Contract Report M10

Mushroom Compost: Development of a rapid diagnostic test for colonising forms of *Trichoderma*

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Contents

Relevance to growers and practical application

Application

The objective of Project M10 was to use DNA-based molecular techniques to differentiate 'aggressive' and 'non-aggressive' colonising forms of *Trichoderma harzianum* from mushroom compost. Various techniques successfully distinguished 'aggressive' colonising forms of *Trichoderma harzianum*. Further work is now required to use these data to develop a rapid diagnostic test for use directly on compost samples.

Summary

Trichoderma remains a potential threat to the UK mushroom industry with losses estimated as high as £3-4 million per annum. *Trichoderma harzianum* has most frequently been associated with aggressive colonization of mushroom compost. This species has been differentiated into three biological forms termed Th1, Th2 and Th3. These three forms of the fungus, which are morphologically identical, cannot always be reliably distinguished by variation in specified cultural characteristics. *T. harzianum* isolates differ in their aggressiveness in colonizing compost in competition with *Agaricus bisporus* and early and accurate identification of Th2, the most aggressive form, is of paramount importance to the mushroom industry. For this reason various molecular techniques were used to assess genetic diversity among *T. harzianum* isolates with a view to producing molecular markers for isolates of Th2.

Based on these molecular data, the aggressive colonizing Th2 isolates could be readily distinguished from isolates of the other two groups. Genetic uniformity within the Th2 isolates is indicative of a single clonal type. This supports the hypothesis that isolates obtained from mushroom compost throughout the British Isles originated from a single source, although minor variations in mtDNA could distinguish Irish isolates from those in Great Britain.

The main findings of this project were:

1. Molecular techniques can divide eighty-one isolates of *Trichoderma harzianum* obtained from England, Northern Ireland and Republic of Ireland into three major types (formerly termed Th1, Th2 and Th3).

2. Very little variation was found at a genetic level within the Th2 group (the most aggressive group).

3. Th1, Th2 and Th3 are genetically distinct so it is unlikely that Th2 has evolved recently from either Th1 or Th3 (ie not a contaminant from biological control work).

4. Th2 appears to be unique to mushroom compost ie it has not been found in any other ecological niche.

5. Sufficient molecular data have now been collected to suggest that a laboratory test for Th2 could be developed.

6. Although Th2 was shown to be the major coloniser of compost, a small number of Th1 and Th3 isolates may be able to cause cropping problems.

7. Analysis of aggressive *Trichoderma* isolates from Canada and the USA has identified a further *T. harzianum* (termed Th4). This variation underlines the potential threat to compost from other *Trichoderma* isolates as yet not identified in the UK.

8. Preliminary experiments have shown that a bacterium isolated from compost may prevent growth and sporulation of *Trichoderma* but not *Agaricus*.

Whilst growers are aware of the importance of good hygiene practices to control spread of *T. harzianum*, this project has highlighted the variation in aggressive isolates from other continents. Care should be taken to avoid global spread of non-indigenous isolates.

Eventual control of *Trichoderma* in mushroom compost will, in part, be due to accurate identification of aggressive isolates.

1. 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 *Trichoderma* genus 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. *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 3 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 systematics of phytopathogenic and other (commercially important) fungi to assess intra- and inter-specific variation and to determine phylogenic relationships (Buchko & Klassen, 1990; Chen *et al.*, 1992, Förester *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) have used DNA finger printing techniques to analyse the nine species aggregates of *Trichoderma* and recognised 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.

2. Materials and Methods

2.1 *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.

Fungal cultures were maintained on potato dextrose agar (Oxoid) at 25°C and mycelium for DNA extraction was grown in liquid shake cultures (120 rpm) at 25°C in glucose casamino acid medium (1l contained glucose, 15.0 g; KH_2PO_4, 1.0 g; $MgSO_4$, 0.5 g, casein hydrolysate, 4.6 g and 2 ml trace element solution) for 3 days. DNA was extracted from 300 mg of 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 of freeze-dried mycelial powder. Mitochondrial DNA was purified by caesium chloride/bis-benzimide gradient centrifugation (Beckman, Ti 70 rotor, 25°C, 40,000 rpm, 60 h) following the method of Garber & Yoder (1983).

2.2 *Southern blotting, hybridisation and autoradiography.* DNA was quantified by ethidium bromide fluorescence on a UV transilluminator with known quantities of lambda DNA (Sambrook *et al.*, 1989). Genomic DNA (1-2 µg) was digested with 25U of restriction enzyme until completion at 37°C according to manufacturer's instructions (Promega). Digested DNA was electrophoresed in 0.8% w/v agarose gels from 13 h at 60 V (electrophoresis buffer: 89 mM Tris, 89 mM boric acid, 2 mM EDTA) with *Hind* III-digested lambda 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.

