Examination of *Trichoderma* phylogenies derived from ribosomal DNA sequence data

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Ribosomal DNA sequences were assessed for their usefulness in distinguishing among *Trichoderma* isolates and for their robustness in resolving their phylogenetic relationships. DNA sequences from the D2 region of the 28S rRNA gene were determined for 50 *Trichoderma* isolates representing seven species. Eight distinct sequence types existed, and were mostly consistent with groupings based on morphology. Sequence variability within the D2 region alone was not sufficient to provide a reliable phylogeny. Sequences from the ITS1, 5.8S and ITS2 regions were subsequently determined for 18 of the isolates. Eight distinct ITS sequence types were detected among these 18 isolates. The ITS sequence types were generally consistent with morphology, ITS1 sequence data supported the identification of the Th3 *T. harzianum* group of Muthumeenakshi *et al.* (1994) as *T. atroviride*. The data also confirmed that the biocontrol strains of this study were different from those causing disease problems in the mushroom industry in Europe and North America. Results from the phylogenetic analysis stress the importance of testing the robustness of data used to predict phylogenies. Two ITS sequence data sets for the same group of isolates produced significantly different phylogenies. Congruence analysis detected that *T. inhamatum* (GJS90-90) was corrupting tree topologies and 'first order pruning' was performed by removing its sequence from the two ITS data sets. Subsequent differences in the topologies of pruned ITS1 and ITS2 trees were attributed to a lack of phylogenetic information in the ITS2 sequence region. Although ITS sequences successfully differentiated among morphologically distinct isolates within *Trichoderma*, it did not provide a sufficient phylogenetic signal to resolve all of their relationships.

Trichoderma consists of a number of economically important strains, valued for their biocontrol capabilities and ability to produce beneficial metabolites and enzymes (Papavizas, 1985; Ghisalberti & Sivasithamparam, 1991; Buchert *et al.*, 1992; Schirmbock *et al.*, 1994). In contrast to their usefulness to industry, however, there have also been reports of pathogenic strains that pose a threat to both horticulture and human health (Loeppke *et al.*, 1983; Seaby, 1987; Menzies, 1993).

Differentiation of such isolates within *Trichoderma* has proved difficult due to the limited number of easily identifiable cultural and morphological characters available for comparison (Rifai, 1969; Bissett, 1984, 1991*a–c*, 1992). Consequently, the taxonomy of the genus is currently under review with a variety of morphological, cultural, molecular and biochemical characteristics being used to define isolates (see review by Samuels, 1996).

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For this study, a method was sought that would differentiate *Trichoderma* isolates exhibiting good biological control activity against plant pathogenic *Armillaria* species. In particular, sequence data from the ribosomal gene cluster was investigated because of the successful use of such data in distinguishing among *Trichoderma* isolates (Muthumeenakshi *et al.*, 1994; Ospina-Giraldo *et al.*, 1998). In addition, sequence data may be used to reconstruct the phylogeny of a group of isolates, thus providing valuable insights into their evolutionary relationships. Thus, in turn, may lead to a more efficient selection and use of such isolates for commercial applications. We also wished to use the sequence data to determine if our biocontrol agents were identical to those identified as a problem in the mushroom industry in Europe and North America (Muthumeenakshi *et al.*, 1994; Muthumeenakshi, Brown & Mills, 1998; Ospina-Giraldo *et al.*, 1998).

Previous studies have used sequences from the ribosomal gene cluster to distinguish among, and predict phylogenies

Table 1. Origin, morphological and molecular characterisation of *Trichoderma* isolates

