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RESEARCH ARTICLE

Morphological and molecular characterization of *Trichoderma asperellum* strain *Ta*13

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ABSTRACT: Morphological characters are not sufficient to properly identify different species of *Trichoderma*. Recently, multi-gene phylogeny in combination with morphological characters is used to identify *Trichoderma* at species level. The current study was focused to characterize *Trichoderma asperellum* strain *Ta*13 based on morphology and molecular analysis using genes such as ITS, tef1α, rpb2, act and cal. Light and scanning electron microscopy (SEM) results showed that *Ta*13 has regularly branched, typically paired conidiophores with straight phialides and globose to sub-globose shaped conidia having size of 2.91µm x 2.37µm with inconspicuous ornamentation. Sequence similarity analysis with reference *T. asperellum* isolates available in ISTH database showed 99.30, 90.60, 99.20, 98.70, 100 and 99.80 per cent nucleotide similarity for ITS1 and ITS2, tef1α intron4 (large), tef1α intron5 (short), rpb2, cal and act respectively. Confrontation assay clearly showed that *Ta*13 inhibited several fungal plant pathogens *viz. Rhizoctonia solani* (88.69 per cent), *Fusarium oxysporum* f. sp. *lycopersici* (85.27 per cent), *F. moniliforme* (84.55 per cent), *S. sclerotiorum* (80.17 per cent), *S. rolfsii* (78.26 per cent), and *A. brassicicola* (77.5 per cent).

Key words: Biocontrol agents, confrontation assay, multi-gene analysis, *Trichoderma asperellum*

Trichoderma species (Perfect stage: *Hypocrea*) is a filamentous ascomycetous fungus widely used as bio-control agent (BCA) against plant pathogens. It comprises different economically important species *viz., T. harzianum, T. asperellum, T. viride, T. atroviride* and *T. virens* and *T. reesei* (Sharma *et al*., 2014). The above species are well known for their biocontrol activity, rhizosphere competence and production of hydrolytic enzymes such as cellulases, chitinases, glucanases and proteases as well as many secondary metabolites (Sharma *et al*., 2011; Nicolus *et al*., 2014).

Trichoderma asperellum Samuels Lieckf. & Nirenberg is morphologically indistinguishable from its cryptic species *T. asperelloides* (Samuels *et al.*, 2010). Molecular tools such as multi-gene phylogeny were commonly used to define *Trichoderma* species. Very recently, *Trichoderma viride* is re-classified (Sriram *et al.,* 2013; Mukherjee, 2015). The present work was mainly focused on characterization of *Trichoderma asperellum* strain *Ta*13 based on morphological and molecular analysis.

MATERIALS AND METHODS

Fungal cultures

Fungal biocontrol agent*, T. asperellum* (*Ta*13) (ITCC number-7766) and fungal plant pathogens were obtained from biological control laboratory, Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi. Cultures were routinely grown in potato dextrose agar (PDA) (HIMEDIA Laboratories Pvt. Ltd., India) plates and incubated at 28±2°C for 5-7 days.

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Confrontation assay

Antagonistic potential of *Ta*13 was studied by dual culture assay against various fungal plant pathogens by keeping the pathogen inoculated PDA plates as control (Dennis and Webster, 1971). Six replications of the each culture plates were incubated at 28±2°C for measuring colony growth both in control and dual culture plates. The plates were evaluated based on the grading method (Bell *et al*., 1982). The radial growth of the pathogen was measured after full growth and the percent inhibition was calculated as follows: PI = (C - T) × 100 / C, where PI is the percent inhibition of mycelial growth; C is the radial growth of pathogen in control plates (cm) and T is the radial growth of pathogen in dual culture (cm).

The antagonistic potential was further evaluated by cell-free-culture filtrate assays by growing in liquid cultures. Discs of actively growing fungal mycelium (5mm) was inoculated and then incubated at 28±2°C for 10 days in shaker cultures and filtered through Millipore filters (Pore size 450 nm). Poisoned food technique was followed to analyze the inhibitory properties of *Ta*13 against the pathogen and expressed as per cent of inhibition over the control.

