RAPD profile and rDNA sequence analysis of *Talaromyces flavus* and *Trichoderma* species

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*Talaromyces flavus* isolated from paddy rhizosphere exhibited phosphate solubilizing activity in vitro and positively influenced the growth of *Oryza sativa*, *Cicer arietinum* and *Vigna radiata* under green house conditions. Besides, isolates of *Trichoderma harzianum* and *T. asperellum* obtained from agricultural field of Darjeeling hills showed antifungal activities against fungal pathogens. Phylogenetic analysis of all these isolates were carried out by sequencing the internal transcribed spacer regions (ITS1 & ITS4) of the ribosomal DNA of *T. flavus* and *T. harzianum* using universal primers. The sequences were aligned against ex-type strain sequences from NCBI GenBank. Genetic relatedness among the isolates was studied using random decamer primers. UPGMA cluster analysis showed that the similarity coefficient ranged from 0.37 to 1 for *T. flavus* and 0.68 to 1 for *T. harzianum*. The open reading frame (ORF) analysis of 18S rDNA sequence of *T. harzianum* revealed that the sequence contains in total of four ORFs, encoding 159 amino acids with an estimated molecular mass of 17.84 kDa. The sequences of ITS1 region have been used as the reference sequence, which may be used for future study involving the identification and taxonomy of both *T. flavus* and *Trichoderma* isolates.

**Keywords:** *Talaromyces flavus*, *Trichoderma harzianum*, RAPD, rDNA

**Introduction**

*Talaromyces flavus* [anamorph, *Penicillium dangeardii*; sometimes reported as *Penicillium vermiculatum* (Family: Trichocomaceae; Ascomycota)] is the type species of *Talaromyces* and was first reported from soil of paddy fields in West Bengal, India. This fungus is perhaps the most common species of its genus with a worldwide distribution and is commonly isolated from soil and organic substrates in warmer regions. Some strains of *T. flavus* can produce novel bioactive compounds, such as, actofunicone, deoxy-funicone and vermistatin, which reinforce the anti-*Candida albicans* activity of miconazole. *T. flavus* has shown ability to convert insoluble phosphorus (P) to an accessible form like orthophosphate, which is an important trait for increasing plant yields. Similarly, *Trichoderma* (Family: Dematiaceae; Deuteromycotina) has gained enough importance since last few decades due to its biological control activities against several plant pathogens. Besides, they are of great economic importance as sources of enzymes, antibiotics, plant growth promoters, xenobiotic degraders and most importantly as commercial biofungicides.

Taxonomy of these groups of microorganisms is currently based on morphological characters, such as, conidial form, size, colour, ornamentation and branching pattern, and at times creates confusion. It is at this point that the correct identification of beneficial organisms is needed since other two species of *Talaromyces* (*T. longibrachiatum* and *T. citrinoviride*) are known to be opportunistic pathogens. Similarly, identification of *T. flavus*, at the species level, has also proved to be difficult due to the degree of morphological similarities with various species of *Penicillium*.

Molecular methods have recently been introduced into *Trichoderma* taxonomy with the revision of *Longibrachiatum* and *Trichoderma*, and related teleomorphs. These techniques have been proven to be valuable tools in fungal taxonomy and their application has led to the reconsideration of several genera. The close morphological resemblance that exists between the different species of *Trichoderma* has been resolved clearly without any controversy using molecular and biochemical analysis. Kindermann *et al.* attempted the first phylogenetic analysis of the genus *Trichoderma*, using sequence analysis of the ITS1 region of the rDNA. Nevertheless, the use of phylogenies based on single gene sequences is now normally discredited, especially as regards the use of ITS1 and/or ITS2, as...
some fungi and plants have been shown to contain paralogous copies\textsuperscript{12}. Taylor et al\textsuperscript{13} demonstrated that molecular techniques indicating interrelations among species combined with phenotypic characters can lead to a reliable taxonomy that is reflective of phylogenetic relationships. Internal transcribed spacer sequences of ribosomal DNA (rDNA) analysis and universally primed polymerase chain reaction have been used to categorize the isolates of \textit{Trichoderma}. The Random Amplified Polymorphic DNA (RAPD) method developed by Williams et al\textsuperscript{14} involves simultaneous amplification of several anonymous loci in the genome using primers of arbitrary sequence and has been used for genetic, taxonomic and ecological studies of several fungi.