Isolate morphological groupings	Culture collection reference	Origin	D2 sequence type[a]	D2 GenBank accession no.[b]	ITS1 sequence type[c]	ITS2 sequence type[d]	ITS GenBank accession no.[b]
T. atroviride							
M1057#	HortResearch	N.Z.	1		1	1	
OG3#	HortResearch	N.Z.	1		1	1	
TTS#	HortResearch	N.Z.	1		1	1	
M1037#	HortResearch	N.Z.	1		1	1	
HPP1#	HortResearch	N.Z.	1		1	1	
THF2/3#	HortResearch	N.Z.	1				
TBHPP7#	HortResearch	N.Z.	1		1	1	
TT9/10#	HortResearch	N.Z.	1				
D#	HortResearch	N.Z.	1	AF055203	1	1	AF055212
C52	AUMCC	N.Z.	1				
C53	ICMP 4208	N.Z.	1				
1079	ICMP 1079	N.Z.	1				
D72	AUMCC	N.Z.	1				
D73	AUMCC	N.Z.	1				
D74	AUMCC	N.Z.	1		1	1	
D76	AUMCC	N.Z.	1				
D79	AUMCC	N.Z.	1				
C59	AUMCC	N.Z.	3	AF059512			
T. harzianum I							
NS27	G. Samuels	U.S.A.	3				
TR11	ATCC 48131	U.S.A.	3				
TR108	G. Samuels	U.S.A.	3		3	4	
TR112	G. Samuels	U.S.A.	3	AF055204	3	4	AF055215
US2	HortResearch	U.S.A.	3				
5420	ICMP	N.Z.	3				
1695	ICMP	N.Z.	2	AF059514			
T. harzianum II							
US1	HortResearch	U.S.A.	3		*	2	AF055213
TV#	HortResearch	N.Z.	3				
JD12B	J. Deacon	U.K.	3	AF055204			
TR77	ATCC 58637	U.S.A.	3				
TR10	ATCC 24274	U.S.A.	3				
TR12	G. Samuels	U.S.A.	*	AF055205			
T95	ATCC 60850	U.S.A.	3	AF055204			
T. harzianum III							
JD10	J. Deacon	U.K.	2	AF059514			
T. harzianum IV							
KEK#	HortResearch	N.Z.	3				
G4#	HortResearch	N.Z.	3	AF055204	2	3	AF055216
HEND#	HortResearch	N.Z.	3		2	3	
1696	ICMP 1696	N.Z.	3				
T. cf. hamatum							
OMK#	HortResearch	N.Z.	3	AF059513	*	2	AF055214
JD2	J. Deacon	U.K.	2				
1693	ICMP 1693	N.Z.	2	AF055206			
5411	ICMP 5411	N.Z.	2				
T. inhamatum							
GJS90-89	G. Samuels	U.S.A.	4	AF055207	*	5	AF055218
GJS90-90	G. Samuels	U.S.A.	4		*	5	AF055217
T. cf. koningii							
TR90	G. Samuels	U.S.A.	*	AF055208	*	*	AF055219
T. koningii							
MTM#	HortResearch	N.Z.	*	AF055202	*	*	AF055211
T. cf. virens							
5412	ICMP 5412	N.Z.	*	AF055209			
T. asperellum							
D75	AUMCC	Canada	2				
D78	AUMCC	N.Z.	2				
D77	AUMCC	Canada	2	AF059515			
1697	ICMP 1697	N.Z.	2				

for, isolates of *Trichoderma* (Muthumeenakshi *et al*., 1994; Rehner & Samuels, 1994; Schlick *et al*., 1994; Kuhls *et al*., 1997). There has, however, been no detailed analysis of the adequacy of the data or the robustness of the reconstructed phylogenies. In the present study, we assessed the level of variability and determined the phylogenetic robustness of sequence data from the D2 region of the 28S rRNA gene for 50 *Trichoderma* isolates, including 15 from New Zealand with biocontrol activity. Similarly, sequences from the ITS1, ITS2 and combined ITS1, 5.8S and ITS2 regions were assessed for 18 of these isolates. Our results indicate that ITS sequences may be used to reconstruct the evolutionary relationships amongst isolates of *Trichoderma*. These evolutionary relationships appear to agree closely with those obtained using morphological information. Nonetheless, phylogenies of ITS1 and ITS2 may be incongruent, although much of this may be attributed to the absence of sufficient phylogenetic signal in these regions.

MATERIALS AND METHODS

Fungal cultures

Morphological identity, source, geographical origin, and sequence type of the 50 *Trichoderma* isolates investigated are listed in Table 1. All isolates were identified morphologically to species and sub-species level. *T. harzianum* Rifai isolates were further differentiated into four subgroups I–IV based on morphological characters.