Microscopy

Light microscopy

Single spore culture of *Ta*13 was prepared and recorded for its cultural characters *viz.* growing pattern, colour and texture as well as morphological features *viz*. conidiophores branching pattern, phialides structures, conidial shape and size (Olympus CH20*i,* Olympus opto system India Pvt. Ltd., India).

Table 1. List of multi-genes and their primer sequences used to amplify the *Trichoderma asperellum* (*Ta*13)

Gene code	GenBank Accession Number	Forward (F)/ Reverse (R) primer	Nucleotide sequence (5'-3')	Primer size (bp)	Product size (bp)
cal	KT302160	F	5'-CAGTCGATTTGGTCACTAACCC-3'	22	419
		R	5'-GGTAAGGAACTCTTCACCGCTC-3'	22	
ITS	KT426887	F	5'-TCCGTAGGTGAACCTGCGG-3'	20	602
		R	5'-TCCTCCGCTTATTGATATGC-3'	20	
act	KT302159	F	5'-TACAATGAGCTGCGTGTTGC-3'	20	750
		R	5'-CTGCATACGGTCGGAGAGAC-3'	20	
tef1α	KT302162	F	5'-ATGGGTAAGGAGGACAAGAC-3'	22	1015
		R	5'-GCCATCCTTGGAGATACCAGC-3'	21	
rpb2	KT302161	F	5'-TAAGAAGCGTCTGGATCT-3'	18	1065
		R	5'-GATCACAACCAGGTATGTCAACC-3'	23	

Scanning electron microscopy (SEM)

Actively growing *Ta*13 fungal culture was fixed overnight at 28°C in 0.05M phosphate buffer (pH 7.3) containing 4% glutaraldehyde. Fungal mat was washed three times in phosphate buffer (each for 15 minutes) on the next day and dehydration of the samples were done using a series of graded ethanol (30, 50, 70, 80, 90 and 100%) for 15 minutes in each solution. The fixed and dehydrated samples were further dried with CO_2 for 5 minutes and were fixed on aluminum stubs immediately, sputter-coated with Carbon in a Polaron E-500 sputter coater (Polaron Equipment, Watford, England) and immediately observed under a scanning electron microscope (Zeiss Evoi Maio, Germany).

DNA isolation

A small disc (5 mm) of well grown *Ta*13 was inoculated into a conical flask containing 50 mL potato dextrose broth at 180 rpm for 5-6 days at 28±2°C. Fungal mycelium was filtered by using sterile No. 1 Whatman filter paper (HIMEDIA Labs, India). The mycelial mat was ground with liquid nitrogen and stored at -80°C. Total fungal DNA was extracted from 100 mg of mycelium by CTAB method with minor modifications (Culling, 1992). The purified DNA was dissolved in 50µl TE buffer (Tris 10mM + EDTA 1mM pH 8.0). Integrity of genomic DNA (gDNA) was checked in 1.5 per cent agarose gel (Best Lab International Inc. China). The quality and quantity of DNA was assessed by using NanoDrop spectrophotometer (Thermo Fisher Scientific NanoDrop 2000c, USA). The concentration of DNA was adjusted to 50 ng/µl and stored at 4°C for further use (Sambrook and Russell, 2001).

Multi-gene analysis

Multi-gene analysis was carried out for the following genes *viz.* internal transcribed spacer (ITS), translation elongation factor1α (tef1α), calmodulin (cal), actin (act) and RNA polymerase subunit 2 (rpb2) to characterize *T. asperellum*. The list of the primers is given in the Table 1. Ribosomal DNA (rDNA) region (18S rDNA, ITS1, 5.8S rDNA, ITS2 and 28S rDNA) was amplified using the following protocol of White *et al*. (1990). PCR was performed with initial denaturation for 5 min at 95°C, followed by 30 cycles of 30s denaturation at 95°C, 1 min primer annealing at 60°C, 1 min extension at 72°C, and a final extension period of 7 min at 72°C. tef1α, cal, act and rpb2 genes were amplified using the same programme except for primer annealing at 60°C for 30s (MultigeneTM OptiMax Thermal cycler, Labnet International, Inc., USA).

