Intraspecific molecular variation among *Trichoderma harzianum* isolates colonizing mushroom compost in the British Isles

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The genetic diversity in *Trichoderma harzianum* isolates from mushroom compost was assessed using various molecular techniques. Restriction fragment length polymorphism (RFLP) analysis of ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) divided the 81 isolates into three major groups, 1, 2 and 3. There was no variation within a group in rDNA, while a low degree of polymorphism was detected in mtDNA. Random amplified polymorphic DNA (RAPD) analysis of 30 randomly chosen isolates, with six primers, in general confirmed the RFLP groups. Nucleotide sequence determination of rDNA internal transcribed spacer (ITS) 1 revealed three distinct ITS types, 1, 2 and 3, possessed by isolates from the respective groups 1, 2 and 3. Based on these molecular data, group 2 isolates, which are aggressive colonizers of mushroom compost, could be clearly distinguished from the isolates belonging to the other two groups.

Keywords: *Trichoderma harzianum*, mushroom compost, molecular variation, systematics

INTRODUCTION

Trichoderma, a common soil fungus, remains a potential threat to the mushroom industry following the green mould epidemic in the British Isles during 1985–86 and more recently in late 1990 and 1991. Losses have been estimated at £3–4 million to the UK and Irish mushroom industries (Fletcher, 1990). *Trichoderma harzianum* has most frequently been associated with aggressive colonization of mushroom compost, although *T. viride*, *T. pseudokoningii*, *T. hamatum* and *T. longibrachiatum* have also been isolated from compost (Seaby, 1989). The genus *Trichoderma* lacks a discrete species concept because of variation within and among species groups, defined as species aggregates by Rifai (1969). Bissett (1991) proposed 'sections' to accommodate morphologically similar forms within the species aggregates of Rifai (1969). *T. harzianum* isolates from mushroom compost all conform to the criteria currently used for identification of the species

(Rifai, 1969); however, these have been differentiated into three biological forms termed Th1, Th2 and Th3 by Seaby (1987) and z, x and y strains by Doyle (1991). These three groups of *T. harzianum* differ in their growth rate and time and pattern of sporulation when grown under specified cultural conditions; however, not all isolates can be reliably identified by these characters. *T. harzianum* isolates also differ in their aggressiveness in colonizing the compost in competition with *Agaricus bisporus* (Doyle, 1991).

In recent years, various molecular techniques have been used in the systematics of phytopathogenic and other (commercially important) fungi to assess intra- and interspecific variation and to determine phylogenetic relationships (Buchko & Klassen, 1990; Chen *et al.*, 1992; Förster *et al.*, 1990; Gaudet *et al.*, 1989; Levy *et al.*, 1991; Moody & Tyler, 1990a, b; O'Donnell, 1992; Vaillancourt & Hanau, 1992). Meyer *et al.* (1992) used a DNA fingerprinting technique to analyse the nine species aggregates of *Trichoderma* and recognized only five groups. In the present study, we have used restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) analyses and sequencing of the internal transcribed spacer (ITS) 1 region in the ribosomal DNA gene block to estimate the intraspecific divergence among isolates of *T. harzianum* and to classify the aggressive Th2 isolates.

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Abbreviations: ITS, internal transcribed spacer; mtDNA, mitochondrial DNA; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism.

The EMBL/Genbank/DBJ accession numbers for the nucleotide sequences reported in this paper are X73686 to X73703.

Table 1. Source and rDNA, mtDNA and ITS 1 sequence groupings of *T. harzianum* isolates from mushroom compost