2.3 *Probes.* For ribosomal DNA (rDNA) analysis, Southern blots were probed with plasmid MY60 (pMY60) containing a complete rDNA unit from *Saccharomyces carlsbergensis* Hensen (Verbeet *et al.*, 1983). For mitochondrial genome probing, mtDNA purified from isolates T28JF, ThI and IMI 110150 were 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. Hybridisation was performed in a hybridisation oven (Hybaid) at 65°C following the procedure described by Sambrook *et al.* (1989). The prehybridisation wash was for 3 h and post-hybridisation washes consisted of 3 washes of 30 min each in 2 x SSC and 0.1% SDS and 3 washes of 30 min each in 0.1 x SSC and 0.1% SDS. Finally membranes were wrapped with cling film and exposed to Hyperfilm β-max (Amersham) at -70°C using intensifying screens.

2.4 *PCR amplification.* For RAPD analysis, primers A3 (AGTCAGCCAC), A11 (CAATCGCCGT), A13 (CAGCACCCAC), B6 (TGCTCTGCCC), B7 (GGTGACGCAG) and B10 (CTGCTGGGAC) supplied by Operon Technologies Inc., California were used. Reaction mixtures contained 5 µl of appropriately diluted genomic DNA (20 - 30 ng), 5 µl of Taq 10 x buffer, 8 µl of 100 µM each of deoxynucleotide triphosphase (dNTP) mix, 5 µl of primer (15 µg ml^{-1}), 0.25 µl of Taq DNA polymerase (5Uµl^{-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 visualised in 1.4% w/v agarose gels stained with ethidium bromide (0.4 µg 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).

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 of dNTPs, 0.4 µM each of primers 1 and 2 and 2.5 units of Taq DNA polymerase and were subjected to 30 cycles of 1.5 min at 94°C, 2 min at 45°C and 3 min at 72°C.

2.5 *Sequencing.* Template DNA (100 µl) was immobilized using 100 µl of Dynabeads (Dynal, Norway) according to 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 Gyllensten & Erlich (1988).

For data analysis, the proportion (%) of shared restriction endonuclease fragments (F) and the nucleotide sequence divergence estimates (p) between various RFLP groups were calculated using the 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 x 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. Length of the smaller sequence was used for computation.

3. Results

3.1 *Analysis of rDNA RFLP patterns*

Ribosomal DNA restriction patterns generated by the enzymes *Eco*R I, *Sac* I and *Cla* I 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*R I digestion (Fig. 1a). A second band of approximately 3.6 kb was seen with group 1 isolates and 4.7 kg 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 *Sac* I 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 kb 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 kb with *Sac* I digestion.

Cla I digestion resulted in three bands for all isolates of which two bands of approximately 2.2 kb 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 (*Hin*d III and *Pst* I) yielded a single band for group 1 and 2 isolates (approximately 9 kb and 12 kb respectively) whereas rDNA from group 3 isolates was not digested. Group 3 isolates could be distinguished by the presence of a *Bam*H I site which resulted in a 9 kb fragment whereas no *Bam* HI 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.

3.2 *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 polymorphism within each 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 hybridisations 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.

3.2.1 *Variation in group 1 isolates.* MtDNA from isolate T28JF (approximately 38-40 kb) was used for probe *Hin*d III digests of group 1 isolates. Out of 35 isolates, 24 fell into the subgroup 1a and the remaining 11 isolates formed 6 more subgroups (1b and 1g) (Table 1). Restriction enzyme digestion patterns of a representative isolate from each of the seven subgroups are shown in Fig. 2a *Hin*d III 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. *Eco*R I digestion produced six to eight bands, of which four were common to all subgroups (data not shown) and subgrouping of isolates was similar to that obtained using *Hin*d III.

3.2.2 *Variation in group 2 isolates.* Restriction fragment patterns generated from group 2 isolates, using *Hin*d III 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. *Hin*d III 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 *Eco*R I digestion (data not presented).

3.2.3 *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 *Hin*d III and *Eco*R 1 digestions. Isolates from the Republic of Ireland and England formed subgroups 3c and 3d respectively. Isolates from Northern Ireland were separated into 3 more subgroups (3a, 3b and 3e). *Hin*d III digestion produced 11-13 fragments (Fig. 2c) of which nine were shared by all subgroups with *F* values ranging from 83%-96%.

3.2.4 *Heterologous probing.* In cross hybridisation experiments using heterologous mtDNA probes, only two or three co-migrating fragments were observed between 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 hybridised with heterologous probes (mtDNA from either group 1 or group 3 isolates) very few fragments hybridised and high molecular weight fragments were visible only after prolonged exposure (7 days).

