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AFLP Analysis of *Trichoderma* spp. from India Compared with Sequence and Morphological-based Diagnostics

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Abstract

Trichoderma species offer considerable potential for controlling aflatoxin contamination in groundnut and other crops. Initial classification of 48 *Trichoderma* isolates, derived from four different groundnut cultivation sites in India was based on alignment of 28S rDNA sequences to GenBank sequences of ex-type strains. This was found to be substantially more reliable than our routine morphological characterization, but did not provide a comprehensive diagnostic solution, as unique single nucleotide polymorphism (SNP) haplotypes could not be identified for all species. However, all the *Trichoderma* isolates could be readily distinguished by amplified fragment length polymorphism (AFLP) analysis, based on six primer pair combinations, which generated 234 polymorphic bands. In addition, individual AFLP bands were identified which differentiate closely related species. Similarly, AFLP bands were identified that correlated with different types of antagonism to *Aspergillus flavus*. The implications of these results for the development of simple polymerase chain reaction (PCR)-based diagnostic assays for antagonistic isolates of *Trichoderma* is discussed.

Introduction

The Food and Agriculture Organization (FAO) estimates that up to 25% of losses in food production worldwide occur postharvest, due mainly to mould and mycotoxin contamination (Boutrif, 1998). Aflatoxins are naturally occurring mycotoxins produced by *Aspergillus flavus* Link. ex Fries and *A. parasiticus* (see bibliography at http://www.aflatoxin.info). Aflatoxins were first discovered in groundnuts, but *Aspergillus* colonizes a wide range of dried fruit, nuts, spices and cereals especially maize, and aflatoxins from these foods can pass into human and bovine milk (Bilgrami and Choudhary, 1998; Waliyar et al., 2003). Aflatoxins are both toxic and carcinogenic, and are implicated in human diseases (Bhat, 1989; Reed and Kasali, 1989; Wild et al., 1996; Vasanthi and Bhat, 1998), cause growth retardation in higher animals (Hall and Wild, 1994), and reduce the productivity of pigs, cattle and poultry (Wang et al., 1996). A large percentage of the human population of Africa and Asia have high levels of aflatoxin in their blood (Waliyar et al., 1994, 2003; Gong et al., 2002, 2003). Maize and groundnut are probably the main sources of aflatoxin contamination as they constitute a major part of the diet of people in these regions. Many industrialized countries regulate the level of total aflatoxins (0–50 μg/kg) in imported food products (FAO, 1997), effectively closing the export market to groundnut producers from Asia and Africa (Chandrasekhar, 1989; Coulibaly, 1989; Mehan et al., 1991).

Trichoderma species are widespread saprophytic soilborne or wood-decaying fungi, which appear to be well adapted to diverse abiotic stresses such as salinity and drought (Kubicek et al., 2002). Some species also cause substantial economic losses in commercial mushroom production (Ospina-Giraldo et al., 1999), while others act as biocontrol agents (Samuels, 1996; Hjeljord and Tronsmo, 1998; Hermosa et al., 2004), induce host-defence responses in plants (De Meyer et al., 1998; Yedidia et al., 1999), produce antibiotics (Ghisalberti and Rowland, 1993) and cellulolytic and hydrolytic enzymes (Haran et al., 1996) and degrade organochlorine pesticides (Katayama and Matsumura, 1993). Some *Trichoderma* species present in groundnut-cultivated soils have been shown to be antagonistic or mycoparasitic to *A. flavus* (Srilakshmi et al., 2001) and to prevent the synthesis of aflatoxin B_1 (Shanta, 1999; Desai et al., 2000).

Taxonomic identification of *Trichoderma* species is largely based on morphological descriptors resulting in

the division of the genus into five sections: *Trichoderma*, *Pachybasium*, *Longibrachiatum*, *Saturnisporum* and *Hypocreanum* (Rifai, 1969; Bissett, 1991a,b, 1992). Unfortunately the morphological differentiation between some species requires a high level of expertise and is highly time-consuming. In addition, these characters are not always present in culture, making morphological identification difficult (Kubicek et al., 2002). Attempts to improve the process have been made by combining morphological and physiological data but these do not necessarily reflect phylogeny (Grondona et al., 1997; Hermosa et al., 2000; Kubicek et al., 2002; Kullnig-Gradinger et al., 2002). Molecular approaches such as isozymes (Hermosa et al., 2000), restriction fragment length polymorphism (RFLP) (Muthumeenakshi et al., 1994; Lieckfeldt et al., 2001), polymerase chain reaction (PCR) fingerprinting (Lieckfeldt et al., 2001), random amplified polymorphic DNA (RAPD) (Anjaiah et al., 2001; Wuczkowski et al., 2003) and sequence polymorphism (Kindermann et al., 1998; Kullnig-Gradinger et al., 2002) have been used to identify four clades: clade A *Trichoderma* sect. including *T. hamatum*, *T. pubescens*, *T. koningii* and *T. atroviride*; clade B containing a large heterogeneous mixture representing the *Pachybrasium* sect. including *T. harzianum* and *T. inhamatum*; clade C the species *Longibrachiatum* and clade D containing *T. aureoviride* (Kindermann et al., 1998; Hermosa et al., 2000; Kubicek et al., 2002; Kullnig-Gradinger et al., 2002). A polyphasic approach based on metabolic characteristics, morphological observations and nucleotide sequence information (internal transcribed spacers, ITS1 and ITS2) has also been used (Bisset et al., 2003). Although this appears to offer the most reliable classification of isolates it does not simplify the diagnostic process.

Amplified fragment length polymorphism (AFLP) is a genotyping method based on the selective amplification of a subset of DNA fragments generated by restriction enzyme digest (Vos et al., 1995). This technique is highly discriminatory and reproducible and has been used extensively for the analysis of other micro-organisms (Savelkoul et al., 1999). These advantages have facilitated the compilation of diversity databases providing standard reference AFLP-banding patterns for harmonizing diagnosis by the community at large (Savelkoul et al., 1999).