Amplification of rDNA

The D2 region of the 28S rDNA molecule was amplified and sequenced for all 50 *Trichoderma* isolates (Table 1) as well as for a single representative isolate of *Fusarium oxysporum* (FO). Similarly, the ITS region was amplified and sequenced for 18 of the 50 *Trichoderma* isolates (Table 1), as well as isolate FO. DNA was extracted from cultures using the method described by Crowhurst *et al*. (1991) and further purified with the Wizard™ DNA Clean-Up System (Promega Corporation, Madison, WI, U.S.A.). The D2 region was amplified using the primers P2 (GAAAAGAACTTTGAAAAGAGAGTG) and P3 (CCTTGGTCCGTGTTTCAAGACG), which were based on those described in Guadet *et al*. (1989). Amplification of the ITS1 and ITS2 regions, as well as the 5.8S rDNA region was performed using primers ITS4 and ITS5 (White *et al*., 1990). Amplification reactions were performed in a volume of 100 µl containing 10 mM Tris-HCl pH 8·3, 50 mM KCl, 1·5 mM $MgCl_2$, 100 mM each of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim, Mannheim, Germany), 20 pmol each of the two appropriate primers, 40–100 ng of genomic DNA and 0·5 U of *Taq* DNA polymerase (Life Technologies Inc., Gaithersburg, MD, U.S.A.). Amplification was carried out in an Ericomp Twinblock Easy Cycler programmed for 3 min at 94 °C followed by 30 cycles of 30 s at 94°, 30 s at 55° and 30 s at 72°, followed by a final 7 min at 72°. Two replicate reactions were performed per isolate. Following amplification, the two reactions were pooled and purified using a Wizard™ PCR Clean-Up System (Promega). Purified products were subject to direct sequencing by the Centre for Gene Research, Department of Biochemistry, University of Otago, Dunedin, N.Z. All products were sequenced in both forward and reverse orientations.

Alignment of sequence data and cluster analysis

For each sequence region analysed (D2, ITS1, ITS2 or combined ITS1, 5.8S and ITS2 regions), multiple sequences were aligned using the computer program PILEUP (Wisconsin Sequence Analysis Package, Version 8, Genetics Computer Group Inc., Madison, WI, U.S.A.) and options GapWeight = 2·0 and LengthGapWeight = 0·3, A UPGMA dendrogram was produced for each data set and isolate groupings were assessed visually. Isolates with identical sequences in a particular rDNA region were removed so that each sequence type was represented once only in subsequent analyses.

Outgroup selection

Using the computer program MEGA 1.01 (Kumar, Tamura & Nei, 1993), a preliminary neighbour-joining tree (NJ) (Saitou & Nei, 1987) was constructed for the four data sets (D2, ITS1, ITS2 and combined ITS1, 5.8S and ITS2). The Jukes–Cantor distance model (Jukes & Cantor, 1969) was employed and *F. oxysporum* FO (GenBank accession no. AF055220) used as the outgroup. FO was tested as an outgroup for all data sets in order to assess the level of reliability in using an isolate from the same order (i.e. Hypocreales) to root derived trees. Unrooted topologies were constructed using the five taxa (M1057, MTM, US1, TR90 and GJS90-90) common to all four data sets. Alignment gaps (i.e. putative insertion/deletion events, or indels) were deleted using the 'pairwise-deletion'

All isolates identified by G.J.S. *T. harzianum* isolates were differentiated further into subgroups I–IV based on morphological characters. Subgroup (I) consisting of a miscellaneous group of isolates with general *T. harzianum* morphologies. (II) No odour; subglobose conidia < 3 µm diam., L/W = 1·1–1·2 µm; phialides squat, 7 µm long, L/mid point width = < 2·4 µm. (III) No odour; large warted conidia, 3·8 µm average diam., L/W 1·2 µm; long phialides 8 µm, L/mid point width 2·5 µm. (IV) No odour; yellow pigment on PDA, conidia < 3 µm diam., L/W 1·1–1·3 µm; phialides variable but < 8 µm long, L/mid point width = 2–2·5 µm. Based on morphology, *T. cf. hamatum* isolates belong to the complex that Rifai (1969) referred to as the *T. hamatum* species-aggregate.

(#) Isolates with biocontrol activity against *Armillaria*.

[a, c, d] Isolates with the same number had identical sequence. (*) indicates isolates that possessed unique sequence. Isolates with no numbers were not tested.