Initial screening for phosphate solubilizers revealed that \textit{T. flavus} has high potential for phosphate solubilization. Previous reports have also indicated that it can act as a biocontrol agent\textsuperscript{15}. Taking these into consideration, \textit{T. flavus} was selected for further study and compared with \textit{Trichoderma}, a known biocontrol agent. The present investigation was carried out for rapid identification of \textit{T. flavus} and \textit{T. harzianum} based on sequence analysis of ITS-region of rDNA gene and development of RAPD markers for analysis of genetic variability among the isolates.

**Material and Methods**

**Isolation of Fungi**

Soil samples were collected from rhizosphere soil of plantation crops and agricultural fields of North Bengal. The location of soil samples were recorded through GIS mapping tool (Garmin). \textit{T. flavus} species were isolated following Warcup soil dilution technique\textsuperscript{16} in a specific selective medium containing (g/L): MgSO\textsubscript{4}.7H\textsubscript{2}O, 0.2; K\textsubscript{2}HPO\textsubscript{4}, 0.9; KCl, 0.15; NH\textsubscript{4}NO\textsubscript{3}, 1.0; glucose, 3.0; and agar, 20 [chloramphenicol, 0.25; fenaminosulf, 0.3; pentachloronitrobenzene, 0.2; rose Bengal, 0.15; and captan, 0.02 (post autoclaving)]. A selective medium containing (g/L): Ca(NO\textsubscript{3})\textsubscript{2}, 1.0; KN\textsubscript{O}, 0.26; MgSO\textsubscript{4}.7H\textsubscript{2}O, 0.26; KH\textsubscript{2}PO\textsubscript{4}, 0.12; CaCl\textsubscript{2}.2H\textsubscript{2}O, 1.0; citric acid, 0.05; sucrose, 2.0; and agar, 20.0, chlortetracycline, 0.05 and captan (50% wettable powder)\textsuperscript{17}, was used for the isolation of \textit{Trichoderma} from soil samples.

**Screening for Phosphate Solubilizing Activity**

Screening for primary phosphate solubilizing activity of \textit{T. flavus} was carried out by allowing the fungi to grow in selective Pikovskaya’s agar medium supplemented with 0.5% tricalcium phosphate for 7 to 10 d at 25°C. The appearance of a transparent halo zone around the fungal colony indicated the phosphate solubilizing activity of the fungus\textsuperscript{18}.

**Genomic DNA Extraction**

Genomic DNA was isolated from fungal mycelia by a modified Raeder and Broda method\textsuperscript{19}. Fungal mycelia from 3 to 4-d-old cultures grown on potato dextrose broths was crushed with liquid nitrogen and incubated with lysis buffer, containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH8.0), 100 mM NaCl and 2% SDS, for 4 h at 65°C, followed by centrifugation at 12,000 rpm for 15 min. The supernatant was extracted with equal volume of water saturated phenol, centrifuged at 12,000 rpm for 15 min and further extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) by centrifugation at 12000 rpm for 15 min. The aqueous phase was transferred in a fresh tube and chloroform (in the ration of 1:4 v/v) was added, followed by 0.5 M Na-acetate (in the ration of 1:10 v/v). Next, isopropanol was added to the above mixture (0.7 times the final volume) and centrifuged. The DNA was precipitated from the aqueous phase with chilled ethanol (100%) and pelleted by centrifuging at 12000 rpm for 15 min, followed by washing in 70% ethanol and centrifugation. The pellets were air dried and suspended in TE buffer pH 8.

**Qualitative and Quantitative Estimation of DNA**

Genomic DNA was purified by re-suspending in 100 µL 1× TE buffer, incubated at 37°C for 30 min with RNase (60 µg) and the sample was re-extracted with PCI solution. Finally DNA was precipitated with chilled ethanol. The quality and quantity of DNA were analyzed both spectrophotometrically and in 0.8% agarose gel.