In the present study we have investigated the power of AFLP fingerprinting to differentiate *Trichoderma* isolates from closely related species, and compared this with 28S rDNA (D2) sequence-based diversity analyses and previously reported morphological classification of 48 *Trichoderma* isolates. It is expected that if specific AFLP bands can be identified that provide species differentiation or association with antagonistic behaviour, these can be readily converted into a rapid, routine and low-cost diagnostic tests. Not only would such tests have significant value in *Trichoderma* research, they would also provide a powerful facilitating resource for the development of aflatoxin biocontrol systems.

Materials and Methods

Collection and conventional characterization of *Trichoderma* isolates

The *Trichoderma* isolates used in this study were collected and identified as described previously (Srilakshmi et al., 2001). Rhizosphere soil samples were collected from major groundnut growing areas of two districts in Andhra Pradesh and two districts in Karnataka (all districts in southern India), during the 2000 and 2001 rainy seasons (Table 1). A total of 212 *Trichoderma* isolates were obtained from 386 soil samples and single-spore representatives were generated. A subset of 48 isolates showed a clear inhibition zone in dual-culture studies, of which 35 were characterized for production of volatile (V-type) and diffusible (D-type) antibiotics, referred to as V- and D-type antagonism, respectively (data not presented). These morphological and biochemical characterization data were used for comparative and association analyses in the present study.

DNA isolation

The 48 *Trichoderma* isolates were maintained on solid potato dextrose agar medium (PDA) and incubated at 28°C for 5 days. DNA was extracted from fungal mycelium using a CTAB DNA extraction protocol (Sivaramakrishnan et al., 2002) with the following modifications: extraction buffer with 2.5% CTAB and 0.03% β-mercaptoethanol, the resultant crude DNA was digested with 7.5 mg RNaseA overnight. The purity of the DNA was determined by electrophoretic analysis on 0.8% (w/v) agarose gel containing ethidium bromide. The concentration of fungal DNA was determined by comparing intensities with that of known concentrations of lambda-DNA.

28S rDNA fungal sequence analysis

The MicroSeq D2 large subunit (LSU) rDNA Fungal Identification System (Applied Biosystems, Foster City, CA, USA) was used for the sequence-based identification of *Trichoderma* isolates. This system uses proprietary primers for the amplification and sequence analysis of the LSU-D2 region of rDNA. All reactions were carried out according to the manufacturer's instructions. Sequences were determined with an Applied Biosystems Genetic Analyzer model ABI 3700 with a POP6 gel matrix, filter set E and DT 3700 POP6 dRhodamine v3 mobility file.

Sequence-based phylogenetic analysis

CHROMAS software version 2.2 (Technelysium Pty Ltd, Helensvale, Australia) was used to generate consensus sequences from forward and reverse sequence runs specific to the LSU-D2 region of 28S rDNA for 38 of the 48 genotypes. The resultant sequence data were compared with the NCBI database using BLASTn. These sequences, approximately 250 nucleotides in length resulted in alignments (E values of e^{-146} to e^{-156}) with 92–100% homology to *Trichoderma* spp. in GenBank.

Table 1
Trichoderma isolates used for molecular analysis

Isolate code	District	*Trichoderma* species[a]
T1	Chittoor	*T. pubescens*/*T. hamatum*
T2	Chittoor	*T. pubescens*/*T. hamatum*
T6	Chittoor	*T. harziamum*/*T. inhamatum*
T11	Anantapur	*T. pubescens*/*T. hamatum*
T12	Chittoor	*T. pubescens*/*T. hamatum*
T13	Chittoor	*T. pubescens*/*T. hamatum*
T14	Chittoor	*T. pubescens*/*T. hamatum*
T16	Chittoor	*T. harziamum*/*T. inhamatum*
T20	Chittoor	*T. pubescens*/*T. hamatum*
T21	Chittoor	*T. pubescens*/*T. hamatum*
T24	Chittoor	*T. pubescens*/*T. hamatum*
T29	Chittoor	*T. harziamum*/*T. inhamatum*
T33	Chittoor	No data
T34	Tumkur	*T. harziamum*/*T. inhamatum*
T35	Chittoor	No data
T37	Kolar	No data
T42	Chittoor	*T. pubescens*/*T. hamatum*
T46	Chittoor	No data
T47	Tumkur	*T. atroviride*
T49	Chittoor	No data
T50	Chittoor	*T. pubescens*/*T. hamatum*
T51	Tumkur	*T. pubescens*/*T. hamatum*
T53	Tumkur	No data
T56	Tumkur	*T. pubescens*/*T. hamatum*
T58	Chittoor	*T. pubescens*/*T. hamatum*
T60	Tumkur	No data
T62	Chittoor	*T. atroviride*
T70	Chittoor	*T. harziamum*/*T. inhamatum*
T72	Anantapur	*T. harziamum*/*T. inhamatum*
T73	Tumkur	No data
T74	Tumkur	*T. atroviride*
T83	Anantapur	*T. pubescens*/*T. hamatum*
T86	Tumkur	*T. koningii*
T10	Chittoor	*T. longibrachiatum*
T102	Chittoor	*T. harziamum*/*T. inhamatum*
T109	Chittoor	*T. pubescens*/*T. hamatum*
T110	Chittoor	*T. harziamum*/*T. inhamatum*
T117	Tumkur	*T. pubescens*/*T. hamatum*
T129	Tumkur	*T. longibrachiatum*
T134	Anantapur	No data
T142	Chittoor	*T. harziamum*/*T. inhamatum*
T143	Chittoor	*T. harziamum*/*T. inhamatum*
T161	Tumkur	*T. harziamum*/*T. inhamatum*
T170	Chittoor	*T. harziamum*/*T. inhamatum*
T179	Chittoor	*T. pubescens*/*T. hamatum*
T188	Tumkur	*T. pubescens*/*T. hamatum*
T205	Kolar	*T. pubescens*/*T. hamatum*
T206	Anantapur	No data

[a]Species designation given to isolates based on 28S rDNA sequence comparison with ex-type strains listed in Table 2.