3.3 *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. Figure 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 were divided into three main clusters on the dendrogram (Fig. 4), corroborating the results from RFLP analyses. Within cluster 1, representing group 1 isolates, similarity percentage varied from 72 - 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 suggested a closer relationship between groups 1 and 2 than between either of these two groups and group 3.

3.4 *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 distinct ITS types 1, 2 and 3 (Fig. 4) 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 groups 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. 4). Group 1 isolates 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).

4. Conclusion

A number of *Trichoderma* spp. have been isolated from mushroom compost but aggressive colonisation 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, eg isolates 4, 53, 61 and 65 (Table 1) were identified as group 1 and isolates 33 to 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 here 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 8-9 kb for group 1 and group 3 isolates and 11-12 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 ribosomal RNA 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 and Taylor (1992) reported values of only 2% between *Phytothphora* 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 colonising 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 3 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 intra-specific molecular variation between other members of the genus *Trichoderma.*

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Table 1. *Source and rDNA, mtDNA and ITS 1 sequence groupings of Trichoderma 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	ThI**	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	2e		
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	2e		+
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 350331)	3	3b	3	+

Table 1. Continued.

No.	Isolate code	rDNA† grouping	mtDNA† grouping	ITS 1 sequence type	RAPD analysis
57	TD12	1	1e	1	+
58	TD13	1	1d		+
59	TD15**	1	1a	1	+
	England*				
60	KPNT** (IMI 353384)	2	2e	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 353383)	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	+

* Countries in which isolates were collected.

** Identity confirmed as *T. harzianum* using the criteria of Rifai (1969) by Dr M.A.J. Williams (I.M.I.) and isolates 56, 60 and 71 have been deposited with IMI.

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

\+ Isolates included for RAPD analysis.

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

Probe DNA	Endonuclease	Target DNA		
		Groups 1	2	3
Groups 1	*Hind* III	60-94	23-26	20-25
	*Eco*R I	66-83	12-13	16-18
2	*Hind* III	20-45	78-97	13-22
	*Eco*R I	22-38	70-97	14-21
3	*Hind* III	22-25	17-19	81-95
	*Eco*R I	28-32	29-33	75-92

Isolates in each group are given in Table 1.

Table 3 *Divergence betweeen the nucleotide sequences of internal transcribed spacer (ITS) 1 region of Trichoderma harzianum isolates.*

*ITS 1 types	Transition (Ts)	Transversion (Tv)	**Insertion\ Deletion (I\D)	***% divergence
1→2	6	2	2	4.7
1→3	17	15	7	20.0
2→3	18	17	9	22.9

Isolates representing the ITS types are given in Table 1.