**PCR Amplification of ITS Region**

Genomic DNA was amplified by mixing the template DNA (50 ng) with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase chain reaction was performed in a total volume of 100 µL, containing 78 µL deionized water, 10 µL 10× Taq polymerase buffer, 1 µL of 1U Taq polymerase enzyme, 6 µL 2 mM dNTPs, 1.5 µL of 100 mM reverse and forward primers as follows:

For amplification of the ITS regions of the ribosomal DNA of \textit{T. flavus}, primer pairs, ITS1 and ITS4 (set A) were used, and for \textit{Trichoderma} isolates, primer pair T/ITS1 and T/ITS4 (set B) were used (Table 1).
PCR was programmed with an initial denaturing at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec and extension (for both the primer sets A & B) at 70°C for 2 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (10 µL) was mixed with loading buffer (5 µL) containing 0.25% bromophenol blue, 40% w/v sucrose in water, and then loaded in 1.5% agarose gel with 0.1% ethidium bromide for examination with horizontal electrophoresis. Nucleotide base pairs of the amplicons were determined on the basis of its migration and conformation relative to the molecular size marker (1000 base pair, wide range DNA ladder, Genie, Bangalore). PCR products were sent for sequencing to Genie, Bangalore.

Data Analysis

The sequenced PCR product was aligned with ex-type strain sequences from NCBI GenBank and established fungal taxonomy for identification. Sequences were aligned following the Clustal W algorithm included in the Megalign module (DNASTAR Inc.). Multiple alignment parameters used were gap penalty=10 and gap length penalty=10. Both of these values are aimed to prevent lengthy or excessive numbers of gaps. The default parameters were used for the pairwise alignment. The use of Clustal W determines that once a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were made manually in order to remove artificial gaps. Phylogenetic analyses were completed using the MEGA package (version 4.01; Institute of Molecular Evolutionary Genetics, University Park, PA). Neither gaps (due to insertion-deletion events) nor equivocal sites were considered phylogenetically informative. Hence, complete deletion prevented the use of any of these sites in further analyses. Phylogenetic inference was performed by the UPGMA method. Bootstrap tests with 1,000 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained. There were a total of 138 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4 as described by Tamura et al.

RAPD Analysis of Isolates

RAPD profiling of all the isolates were conducted using eight random primers, viz., OPA-1, OPB-2, OPD-5, OPA-4, AA-05, OPD-6, AA-04 and AA-1 (Table 1). PCR was programmed with an initial denaturing at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 70°C for 90 sec, and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR products were visualized as described earlier.

Scoring of RAPD Bands and Data Analysis

Bands obtained on the agarose gel were documented through Kodac gel documentation system. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed in silico into similarity matrix using NTSYSpc (Numerical Taxonomy System Biostatsics, version 2.11W). The SIMQUAL program was used to calculate the Jaccard’s coefficients. The RAPD patterns of each isolate was evaluated, assigning character state “1” to indicate the presence of band in the gel and “0” for its absence in the gel. Thus, a data matrix was created which was used to calculate the Jaccard similarity coefficient for each pairwise comparison. Jaccard coefficients were clustered to generate dendograms using the SHAN clustering program, selecting the unweighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYS pc.

Results and Discussion

Isolation and Identification of Microorganisms

Soil inhabiting fungus T. flavus (RHS/P_51) was isolated from the paddy growing soil. Microscopic...
observation revealed that the fungus was filamentous and possessed white to yellow ascoma composed of loose hyphae; ascospores were yellow, thick walled, subglobose to ovoid, 3.5-4 × 2.7-3.5 µm². The fungus produced colonies reaching upto 5-7 cm diam in 7 d at 25-30°C of incubation on PDA. The descriptions and other results were in accordance to the description of Malloch and Cain²⁵, where the species was described as anamorphs mostly belonging to *Penicillium*. Four isolates of *T. harzianum* and 3 isolates of *T. asperellum* were obtained on TSM medium from soils of higher altitude regions of North Bengal.

**Phosphate Solubilization**

*T. flavus* was found to solubilize phosphate in vitro, which was indicated by the formation of halo zone on PVK medium around the colony after incubation for 7 to 10 d at 25°C.