[b] For a particular species, each unique sequence type is represented only once in GenBank.

HortResearch, N.Z. – collection of R. Hill, Horticultural and Food Research Institute of New Zealand Ltd, Rurakura Research Centre, Private Bag 3123, Hamilton, N.Z.; ICMP – International Collection of Microorganisms, Landcare Research Ltd, Mt Albert Research Centre, Private Bag, 92169, Auckland, N.Z.; AUMCC – Auckland University Microbial Culture Collection, School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, N.Z.; J. Deacon – collection of J. Deacon, Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh, EH9 3JH, U.K.: G. Samuels – collection of G. J. Samuels, USDA–ARS, Systematic Botany and Mycology Laboratory, Beltsville, Maryland 20705, U.S.A.

option in MEGA. An indication of the level of confidence given for interior branches was achieved by generating 1000 bootstrap pseudoreplicates (Felsenstein, 1985). The rooted and unrooted topologies constructed using different data sets were compared visually.

To find suitable outgroup sequences for the ITS data sets, a Blast search (Altschul *et al*., 1990) was performed using ITS1, 5.8S and ITS2 sequence of the *T. atroviride* Karst. isolate, M1057. Phylogenetic signal was then determined for 14 sequences identified as most similar to M1057 by calculating the mean number of transition substitutions between each of these sequences and every ingroup sequence (Hillis, Allard & Miyamoto, 1993).

To determine whether the use of four outgroups would be more reliable than one, topologies were compared for a second set of preliminary NJ trees, constructed for each of the three ITS data sets. In this instance, *Neotyphodium uncinatum* (GenBank L20305), *Neotyphodium* sp. (L07134), *Epichloë* sp. (L07131) and *F. oxysporum* (AF055220) were specified as the outgroups. FO was included for consistency among data sets.

Neighbour-joining analysis. NJ trees were constructed for each of the four data sets (D2, ITS1, ITS2 or combined ITS1, 5.8S and ITS2 sequence) using the Kimura-2-parameter distance model (Kimura, 1980) and the pairwise deletion of gaps option. For the tree constructed from the D2 sequence data, FO was specified as the outgroup. For trees constructed from the three ITS sequence data sets, isolate FO plus *N. uncinatum*, *Neotyphodium* sp., *Epichloë* sp. (see above) were specified as the outgroups. One thousand bootstrap replications were performed to assess the level of support for each node. Nodes with bootstrap support of $< 80\%$ were considered to be not significantly supported.

Parsimony analysis. Most parsimonious (MP) estimates of the phylogenetic tree were generated for the D2 and the three ITS sequence data sets using PAUP 3.1.1 (Swofford, 1993). Twenty initial trees were generated using random sequence addition. Branch swapping was performed using the tree-bisection-reconnection algorithm. One thousand bootstrap replicates were also generated using these options. Gaps were treated as missing data. Outgroups were specified for each data set as described in the NJ analysis. Where four outgroups were employed, the ingroup was specified as monophyletic and the outgroup paraphyletic. A 50% majority rule consensus tree (Margush & McMorris, 1981) was calculated from the equally parsimonious trees generated for each data set. Consistency index (CI) (Kluge & Farris, 1969) was calculated for each consensus tree to give an indication of the robustness of tree topologies.

Congruence studies of ITS1 and ITS2 sequence data

Comparison of tree topologies. Initially, both rooted and unrooted topologies of all the phylogenetic trees were compared to determine if the different data sets and different tree building methods produced the same phylogeny. Unrooted trees for all phylogenetic trees were constructed using the isolates present in all four data sets (M1057, MTM, GJS90-90, TR90 and US1), excluding the outgroups.

Following this, a test based on that of Rodrigo *et al*. (1993) was performed to identify if differences observed in the topologies of the phylogenetic trees generated from the ITS1 and ITS2 data sets were significantly different or the result of sampling error. MP trees were generated from both the ITS1 and ITS2 sequence data sets using the seven taxa common to both data sets (MTM, M1057, GJS90-90, TR112, US1, HEND and TR90), as well as the four outgroups mentioned previously. These trees were generated using the heuristic search method within PAUP 3.1.1 and using 20 bootstrap test replications. Equally parsimonious trees generated from the two data sets were then compared to determine if the two data sets shared trees in common. Zero tree to tree distance values were used to determine trees common to both data sets.