It was not possible to obtain good quality sequence data from the remaining 10 isolates in this study. All 38 sequences aligned with one of the following species groups *T. pubescens–T. hamatum*, *T. harzianum–T. inhamatum*, *T. atroviride*, *T. koningii* and *T. longibrachiatum*. The nucleotide sequences from the most homologous BLASTn alignments were retrieved from NCBI (Table 2) and used as reference representatives for phylogenetic analysis. If a 28S rDNA sequence was available for an ex-type strain, this sequence was used preferentially for the analysis, otherwise, strains reported as typical of the species based on morphology, physiology and molecular genetic characterization (Kindermann et al., 1998; Hermosa et al., 2000; Kullnig-Gradinger et al., 2002) were used. The *T. pubescens* sequence used in this respect is listed in GenBank with an incorrect culture number, DAOM 162.162 should be DAOM 166.162 (C. P. Kubicek, personal communication). Sequences in Fasta format were aligned with reference representatives for each of the major *Trichoderma* spp., using the multiple sequence alignment algorithm and default options in CLUSTAL X software version 1.81 (Thompson et al., 1997). Sequences were then adjusted by eye to accommodate gaps. The ends, consisting largely of unaligned regions were trimmed to yield a sequence of 218 nucleotides with no gaps. Sequences generated from the 38 isolates have been deposited in GenBank under the following accession numbers AY994262–AY994288 (Fig. 1). As some clustering alignment programmes are sensitive to data input order (Hickson et al., 2000), various input orders were compared. When a sequence derived from any of

Table 2
Trichoderma spp. representatives used in the phylogeny study including collection number and gene sequence accession numbers

Species name	Collection and number	Geographical origin and habitat	GenBank accession number for 28S rDNA sequence
T. pubescens (ex-type strain)	DAOM 166.162	USA soil	AF399245
T. hamatum (ex-type strain)	DAOM 167057	Canada soil	AF399235
T. koningii	CBS 979.70	Netherlands tree	AF399239
T. viride	ATCC 28020	USA soil	AF127150
T. atroviride	DAOM 165779	USA	AF399227
T. longibrachiatum (ex-type strain)	CBS 816.68[a]	USA	AF399240
T. inhamatum (ex-type strain)	CBS 273.78[a]	Colombia soil	AF399237
T. harzianum (ex-neotype strain)	CBS 226.95	UK	AF399236

ATCC: American Type Culture Collection, Manassas; CBS: Centraalbureau voor Schimmelcultures, Utrecht; DAOM: Department of Agriculture (Mycology), Ottawa, Canada.
[a]Reference strains also used in the MicroSeq Fungal Database v.0050c for fungal identification (http://www.appliedbiosystems.com/techsupp/swpps/MSsw.html).

Trichoderma isolates &	Positions of 28S-rDNA single nucleotide polymorphisms													
Genbank accession no.	23	25	36	55	59	60	62	94	112	113	116	133	150	204
T1 - AY994289	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T109 - AY994292	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T11 - AY994293	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T12 - AY994295	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T13 - AY994297	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T14 - AY994298	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T179 - AY994266	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T188 - AY994267	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T2 - AY994268	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T20 - AY994269	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T205 - AY994270	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T. pubescens	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T. hamatum	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T42 - AY994285	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T117 - AY994286	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T83 - AY994284	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T58 - AY994279	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T56 - AY994278	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T51 - AY994277	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T50 - AY994276	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T24 - AY994272	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T21 - AY994271	C	G	T	C	G	A	G	A	G	A	G	A	C	G
T47 - AY994275	G	G	T	C	G	A	G	C	G	A	A	G	C	G
T62 - AY994281	G	G	T	C	G	A	G	C	G	A	A	G	C	G
T74 - AY994283	G	G	T	C	G	A	G	C	G	A	A	G	C	G
T. atroviride	G	G	T	C	G	A	G	C	G	A	A	G	C	G
T. viride	G	A	T	C	G	A	G	C	G	A	G	G	C	G
T86 - AY994287	G	G	T	G	G	A	C	C	G	A	G	A	C	G
T. koningii	G	G	T	G	G	A	C	C	G	A	A	A	T	G
T10 - AY994290	C	G	C	C	A	G	G	A	G	G	G	G	C	A
T129 - AY994296	C	G	C	C	A	G	G	A	G	G	G	G	C	A
T. longibrachiatum	C	G	C	C	A	G	G	A	G	G	G	G	C	A
T102 - AY994291	C	G	T	C	G	A	G	A	A	G	G	A	C	A
T. harzianum	C	G	T	C	G	A	G	A	A	G	G	A	C	A
T110 - AY994294	C	G	T	C	G	A	G	A	A	G	G	A	C	A
T142 - AY994299	C	G	T	C	G	A	G	A	A	G	G	A	C	A
T143 - AY994262	C	G	T	C	G	A	G	A	A	G	G	A	C	A
T16 - AY994263	C	G	T	C	G	A	G	A	A	G	G	A	C	A
T161 - AY994264	C	G	T	C	G	A	G	A	A	G	G	A	C	A
T170 - AY994265	C	G	T	C	G	A	G	A	A	G	G	A	C	A
T29 - AY994273	C	G	T	C	G	A	G	A	A	G	G	A	C	A
T34 - AY994274	C	G	T	C	G	A	G	A	A	G	G	A	C	A
T6 - AY994280	C	G	T	C	G	A	G	A	A	G	G	A	C	A
T70 - AY994282	C	G	T	C	G	A	G	A	A	G	G	A	C	A
T72 - AY994288	C	G	T	C	G	A	G	A	A	G	G	A	C	A
T. inhamatum	C	G	T	C	G	A	G	A	A	G	G	A	C	A

Fig. 1 Comparative analysis of 28S rDNA (D2) sequences from 38 *Trichoderma* isolates (identified by collect 'T' numbers), GenBank accession numbers and eight reference isolates (identified by species name) showing single nucleotide polymorphisms (shaded)

the isolates used in this study (as opposed to a GenBank accession sequence) was entered first there were no gaps in the alignment and the phylogenetic trees were consistent.

