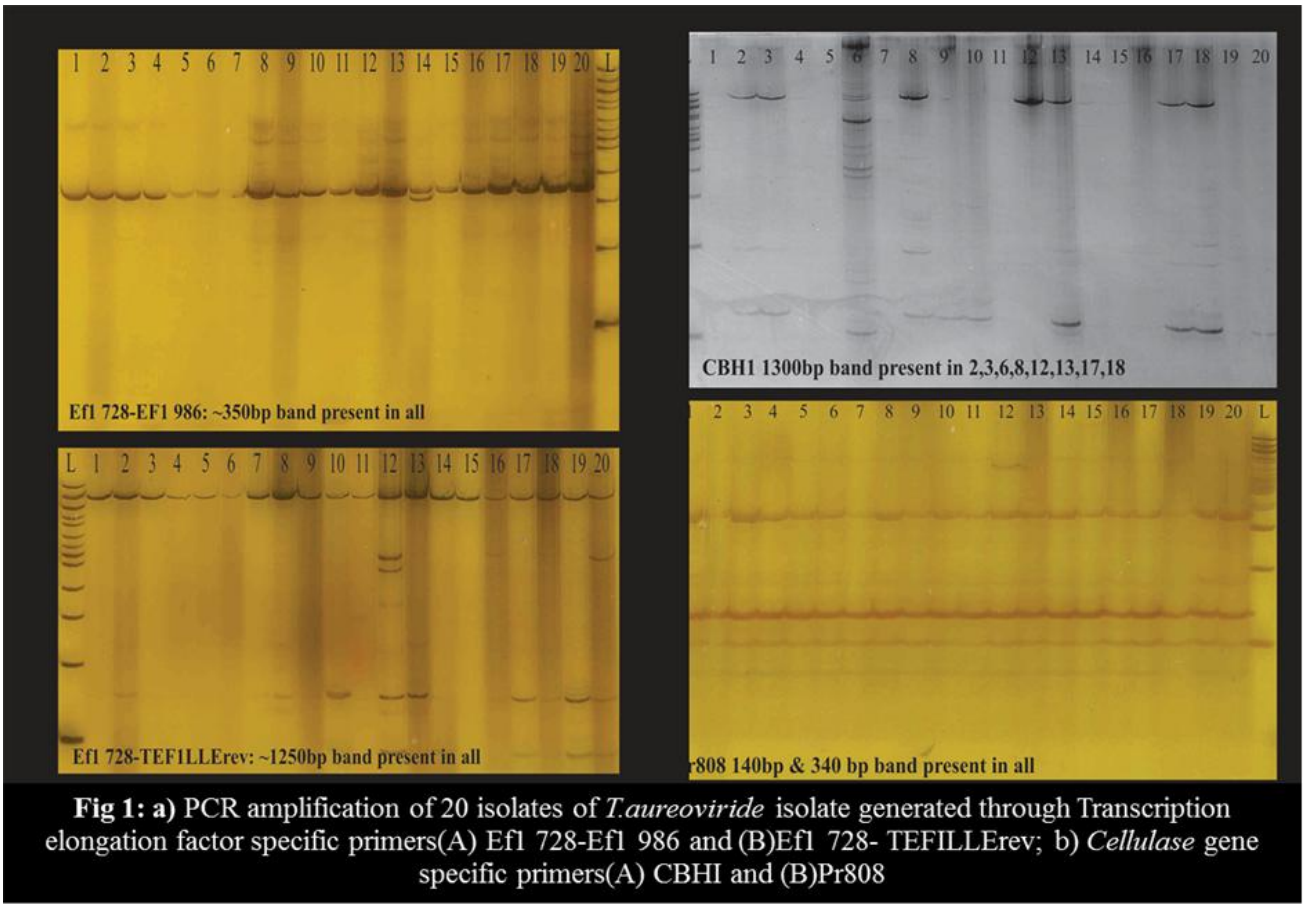

Fig 1: a) PCR amplification of 20 isolates of *T.aureoviride* isolate generated through Transcription elongation factor specific primers(A) Ef1 728-Ef1 986 and (B)Ef1 728- TEFILLErev; b) *Cellulase* gene specific primers(A) CBHI and (B)Pr808