**RAPD Analysis of *T. flavus* and Trichoderma Isolates**

The genetic relatedness among all the isolates of *Trichoderma* was analyzed by six random primers OPA-1, OPD-6, OPA-4, A-5, AA-04 and AA-11 to generate reproducible polymorphisms. In case of *T. flavus* its genetic relatedness was compared with different species of *Aspergillus*, viz., *A. niger*, *A. melleus* and *A. clavatus*, which were also reported as phosphate solubilizing isolates obtained from soil²⁴. All amplified products with the primers had shown polymorphic and distinguishable banding patterns, which indicate the genetic diversity. RAPD profiles were scored by visually comparing RAPD amplification profiles and scoring the presence or absence of each band in each profile. Basically, the formation obtained from agarose gel electrophoresis was digitalized by hand to a two discrete character matrix (0 and 1 for absence and presence of RAPD markers). In case of *T. flavus* and other phosphate solubilizing isolates, 42 reproducible and scorable bands generated with primer A-5 were scored (Fig. 1). In case of *Trichoderma* isolates, 34 reproducible and scorable bands generated with primer A-11 were scored (Fig. 2). The dendrograms were generated by UPGMA using NTSYSpc software (Figs 3 & 4).

Similarity, coefficient among the phosphate solubilizers and *T. flavus* ranged from 0.37 to 1. All the isolates were divided into three main clusters at 0.37 similarity level. In case of *Trichoderma* isolates the similarity coefficient ranged from 0.68 to 1 and all the seven isolates could be grouped into two main clusters at 0.68 similarity level where one cluster represents isolate *T. asperellum*, while the other cluster represents isolates of *T. harzianum*.

**rDNA Sequence Analysis**

The ribosomal DNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. rDNAs are highly stable and exhibit a mosaic of conserved and
diverse regions within the genome [25]. They also occur in multiple copies with up to 200 copies per haploid genome [26,27], arranged in tandem repeats with each repeat consisting of 18S small subunit (SSU), and 5.8S and 28S large subunit (LSU) genes. Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species [28]. In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies. In the present study, we focused on the ITS regions of ribosomal genes for the construction of primers that can be used to identify *T. flavus* and *Trichoderma* isolates. In case of *T. flavus*, ITS region of rDNA was amplified using genus specific ITS1 and ITS4 primers. Amplified product size in the range of 800 bp was obtained for *T. flavus*, whereas uniform PCR product of 600 bp was obtained for *Trichoderma* isolates (Fig. 5). All these PCR products were sequenced that could be aligned and showed satisfactory homology with ex-type strains of both *T. flavus* and *T. harzianum*.
sequences from the NCBI GenBank data base as analysed by BLAST (Tables 2 & 3).

The present results are in accordance with those of previous study on the identification and genetic variability of the *Trichoderma* isolates. These results are also in accordance with several workers who observed the amplified rDNA fragment of approximately 500 to 600 bp by ITS-PCR in *Trichoderma*. ITS sequences were submitted to the NCBI GenBank database under the accession number GU324073 for *T. flavus* and HQ334995 for *T. harzianum*.

From the sequence alignment observed between isolates of both the species and their other ex-type isolates, the evolutionary history was inferred using the UPGMA method. In case of ITS sequences of *T. flavus*, the optimal tree with the sum of branch length=0.48437862 was obtained (Fig. 6); there was a total of 463 positions in the final dataset. Whereas in case of *T. harzianum*, the optimal tree with the sum of branch length=0.12242726 was obtained (Fig. 7) and there were a total of 444 positions in the final dataset. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches.

### Table 2—NCBI GenBank accession numbers of ex-type *T. flavus* strains used for identification of isolate RHS/P 51 and phylogenetic analysis

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>GenBank acc. no.</th>
<th>Identified as</th>
<th>Country of origin</th>
<th>Identity (%)</th>
</tr>
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<td>RHS/P 51</td>
<td>GU324073</td>
<td><em>T. flavus</em></td>
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<td>South-West0092</td>
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<td><em>T. flavus</em></td>
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<tr>
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<td>EU021596.1</td>
<td><em>T. flavus</em></td>
<td>USA</td>
<td>100</td>
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### Table 3—NCBI GenBank accession numbers of ex-type *T. harzianum* strains used for identification and phylogenetic analysis

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<th>Identified as</th>
<th>Country of origin</th>
<th>Identity (%)</th>
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<td>HQ115702</td>
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<td>Austria</td>
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![Fig. 6](image1.png) — The phylogenetic analyses conducted using UPGMA method among isolates of *T. flavus* (RHS/P 51) with other ex-type isolates of *T. flavus* by MEGA4.1 software.