No.	Isolate code	rDNA grouping*	mtDNA grouping*	ITS 1 sequence type	RAPD analysis
Northern Ireland†					
1	Th2A	2	2d	2	+
2	Th2F	2	2b		
3	Th1H	1	1a		
4	Th1‡	2	2b	2	+
5	Th3K	1	1a		
6	ThL	1	1g		+
7	Th1M	1	1a		+
8–9	Th1 (a, b)	1	1a		
10	Th1 (c)	1	1a		+
11	Th2a	2	2c		
12	Th2b	2	2a		
13	Th3e	1	1a		
14–19	Th1 (1–6)	1	1a		
20	Th1 (7)	1	1f		+
21	Th1 (8)	1	1f		
22	Th2 (1)	2	2a	2	+
23–31	Th2 (2–10)	2	2a		
32	Th2 shiitake	2	2a		+
33–39	Th3 (1, 3, 6, 8, 9, 10, 11)	2	2a		
40	Th3 (4)	3	3a	3	+
41	Th3 (5)‡	2	2c		+
42	Th3 (7)	3	3a	3	
43	Th3 McG	3	3e	3	+
44–47	S (1, 3, 4, 5)	1	1a		
48	S (6)	2	2d		
49–50	S (7, 9)	2	2a		
51	TD1	1	1g	1	
52	TD2	1	1a		
53	TD3	2	2a		
54	TD5	3	3b		
55	TD6	3	3a		+
56	TD7‡ (IMI 359825)	3	3b	3	+
57	TD12	1	1e	1	+
58	TD13	1	1d		+
59	TD15‡	1	1a	1	+
England†					
60	KPNT‡ (IMI 359824)	2	2c	2	+
61	T7JF	2	2e		
62	T8JF	2	2e		
63	T9JF	2	2e		+
64	T11JF	3	3d	3	+
65	T13JF	2	2c		+
66	T18JF	3	3d		+
67	T20JF	1	1a		
68	T23JF	1	1b		+
69	T24JF	2	2d		
70	T26JF	1	1a		
71	T28JF‡ (IMI 359823)	1	1a	1	+
72	T35AJF	1	1c		
73	T35BJF	1	1c		
74	T41JF	1	1c	1	+
Republic of Ireland†					
75	A006022	3	3c	3	+
76	A006027	3	3c		+
77	A007036	1	1a	1	
78	A007040	1	1a		+
79	D010011	2	2a	2	+
80	Z021003	2	2a		+
81	UCD13	1	1b	1	+

* RFLP groups 1, 2 and 3 correspond to biological forms Th1, Th2 and Th3, respectively (Seaby, 1987).

† Countries in which isolates were collected.

‡ Identity confirmed as *T. harzianum* using the criteria of Rifai (1969) by Dr M. A. J. Williams (International Mycological Institute [IMI], Egham, UK) and isolates 56, 60 and 71 have been deposited with IMI.

+, Isolates included for RAPD analysis.

METHODS

Fungal growth conditions and DNA extraction. Eighty-one isolates of *T. harzianum* (Table 1) were used in this study. All isolates were identified as *T. harzianum* according to the criteria of Rifai (1969). Isolates 1–59 came from Northern Ireland. Isolates 60–74 came from England and were provided by Dr John Fletcher, ADAS, Wye. Isolates 75–81 came from the Republic of Ireland and were provided by Dr Owen Doyle, UCD, Dublin. Isolate IMI 110150 was obtained from the International Mycological Institute and used only to extract mtDNA for use as a probe for RFLP analysis.

Fungal cultures were maintained on potato dextrose agar (Oxoid) at 25 °C and mycelium for DNA extraction was grown in liquid shake cultures (120 r.p.m.) at 25 °C in glucose Casamino acid medium (1 l contained glucose, 15.0 g; KH_2PO_4 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; casein hydrolysate, 4.6 g and 2 ml trace element solution) for 3 d. DNA was extracted from 300 mg freeze-dried mycelial powder essentially following the method of Raeder & Broda (1985) but with an additional phenol/chloroform extraction step.

For mitochondrial DNA (mtDNA) isolation, total nucleic acid was extracted from 6 g freeze-dried mycelial powder. Mitochondrial DNA was purified by caesium chloride/bisbenzimidazole gradient centrifugation (Beckman Ti 70 rotor, 25 °C, 40000 r.p.m., 60 h) following the method of Garber & Yoder (1983).

Southern blotting, hybridization and autoradiography. DNA was quantified by ethidium bromide fluorescence on a UV transilluminator with known quantities of λ DNA (Sambrook *et al.*, 1989). Genomic DNA (1–2 μg) was digested with 25 U restriction enzyme until completion at 37 °C according to the manufacturer's instructions (Promega). Digested DNA was electrophoresed in 0.8% (w/v) agarose gels for 13 h at 60 V (electrophoresis buffer: 89 mM Tris, 89 mM boric acid, 2 mM EDTA) with *Hind*III-digested λ DNA and/or digested pGEM as molecular size markers (Promega), transferred to a nylon membrane (Hybond N, Amersham) by capillary transfer (Sambrook *et al.*, 1989) and immobilized by baking at 80 °C for 90 min.