All PCR reactions were performed for 50 µL comprising of PCR buffer (1X); $MgCl_2$ (1.5 mM); dNTP's (200 µM); primers (10 picomoles ends); Taq polymerase (1 U) and genomic DNA (50 ng). The amplified fragments were separated electrophoretically (Best Lab International Inc., China) in 1.2 per cent (w/v) agarose gel containing ethidium bromide (0.5µg/µl) for 60 min at constant 80 V (Sambrook and Russell 2001). Amplicon was visualized in gel documentation system (BioRad). Gel purification of PCR products were done using NucleoSpin® Gel and PCR Clean-up (A Takara Bio Company, CA, USA) and subsequently cloned in TA cloning vector (RBC, UK). Ligations, transformations in *Escherichia coli* DH5α strain and plasmid isolations were carried out by following the standard procedures (Sambrook and Russell, 2001). Positive colonies were confirmed by colony PCR (using gene specific primers) as well as restriction digestion. Positive confirmed clones were sequenced using universal primers (M13 F and R). Sequences obtained were end trimmed for vector sequences and identity confirmed using BLAST tool in NCBI (http://www.ncbi.nlm.nih.gov/). Further confirmation was done using TrichoBlast and TrichoKey in International sub-commission for *Trichoderma* and *Hypocrea* (ISTH) database (Druzhinina *et al*., 2005).

Sequence similarity analysis

Partial nucleotide sequences of ITS, tef1α, rpb2, cal and act of reference *T. asperellum* isolates [CBS 433.97 and *Tr*3 (synonym of CBS 433.97)] were downloaded from ISTH database (www.isth.info). All the sequences

were aligned and end trimmed for uniform length. Bioedit sequence alignment tool was used for nucleotide sequence alignment and analysis.

Statistical analysis

All experiments were repeated atleast once. Data were analyzed by one-way analysis of variance (ANOVA) using OPSTAT software (www.hau.ernet.in/about/opstat.php). Differences between treatment mean values were determined following least significant difference (LSD) test at P <0.05.

RESULTS

Microscopic assay

Microscopic analysis of *Ta*13 revealed regularly branched typically paired conidiophores with straight phialides. Conidia were globose to sub-globose shaped and measuring size ranging from 2.56 µm to 3.09 µm in length and 2.15 µm to 2.62 µm width with inconspicuous ornamentation (Fig. 1). SEM analysis revealed further surface features like phialides arising in whorls and straight in shape on which conidia was produced having inconspicuous ornamentation (Fig. 2). Morphological features of *Ta*13 were enough to define precisely as *T. asperellum.* However, molecular based multi-gene analysis was used for reconfirmation of the identity as *T. asperellum*.

Confrontation assay

Confrontation assay results indicated that *T. asperellum* (*Ta*13) was able to overgrow all the fungal plant pathogens tested by exhibiting Class 1 level of antagonism (Fig. 3). Percentage of Inhibition (PI) against the pathogens were observed as 88.99 per cent *in R. solani*, 85.27 per cent in *Fusarium oxysporum* f. sp. *lycopersici*, 84.55 per cent in *F. moniliforme*, 80.17 per cent in *S. sclerotiorum*, 78.26 per cent in *S. rolfsii* and 77.5 per cent in *A. brassicicola*. Confrontation assay results clearly stated that *Ta*13 displayed antagonistic activity on fungal pathogens significantly. In cell-free