* ITS 1 types 1, 2 and 3 relate to RFLP and RAPD groups 1, 2 and 3

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

*** % divergence = Ts + Tv + I\D/sequence length x 100.

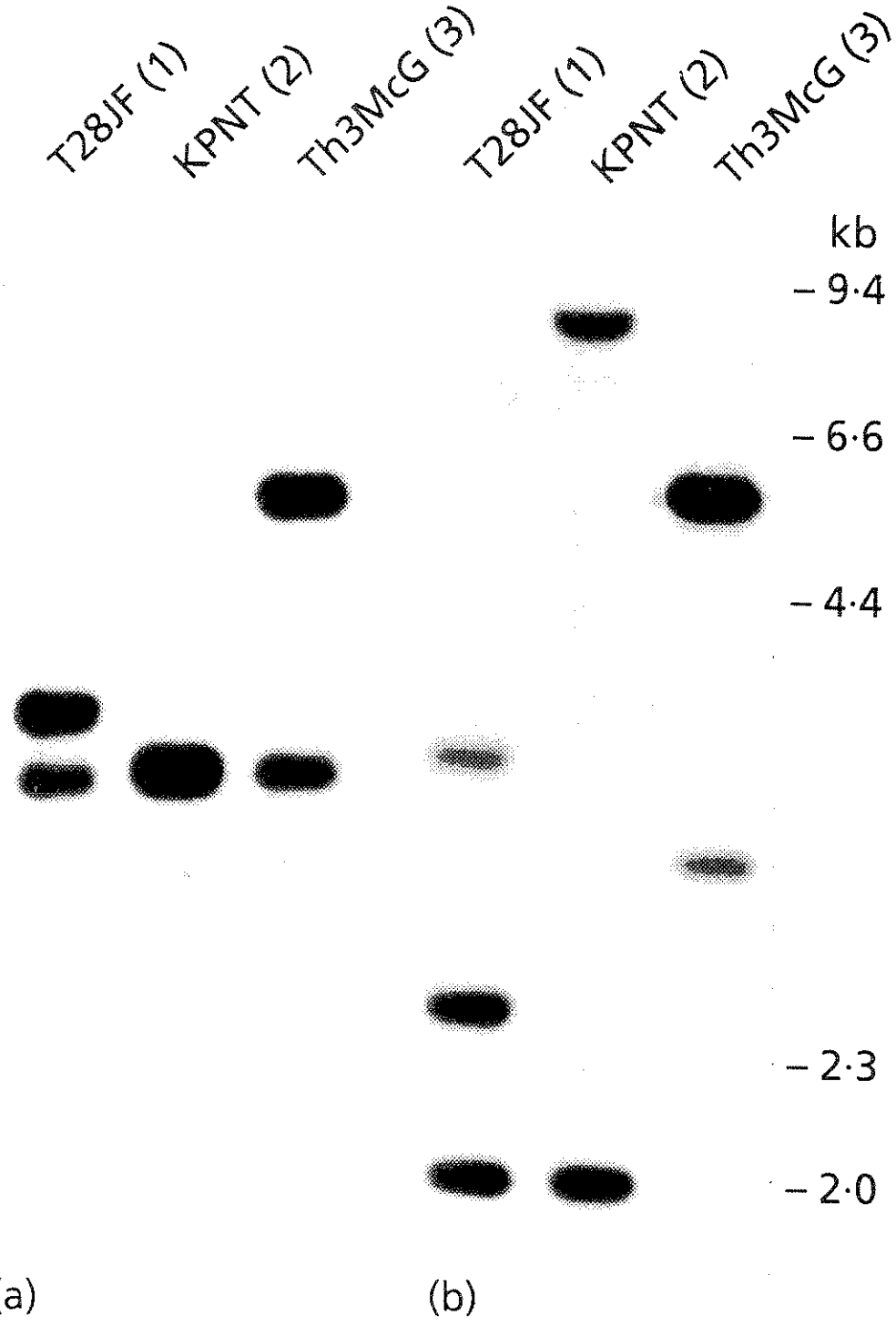

Fig. 1. Restriction fragment patterns of rDNA from *T. harzianum* isolates digested with (a) *Eco*RI and (b) *Sac*I. Molecular size markers refer to *Hind*III-digested λ DNA. The *T. harzianum* rDNA group numbers of the isolates are given in parentheses. Isolates in each group are given in Table 1.