Probes. For ribosomal DNA (rDNA) analysis, Southern blots were probed with plasmid pMY60 containing a complete rDNA unit from *Saccharomyces carlsbergensis* Hansen (Verbeet *et al.*, 1983). For mitochondrial genome probing, mtDNA purified from isolates T28JF, ThI and IMI 110150 was used.

Probes were labelled with [α - ^{32}P]deoxyadenosine 5'-triphosphate (Amersham) using the Prime-a-Gene labelling system (Promega) and separated from unincorporated nucleotides on a G-50 Sephadex column. Hybridization was performed in a hybridization oven (Hybaid) at 65 °C following the procedure described by Sambrook *et al.* (1989). The prehybridization wash was for 3 h and post-hybridization washes consisted of three washes of 30 min each in $2 \times \text{SSC}$ and 0.1% SDS, and three washes of 30 min each in $0.1 \times \text{SSC}$ and 0.1% SDS. Finally membranes were wrapped with clingfilm and exposed to Hyperfilm β -max (Amersham) at -70 °C using intensifying screens.

PCR amplification. For RAPD analysis, primers A3 (AGTCAGCCAC), A11 (CAATCGCCGT), A13 (CAGCACCAC), B6 (TGCTCTGCC), B7 (GGTGACGAG) and B10 (CTGCTGGGAC), supplied by Operon Technologies, CA, USA, were used. Reaction mixtures contained 5 μl of appropriately diluted genomic DNA (20–30 ng), 5 μl of *Taq* 10 $\times$

buffer, 8 μl of 100 μM each of deoxynucleotide triphosphate (dNTP) mix, 5 μl of primer (15 $\mu\text{g ml}^{-1}$), 0.25 μl of *Taq* DNA polymerase (5 U μl^{-1}) and sterile distilled water (up to a total volume of 50 μl). Amplification reactions consisted of 45 cycles in a thermal cycler (Perkin-Elmer-Cetus). Each cycle consisted of 1.5 min at 94 °C, 2 min at 30 °C and 3 min at 72 °C, followed by a final extension time of 7 min at 72 °C. PCR products (20 μl) were visualized in 1.4% (w/v) agarose gels stained with ethidium bromide (0.4 $\mu\text{g ml}^{-1}$).

For data analysis, each amplified fragment with all six primers was treated as a separate character. DNA fragments of the same size were assumed to represent the same genetic locus and scored as either present or absent. The cluster analysis of the data was done based on a similarity matrix derived from the formula: number of shared characters/total number of characters. The dendrogram was generated by the 'group average' method on the program GENSTAT 5 (Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, UK).

The ITS 1 region of rDNA was amplified using biotinylated ITS 1 and ITS 2 primers (White *et al.*, 1990) supplied by Operon. For each isolate, two 100 μl reactions using one of the biotinylated primers with appropriate non-biotinylated primers were performed. PCR mixtures contained diluted genomic DNA (40–50 ng), reaction buffer, 200 μM of each dNTP, 0.4 μM each of primers 1 and 2, and 2.5 units of *Taq* DNA polymerase; they were subjected to 30 cycles of 1.5 min at 94 °C, 2 min at 45 °C and 3 min at 72 °C.

Sequencing. Template DNA (100 μl) was immobilized using 100 μl Dynabeads (Dynal, Norway) according to the manufacturer's instructions. Direct solid-phase sequencing (Hultman *et al.*, 1989) was done by the chain-termination method (Sanger *et al.*, 1977) using the non-biotinylated primer as the sequencing primer with a T7 DNA polymerase sequencing kit (Pharmacia). Sequencing reactions and electrophoresis were carried out as described by Gyllenstein & Erlich (1988).

For data analysis, the percentage of shared restriction endonuclease fragments (F) and the nucleotide sequence divergence estimates (p) between various RFLP groups were calculated using the following formulae: $F = (2N_{xy})/N_x + N_y$ and $p = (-\ln F)/r$, where N_{xy} is the number of shared fragments between two samplings, N_x and N_y are the total number of fragments in each of the samples and r is the number of nucleotide bases for the restriction enzyme (Nei & Li, 1979; Nei & Tajima, 1983). The sequence data were aligned using the CLUSTAL V package (Higgins *et al.*, 1992). Percentage divergence was calculated as follows: $[(Ts + Tv + I/D)/(sequence length)] \times 100$, where Ts is transition, Tv is transversion, I is insertion and D is deletion. One or more base I/D was scored as one. The length of the smaller sequence was used for computation.