![Fig. 7](image2.png) — The phylogenetic analyses conducted using the UPGMA method among the isolate of *T. harzianum* (RHS/S 560) with other ex-type isolates of *T. harzianum* by MEGA4.1 software.
units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method\textsuperscript{21} and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

ITS4 region of *T. harzianum*, obtained from higher altitude regions of Darjeeling hills of North Bengal, was aligned with other ex-type isolate sequences obtained from NCBI GenBank database using the bioinformatic tool BioEdit. Multiple sequence alignment revealed that quite a number of gaps were introduced in the alignment within the ITS region of 18S rDNA. However, similar sequences among the isolates were closely related due to their conserved regions shown in colours (Fig. 8). Combinations and percentage of occurrence of different nucleotide in the entire sequences were calculated using the bioinformatics algorithm from the website http://www.ualberta.ca/~stothard/javascript/dna_stats.html (Table 4).

The sequence of DNA fragments of ITS region for 554 residue sequence "560_ITS4" starting with "GGATATGCTT", presented below, following this protocol revealed that the guanine ‘G’ content of the sequence is maximum (30.14 %) with highest repetition of 167. Combinations like GC were also

Fig. 8—18S rDNA sequence alignments of *T. harzianum*. Data for other species were gathered from NCBI. The conserved regions of the gene are demonstrated in different colour.
maximum at the level 55.42%, which occurred at least 307 times in the entire sequence.

The mol wt of DNA residue sequence "560_ITS4" starting "GGATATGCTT" is 171662.84 Da (http://www.ualberta.ca/~stothard/javascript/dna_mw.html). A total of 4 open reading frames for the designated sequence were calculated with the help of ORF finder available from http://www.ualberta.ca/~stothard/javascript/orf_find.html for residue sequence of T. harzianum RHS/S 560 starting at "GGATATGCTT".

ORF number 1 in reading frame 1 on the direct strand extends from base 1 to base 186:

GGATATGCTTTTCAGAAAGTGTTTGTATC
GGACGTGGACGCGCCGCGCTCCCGGTGCGAG
TTGTGCAAACTACTGCGCAGGAGAGGCTGCG
GCGAGACCGCCACTGTATTTCGGGGCCGGCA
CCCGTGTGAGGGGTCCCGATCCCCAACGCCG
ATCCCCCGAGGGGCTGTCGCGCGATGCC

ORF number 2 in reading frame 1 on the direct strand extends from base 250 to base 351:

TTCACTGAATTCTGCAATTCACATTACTTAC
GCATTTCGCTGCGTTCTTCATCGATGCCAGAA
CCAAGAGATCCGTTGTTGAAAGTTTTGATTCA
TTTTGAG

ORF number 3 in reading frame 1 on the direct strand extends from base 352 to base 462:

ATTTTGTTCAGAGCTGTAAGAATACGTTCC
GGCAGGGGACTACAGAAAGAGTTTGGTTGGGCT
TCCTTCGGCAGGGCGCCTGTTCCGGGGCTC
AGCAACCCGGGGCGTG

ORF number 4 in reading frame 1 on the direct strand extends from base 463 to base 552:

CCCCGCCCGAGGCAACAGTTTGTTGAACGTTCA
CATTTGGGTTTGGGAGTTGTAAACTCGGTAATG
ATCCCTCCGCAAGGTTCTCCTACCTAAC

We have demonstrated that the analysis of aligned rDNA sequences is a reliable clustering strategy for identification purposes in a variety of taxonomic groups and systemic levels. While this approach was previously applied in analyzing complete genome data, the present study shows that it is also applicable in analyzing much shorter DNA sequences from a single gene, which is going to be the fundamental block in the massive rDNA database. This analysis could have other applications in DNA bar-coding, besides cluster analysis. The determination of frequencies of DNA strings would enable easy identification of taxon-specific strings that can be used as taxon specific probes in DNA chip for species identification. In conclusion, above results indicated that the T. favus and T. harzianum under study could be identified very easily on the basis of similar "rDNA-based sequence" from the available database.

Acknowledgement

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References

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