

*tata*, which happen to be a new addition to the existing rice stem borers of Andaman islands.

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## PCR-fingerprinting of some *Trichoderma* isolates from two Indian type culture collections – a need for re-identification of these economically important fungi

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<sup>1</sup>. Two species (*T. longibrachiatum* and *T. citrinoviride*) are also known to be opportunistic pathogens on immunosuppressed patients<sup>2</sup>. Until recently, *Trichoderma* spp. were being identified based on morphological data like cultural characteristics, structure of conidiophores/conidia, etc.<sup>3,4</sup>. However, subsequent molecular analysis of several strains, including some ex-type strains revealed that classification based on morphological data has been, to a great extent, erroneous resulting in re-classification of several isolates and species<sup>5–12</sup>. 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.*<sup>13</sup>. 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 the 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 (RAPD – random amplified polymorphic DNA, and restriction analysis of the amplified ITS1–5.8S–ITS2 region of the nuclear ribosomal DNA).

Two isolates each of six species of *Trichoderma* (*T. virens*, *T. pseudokoningii*, *T. hamatum*, *T. harzianum*, *T. viride* and *T. koningii*) were procured from either Indian Type Culture Collection (ITCC), New Delhi or the Microbial Type Culture Collection (MTCC),

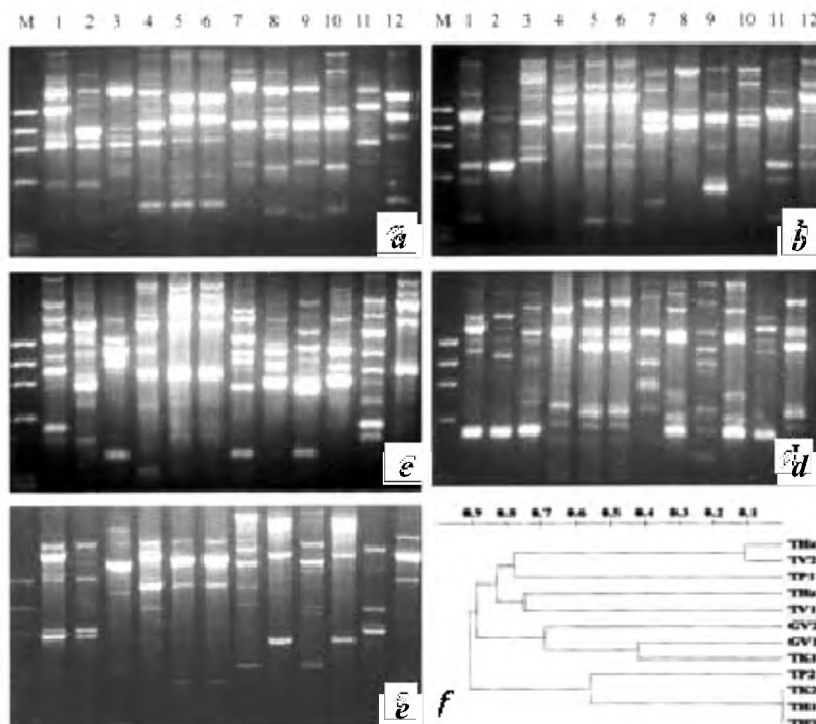
Chandigarh (Table 1). The isolates were grown in potato dextrose broth at ambient temperature and total genomic DNA extracted as described earlier<sup>14</sup>. For all the PCR amplifications, 50 ng of DNA, 0.5 U of Taq DNA polymerase (Bangalore Genei) and 0.1 mM each of the dNTPs were used. Amplifications were performed in 25 µl reaction volume in an Eppendorf Mastercycler Personal thermal cycler. For RAPD, amplifications were performed with five random decamers from Operon Technologies kit A (OPA1–CAGGCCCTTC, OPA2–

Table 1. Isolates of *Trichoderma* spp. used in this study

Species	Isolate designation	Catalogue no.	Isolated from/locality*
<i>T. virens</i>	GV1	MTCC 794	Soil/Pantnagar
	GV2	ITCC 4177	Mango/Lucknow
<i>T. pseudokoningii</i>	TP1	MTCC 3011	Ginger/Mumbai
	TP2	MTCC 2049	–/–**
<i>T. hamatum</i>	TH1	ITCC 2084	Soil/South India
	TH2	ITCC 3380	Mushroom/Hisar
<i>T. harzianum</i>	THz1	ITCC 4532	Peas/Palampur
	THz2	MTCC792	Soil/Pantnagar
<i>T. viride</i>	TV1	ITCC 2109	–/Assam
	TV2	MTCC 793	Soil/Pantnagar
<i>T. koningii</i>	TK1	ITCC 4303	–/Solani
	TK2	ITCC 2170	Soil/Assam

\*As mentioned in the catalogue; \*\*Information not available.

MTCC, Microbial Type Culture Collection, Chandigarh; ITCC, Indian Type Culture Collection, New Delhi.



**Figure 1.** RAPD analysis of *Trichoderma* isolates based on OPA1 (a), OPA2 (b), OPA3 (c), OPA4 (d) and OPA9 (e). Lane 1, *T. virens* 1; lane 2, *T. virens* 2; lane 3, *T. pseudokoningii* 1; lane 4, *T. pseudokoningii* 2; lane 5, *T. hamatum* 1; lane 6, *T. hamatum* 2; lane 7, *T. harzianum* 1; lane 8, *T. harzianum* 2; lane 9, *T. viride* 1; lane 10, *T. viride* 2; lane 11, *T. koningii* 1; lane 12, *T. koningii* 2; M, Molecular weight marker. Cluster analysis (f) was done based on a total of 205 polymorphic bands.