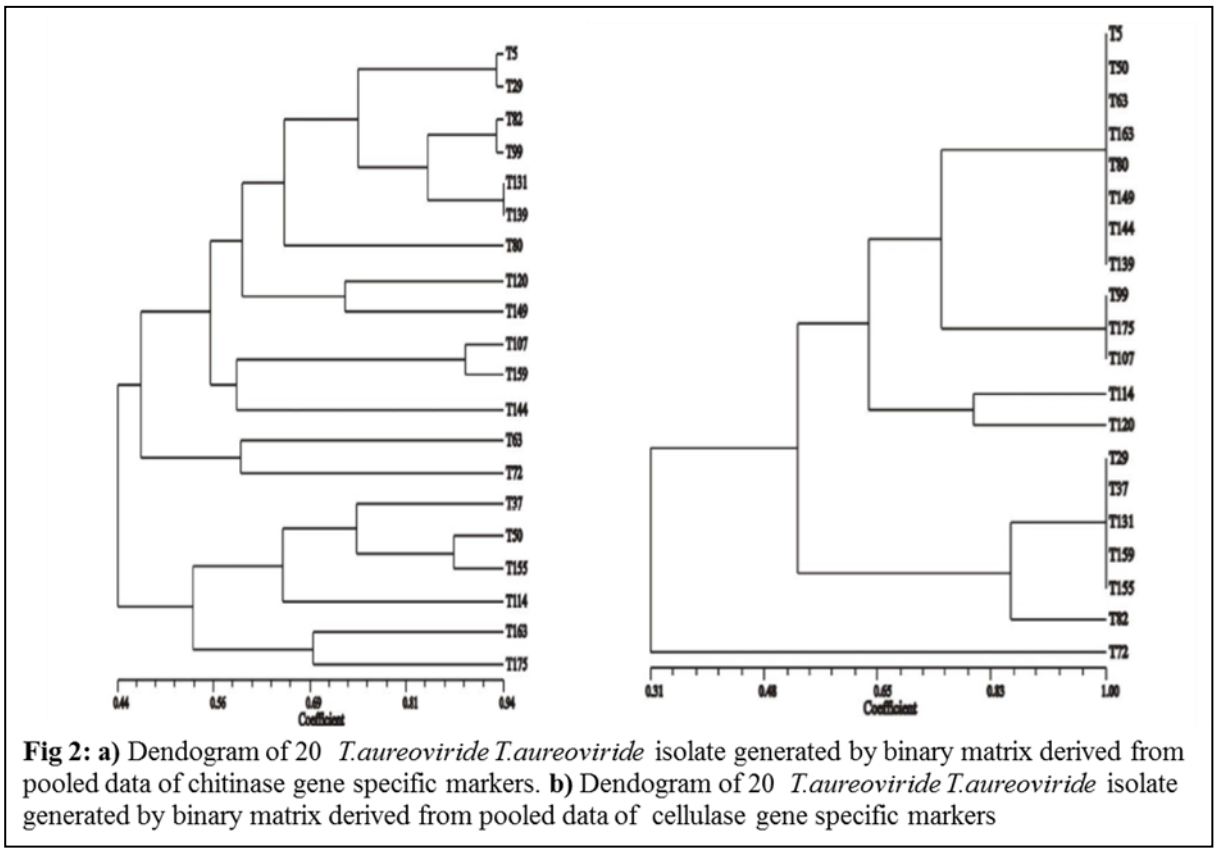

Fig 2: a) Dendogram of 20 *T.aureoviride T.aureoviride* isolate generated by binary matrix derived from pooled data of chitinase gene specific markers. **b)** Dendogram of 20 *T.aureoviride T.aureoviride* isolate generated by binary matrix derived from pooled data of cellulase gene specific markers

Conclusion

PCR amplification with ITS primers showed homogeneity of all twenty *T. aureoviride* isolates indicating that they belong to the same species. Almost all the isolates showed the presence of endochitinase and cellulose gene fragments.

