Molecular Characterization of ex-type Strains of *Trichoderma* spp. from two Indian Type Culture Collections

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**Abstract**

Species in the genus *Trichoderma* are important as commercial sources of several enzymes and as biofungicides/growth-promoters. Correct identification of species/isolates is important not only because several traits are species-specific, but also because two of the species are opportunistic pathogens on immuno-suppressed patients. We have examined two isolates each of six species of *Trichoderma* collected from MTCC, Chandigarh, and ITCC, New Delhi, using RAPD and rDNA polymorphism analysis. A set of four random primers were used for characterization of twelve isolates of *Trichoderma* spp., and the cluster analysis showed that the isolates, except *T. hamatum* isolates, were different from each other, and there were some overlapping with regard to species identification. Analysis of the ITS1-5.8S-ITS2 region of the rDNA showed that the approximate size of the region was 600 bp and size variation was observed. One of the *T. viride* isolates showed two amplicons against single band in all the other isolates. Restriction analysis of the amplified ITS1-5.8S-ITS2 region showed inter- and intra-specific polymorphism. One of the *T. koningii* isolates was exactly the same as *T. hamatum* isolates in RAPD and rDNA polymorphism. The results indicate the need for re-examination and re-classification of *Trichoderma* spp. strains deposited in Indian culture collections.

**Introduction**

Species in the fungal genus *Trichoderma* (Ascomycetes, Hypocreales) are of great economic importance as sources of enzymes, antibiotics, as plant growth promoters, xenobiotic degraders, and most importantly, as commercial biofungicides (Mukherjee, 1999). Two species (*T. longibrachiatum* and *T. citrinoviride*) are also known to be opportunistic pathogens on immuno-suppressed patients (Kuhls et al., 1999). Until recently, *Trichoderma* spp. were being identified based on morphological data like cultural characteristics, structure of conidiophores/conidia, etc. (Rifai, M.A., 1969; Bissett, J., 1984, 1991a, 1991b, 1991c, 1992). However, subsequent molecular analysis of several strains, including some ex-type strains revealed that classification based on morphological data have been, to a great extent, erroneous resulting in re-classification of several isolates and species (Meyer et al., 1994; Rehner et al. 1994; Kuhls et al. 1996, 1997; Bulat et al. 1998; Castle et al. 1998; Kindermann et al. 1998; Lieckfeldt et al. 1999; Hermosa et al. 2000). For example, three Indian isolates of *Trichoderma*, that are deposited at Microbial Type Culture Collection, Chandigarh, as *T. harzianum*, were examined by Hermosa et al. 2000. Two of them were found to be *T. inhamatum*, and one was classified as *T. longibrachiatum*, using molecular tools. Correct identification of *Trichoderma* spp. is important both from commercial point of view (as several traits are species-specific), and from safety point of view. This is particularly important for the type cultures as these are used by several workers and are taken as authentic samples. We therefore examined some representative isolates of this commercially important fungal genus using two PCR-based techniques viz. RAPD- Random Amplification of Polymorphic DNA, and restriction analysis of the amplified ITS1-5.8S-ITS2 region of the nuclear ribosomal DNA.

**Materials and Methods**

Two isolates each of six species of *Trichoderma* (*T. virens*, *T. pseudokoningii*, *T. hamatum*, *T. harzianum*, *T. viride* and *T.
The isolates were procured from either Indian Type Culture Collection (ITCC), New Delhi, or the Microbial Type Culture Collection (MTCC), Chandigarh (Table 1). The isolates were grown in potato dextrose broth at ambient temperature and total genomic DNA extracted as described earlier (Mukherjee, P.K., 1999). For all the PCR amplifications, 50 ng of DNA, 0.5 U of Taq DNA polymerase (Bangalore Genei) and 0.1 mM each of the dNTPs were used. Amplifications were performed in 25 μl reaction volume in an Eppendorf Mastercycler Personal thermal cycler. For RAPD, amplifications were performed with 5 random decamers from Operon Technologies kit A (OPA1-CAGGCCTTC, OPA2- TGCCGAGCTG, OPA3-AGTCAGCCAC, OPA4-AATCGGGCTG, OPA9-CGGTAACGCC) at 37°C annealing (1'), 94°C denaturation (1') and 72°C extension (2') - 35 cycles. Amplification products were size separated in 2 % agarose gel, visualized after staining with ethidium bromide and photographed with a UVP polaroid camera. The isolates were clustered using TreeconW programme based on binary score for the presence (1) or absence (0) of a band. For amplification of the ITS1-5.8S-ITS2 region, the primer pair ITS1 (TCTGTAGGTGAACCTGCGG) - ITS4 (TCCTCCGCTTTATTGATATGC) was used (White et al., 1990). To optimize the annealing temperature, amplifications were performed at 56°C, 58°C and 59°C. For restriction analysis, 20 μl of the amplification-product (amplified at 59°C annealing) was digested with 5 units of restriction enzymes (all from Bangalore Genei) for 2 hours and size separated in 2 % agarose gel, stained and photographed as described above.