RESULTS

Analysis of rDNA RFLP patterns

Ribosomal DNA restriction patterns generated by the enzymes *Eco*RI, *Sac*I and *Clal* placed the 81 isolates of *T. harzianum* into three distinct groups (Table 1). Within each group all isolates gave a similar pattern with each of the enzymes tested. All three groups shared a band of approximately 3.1 kb in *Eco*RI digestion (Fig. 1a). A

Fig. 1. Restriction fragment patterns of rDNA from *T. harzianum* isolates digested with (a) *EcoRI* and (b) *SacI*. Molecular size markers refer to *HindIII*-digested λ DNA. The *T. harzianum* rDNA group numbers of the isolates are given in parentheses. Isolates in each group are given in Table 1.

second band of approximately 3.6 kb was seen with group 1 isolates and of approximately 4.7 kb with group 3 isolates. With group 2 isolates, a second band was not detected, but the signal intensity of the 3.1 kb band was strong, suggesting the presence of two co-migrating fragments. With *SacI* digestion (Fig. 1b), a common band of approximately 2 kb was observed with group 1 and 2 isolates. Group 1 isolates differed from group 2 isolates in having two more bands of 3.3 and 2.4 kb, whereas group 2 isolates produced a second band of 6.9 kb. Group 3 isolates yielded two bands of 4.7 and 2.7 kb with *SacI* digestion.

ClaI digestion resulted in three bands for all isolates, of which two bands of approximately 2.2 and 1.1 kb were common to all isolates. The third band was polymorphic and confirmed the groupings (data not shown).

Two out of the six enzymes used (*HindIII* and *PstI*) yielded a single band for group 1 and 2 isolates (approximately 9 and 12 kb respectively), whereas rDNA from group 3 isolates was not digested. Group 3 isolates could be distinguished by the presence of a *BamHI* site which resulted in a 9 kb fragment, whereas no *BamHI* site was found in the ribosomal repeat units of either group 1 or group 2 isolates.

By summation of the size of fragments from various digestions, the approximate unit length of rDNA was

calculated to be 8–9 kb for group 1 and 3 isolates and 11–12 kb for group 2 isolates.

Polymorphisms in mtDNA

Mitochondrial DNA RFLPs divided the 81 *T. harzianum* isolates into three major groups (1, 2 and 3) when probed with mtDNA from the isolate Th1. Groupings were the same as those identified by rDNA analysis. For further analysis, mtDNA extracted from one isolate in each major group was used as a probe to determine the polymorphisms within the group.

Isolates were placed in subgroups according to the limited degree of variation observed within a group (Table 1). Representative isolates of each subgroup from all three major groups were chosen and heterologous hybridizations were carried out. The size of the mitochondrial genome of each group was calculated by summation of fragment sizes (from various digestions) on ethidium bromide stained agarose gels.

Variation in group 1 isolates. mtDNA from isolate T28JF (approximately 38–40 kb) was used to probe *HindIII* digests of group 1 isolates. Out of 35 isolates, 24 fell into the subgroup 1a and the remaining 11 isolates formed six more subgroups (1b–1g) (Table 1). Restriction enzyme digestion patterns of a representative isolate from each of the seven subgroups are shown in Fig. 2a. *HindIII* digestion yielded 9–11 fragments, of which seven bands were shared by all seven subgroups. The proportion of shared fragments (*F*) was between 61 % and 95 % for the various subgroups. *EcoRI* digestion produced six to eight bands, of which four were common to all subgroups (data not shown); the subgrouping of isolates was similar to that obtained using *HindIII*.

Variation in group 2 isolates. Restriction fragment patterns generated for group 2 isolates, using *HindIII* and mtDNA from isolate Th1 as probe (approximately 60–62 kb), divided the 36 isolates into five subgroups (2a–2e). Twenty-four isolates (all from Northern Ireland and the Republic of Ireland) formed the large subgroup 2a. *HindIII* digestion of group 2 isolates generated 20–23 bands (Fig. 2b) with 16 bands common to all subgroups. The *F* values ranged from 78 % to 98 % between the various subgroups. These subgroupings were confirmed by *EcoRI* digestion (data not presented).