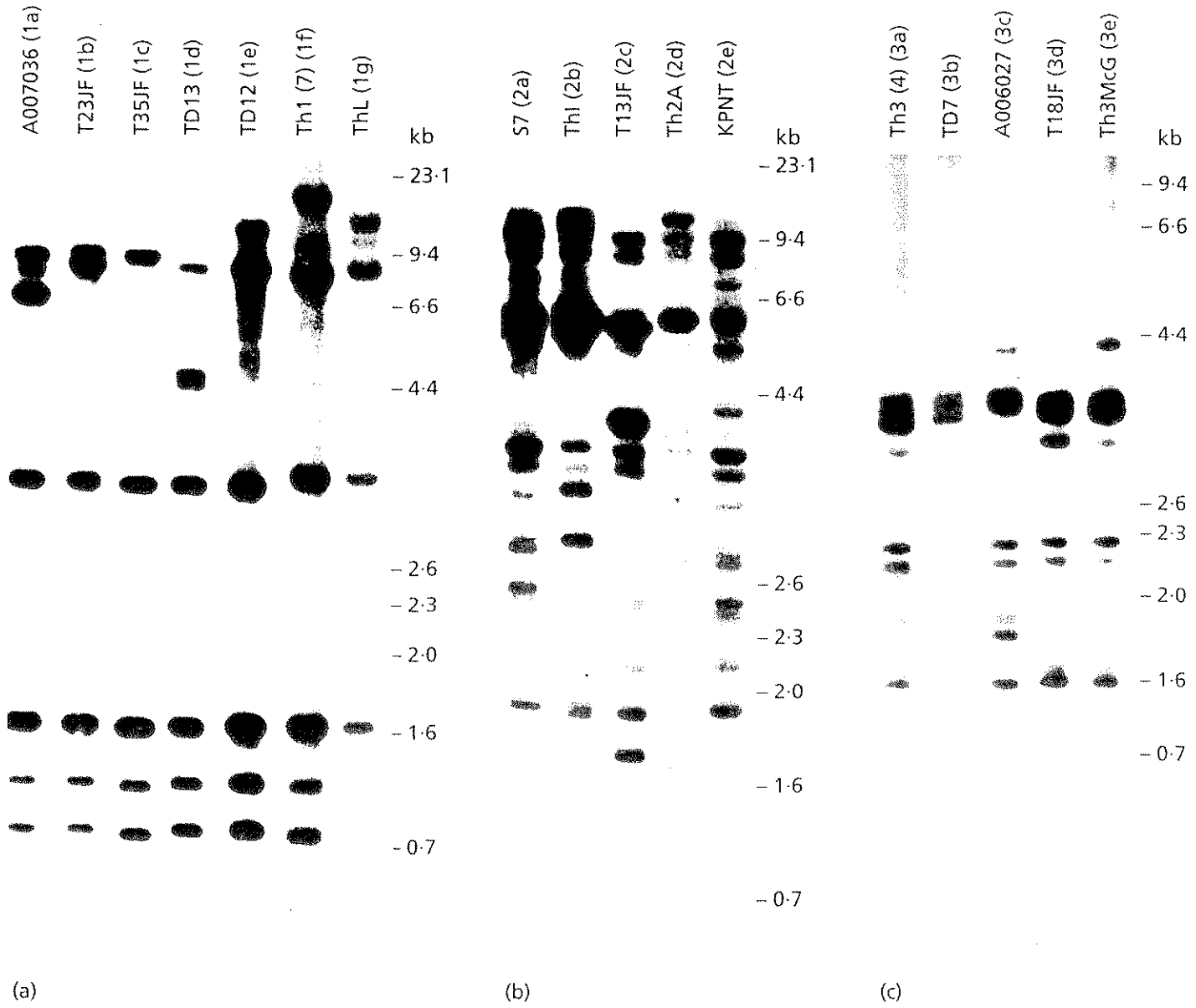

Fig. 2. mtDNA RFLP patterns of *T. harzianum* isolates digested with *Hin*dIII: (a) group 1 isolates probed with mtDNA from the group 1 isolate T28JF; (b) group 2 isolates probed with mtDNA from the group 2 isolate ThI; (c) group 3 isolates probed with mtDNA from the group 3 isolate IMI 110150. Subgroupings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