Nucleotide divergence was estimated using the Kimura's two-parameter (K2P) method (Kimura, 1980) and phylogenetic inference was performed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) and unweighted pair-group method analysis (UPGMA) as implemented by the MEGA software version 2.1 (Kumar et al., 2001). Bootstrap analysis was used to resample the data set (1000 resamplings) to provide a statistical test of the clusters/clades in the UPGMA and NJ tree (values shown at nodes).

AFLP fingerprinting

AFLP preselective and selective reactions were performed according to the manufacturer's instructions (Invitrogen, Grand Island, NY, USA) with some minor modifications: fungal DNA (400 ng) was cleaved with restriction enzymes *Eco*RI and *Mse*I at 37°C for 2 h; preselective amplification of a 10-fold dilution of ligated DNA product was carried out using *Eco*RI and *Mse*I primers; preselective amplification products were diluted 1 : 50 prior to selective amplification. The *Eco*RI selective primers were labelled with [γ-^{32}P]-ATP (3000 Ci/mmol).

A total of 36 primer pairs were screened on four *Trichoderma* isolates for protocol optimization and to identify the primer pairs that produced the most polymorphic fragments. All 48 isolates were screened with six AFLP primer combinations: E-AC/M-CTG, E-AG/M-CTG, E-AT/M-CAG, E-TA/M-CAC, E-TG/M-CTA and E-TA/M-CTC. DNA fragments generated from selective amplifications were separated on denaturing 6% polyacrylamide sequencing gels containing 7.5 M urea. The dried gels were exposed overnight at room temperature to X-ray films (Kodak X-Omat, Eastman Kodak Company, Rochester, New York) with one intensifying screen sandwiched in a film-cassette.

AFLP data collection and diversity analysis

The AFLP autoradiographs were scored for the presence (1) or absence (0) of all polymorphic bands generated a 48×250 binary data matrix from six primer combinations. The phylogenetic relationships among isolates obtained from sequence data were used to group the AFLP data set into four subpopulations to assess the extent to which the sequence-based clustering matched with that of AFLP-based clustering of these subpopulations. For this purpose, the AFLP data set was subjected to Nei's gene diversity index (H) to quantify intrapopulation variability and to investigate which subpopulations are genetically close to each other. Allele frequency-based Nei's genetic distance and UPGMA clustering methods were employed using Tools for Population Genetic Analysis (TFPGA) software version 1.3 (Miller, 1997).

Pairwise genetic similarities based on Jaccard's (1912) coefficient were applied to the AFLP data-subsets associated with isolates belonging to the clades: *T. pubescens–T. hamatum* and *T. harzianum–T. inhamatum*. The similarity matrices were subjected to sequential agglomerative hierarchical nested (SAHN) clustering using UPGMA in NTSYS-pc software version 2.0 (Rohlf, 1998).

AFLP marker association analysis

Bands in AFLP gels can potentially be excised, sequenced, cloned and converted to specific PCR markers that are referred to hereafter as simple PCR markers. The frequency of bands specific to a species was computed to identify markers that could differentiate between *T. harzianum* and *T. inhamatum* (12 individuals, 32 pairwise combinations) and between *T. pubescens* and *T. hamatum* (20 individuals, 99 pairwise combinations). The degree to which a particular marker differentiated the two species was then estimated as the ratio $D = [\#(0_a,1_b) + \#(1_a,0_b)/\text{Total } \# \text{ pairs}] \times 100$, where '*a*' is the first individual and '*b*' is the second individual in pairwise comparisons.

To identify markers that may be significantly associated with D- and V-type antagonism, the data on each marker were subjected to linear regression analysis using the model

$$y_i = \alpha + \beta x_i + \varepsilon_i$$

where, y_i is the antagonism value of individual i, α is the intercept, β is the linear regression coefficient, x_i takes value 1 for the presence of a band and 0 otherwise, ε_i is random error assumed to follow a normal distribution with mean 0 and constant variance σ^2_ε. Due to the binary nature of the independent variable x_i, the regression coefficient $\beta = \mu_1 - \mu_0$, where μ_1 and μ_0 are mean values of antagonism values corresponding to the presence and the absence of bands respectively for the markers concerned. Sixteen AFLP bands were removed from the data set prior to analysis because of their duplicate binary nature across all 48 isolates.

Results

28S rDNA phylogenetic analysis

Fourteen single nucleotide polymorphisms (SNPs) were observed in the 28S rDNA region across the 38 isolates tested, most of which were transversions (Fig. 1). There was no variation of the 28S rDNA region among isolates of the same *Trichoderma* species, suggesting a high level of sequencing accuracy and sequence conservation within species in this region of the genome. Nevertheless, clear haplotypes were observed for all species apart from *T. pubescens–T. hamatum* and *T. harzianum–T. inhamatum* groups. Certain individual SNPs and groups of SNPs were common across pairs of species (see below).

The NJ analysis of 28S rDNA sequence data from 38 isolates and eight *Trichoderma* spp. reference strains identified four distinct clades (Fig. 2). UPGMA analysis generated identical clusters but with higher bootstrap values of 90–95% (figure not shown) providing some level of validation of the putative phylogenetic