Variation in group 3 isolates. Homologous probing with mtDNA from the isolate IMI 110150 (approximately 28–30 kb) separated the 13 isolates of group 3 into five subgroups (3a–3e) in both *HindIII* and *EcoRI* digestions. Isolates from the Republic of Ireland and England formed subgroups 3c and 3d respectively. Isolates from Northern Ireland were separated into three more subgroups (3a, 3b and 3e). *HindIII* digestion produced 11–13 fragments (Fig. 2c), of which nine were shared by all subgroups with *F* values ranging from 83 % to 96 %.

Heterologous probing. In cross-hybridization experiments using heterologous mtDNA probes, only two or three co-migrating fragments were observed between

Fig. 2. mtDNA RFLP patterns of *T. harzianum* isolates digested with *Hind*III: (a) group 1 isolates probed with mtDNA from the group 1 isolate T28JF; (b) group 2 isolates probed with mtDNA from the group 2 isolate Th1; (c) group 3 isolates probed with mtDNA from the group 3 isolate IMI 110150. Subgroupings of the isolates are given in parentheses; isolates in each group and subgroup are given in Table 1. Molecular size markers refer to *Hind*III-digested λ DNA and digested pGEM.

groups 1, 2 and 3, resulting in low *F* values (Table 2). The signal intensity was reduced relative to homologous probings. When group 2 isolates were hybridized with heterologous probes (mtDNA from either group 1 or group 3 isolates) very few fragments hybridized and high molecular mass fragments were visible only after prolonged exposure (7 d).

RAPD analysis of *T. harzianum* isolates

To estimate the intraspecific diversity of *T. harzianum* isolates from mushroom compost, six RAPD primers were used with 30 randomly chosen isolates. These represented all three major RFLP groups as indicated in Table 1. The RAPD patterns produced by all six primers differentiated the 30 isolates, with some exceptions, into the same three groups as revealed by RFLP analyses. None of the primers used unified the three groups. Fig. 3

illustrates the RAPD pattern of eight isolates from each group with primers A3 and A13 (data for primers A11, B6, B7 and B10 are not presented).

The isolates formed three main clusters on the dendrogram (Fig. 4), corroborating the results from RFLP analyses. Within cluster 1, representing group 1 isolates, similarity varied from 72% to 100%. Group 2 isolates in cluster 2 were identical. The third cluster, representing group 3 isolates, showed similar variation to group 1 isolates, of 70% to 100%. Cluster analysis suggests a closer relationship between groups 1 and 2 than between either of these two groups and group 3.

Sequence analysis of the ITS 1 region

Sequencing of the ITS 1 region of 18 *T. harzianum* isolates (indicated in Table 1) revealed that there were three

Fig. 3. Randomly amplified polymorphic DNA patterns of *T. harzianum* isolates with primers A13 and A3 (M, digested pGEM used as molecular size marker).

distinct ITS types 1, 2 and 3 (Fig. 5), corresponding to the groups 1, 2 and 3 from RFLP and RAPD analyses. The ITS 1 sequence of all five group 2 isolates (202 bp) was identical. The ITS 1 sequence of all six group 3 isolates (182 bp) was identical. Within the seven group 1 isolates (201–203 bp), insertion/deletion of two thymines (at positions 115 and/or 151 and 168) was the only variation observed within the group (Fig. 5). Group 1 isolates

Table 2. Percentage of shared fragments (*F*) within and between the *T. harzianum* groups from homologous and heterologous hybridizations of mtDNA

Isolates in each group are given in Table 1.

Probe DNA	Endonuclease	Target DNA		
		Group 1	Group 2	Group 3
Group 1	<i>Hind</i> III	60–94	23–26	20–25
	<i>Eco</i> RI	66–83	12–13	16–18
Group 2	<i>Hind</i> III	20–45	78–97	13–22
	<i>Eco</i> RI	22–38	70–97	14–21
Group 3	<i>Hind</i> III	22–25	17–19	81–95
	<i>Eco</i> RI	28–32	29–33	75–92

Fig. 4. Clustering of isolates of *T. harzianum* from pairwise comparison of RAPDs by the group average method (see Table 1 for details of isolates). Isolate UCD13 in cluster 1 is representative of group 1 isolates ThL, A001040 and Th1M. Isolate ThI in cluster 2 is representative of group 2 isolates Th2 shiitake, Th2(1), Th3(5), D010011, Z021003, T9JF, KPNT, T13JF and Th2A.