First-order pruning. This is a procedure where the sequence data for a single taxon are removed from both data sets to remove possible misinformative data (Rodrigo *et al*., 1993). This procedure was performed by first generating a 50% majority rule consensus tree, from the equally parsimonious trees generated in the above topology analysis, for each of the ITS sequence data sets. ITS1 and ITS2 consensus trees were then compared to determine if the placement of one (or a few) taxon was responsible for the differences observed between the tree topologies. Sequence for GJS90-90 was subsequently removed from each of the two ITS data sets and a second bootstrapped heuristic search performed on each of the pruned data sets. Trees generated for the two data sets were again compared to determine if they shared topologies in common using zero tree to tree distance values. Average, maximum and minimum tree to tree distance values were also calculated.

A comparison was then undertaken to determine whether the differences observed between trees of the two pruned data sets fell within the range of natural variation observed among trees of a single data set. Initially, the level of variation existing between trees of the two data sets was calculated. A non-bootstrapped heuristic search was subsequently performed on each of the pruned ITS1 and ITS2 data sets separately, using TBR branch swapping and 20 replications of random taxon addition options within PAUP 3.1.1. Pruned ITS1 trees were then compared to pruned ITS2 trees using tree to tree distance values. Average, maximum and minimum distance values were also calculated.

Secondly, the level of variation within each data set was determined. Pairwise distances were calculated between trees within each of the two ITS data sets. For each data set, a maximum tree to tree distance value was calculated, as well as the percentage of the tree to tree distances that were greater than the average tree to tree distance observed between the two data sets.

Finally, the number of steps (i.e. increase in tree length) required to produce the trees of one data set from the sequence data of the other, was calculated. For this step, one of the pruned ITS sequence data sets was 'executed' under PAUP 3.1.1 while the trees generated from the heuristic search of the other pruned ITS data set, were loaded using the

'load constraints' option. The new tree length was then determined using the 'describe trees' option, and the difference in tree length between these trees and those of the data set whose sequence data was 'executed' was calculated. This calculation was performed for both ITS data sets.

RESULTS

Sequence data

Eight distinct sequence types were found in the D2 region of the 28S rDNA molecule for 50 *Trichoderma* isolates (Table 1). Eighteen representative isolates were selected to determine whether sequences from the ITS region gave greater differentiation between isolates than D2 sequences. Representatives were mainly from the two biggest D2 sequence groups (Groups 1 and 3), but also included the two *T. inhamatum* Veerkamp & W. Gams isolates and the five with unique D2 sequences. Isolates were selected to provide the greatest information for a small sample size. Eight distinct ITS1 and seven distinct ITS2 sequence types were determined for the 18 isolates (Table 1). In contrast, 5.8S rDNA sequence was totally conserved among these 18 *Trichoderma* isolates but was used to assist in the alignment of non-homologous ITS regions among the 18 isolates.

ITS1 sequence data was the most variable of the three regions investigated, followed by ITS2 and then the D2 regions (Table 2). All but ITS1 sequences appeared to have a GC base bias according to the χ^2 test ($P = 0{\cdot}01$). The ITS2 sequence showed the greatest bias with 65% GC content, followed by D2 (61%) and finally the combined ITS and 5.8S region (56%).

Cluster analysis

D2 sequence. Cluster analysis of sequence data from the D2 region revealed four main sequence types and four unique sequences among the 50 isolates. D2 sequence types were generally consistent with morphologically based groupings but not with isolate origin since the origin of isolates with Type 2 and 3 sequence overlapped (Table 1).

ITS sequence. When the ITS sequence of isolates with identical D2 sequence was analysed, isolates with Type 1, 3 and 4 D2 sequence all formed their own discrete clusters within each of the three ITS data sets (i.e. data sets consisting of ITS1, ITS2 or combined ITS1, 5.8S and ITS2 sequence). None of the ITS sequence data sets further differentiated the *T. atroviride* isolates with Type 1 D2 sequence. In contrast, Type 3 D2 isolates were further differentiated with ITS1 and, for the most part, ITS2 sequence and their groupings were consistent with morphology.