**Figure 1:** RAPD analysis of the Trichoderma isolates based on OPA1 (A), OPA2 (B), OPA3 (C), OPA4 (D) and OPA9 (E). Lanes 1= T. viridescens, 2= T. viride, 3= T. pseudokoningii, 4= T. pseudokoningii, 5= T. hamatum, 6= T. hamatum, 7= T. harzianum, 8= T. harzianum, 9= T. viridescens, 10= T. viridescens, 11= T. koningii, 12= T. koningii. M= Molecular weight marker. The cluster analysis (F) was done based on a total of 205 polymorphic bands.
Results and Discussion

RAPD analysis based on 5 random primers (selected out of 10 primers after an initial screening) revealed a great deal of intra- and inter-specific variability amongst *Trichoderma* isolates examined except for the two *T. hamatum* isolates that were exactly identical (Fig. 1). Surprisingly, one *T. koningii* isolate (TK2) was also exactly identical to both the *T. hamatum* isolates. The RAPD fingerprinting data (Fig. 1) clearly indicate that there is mixing-up as far as species identification is concerned. For example, *T. harzianum* isolate no. 1 clusters with *T. viride* isolate no. 1, while *T. harzianum* isolate no. 2 clusters with *T. viride* isolate no. 2. Similarly, *T. viride* isolate no. 1 is more close to *T. koningii* isolate no. 1, than to *T. viride* isolate no. 2, while *T. pseudokoningii* isolate no. 2 is more close to *T. hamatum* isolates, than to *T. pseudokoningii* isolate no. 1. Since RAPD is often considered to be less reliable than RFLP data, we also analyzed the RFLP in the amplified ITS1-5.8S-ITS2 region. An annealing temperature of 59°C was found to be suitable for the amplification of ITS1-5.8S-ITS2 region from all the isolates with good product yield and minimum non-specific amplifications. The product size was approximately 600 bp, and there was size variation across the isolates (Fig. 2). At all the three annealing temperatures, we could see two bands only with the *T. viride* isolate no. 1. Digestion of this product with five tetra-base cutters (*MboI*, *HaeIII*, *TaqI*, *Sau3AI*, *MspI*) revealed polymorphism in the ITS1-5.8S-ITS2 region (Fig. 2). All the isolates could be grouped into broadly four groups (Table 2), which again showed the overlapping in species identification of these strains, e.g., *T. pseudokoningii* isolate no. 2, and *T. koningii* isolate no. 2 grouped with *T. hamatum* isolates.

The present analysis questions the identity of *Trichoderma* isolates maintained in two of the Indian type culture collections. This is not surprising given the fact that these were identified using morphological data, which, as a taxonomic tool for *Trichoderma* spp., has been confusing. It is therefore, proposed...
Table 1: Isolates of *Trichoderma* spp. used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate Designation</th>
<th>Catalogue no.</th>
<th>Isolated from/Locality*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. virens</em></td>
<td>GV1, GV2</td>
<td>MTCC 794, ITCC 4177</td>
<td>Soil/ Panntagar, Mango/Lucknow</td>
</tr>
<tr>
<td><em>T. pseudokoningii</em></td>
<td>TP1, TP2</td>
<td>MTCC 3011, MTCC 2049</td>
<td>Ginger/Mumbai, .-.**</td>
</tr>
<tr>
<td><em>T. hamatum</em></td>
<td>TH1, TH2</td>
<td>ITCC 2084, ITCC 3380</td>
<td>Soil/South India, Mushroom/Hisar</td>
</tr>
<tr>
<td><em>T. harzianum</em></td>
<td>THz1, THz2</td>
<td>ITCC 4532, MTCC 792</td>
<td>Peas/Palampur, Soil/Panptagar</td>
</tr>
<tr>
<td><em>T. viride</em></td>
<td>TV1, TV2</td>
<td>ITCC 2109, MTCC 793</td>
<td>.-/Assam, Soil/Panptagar</td>
</tr>
<tr>
<td><em>T. koningii</em></td>
<td>TK1, TK2</td>
<td>ITCC 4303, ITCC 2170</td>
<td>.-/Solan, Soil/Assam</td>
</tr>
</tbody>
</table>

*As mentioned in the catalogue; **Information not available
MTCC- Microbial Type Culture Collection, Chandigarh; ITCC- Indian Type Culture Collection, New Delhi.

Table 2: Grouping of *Trichoderma* isolates based on restriction analysis of the ITS1-5.8S-ITS2 region

<table>
<thead>
<tr>
<th>MboI/HaeIII</th>
<th>TaqI/Sau3AI</th>
<th>MspI</th>
</tr>
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<tr>
<td>GV1, GV2, TP1, THz1, THz2, TK1, TV2</td>
<td>GV1, GV2, TP1</td>
<td>GV1, GV2, TK1</td>
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<tr>
<td>TV1</td>
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<td>THz1, THz2, TP1, TV2</td>
</tr>
<tr>
<td>TP2, TH1, TH2, TK2</td>
<td>TP2, TH1, TH2, TK2</td>
<td>TP2, TH1, TH2, TK2</td>
</tr>
</tbody>
</table>

that all the isolates of *Trichoderma* spp. deposited in Indian type culture collections be re-identified using now available molecular tools (e.g., sequencing of the part of rDNA), in order to effectively utilize these fungi of immense agricultural, biotechnological and industrial importance.

References


This paper received the Best Poster Award at the 70th Annual Meeting of the Society of Biological Chemists (India), held at Dept. of Biochemistry, Osmania University, Hyderabad, during December 27-29, 2001.

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