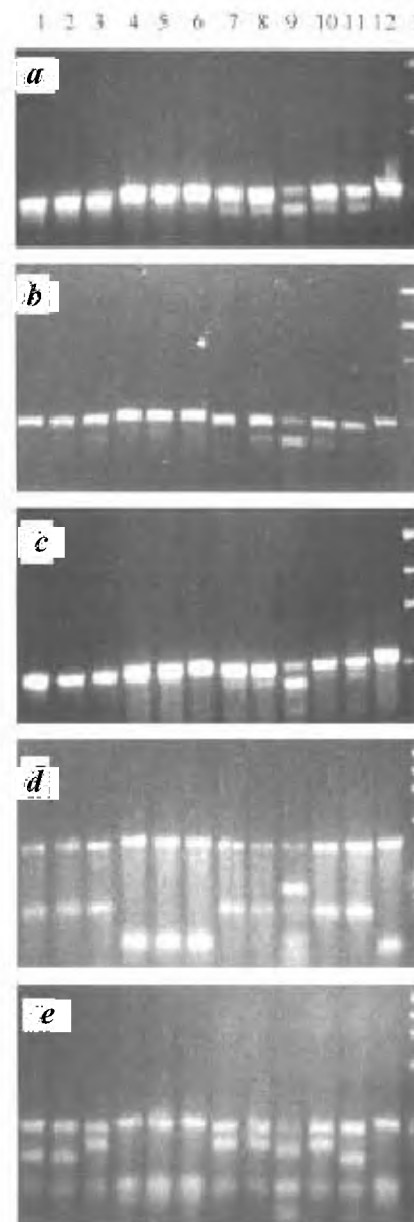
**Table 2.** Grouping of *Trichoderma* isolates based on restriction analysis of the ITS1–5.8S–ITS2 region

<i>Mbo</i> II/ <i>Hae</i> III	<i>Taq</i> I/ <i>Sau</i> 3AI	<i>Msp</i> I
GV1, GV2, TP1, THz1, THz2, TK1, TV2	GV1, GV2, TP1 THz1, THz2, TK1, TV2	GV1, GV2, TK1 THz1, THz2, TP1, TV2
TP2, TH1, TH2, TK2	TP2, TH1, TH2, TK2	TP2, TH1, TH2, TK2
TV1	TV1	TV1

TGCCGAGCTG, OPA3–AGTCAGCC–AC, 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 program 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 (TCCTCCGCTTAT–TGATATGC) was used<sup>15</sup>. To optimize the annealing temperature, amplifications were performed at 56, 58 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 h and size-separated in 2% agarose gel, stained and photographed as described above.

RAPD analysis based on five random primers (selected out of ten 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 (Figure 1). Surprisingly, one *T. koningii* isolate (TK2) was also exactly identical to both the *T. hamatum* isolates. The RAPD fingerprinting data (Figure 1) clearly indi-



**Figure 2.** Analysis of the ITS1–5.8S–ITS2 region of *Trichoderma* isolates. (Lane 1, *T. virens* 1; lane 2, *T. virens* 2; lane 3, *T. pseudokoningii* 1; lane 4, *T. pseudokoningii* 2; lane 5, *T. hamatum* 1; lane 6, *T. hamatum* 2; lane 7, *T. harzianum* 1; lane 8, *T. harzianum* 2; lane 9, *T. viride* 1; lane 10, *T. viride* 2; lane 11, *T. koningii* 1; lane 12, *T. koningii* 2; M, Molecular weight marker). a, Amplification at 56°C, b, Amplification at 58°C, c, Amplification at 59°C, d, Digestion of the ITS1–5.8S–ITS2 region with *Hae*III; and e, Digestion of the ITS1–5.8S–ITS2 region with *Msp*I.

cate 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. virens* isolate no. 1 is more close to *T. koningii* isolate no. 1, than to *T. virens* 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 analysed 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 (Figure 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 (*Mbo*I, *Hae*III, *Taq*I, *Sau*3AI, *Msp*I) revealed polymorphism in the ITS1–5.8S–ITS2 region (Figure 2). All the isolates could be divided 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., have been confusing. It is therefore proposed that all the isolates of *Trichoderma* spp. deposited in Indian type culture collections be re-identified using the currently 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.

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## Phenolics in elephant dung: a complex zoochoric system

While anemochory has brought in its wake a wide assortment of engineering perfection in wind-borne seeds, zoochory involves many complex aspects of chemistry, for example, the chemistry of fruit pulp<sup>1–3</sup>. Even seed dispersal through elephant dung<sup>4–6</sup> is intricately associated with the chemical properties of the dung. For example, herbivore dung is known to contain phenolics and these compounds influence growth.

Apart from dispersal per se, the effect of dung as manure on the germinated

seedlings has been considered<sup>4</sup>. But elephant dung is a very complex system, with so many possible chemical agents which might act as stimulators or inhibitors of germination and/or seed growth. These may be the metabolic products of the elephant's own physiological system and chemicals from the very large amount of undigested/partially digested vegetal remains, a characteristic feature of elephant and rhino dung. Such dung are expected to contain phenolics. Since a large quantity and variety of phenolic

substances occur in the plant world, we have investigated the possibility of phenolics in elephant dung exerting an influence on seedling growth. This would be apart from any nutritive effect that the dung as a source of manure might exert.

Elephant dung was collected from Chandak Elephant Reserve, Orissa and the aqueous extract was filtered and tested on IET rice grains showing 100% germination. A standard volume of water (10 ml) was put in petri dishes containing Whatman filter paper on which the