Table 3. Mean transition frequencies for pairwise comparisons between sequence from each outgroup test organism and sequences of the 18 *Trichoderma* isolates in each of the three ITS sequence data sets

	GenBank accession no.	Transition frequencies		
		ITS1	ITS2	ITS1, 5.8S+ITS2
Cladosporium sphaerospermum Penz.	L25433	0·47	0·27	0·40
Neotyphodium uncinatum (W. Gams, Petrini & D. Schmidt) Glenn, Bacon & Hamlin	L20305	0·45	0·48	0·47
N. uncinatum	L07135	0·42	0·49	0·42
Neotyphodium sp.	L07134	0·41	0·49	0·42
Fusarium oxysporum Schltdl.*	AF055220	0·38	0·37	0·41
N. uncinatum	L07128	0·37	0·48	0·46
Epichloë sp.	L07131	0·37	0·43	0·39
Fusarium sambucinum Fuckel	X65480	0·34	0·47	0·45
E. typhina (Pers.) Tul	L07133	0·29	0·48	0·38
Gaeumannomyces graminis (Sacc.) Arx & D. L. Olivier	U17207	0·27	0·41	0·41
E. typhina	L07132	0·25	0·48	0·38
E. amarillans J. F. White	L07129	0·25	0·45	0·37
Magnaporthe grisea (T. T. Hebert) M. E. Barr	U17329	0·25	0·44	0·41
Phialophora graminicola (Deacon) J. Walker	U17217	0·24	0·35	0·31
Acremonium lolii Latch, M.J.C. & Samuels	L07130	0·23	0·47	0·35

* Sequence alignments available from Sarah Dodd, Soil Plant and Ecological Sciences Division, Lincoln University, N.Z.

Table 2. Properties of *Trichoderma* sequence data*

Sequence region	Length of sequence (bp)	Sequence types shared by > 1 isolate (%)	Variable sites (%)	Alignment gaps (no.)	G+C (%)
D2	199	50	11	6	61
ITS1	213	50	24	88	53
ITS2	180	71	18	44	65
ITS1, 5.8S and ITS2	557	50	16	132	56

* Isolate whose ITS sequence was obtained as described in Materials and Methods.

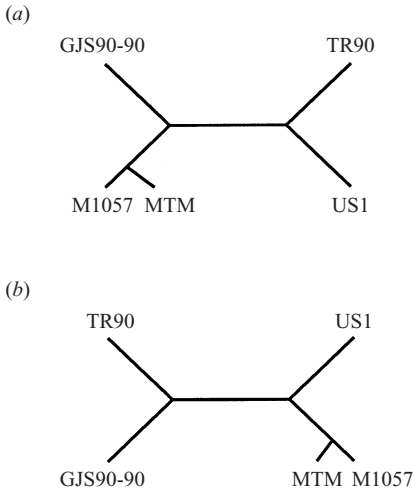

Fig. 1. The two unrooted topologies derived from all phylogenetic trees using the taxa common to all data sets, excluding the outgroup *Fusarium oxysporum*. Tree (*a*) represents the unrooted topology of all D2, ITS1 and ITS2 neighbour-joining trees and the neighbour-joining tree of the combined ITS sequence data set where *F. oxysporum* was specified as the outgroup. It also represents the unrooted topology of ITS1 and ITS2 parsimony trees. Tree (*b*) represents the unrooted topology of the neighbour-joining tree of the combined ITS sequence data set where four outgroups were specified.

The two *T. inhamatum* isolates (GJS90-89 and GJS90-90) with Type 4 D2 sequence differed from one another by only one nucleotide in the ITS1 region. The three isolates with unique D2 sequence (MTM, TR90 and FO) also possessed unique sequence in both ITS regions.

Outgroup selection

To ensure the correct placement of an outgroup within a tree, the outgroup sequence must contain sufficient comparable phylogenetic signal to the ingroup sequences. The level of phylogenetic signal is considered sufficient for analysis if the mean number of transition substitutions between the outgroup sequence and each of the ingroup sequences is greater than 50% (Hillis *et al.*, 1993). A mean transition frequency of 54% existed between D2 sequence data from the *F. oxysporum* outgroup and that of the *Trichoderma* ingroup. It was, therefore, assumed that sufficient phylogenetic signal existed between these sequences. In contrast, sequence of *F. oxysporum* produced mean transition frequencies of less than 50% with all three ITS sequence data sets (Table 3) indicating insufficient signal for rooting. Lack of signal was also indicated by the fact that differences were observed among the topologies of the four preliminary NJ trees (not shown), yet all four data sets produced the same unrooted topology (Fig. 1*a*).