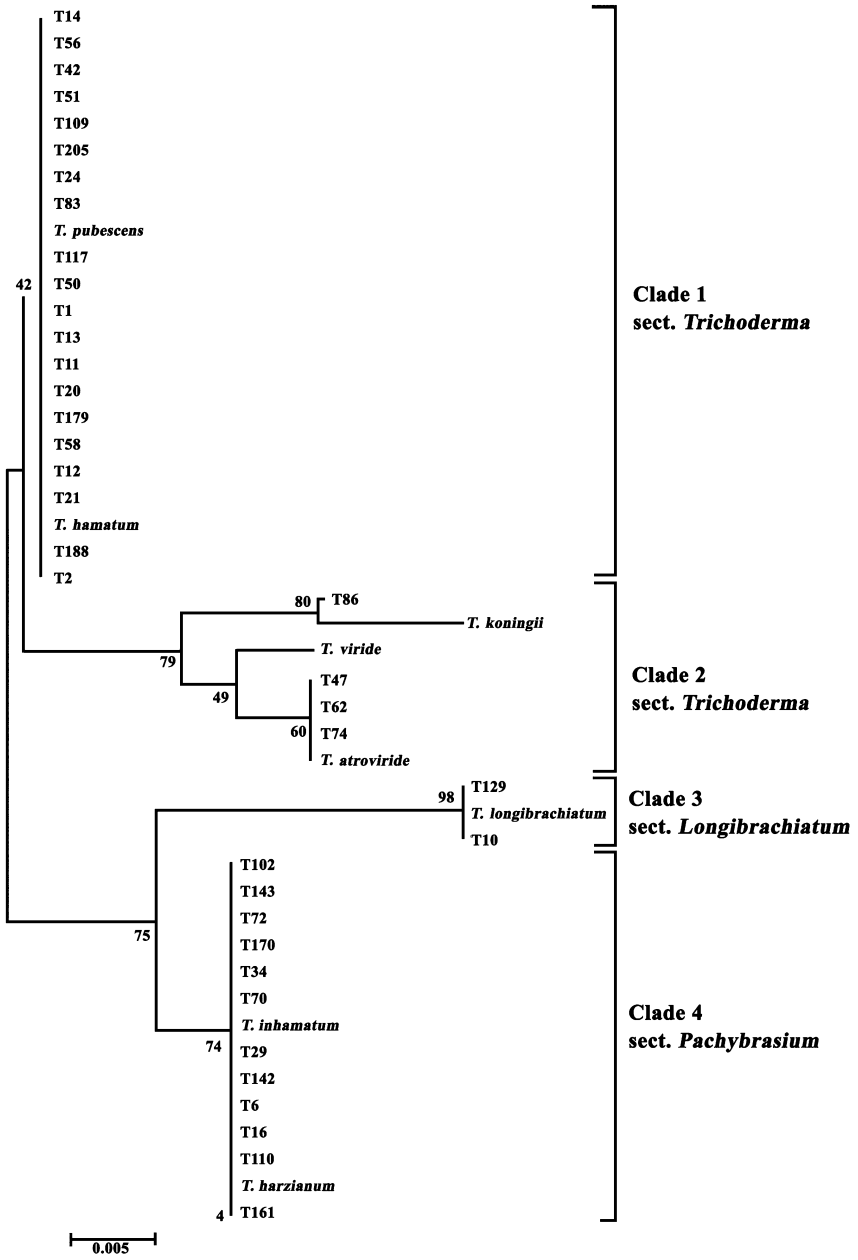

Fig. 2 Phylogenetic tree of 38 isolates inferred by neighbor-joining analysis of 28S rDNA sequences. The numbers given over branches indicate the percentage of 1000 bootstrap resampled data sets supporting the clades. Clustering was based on the Kimura's two-parameter

relationships from NJ analysis. Clade 1 included 20 isolates, *T. pubescens* DAOM 166.162 (ex-type strain), and *T. hamatum* DAOM 167057 (ex-neotype strain), all with identical 28S rDNA sequences. Clade 2 included four isolates, *T. koningii*, *T. atroviride* and *T. viride*, with seven SNPs each differentiating one or two of the species (Fig. 2). This clade had three subgroups, the first with one isolate clustering with *T. koningii* (CBS 979.70), the second with three isolates clustering with *T. atroviride* (DAOM 165779) and the third with *T. viride* (ATCC 28020) alone. The *T. koningii* cluster was differentiated by two unique SNPs (positions 55 and 62) but there were also two SNPs between the isolate and the reference strain at positions 116 and 150. There were only two SNPs (positions 25 and 116) between *T. viride* (ATCC 28020) from *T. atroviride* (DAOM 165779), and none of the other isolates in this study had those SNPs. There were four SNPs in *T. atroviride* but none of these was unique. Clade 3 contained the ex-type strain of *T. longibrachiatum* (CBS 816.68) and two isolates with six SNPs of which three were unique to this species clade (positions 36, 59 and 60). Clade 4 includes *T. inhamatum* CBS 273.78 (ex-type strain), *T. harzianum* CBS 226.95 (ex-neotype strain) and 12 isolates with three SNPs in common, one of which (position 112) was unique to this clade.

AFLP clade validations

A total of 250 reliable polymorphic bands were observed in the six AFLP gels, of which 16 were monomorphic across all 48 isolates. The large number of bands observed demonstrates that AFLP analysis is a robust and efficient method for detecting differences between *Trichoderma* isolates (see Fig. 3). The number

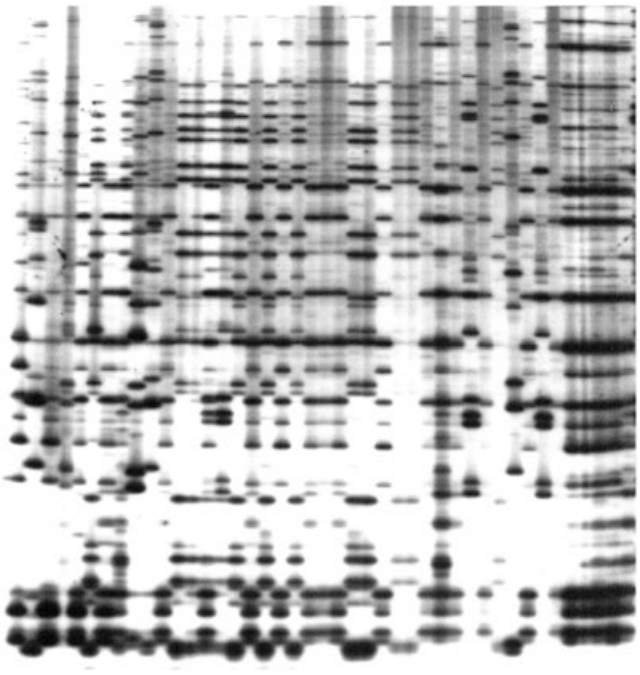

Fig. 3 Amplified fragment length polymorphism (AFLP) pattern of 48 *Trichoderma* isolates produced with E-AG and M-CTG selective primers. The order of the isolates is as presented in Table 1