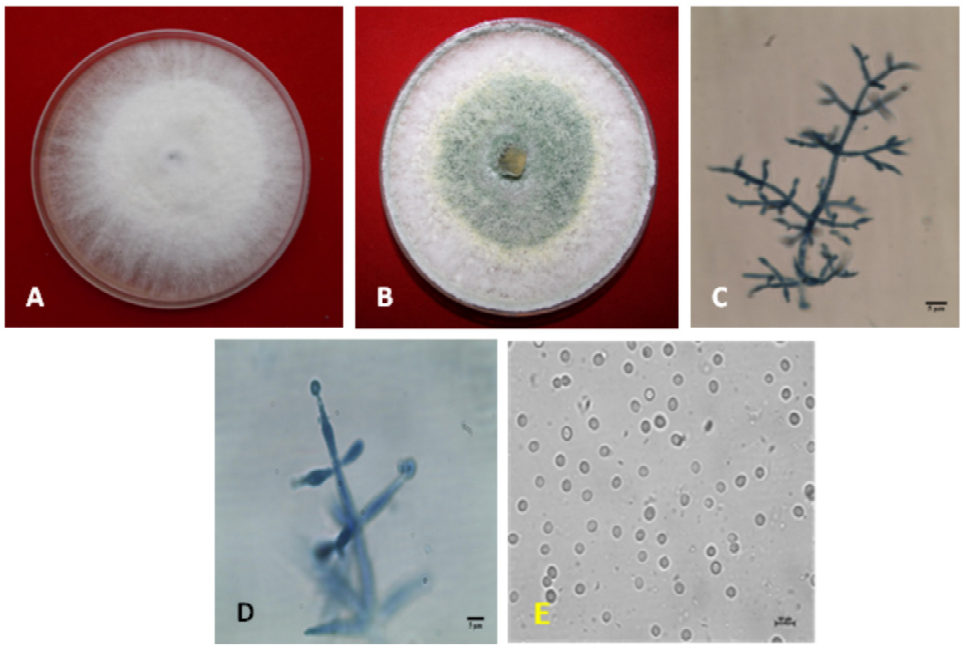

Fig. 1. Culture plates showing (A) 3 days old *Trichoderma asperellum* (*Ta*13); (B) 7 days old *Trichoderma asperellum* (*Ta*13); (C) Microscopic images showing Conidiophores structures and branching pattern (Magnification at 40X); (D) Phialides arrangement with conidia (Magnification at 100X) (E) Conidia (Magnification at 100X)

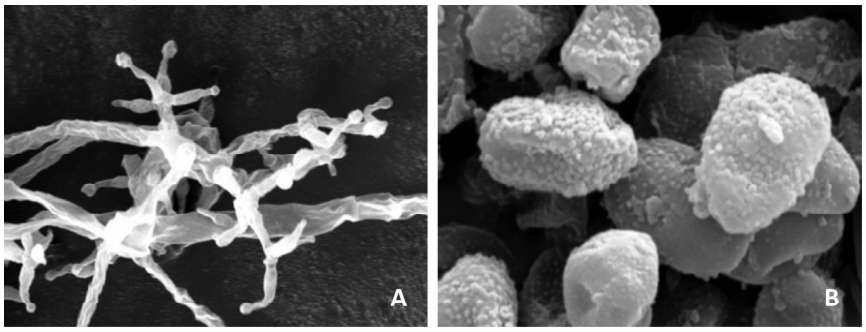

Fig. 2. Scanning electron microscopic (SEM) images of *Trichoderma asperellum* (*Ta*13) showing (A) Conidiophores structures and branching pattern (Magnification at 3.64 KX) (B) Conidia morphology (Magnification at 25.00 KX)