The 14 sequences selected from the GenBank database search, and showing the greatest sequence similarity to the ITS sequence of M1057, were found to all have mean transition frequencies of less than 50% with the 18 corresponding *Trichoderma* sequences (Table 3). When the three fungi that gave the best overall transition frequencies for all three ITS data sets (*Neotyphodium uncinatum*, *Neotyphodium* sp. and *Epichloë* sp.) plus *F. oxysporum* were used as an outgroup, they were all placed in the same position for each of the three ITS trees (not shown). This result indicated an acceptable level of reliability.

Neighbour-joining analysis

D2 sequence. D2 sequence data produced a NJ tree in which only two clades were resolved (Fig. 2*a*), as indicated by a greater than 80% bootstrap support. These were the clade consisting of *T. atroviride* M1057 and *T. koningii* Oudem. MTM, and the clade with *T. cf. virens* (J. H. Mill., Giddens & A. A. Foster) Arx 5412. Phylogenetic relationships among all other groups were not resolved.

ITS sequence. Sequence from the ITS1 region provided the greatest amount of phylogenetic information out of the three regions investigated. The ITS1 tree was able to resolve five clades (Fig. 2*b*), whereas the ITS2 (Fig. 2*c*) and combined ITS sequence trees (Fig. 2*d*) could only resolve three.

Like the D2 NJ tree, all three ITS data sets gave high bootstrap support for the clade consisting of *T. atroviride* M1057 and *T. koningii* MTM. In addition, the three ITS trees all gave high bootstrap support for the clade consisting of isolates with Type 3 D2 sequence (Table 1, Fig. 2), *T. harzianum* I (TR112), *T. harzianum* II (US1) and *T. harzianum* IV (HEND). *T. cf. hamatum* (Bonord.) Bainier OMK was also included in this clade for the ITS1 and combined data sets. Although ITS1 sequence was unable to further resolve the members of this clade, ITS2 sequence and combined ITS was able to distinguish the *T. harzianum* IV isolate HEND from the other members of the clade.

The ITS1 tree also distinguished *T. inhamatum* (GJS90-89 and GJS90-90 from one another and *T. cf. koningii* TR90 from all other isolates. Similarly, the combined ITS tree was able to distinguish the *T. inhamatum* group.

Comparison of the topologies of the three ITS NJ trees revealed differences in the placement of taxa within the trees. The placement of taxa within ITS2 NJ tree appeared to be in the reverse order to that produced in the ITS1 and combined ITS NJ trees. Only two of the main clades were, however, resolved within the ITS2 tree as opposed to four in the ITS1 tree, thus giving less support for the order of taxa within the ITS2 tree.

Parsimony analysis

The MP trees essentially produced the same isolate groupings and order of clades as the NJ trees for the corresponding data sets (Fig. 3). All four trees had high consistency indices indicating a high level of support for their topologies. Fewer groups were, however, resolved within each of the trees compared to the NJ method, as indicated by fewer clades with significant bootstrap support ($> 80\%$). As with NJ trees, all four MP trees gave high bootstrap support fo the *T. atroviride* (M1057) and *T. koningii* (MTM) clade. Similarly, the MP D2 tree distinguished *T. cf. virens* 5412 from all other isolates, the