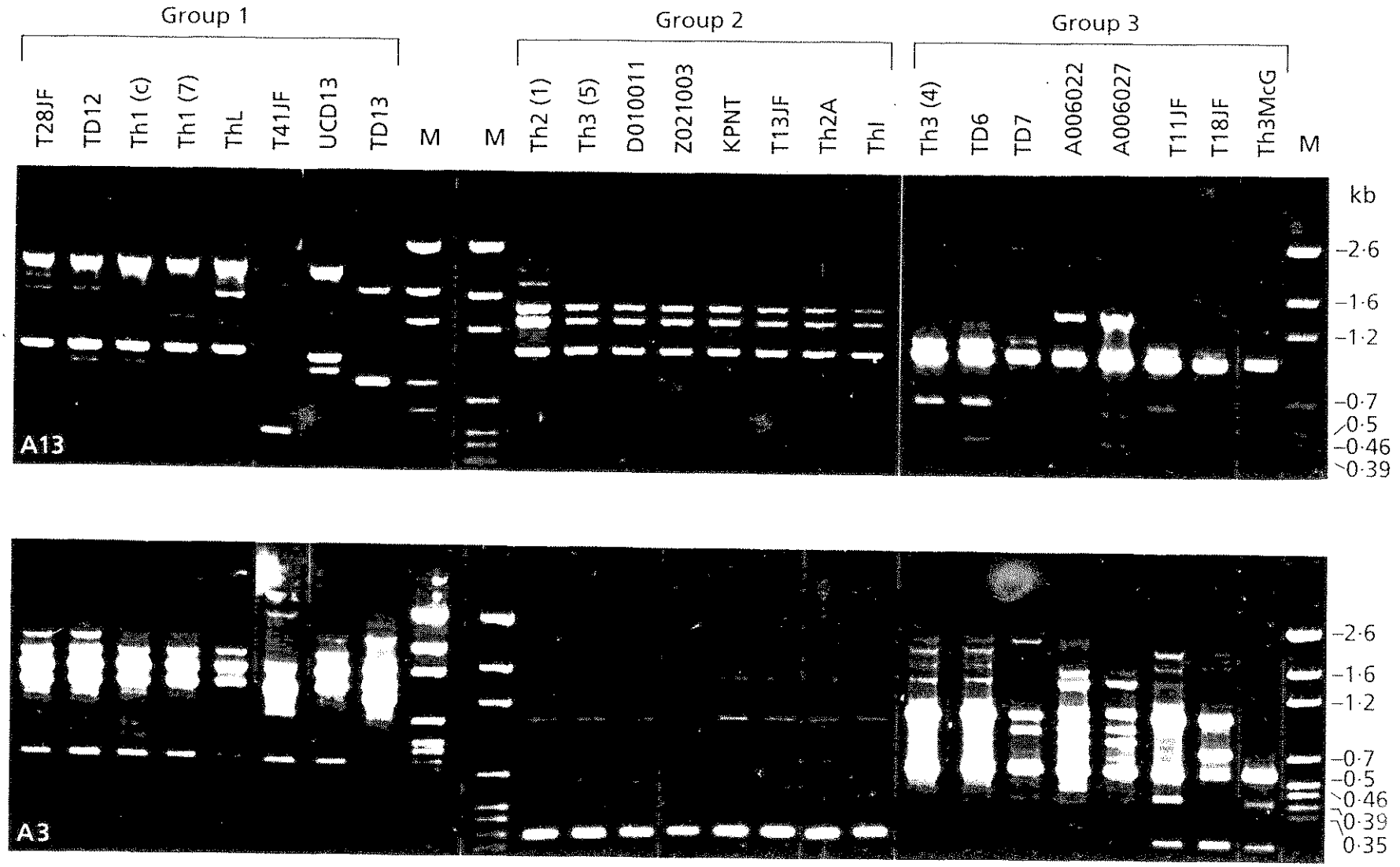

Fig. 3. Randomly amplified polymorphic DNA patterns of *T. harzianum* isolates with primers A13 and A3 (M, digested pGEM used as molecular size marker).

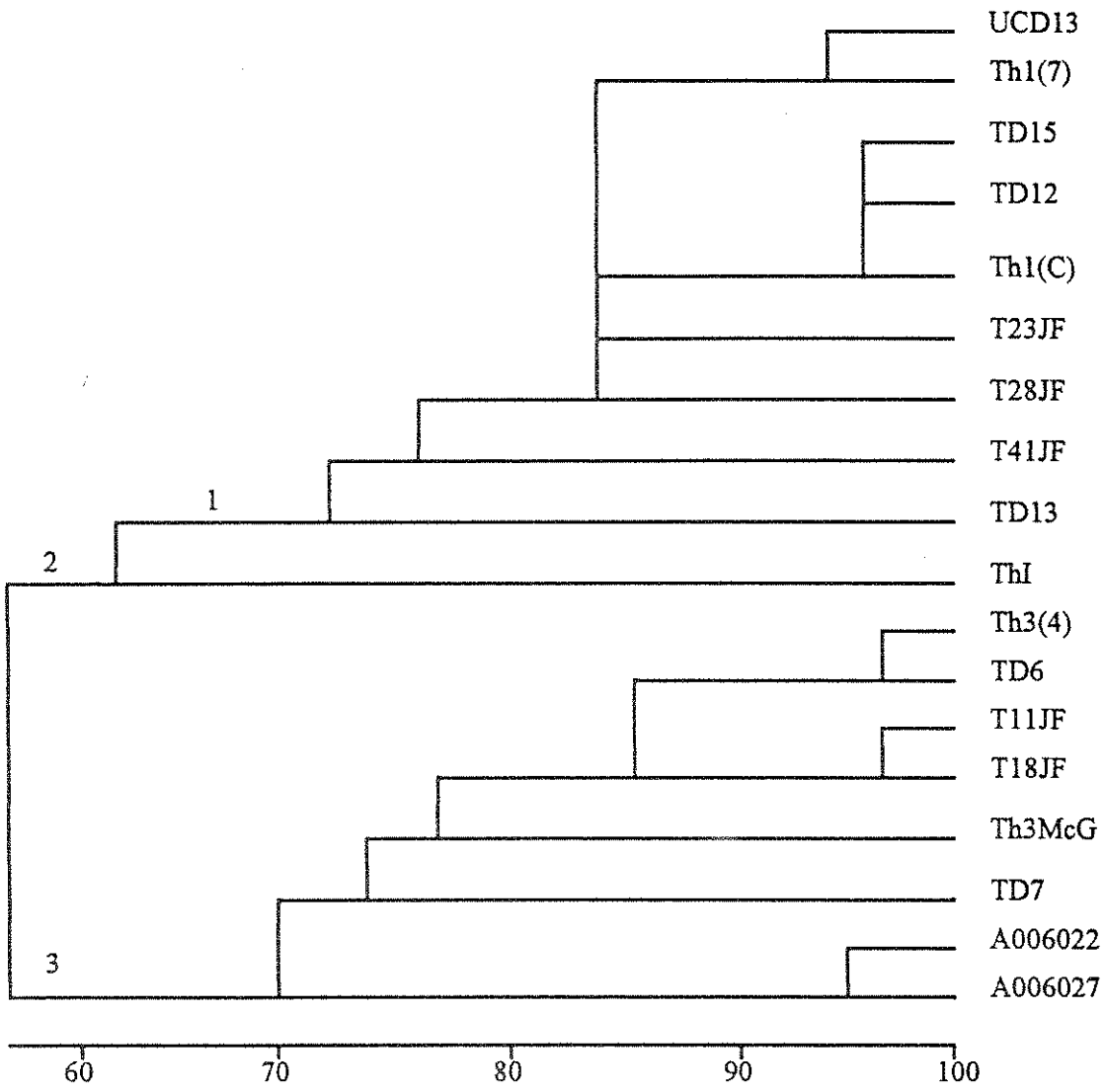

Fig.4 Clustering of isolates of Trichoderma harzianum from pairwise comparison of RAPDs by the group average method (see Table 1 for details of isolates). Isolate UCD13 in cluster 1 is representative of group 1 isolates ThL, A001040 and Th1M. Isolate ThI in cluster 2 isolates Th2 shiitake, Th2(1), Th3(5),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