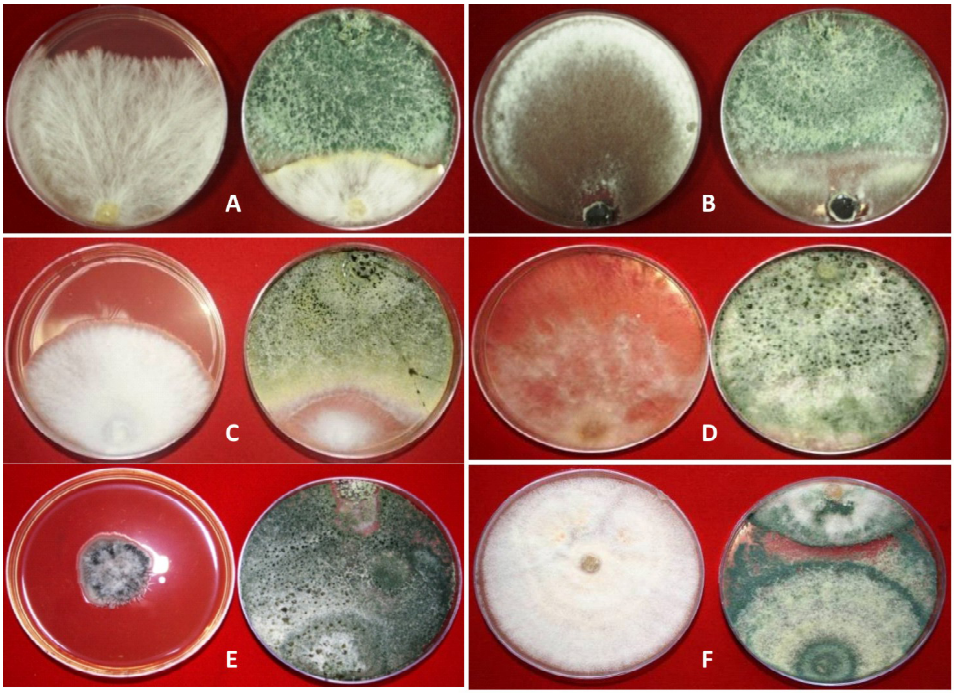

Fig. 3. Antagonistic activity of *Trichoderma asperellum* (*Ta*13) against fungal plant pathogens on PDA plates (A) *Sclerotium rolfsii*; (B) *Sclerotinia sclerotiorum*; (C) *Fusarium oxysporum* f. sp. *Lycopersicii*; (D) *Rhizoctonia solani*; (E) *Alternaria brassicicola*, (F) *Fusarium moniliforme*

culture filtrate assays, *Ta*13 exhibited maximum inhibition (>75 per cent) on plant pathogens as compared to control that clearly confirmed its biocontrol potential.

Multi-gene analysis

Total genomic DNA was isolated, and PCR amplified with the following gene specific primers *viz.* cal F/R, ITS 1/4, act F/R, tef1α F/R and rpb2 F/R. The amplified fragments were gel eluted, cloned and sequenced. Contig length of 419 bp (cal), 602 bp (ITS), 750 bp (act), 1015 bp (tef1α) and 1065 bp (rpb2) were obtained and used in the analysis. The gene sequences were assigned accession numbers and published in NCBI data base (Fig. 4). Trichokey and TrichoBlast analysis confirmed the gene sequences as belongs to *T. asperellum* (Table 1).

Sequence similarity analysis

Sequence similarity analysis was done for ITS1 and ITS2 region of internal transcribed spacer gene with reference *T. asperellum* (CBS_433.97). *Ta*13 showed 99.30 per cent nucleotide similarity with CBS-433.97. But it matched 96.00 per cent with closely related *T. viride* (GJS-91-62) gene sequence. Hence, comparison of some other genes *viz*. translation elongation factor1α [tef1α intron4 (large), and tef1α intron5 (short)] was carried out with reference *T. asperellum* (CBS 433.97) gene sequences. Nucleotide sequence similarity for *tef*1α intron4 (large) revealed that *Ta*13 was matching 90.6 per cent with reference *T. asperellum* (CBS_433.97) and 71.90 per cent with *T. viride* (CBS_101526, and CBS_240.63). Also nucleotide similarity for tef1α intron4 (large) showed that *Ta*13 was 99.2 per cent matching with reference *T. asperellum* (CBS_433.97) sequence and 79.30 per cent with *T. viride* (ATCC_28020). For rpb2 gene, the matching per cent is 98.70 with reference *T. asperellum* isolate *Tr*3 (Synonym of CBS 433.97) sequence, and 92.50 with standard *T. viride* (GJS_89-127). Nucleotide sequence analysis for cal and act gene partial CDS resulted in 100 and 99.80 per cent similarity with the reference *T. asperellum* isolate *Tr*3 (Synonym of CBS 433.97) (Table 2).