of polymorphic bands per primer varied from 24 to 64: E-AC/M-CTG (24); E-AG/M-CTG (64); E-TA/M-CAC (40); E-AT/M-CAG (48); E-TA/M-CTC (28) and E-TG/M-CTA (46). TFPGA is a population analysis tool that allows the user to predefine the hierarchical population structure and, then using allele frequencies, to investigate the genetic distance between and within those defined subpopulations (Miller, 1997). In the present study, subpopulations were defined on the basis 28S rDNA sequence analysis which provided species or species complex designations. Based on this imposed subpopulation structure, the AFLP data set was subjected to Nei's gene diversity index (*H*) to quantify intrapopulation variability and to investigate which subpopulations are genetically close to each other. The resultant UPGMA dendrogram from this analysis (Fig. 4) has very high bootstrap values for all subpopulations (96–100). In contrast, when this analytical approach was repeated using our morphological-based species designations, the AFLP data did not support the resultant hierarchical population structure (data not shown).

AFLP marker association analysis

Genetic similarity coefficients were obtained using the Jaccard (1912) algorithm. This analysis does not consider '0,0' matches which is an important aspect in view of the likelihood that the absence of PCR bands (particularly in AFLP analysis) can be due to a number of reasons not directly related to genetic diversity. This analysis does not include direct comparison with reference strains from each of the *Trichoderma* species as it was impossible to include these due to quarantine restrictions. However, analysis of the AFLP data set, regarding the two major clades *T. pubescens–T. hamatum* and *T. harzianum–T. inhamatum*, suggests that there are two distinct subgroups within the *T. pubescens–T. hamatum* clade separated at the 20% similarity level (Fig. 5a). Similarly, there appears to be two distinct clusters within the *T. harzianum–T. inhamatum* clade separated at the 28% similarity level (Fig. 5b). On this basis we attempted identifying candidate AFLP markers able to differentiate amongst these subgroups (*T. pubescens–T. hamatum* and *T. harzianum–T. inhamatum*). For the 12 isolates within the *T. harzianum–T. inhamatum* clade (eight isolates in subgroup 1 and four isolates in subgroup 2) eight markers: M47, M48, M49, M80, M85, M131, M132 and M134 provided 100% differentiation between the two groups. Similarly, for the 20 isolates within *T. pubescens–T. hamatum* clade (nine isolates in subgroup 1 and 11 in subgroup 2), one marker M74 provided 100% differentiation between the two groups. Six of the nine markers were generated by primer combination E-AG/M-CTG the rest were generated by primer combination E-AT/M-CAG.

We also attempted to identify candidate AFLP markers associated with antagonistic characteristics. V-type and D-type antagonism data for 35 *Trichoderma* isolates was compared with the AFLP data set of 234 bands. Nine AFLP markers were significantly ($P = 0.05$) correlated with the D-type antagonism (Table 3a). The two (independent) markers M25 and M40 exhibit the tightest association with D-type antagonism. Eight markers were significantly correlated with

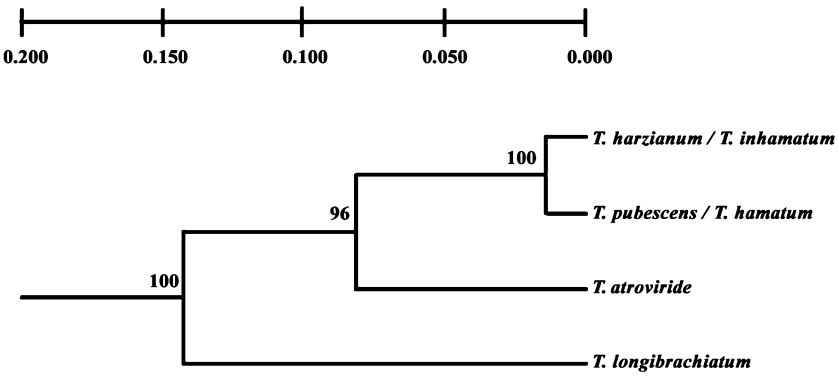

Fig. 4 Genetic relationship of *Trichoderma* subpopulations inferred through unweighted pair-group method analysis (UPGMA) clustering of the amplified fragment length polymorphism (AFLP) data set using Nei's genetic distance. The subpopulations were defined on the basis of 28S rDNA phylogenetic sequence analysis

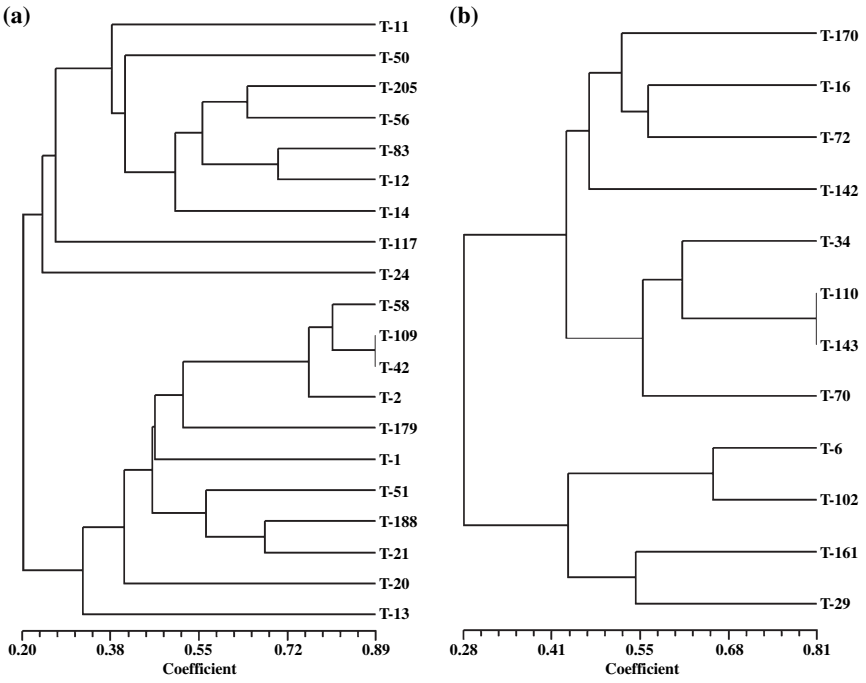

Fig. 5 (a) Genetic relationship inferred through unweighted pair-group method analysis (UPGMA) clustering of amplified fragment length polymorphism (AFLP) data for 20 isolates belonging to *Trichoderma pubescens–T. hamatum* using Jaccard (1912) similarity coefficient. (b) Genetic relationship inferred through UPGMA clustering of AFLP data for 12 isolates belonging to *T. harzianum–T. inhamatum* using Jaccard (1912) similarity coefficient