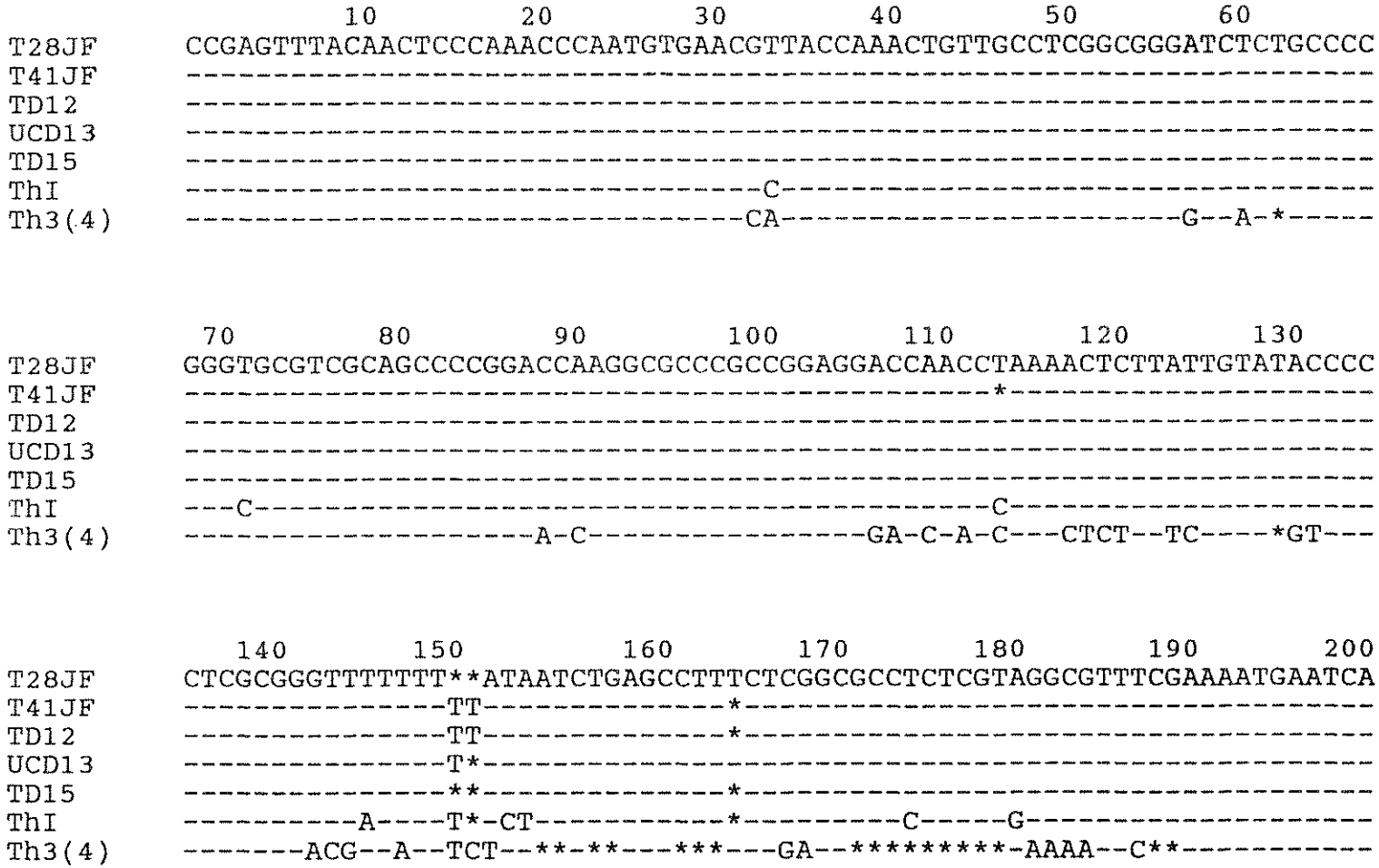

Fig.5 Nucleotide sequence of the ITS 1 region of Trichoderma harzianum isolates. (a) Representatives of group 1 isolates (type 1 sequence). Isolate A007036 had an identical sequence to isolate T28JF. Isolate TD1 had an identical sequence to isolate TD15. (b) Representative of group 2 isolates (type 2 sequence). Th2A, ThI, 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.

Contract between Queens University of Belfast (hereinafter called the "Contractor") and the Horticultural Development Council (hereinafter called the "Council") for a research/development project.