Fig. 5. Nucleotide sequence of the ITS 1 region of *T. harzianum* isolates. (a) Representatives of group 1 isolates (type 1 sequences). Isolate A007036 had an identical sequence to isolate T28JF. Isolate TD1 had an identical sequence to isolate TD15. (b) Representative of group 2 isolates (type 2 sequence). Th2A, Th1, Th2(1), KPNT and D010011 had an identical sequence. (c) Representative of group 3 isolates (type 3 sequence). Th3(4), Th3(7), Th3McG, TD7, T11JF, and A006022 had an identical sequence. Identical bases are indicated by -. For list of isolates, see Table 1.

Table 3. Divergence between the nucleotide sequences of the ITS 1 region of *T. harzianum* isolates

ITS 1 type*	Transition (Ts)	Transversion (Tv)	Insertion/Deletion (I/D)†	Percentage divergence‡
1 → 2	6	2	2	4.7
1 → 3	17	15	7	20.0
2 → 3	18	17	9	22.9

*ITS 1 types 1, 2 and 3 relate to RFLP and RAPD groups 1, 2 and 3. Isolates representing the ITS types are given in Table 1.

†One or more base length differences were scored as one.

‡Percentage divergence = [(Ts + Tv + I/D)/(sequence length)] × 100.

showed 4.7% divergence from group 2 isolates, and group 1 and group 2 isolates showed 20.0% and 22.9% divergence from group 3 isolates respectively (Table 3).

DISCUSSION

A number of *Trichoderma* spp. have been isolated from mushroom compost but aggressive colonization appears to be restricted to some isolates of *T. harzianum* (Seaby, 1987; Doyle, 1991). *T. harzianum* isolates from mushroom compost have been differentiated into three biological

forms by Seaby (1987) and Doyle (1991). However, delineation of the three forms is not always reliable using biological characteristics, and on many occasions group 2 isolates were incorrectly identified as group 1 and group 3 on the basis of time and pattern of sporulation, e.g. isolates 4, 53, 61 and 65 (Table 1) were identified as group 1 and isolates 33, 39 and 41 (Table 1) were identified as group 3. Unambiguous differentiation of aggressive Th2 isolates from morphologically identical non- (or less) aggressive Th1 and Th3 forms is essential when assessing the risk to mushroom compost from Th2 isolates or when assessing the biological variation within Th2 isolates.

The results reported in the present paper indicate the value of molecular techniques in isolate differentiation for *T. harzianum*. RFLP analysis of the generally conserved ribosomal RNA (rDNA) gene block separated *T. harzianum* into three distinct groups within which there was no variation. The size of the rDNA gene block varied between 8 and 9 kb for group 1 and group 3 isolates, and between 11 and 12 kb for group 2 isolates. This level of size variation within a species is unusual but similar variation has been reported for *Colletotrichum gloeosporioides* (Braithwaite *et al.*, 1990; Hodson *et al.*, 1993), where again species concept is ambiguous.

The level of variation between groups was greatest when comparisons were made using mtDNA. As with rDNA RFLP and RAPD analyses, mtDNA restriction enzyme fragment patterns separated *T. harzianum* isolates into the same three groups. Within a group the variation was

generally low, with *p* values similar to those reported within species in genera such as *Phytophthora* (Förster *et al.*, 1988), *Aspergillus* (Moody & Tyler, 1990b) and *Colletotrichum* (Sreenivasaprasad *et al.*, 1992). Between groups the variation was high, with few shared bands, resulting in *F* values generally lower than or similar to those reported for different species of *Phytophthora* of 43–68% (Förster *et al.*, 1988) and *Pythium* spp. of 20–67% (Martin & Kistler, 1990).