V-type antagonism, with markers M21, M155 and M196 being the most tightly associated (Table 3a). Several of these markers are significantly associated with each other based on Fisher's exact probability test suggesting these markers may be genetically linked on the same chromosome (Table 3b). These markers associated with antagonism can be considered for conversion to simple PCR-based assays (Brugmans et al., 2003).

Discussion

The exact characterization and identification of *Trichoderma* strains at the species level is an important first step in systematically utilizing the full potential of fungi in specific applications (Lieckfeldt et al., 2001). Species designations based on the evaluation of 40 or more morphological traits (Hermosa et al., 2000; Lieckfeldt et al., 2001) have been shown to provide a reliable diagnostic method in expert hands. However, this presents a major time and cost bottleneck for rapid large-scale screening to identify agriculturally useful isolates (Kubicek et al., 2002). We have tried to use the evaluation of six morphological characteristics as a short cut diagnostic process (Srilakshmi et al., 2001). However, when this data set (appropriately separated into quantitative and qualitative parameters in the current study) was subjected to UPGMA diversity analysis, the resultant plot (not shown) failed to differentiate 19 of the 48 isolates which fell into identical clusters of two, three or four isolates, while a further six clusters were only differentiated at the 99% similarity level. Thus, we have concluded, in accordance with others (Kindermann et al., 1998; Kullnig-Gradinger et al., 2002) that routine morphological characterization does not provide an accurate means of identifying *Trichoderma* species.

Several groups are investigating the molecular phylogeny of *Trichoderma*, with particular progress recently made in the *T. harzianum*/*T. inhamatum* clade by Chaverri et al. (2003). For genus-wide studies, the molecular classification proposed by Kullnig-Gradinger et al. (2002) is widely used as the benchmark for *Trichoderma* classification. Sequence-based diagnostic analysis has been useful in establishing coarse-scale phylogeny of Ascomycetes and defining family-level groups (Berbee et al., 1995). In addition, it has been shown that single gene trees are highly correlated with multiple gene trees at the clade level (Kullnig-Gradinger et al., 2002). Thus, in this study we have pursued the use of the commercially available MicroSeq Fungal Identification System for the classification of isolates obtained from four different groundnut cultivation sites in southern India. By using ex-type strain sequences from GenBank as references, it has been possible to classify the 48 isolates into four phylogenetic clades. This approach appears to be considerably more reliable than the use of routine morphological characterization. In many cases, SNPs unique to a given clade have been validated amongst multiple isolates in this study (clades 1 and 4). These are now good candidates for conversion into multiplexed PCR-based SNP assays to form the basis of a more simple diagnostic process (Van Eijk et al., 2004). Other potential clade-specific SNPs identified in this study still require validation amongst a larger selection of isolates (clades 2 and 3). However, the primary goal of this study was to contribute to the development of a practical solution for a rapid and cost-effective yet accurate and precise method of classifying newly collected isolates of *Trichoderma*. In this study we have been unable to differentiate the *T. harzianum–T. inhamatum* and *T. pubescens–T. hamatum* clades based on 28S rDNA (Kullnig-Gradinger

Table 3
(a) AFLP markers significantly associated with antagonism; (b) association between AFLP markers based on Fisher's exact test

Marker	Primer and approximate band size (bp)	Intercept	Slope	T-Prob	Adjusted R^2	Duplicate markers	Linked markers
(a) D-type ($n = 14$)							
M199	E-TA/M-CTC 550	2.02	1.73	0.049	22.6		
M44	E-AG/M-CTG 600	1.69	1.61	0.008	41.7		M40
M105	E-TA/M-CAC 575	1.78	1.36	0.032	27.5		
M152	E-AT/M-CAG 750	2.79	−1.22	0.050	22.3		M165
M165	E-AT/M-CAG 475	2.93	−1.33	0.028	28.7		M152
M70	E-AG/M-CTG 625	1.87	1.38	0.042	24.3		
M40	E-AG/M-CTG 2000	3.36	−1.70	0.004[a]	47.7	M173	M44
M25	E-AG/M-CTG >2000	4.50	−2.61	0.001[a]	61.7		M27
M27	E-AG/M-CTG >2000	3.60	−1.70	0.019	32.6		M25
V-type ($n = 21$)							
M21	E-AC/M-CTG 300	3.14	1.20	0.026[a]	20.4		M35, M155, M196
M155	E-AT/M-CAG 600	3.14	1.19	0.022[a]	20.7	M191	M21, M35, M196
M196	E-TA/M-CTC 590	3.21	1.13	0.026[a]	20.6		M21, M35, M155
M187	E-TA/M-CTC 900	3.14	0.80	0.045	16.0		
M35	E-AG/M-CTG >2000	3.05	0.78	0.048	14.8		M21, M155, M196
M190	E-TA/M-CTC 725	3.67	−0.73	0.051	15.0		
M239	E-TG/M-CTA 500	3.61	−0.72	0.052	14.9		
M112	E-TA/M-CAC 400	3.77	−0.62	0.045	16.2		

	M199	M44	M105	M152	M165	M70	M40	M25	M27
(b) D-type ($n = 14$)									
M199									
M44	0.110								
M105	0.604	0.203							
M152	0.692	0.657	0.238						
M165	0.769	0.500	0.133	***0.002***					
M70	0.066	0.095	0.095	0.594	0.720				
M40	0.110	***0.023***	0.203	0.238	0.133	0.095			
M25	0.275	0.110	0.110	0.308	0.231	0.066	0.110		
M27	0.396	0.275	0.275	0.154	0.500	0.176	0.275	***0.033***	