PROPOSAL

1. TITLE OF PROJECT **Contract No: M10**

MUSHROOM COMPOST: DEVELOPMENT OF A RAPID DIAGNOSTIC TEST FOR COLONISING FORMS OF *TRICHODERMA*

2 BACKGROUND AND COMMERCIAL OBJECTIVE

Trichoderma species affect mushrooms in various ways producing cap spotting and also colonising the compost and interfering with mushroom mycelial growth. Recently *T.harzianum* has been found to be associated with the compost problem. Different forms of *T. harzianum* have been recognised by Seaby (1985, 1987) and Doyle (1991). Forms of *T. harzianum* are indistinguishable morphologically but differ in their ability to grow in mushroom compost. Differentiating between the "agressive and non-agressive" form requires precise laboratory work and takes about a week.

Rapid identification of damaging forms of *Trichoderma* will result in a higher health status of compost which should lead to lower yield losses associated with this contamination problem.

3. POTENTIAL FINANCIAL BENEFIT TO THE INDUSTRY

This is difficult to assess but a diagnostic test, based on the results of this project, could easily be used by ADAS for a relatively small capital outlay (£3,000).

4. SCIENTIFIC/TECHNICAL TARGET OF THE WORK

New techniques enable very precise identification of fungal strains. These are based on techniques which utilise certain biochemical reactions and the ultimate test (PCR test) is very specific and quick. PCR tests can be developed for *Trichoderma* species and possibly strains. The objective of this work is to develop such a test to identify damaging strains. This would have the major advantages over existing methods of speed and accuracy. Such tests would have to be compared with the standard agar procedures for verification.

5. CLOSELY RELATED WORK

Work on *Trichoderma* spp is already in progress in this department. A large number of isolates of all *Trichoderma* species have been collected from a range of sources including GB, New Zealand and India. DNA has been extracted from all isolates and comparisons of restriction

fragment length polymorphisms (RFLPs) made. From this work a crude taxonomic relationship has been determined for *Trichoderma* species.

Similar projects are in progress for other fungal pathogens including *Colletotrichum gloesporiodes, C. acutatum, Nectria galligena* and *Phytophthora fragariae*. Results from these projects have suggested that species-specific tests can be developed for identification of fungi.

This department has also considerable experience in the biological aspects of compost colonising forms of *Trichoderma* spp. The project will hopefully integrate with the HDC-funded project of Dr John Fletcher (ADAS, Wye).

6. DESCRIPTION OF THE WORK

The aims of this project are:-

1. To extract DNA from mycelial cultures of *Trichoderma* spp grown in liquid media.

2. To digest DNA with a range of restriction endonucleases, to separate DNA fragment on agarose gels and Southern blot onto suitable membranes. These membranes will be probed with a ribosomal DNA (rDNA) clone from *Saccharomyces carlsbergensis* to compare rDNA fragments from different *Trichoderma* species.

3. To separate mitochondrial DNA and prepare probe(s) for comparison of mitochondrial DNA fragments.

4. To amplify specific parts of the fungal genome using the polymerase chain reaction (PCR) in order to study the internally transcribed spacer regions and 5.8s gene, in different *Trichoderma* spp. The internally transcribed spacer region 1 will be cloned and the DNA sequenced. This should allow synthesis of primers suitable for species-specific PCR.

5. To assess the potential of PCR using randomly-generated primers to develop a diagnostic test to differentiate *Trichoderma* spp.

   Some help will be provided by Dr John Fletcher in the form of provision of *T. harzianum* isolates from mushroom compost, and Dr Michele Williams, IMI, CAB, Kew in isolate identification.

7. COMMENCEMENT DATE AND DURATION

Start date 1.11.91; duration 12 months.

8. STAFF RESPONSIBILITIES

Project Leader: - Dr Peter R Mills

Other staff: - one research assistant (QUB)

Mrs Meena Prasad

Dr John Fletcher
ADAS
Wye

Dr Michele Williams
IMI (CAB)
Kew

9. LOCATION

Department of Mycology and Plant Pathology
Queen's University of Belfast
Newforge Lane
Belfast BT9 5PX

11. PAYMENT

On each quarter day the Council will pay to the Contractor in accordance with the following schedule:

Quarter/Year	1991	1992	1993
1	-	2950	-
2	-	2950	-
3	-	2950	-
4	1967	983	-

Contract No: M/10

TERMS AND CONDITIONS

The Council's standard terms and conditions of contract shall apply.

Signed for the Contractor(s)

Signature.....

Position...B.U.R.S.A.R. THE QUEEN'S UNIVERSITY OF BELFAST

Date....31...January...1992.....

Signed for the Contractor(s)

Signature.....

Position.....

Date.....

Signed for the Council

Signature.....

Position.....CHIEF EXECUTIVE

Date.....17.1.92.....