DISCUSSION

Trichoderma spp. is having enormous applications in industries as enzyme and secondary metabolites producers and agricultural fields as biofungicides. It is

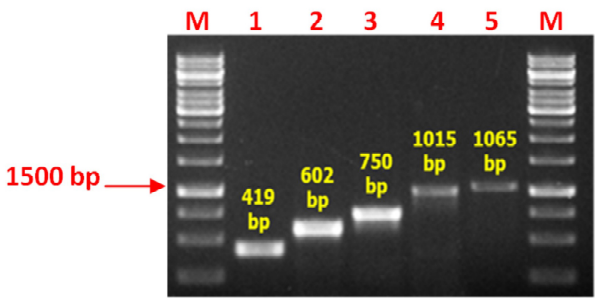

Fig. 4. Amplification profile of multi-genes from *Trichoderma asperellum* (*Ta*13). M - 1 Kb DNA ladder; Lane 1 - Calmodulin; Lane 2 - Internal transcribed spacer (ITS); Lane 3 - Actin; Lane 4 - Translation elongation factor1α; and Lane 5 - RNA polymerase subunit 2

Table 2. Sequence comparison of *Trichoderma asperellum Ta*13 with the reference strain CBS 433.97 for various genes

Gene	Region used for the study	Nucleotide length used (bp)	Sequence similarity index (%)
Internal Transcribed Spacer (ITS)	ITS1 and ITS2	445	99.30
Translation elongation factor1α (tef1α)	Intron4 (Large)	300	90.60
	Intron5 (Short)	130	99.20
RNA polymerase subunit2 (rpb2)	rpb2 exon	827	98.70
Calmodulin (cal)	Partial CDS	452	100.00
Actin (act)	Partial CDS	580	99.80

well known that *Trichoderma* spp. uses diverse mechanisms to overcome and/or reduces the effects of a pathogen of which, cell wall degradation and mycoparasitism are important events. To claim *Ta*13 as a successful biocontrol agent it has to outplay the fungal targets in the culture plates itself when used in confrontation assay. Our results revealed that *Ta*13 inhibited all the tested fungal plant pathogens efficiently and showed good antagonism (Class 1) under *in vitro*. Results are in agreement with many other works who indicated that *T. asperellum* is a successful biocontrol agent against *F. oxysporum*. f. sp. *lycopersici* (El Komy *et al.,* 2015), *R. solani*, *S. rolfsii* (Hamed *et al.,* 2015), and *Gibberella fugikorai* (Watanabe *et al.,* 2007).

Correct taxonomic identification at the species/sub-species level is of outmost importance for safe and effective use of *Trichoderma* spp. But over lapping morphological features among the closely related species have created difficulty in precise identification (Prabhakaran *et al*., 2014). Recently, few widely used commercial *Trichoderma* strains have been re-classified (Mukherjee *et al*., 2013; Sriram *et al*., 2013; Chaverri *et al*., 2015). Many of the strains at the culture collections and gene sequences in NCBI need to be updated. In the present classification scheme, in addition to morphological characterization, molecular confirmation using multi-genes are also routinely performed to identify and categorize the species (Atanasova *et al*., 2013).