Sequence analyses of the rRNA genes and the non-coding spacer regions have provided valuable taxonomic information for a range of organisms (Förster *et al.*, 1990; Illingworth *et al.*, 1991; Olsen *et al.*, 1986; Sreenivasaprasad *et al.*, 1992). Analysis of the ITS 1 region of 18 *T. harzianum* isolates showed no sequence variation within either group 2 isolates or group 3 isolates and only limited variation (two insertions/deletions) among group 1 isolates. Divergence values between groups suggest a closer relationship between groups 1 and 2 (4.7%) than the more distant relationship between these two groups and group 3 (approximately 20%). Divergence values for other genera vary. Lee & Taylor (1992) reported values of only 2% between *Phytophthora* spp. but values of 6% and 15% have been reported between isolates of *Colletotrichum acutatum* (Sreenivasaprasad *et al.*, 1992) and *Fusarium sambucinum* (O'Donnell, 1992) respectively.

Genetic uniformity within the aggressive colonizing group 2 isolates may support the hypothesis that outbreaks of *Trichoderma* within the British Isles could have originated from a single source, although minor differences in mtDNA could distinguish Irish isolates from those in Great Britain. It is interesting to note that international culture collections contain isolates of *T. harzianum* that are identical to either group 1 or group 3 isolates but no *Trichoderma* cultures received from these collections showed similarity to group 2 isolates (S. Muthumeenakshi, unpublished). These data also refute the view that the aggressive group 2 forms of *T. harzianum* have been selected from populations of group 1 or 3 isolates.

Although the morphotaxonomic characteristics of isolates of these three groups fall within the broad range of variation described for the species aggregate *T. harzianum* (Rifai, 1969), the molecular data could suggest their separation into three DNA-based species (Reynolds & Taylor, 1991). An example of each group has been deposited with the International Mycological Institute. Sexual compatibility studies could possibly be used to support this hypothesis; however, the teleomorph stage is difficult to induce in culture. Further supporting evidence may be provided by assessing the level of inter- and intraspecific molecular variation between other members of the genus *Trichoderma*.

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REFERENCES

- Bissett, J. (1991). A revision of the genus *Trichoderma*. II. Infrageneric classification. *Can J Bot* **69**, 2357–2372.
- Braithwaite, K. S., Irwin, J. A. G. & Manners, J. M. (1990). Restriction fragment length polymorphisms in *Colletotrichum gloeosporioides* infecting *Stylosanthes* spp. in Australia. *Mycol Res* **94**, 1129–1137.
- Buchko, J. & Klassen, G. R. (1990). Detection of length heterogeneity in the ribosomal DNA of *Pythium ultimum* by amplification of the intergenic region. *Curr Genet* **18**, 203–205.
- Chen, W., Hoy, W. & Schneider, R. W. (1992). Species-specific polymorphisms in transcribed ribosomal DNA of five *Pythium* species. *Exp Mycol* **16**, 22–34.
- Doyle, O. (1991). *Trichoderma* green mould – update. *Irish Mushroom Rev* **3**, 13–17.
- Fletcher, J. T. (1990). *Trichoderma* and *Penicillium* diseases of *Agaricus bisporus*. A literature review for the Horticultural Development Council. London: ADAS.
- Förster, H., Kinscherf, T. G., Leong, S. A. & Maxwell, D. P. (1988). Estimation of relatedness between *Phytophthora* species by analysis of mitochondrial DNA. *Mycologia* **80**, 466–478.
- Förster, H., Coffey, M. D., Ellwood, H. & Sogin, M. L. (1990). Sequence analysis of the small subunit ribosomal RNAs of three zoospore fungi and implications for fungal evolution. *Mycologia* **82**, 306–312.
- Garber, R. C. & Yoder, O. C. (1983). Isolation of DNA from filamentous fungi and separation into nuclear, mitochondrial, ribosomal and plasmid components. *Anal Biochem* **135**, 416–422.
- Gaudet, J., Julien, J., Lafay, J. F. & Brygoo, Y. (1989). Phylogeny of some *Fusarium* species, as determined by large-subunit rRNA sequence comparison. *Mol Biol Evol* **6**, 227–242.
- Gyllenstein, U. B. & Erlich, H. A. (1988). Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. *Proc Natl Acad Sci USA* **85**, 7652–7656.
- Higgins, D. G., Bleasby, A. J. & Fuchs, R. (1992). CLUSTAL V: improved software for multiple sequence alignment. *Comput Appl Biosci* **8**, 189–191.
- Hodson, A., Mills, P. R. & Brown, A. E. (1993). Ribosomal and mitochondrial DNA polymorphisms in *Colletotrichum gloeosporioides* isolated from tropical fruits. *Mycol Res* **97**, 329–335.
- Hultman, T., Ståhl, S., Hornes, E. & Uhlén, M. (1989). Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support. *Nucleic Acids Res* **17**, 4937–4946.
- Illingworth, C. A., Andrews, J. H., Bibeau, C. & Sogin, M. L. (1991). Phylogenetic placement of *Athelia bombacina*, *Aureobasidium pullulans* and *Colletotrichum gloeosporioides* inferred from sequence comparisons of small-subunit ribosomal RNAs. *Exp Mycol* **15**, 65–75.
- Lee, S. B. & Taylor, J. W. (1992). Phylogeny of five fungus-like protist *Phytophthora* species inferred from the internal transcribed spacers of ribosomal DNA. *Mol Biol Evol* **9**, 636–653.
- Levy, M., Romao, J., Marchetti, M. A. & Hamer, J. E. (1991). DNA fingerprinting with a dispersed repeated sequence resolves pathotype diversity in the rice blast fungus. *Plant Cell* **3**, 95–102.
- Martin, F. N. & Kistler, H. C. (1990). Species-specific banding