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References

1. Bissett J. A revision of the genus *Trichoderma*. I. Sect. *Longibrachiatum* sect. Canadian Journal Botany. 1984; 62:924-931.
2. Bissett J. A revision of the genus *Trichoderma*. IV. Additional notes on section *Longibrachiatum*. Canadian Journal Botany. 1991a; 69:2418-2420.
3. Bissett J. A revision of the genus *Trichoderma*. III. Sect. Pachybasium. Canadian Journal of Botany. 1991b; 69(11):2373-2417.
4. Bissett J. *Trichoderma atroviride*. Canadian Journal of Botany. 1992; 70:639-641.
5. Bulat SA, Lübeck M, Mironenko N, Jensen DF, Lübeck PS. *Mycol. Res*. 1998; 102:933-943.
6. Chaverri P, Castlebury LA, Overton BE, Samuels GJ. *Hypocrea*/*Trichoderma*: species with conidiophore elongations and green conidia. Mycologia. 2003b; 95:1100-1140.
7. Chaverri P, Samuels GJ, Stewart EL. *Hypocrea virens* sp. nov., the teleomorph of *Trichoderma virens*. Mycologia. 2001; 93:1113-1124.
8. Danielson RM, Davey CB. Carbon and nitrogen nutrition of *Trichoderma*. *Soil Biology and Biochemistry*. 1973; 5:505-515.
9. Druzhinina IS, Kopchinskiy AG, Komon´M, Bissett J, Szakacs G, Kubicek CP. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. *Fungal Genet Biol*. 2005; 42:813-828.
10. Druzhinina IS, Kopchinskiy AG, Kubicek CP. The first one hundred of *Trichoderma* species is characterised by molecular data. *Mycoscience*. 2006; 47:55-64.
11. Gams W, Bissett J. Morphology and identification of *Trichoderma*. Pages 3-34 in: *Trichoderma* and *Gliocladium* Basic Biology, Taxonomy and Genetics. C. P. Kubicek and G. E. Harman, eds. Taylor and Francis, London, 1998, 1.
12. Garcia I, Lora JM, de la Cruz J, Benitez T, Llobell A, Pintor-Toro JA. Cloning and characterization of a chitinase (*chit42*) cDNA from the mycoparasitic fungus *Trichoderma harzianum*. Current Genetics. 1994; 27:83-89.
13. Hayes CKS, Klemsdal M, Lorito A, Di Pietro CK, Peterbauer JP, Nakas A *et al*. Isolation and sequence of an endochitinase-encoding gene from a cDNA library of *Trichoderma harzianum*. *Gene* 1994; 138:143-148.
14. Hermosa MR, Grondona I, Iturriaga EA, Diaz-Minguez JM, Castro C, Monte E *et al*. Molecular characterization of biocontrol isolates of Trichoderma. Appl Environ Microbiol. 2000; 66:1890-1898.
15. Hjeljord L, Tronsmo A. *Trichoderma and Gliocladium* in biological control: an overview. In Trichoderma and Gliocladium. 1998; 2:131-152.
16. James TY, Kauff F, Schoch C. (and 69 additional co-authors). Reconstructing the early evolution of the fungi using a six gene phylogeny. *Nature*, in press, 2006.
17. Karlsson J, Saloheimo M, Siika-aho M, Tenkanen M, Penttil M, Tjerned F. Homologous expression and characterization of Cel61A (EG IV) of *Trichoderma reesei*, 2001; 268(24):6329-6650
18. Kindermann J, El-Ayouti Y, Samuels GJ, Kubicek CP. Phylogeny of the genus *Trichoderma* based on sequence analysis of the internal transcribed spacer region 1 of the rDNA cluster. Fungal Genet. Biol. 1998; 24:298-309.
19. Klein D, Eveleigh DE. Ecology of *Trichoderma*: Kubicek