	M21	M187	M35	M190	M239	M112	M155	M196
V-type ($n = 21$)								
M21								
M187	0.222							
M35	***0.031***	0.664						
M190	0.624	0.455	0.392					
M239	0.211	0.556	0.314	0.255				
M112	0.458	0.259	0.102	0.430	0.414			
M155	***0.001***	0.202	***0.026***	0.656	0.237	0.421		
M196	***0.001***	0.202	***0.031***	0.656	0.263	0.458	***0.001***	

Duplicate markers: markers that have exactly the same 0–1 data pattern across isolates.
Linked Markers as inferred from Fisher's exact probability test (Table 3b).
[a]Markers with most significant association with D- and V-type antagonism.
Bold italics indicate markers significantly associated with each other.
AFLP, amplified fragment length polymorphism.

et al., 2002). *Trichoderma harzianum* and *T. inhamatum* have been consistently troublesome to differentiate based on sequence-based molecular phylogeny, physiological or morphological characters (Hermosa et al., 2000; Kubicek et al., 2002; Samuels et al., 2002; Chaverri et al., 2003). In contrast, *T. pubescens–T. hamatum* has been differentiated using ITS1 and ITS2 in other studies (Kindermann et al., 1998).

To our knowledge this is the first report to test the value of AFLP fingerprints as the basis of a diagnostic process for *Trichoderma* isolates. We have shown AFLP analysis to offer a very high level of differentiation, far beyond any other single diagnostic process. AFLP analysis has differentiated all isolates tested in this study. One of the main advantages of AFLP analysis is that it is able to simultaneously survey many independent points on the genome; 234 polymorphic bands in this study from just six primer combinations. This means that AFLP is a powerful tool in molecular fingerprinting of isolates and for studying relationships amongst isolates at the population and species level (Brown, 1996). In contrast, sequence-based analysis (based on gene genealogies) is more useful in defining higher order phylogenetic relationships between clades (Berbee et al., 1995). As the two approaches reflect independent evolutionary processes, complementing sequence data with AFLP data may provide the most robust means of characterizing new isolates. In the

current study, *a priori* population structure (based on sequence data) was applied to the AFLP data set before analysis based on Nei's gene diversity index. The relationship between the resultant clades was supported by very high (96–100) bootstrap values (Fig. 4). This infers that classification of *Trichoderma* isolates based on AFLP diversity analysis is broadly congruent with sequence-based species designations, as previously reported for *Fusarium oxysporum* (Baayen et al., 2000). When this analytical approach was repeated using our morphological-based species designations, the AFLP data did not support the resultant hierarchical population structure. Thus, this further supports our earlier conclusion that the reduced morphological classification of species employed in this study is not reliable.

AFLP analysis of individual sequence-based clades generates clear subgroups within *T. harzianum–T. inhamatum* and *T. pubescens–T. hamatum* (Fig. 5a,b), which may relate to the two different species in each pair. Moreover, we have shown the usefulness of AFLP for high-resolution studies by identifying candidate AFLP bands that differentiate between the *T. harzianum–T. inhamatum* and *T. pubescens–T. hamatum* isolates in this study. However, none of these markers was correlated with the two antagonistic characteristics assessed.

It is widely reported that *T. harzianum–T. inhamatum* is the most abundant taxon in virtually all habitats (Kubicek et al., 2002). However, of the isolates investigated in this study (preselected on the basis of antagonism to *Aspergillus*), we found that the predominant taxon was *T. pubescens–T. hamatum* (52% of isolates) followed by *T. harzianum–T. inhamatum* (32%). Abundance of *A. flavus* antagonistic *Trichoderma* species in groundnut fields could provide an effective biocontrol strategy for reducing *A. flavus* populations and consequently the aflatoxin contamination of groundnut kernels. Although, further studies would be needed to understand the interaction between specific *Trichoderma* species and *A. flavus* populations in groundnut fields. The global *Trichoderma* biodiversity initiative has reported the identification of a high proportion of new species from collections in Asia and Europe (Kubicek et al., 2002; Kullnig-Gradinger et al., 2002; Bisset et al., 2003). However, all the isolates studied here cluster with an already well-defined species. As we have only studied the 48 most antagonistic isolates from the collection of 212 isolates, this may suggest that the best *Trichoderma* biocontrol agents against *A. flavus* are to be found in already defined species: predominantly *T. harzianum–T. inhamatum* and *T. pubescens–T. hamatum*.

The search for the most effective *Trichoderma* spp. and isolates as biocontrol agents for reducing toxigenic *Aspergillus* spp. would be significantly enhanced by the development of rapid and precise diagnostics for *Trichoderma* species and for diversity groups with high levels of antagonistic behaviour against *Aspergillus* species. The current study suggests that AFLP may prove to be an appropriate means of detecting species-specific variation in the *Trichoderma* genus. Similarly a number of AFLP bands have been associated with the two types of antagonistic behaviour (V- and D-type). Once robust species and/or antagonism-specific marker associations have been widely validated, these should be primary candidates for conversion into simple PCR assays. It is likely that PCR diagnostics based on SNPs observed with these AFLP bands would provide a simple and cost-effective system. In particular, by sequencing bands from different species it will be possible to remove those co-migrating bands that do not share a common ancestry. It may be that in the first instance this approach should be followed on a species by species basis. Similarly, increased robustness may be achieved by multiplexing these markers with SNPs identified in 28S rDNA analysis.

The results from this study suggest that AFLP and ITS amplification products may be useful material for the development of simple PCR-based diagnostic assays that can assist in the development of *Aspergillus* biocontrol systems for groundnut and other crops. As *Trichoderma* is used as a biocontrol agent in many cropping systems (Hjeljord and Tronsmo, 1998; Samuels et al., 2002; Hermosa et al., 2004), it is likely that simple diagnostics for *Trichoderma* species identification will have impacts far beyond control of aflatoxin in groundnut.

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