patterns of restriction endonuclease-digested mitochondrial DNA from the genus *Pythium*. *Exp Mycol* **14**, 32–46.

Meyer, W., Morawetz, R., Börner, T. & Kubicek, C. P. (1992). The use of DNA-fingerprint analysis in the classification of some species of the *Trichoderma* aggregate. *Curr Genet* **21**, 27–30.

Moody, S. F. & Tyler, B. M. (1990a). Restriction enzyme analysis of mitochondrial DNA of the *Aspergillus flavus* group: *A. flavus*, *A. parasiticus* and *A. nomius*. *Appl Environ Microbiol* **56**, 2441–2452.

Moody, S. F. & Tyler, B. M. (1990b). Use of nuclear DNA restriction fragment length polymorphisms to analyze the diversity of the *Aspergillus flavus* group: *A. flavus*, *A. parasiticus* and *A. nomius*. *Appl Environ Microbiol* **56**, 2453–2461.

Nei, M. & Li, W. H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci USA* **76**, 5269–5273.

Nei, M. & Tajima, F. (1983). Maximum likelihood estimation of the number of nucleotide substitutions from restriction sites data. *Genetics* **105**, 207–217.

O'Donnell, K. (1992). Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). *Curr Genet* **22**, 213–220.

Olsen, G. J., Lane, D. J., Giovannoni, S. J., Pace, N. R. & Stahl, D. A. (1986). Microbial ecology and evolution: a ribosomal RNA approach. *Annu Rev Microbiol* **40**, 337–365.

Raeder, U. & Broda, P. (1985). Rapid preparation of DNA from filamentous fungi. *Lett Appl Microbiol* **1**, 17–20.

Reynolds, D. R. & Taylor, J. W. (1991). DNA specimens and the 'International code of botanical nomenclature'. *Taxon* **40**, 311–315.

Rifai, M. A. (1969). A revision of the genus *Trichoderma*. *Commonw Mycol Inst Mycol Pap* no. 116, pp. 1–56.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sanger, F. S., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.

Seaby, D. A. (1987). Infection of mushroom compost by *Trichoderma* species. *Mushroom J* **179**, 355–361.

Seaby, D. A. (1989). Further observations on *Trichoderma*. *Mushroom J* **197**, 147–151.

Sreenivasaprasad, S., Brown, A. E. & Mills, P. R. (1992). DNA sequence variation and interrelationships among *Colletotrichum* species causing strawberry anthracnose. *Physiol Mol Plant Pathol* **41**, 265–281.

Vaillancourt, L. J. & Hanau, R. M. (1992). Genetic and morphological comparisons of *Glomerella* (*Colletotrichum*) isolates from maize and from sorghum. *Exp Mycol* **16**, 219–229.

Verbeet, M. Ph., Klootwijk, J., Heerikhuizen, H., Fontijn, R., Vreugdenhil, E. & Planta, R. J. (1983). Molecular cloning of the rDNA of *Saccharomyces rosei* and comparison of its transcription initiation region with that of *Saccharomyces carlsbergensis*. *Gene* **23**, 53–63.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols. A Guide to Methods and Applications*, pp. 315–322. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: Academic Press.

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