C. P., Harman G.E. (Eds.), *Trichoderma* and *Giocladium.* Basic Biology, Taxonomy and Genetics. Taylor and Francis Ltd., London, 1998; 1:57-74.
20. Kubicek CP, Penttilä ME. Regulation of production of plant polysaccharide degrading enzymes by *Trichoderma*: In *Trichoderma* and Gliocladium. vol 2, Enzymes, biological control and commercial applications. Edited by Kubicek CP, Harman GE, Taylor and Francis, London, UK. 1998, 49-71.
21. Kuhls K, Lieckfeldt E, Samuels GJ, Kovacs W, Meyer W, Petrini O *et al*. Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. *Proc. Natl. Acad. Sci*. USA. 1996; 93:7755-7760.
22. Kullnig-Gradinger CM, Szakacs G, Kubicek CP. Phylogeny and evolution of the genus *Trichoderma:* a multigene approach. *Mycol Res.* 2002; 106(7):757-767.
23. Liu YL, Whelen S, Hall BD. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. *Molecular Biology Evol*. 1999; 16:1799-1808.
24. Lord NS, Kaplan CW, Shank P, Kitts CL, Elrod SL. Assessment of fungal diversity using terminal restriction fragment (TRF) pattern analysis: comparison of 18S and ITS ribosomal regions. FEMS Microbiol Ecol. 2002; 42(3):327-37
25. Lutzoni F, Kauff F, Cox CJ. Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits. American Journal of Botany. 2004; 91:1446-1480.
26. Monte E. Understanding *Trichoderma*: between biotechnology and microbial ecology. *Int Microbiol.* 2001; 4:1-4.
27. Nagy V, Seidl V, Szakacs G, Komon-Zelazowska M, Kubicek CP, Druzhinina IS. Application of DNA bar codes for screening of industrially important fungi: the haplotype of *Trichoderma harzianum* sensu stricto indicates superior chitinase formation. Appl. Environ. Microbiol. 2007; 73:7048-58.
28. Rifai MA. A revision of the genus *Trichoderma* . *Mycol Pap*. 1969; 116:1-116.
29. Rohlf FJ. NTSYSpc Numerical Taxonomy and Multivariate Analysis System Version 2.0 User Guide. *Applied Biostatistics Inc.*, Setauket, New York. 1998, 37.
30. Roiger DJ, Jeffers SN, Caidwell RW. Occurrence of *Trichoderma* species in apple orchard and woodland soils. Soil Bio L Biochem. 1991; 23:353-359.
31. Rossman AY, Samuels GJ, Rogerson CT, Lowen R. Genera of *Bionectriaceae*, *Hypocreaceae* and *Nectriaceae* (*Hypocreales, Ascomycetes*), Studies in Mycology, 42, Centraalbureau voor Schimmelcultures (CBS), Utrecht, 1999, 248.
32. Samuels GJ, Petrini O, Kuhls K, Lieckfeldt E, Kubicek CP. The *Hypocrea schweinitzii* complex and *Trichoderma* sect. *Longibrachiatum*. *Stud Mycol*. 1998; 41:1-54.
33. Seidl V, Huemer B, Seiboth B, Kubicek CP. A complete survey of *Trichoderma* chitinases reveals three distinct subgroups of family 18 chitinases. *FEBS J.* 2005; 272:5923-39.
34. Sivasithamparam K, Ghisalberti EL. Secondary Metabolism in *Trichoderma* and *Gliocladium* . In: Kubicek CP, Harman GE, editors. *Trichoderma* and *Gliocladium*. Vol. 1. Basic Biology, Taxonomy and Genetics. *London: Taylor and Francis Ltd*. 1998, 139-191.
35. Sneath PHA, Sokal RR. Numerical taxonomy. W. H. Freeman and Company, San Francisco, 1973.
36. Wardle DA, Parkinson D, Waller JE. Interspecific competitive interactions between pairs of fungal species in natural substrates. Oecologia. 1993; 94:165-172.