T. asperellum strain *Ta*13 was identified based on morphology and molecular sequencing of ribosomal DNA (rDNA) region. An attempt was also made to characterize *Ta*13 by multi-gene analysis using sequence of cal, act, tef1α and rpb2. Light microscopy and SEM analysis has confirmed that *Ta*13 was found to have regularly branched, typically paired conidiophores with straight phialides and globose to sub-globose shaped conidia with inconspicuous ornamentation indication of *T*. *asperellum*. Multi-gene phylogeny analysis has shown that *Ta*13 formed a separate sub-cluster with reference *T. asperellum* (CBS_433.97) gene sequences for ITS, tef1α and rpb2. *T. viride* formed another sub-cluster and together with *Ta*13 sub-cluster formed the major cluster. Rest of the *Trichoderma* species used were formed a second major cluster. The results were matching with the study of Sriram *et al*. (2013) who used morphological and oligonucleotide barcode (ITS and tef1α) for correct identification of *T. asperellum* and reclassified most of the *T. viride* isolates as *T. asperellum.* Samuels *et al*. (2010) also used multi-gene analysis (act, tef1, ITS and rpb2) and morphological studies of different *T. asperellum* strains collected from various parts of the world and concluded there are two morphologically indistinguishable species *viz., T. asperellum* and *T. asperelloides* as well as morphologically distinct but molecularly closely related species *viz., T. yunnanense*.

In this study, ITS, tef1α and rpb2 gene sequences were downloaded from NCBI and used for multiple sequence alignment as well as sequence similarity index with the reference *T. asperellum* [CBS_433.97 and *Tr*3 (Synonym of CBS 433.97)]. Comparison of any unknown *Trichoderma* gene sequence with that of standard reference sequence from ISTH database is a pre requisite to confirm the species identity.

The species confirmation was fine tuned again with sequence similarity index between *Ta*13 and other standard reference *T. asperellum* and closely-related *T. viride* from ISTH database. Moreover, analysis of internal transcribed spacer region alone will not full fill the requirement of species identification; since *Ta*13 shared 99.00 per cent sequence similarity with CBS_433.97 and 96.30 per cent with closely related species *T. viride* (GJS_91-62). Similarly, for rpb2 genes the similarity was observed to be 98.70 per cent with *T. asperellum* isolate *Tr*3 (Synonym of CBS 433.97) and 92.50 per cent with *T. viride* GJS_89-127. But analysis with tef1α gene i.e. intron4 (large), and intron5 (short) showed 90.60 and 99.20 per cent sequence similarity with reference *T. asperellum* CBS_433.97 respectively. Whereas, *Ta*13 showed 71.90 and 79.30 per cent similarity with standard *T. viride* isolates CBS_240.63 and CBS_101526 [intron4 (large)] and ATCC_28020 [intron5 (short)]. Nucleotide sequence similarity analysis for *Ta*13 act and cal sequences shown 100 and 99.80 per cent similarity with the reference *T. asperellum* isolate *Tr*3 (Synonym of CBS 433.97). *Ta*13 also showed 94.80 and 87.10 per cent nucleotide similarity with *T. viride* strain ATCC_28038 and CBS_101526 respectively. This clearly showed that tef1α and cal can be used for *Trichoderma* species identification. Since both the genes showed highest similarity percentage with reference *T. asperellum* isolate CBS_433.97 and low similarity with that of *T. viride* reference isolate CBS_240.63 [intron4 (large)],

CBS_101526 [intron4 (large) and calmodulin], and ATCC_28020 [intron5 (short)]. Moreover, original nucleotide sequences should be analyzed in ISTH database (TrichoMark, TrichoKey and TrichoBlast) before proceeding for species confirmation. Finally, different tools like critical observations of the fine structures at morphological level, use of multi-gene analysis and sequence comparison with reference strains helps in ease and correct identification of the species in *Trichoderma*.

To conclude that *Ta*13 has been characterized using polyphasic morphological analysis including light microscopy and scanning electron microscopy. Molecular analysis with five different genes (ITS, cal, act, tef1α and rpb2) has confirmed *Ta*13 as *T. asperellum.*

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