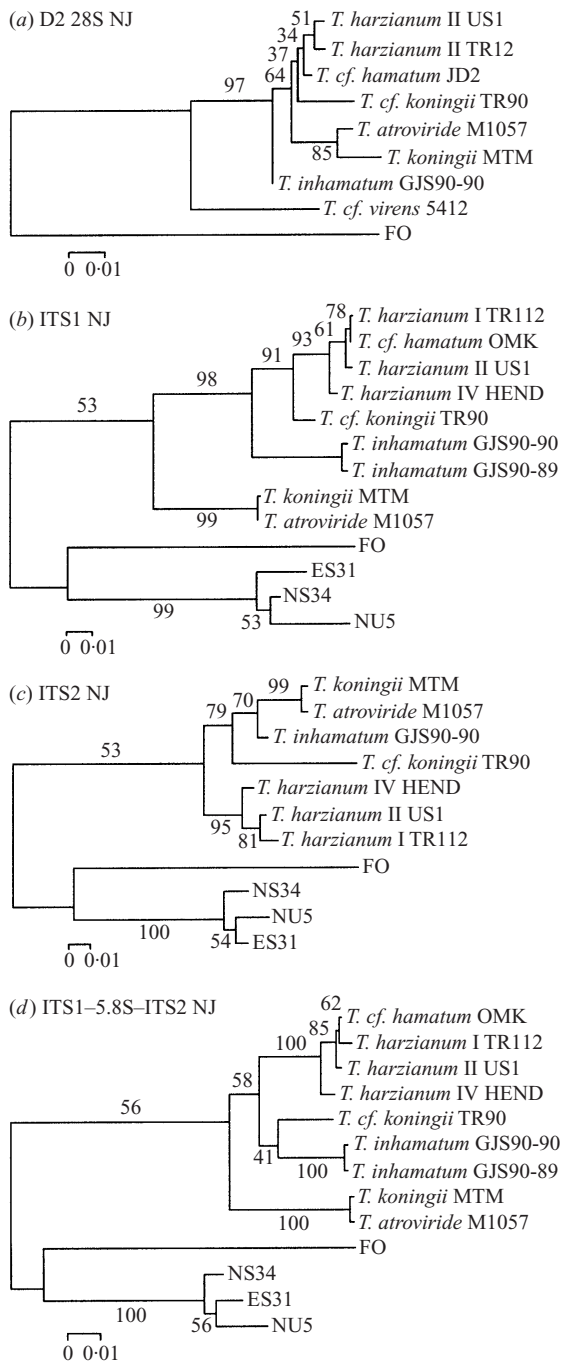

Fig. 2. Neighbour-joining (NJ) estimates of *Trichoderma* isolate phylogeny for sequence data from the (*a*) D2 region of the 28S ribosomal gene, (*b*) ITS1 region, (*c*) ITS2 region and (*d*) ITS1, 5.8S and ITS2 combined regions of the ribosomal genes. Trees were generated using the Kimura-2-parameter distance model with *Fusarium oxysporum* (FO) specified as the outgroup for the D2 sequence tree. *Neotyphodium uncinatum* (NU5), *Neotyphodium* sp. (NS34), *Epichloë* sp. (ES31) and *F. oxysporum* (FO) were specified as the outgroups for the ITS sequence trees. Values associated with branches indicate the degree of bootstrap support expressed as percentage of 1000 bootstrapped trees in which the corresponding clades are present. Scale bars indicate horizontal branch length, expressed as the hypothesised number of nucleotide substitutions per site.

ITS1 tree distinguished the *T. inhamatum* GJS90-89 and GJS90-90 from other isolates and the ITS2 tree supported the clade of isolates (US1, TR112 and HEND) with Group 3 type D2 sequence (Table 1, Fig. 3). Apart from these examples, no other groups were resolved within the four MP trees.

Congruence studies for ITS1 and ITS2 sequence data

Comparison of the unrooted topologies of each of the NJ and MP phylogenetic trees revealed two different topologies for the five taxa in common (Fig. 1). This suggests that the differences in topology were attributed to something other than the unreliable placement of outgroups. Note, unrooted trees could not be constructed from the D2 and combined ITS parsimony trees because two (US1 and TR90) of the five taxa were unresolved within these trees.

Comparison of tree topologies. No zero tree to tree distance values were generated when the equally parsimonious ITS1 trees were compared to those of the ITS2 data set (i.e. no trees were found that were common to both data sets). Differences observed in the topologies were, therefore, significant and not due to sampling error.

First-order pruning. Inspection of the ITS1 and ITS2 consensus parsimony trees (Fig. 4) revealed the placement of taxon GJS90-90 was corrupting their topologies. Sequence data for GJS90-90 was subsequently pruned from both data sets and each set reanalysed (Fig. 5). Comparison of pruned ITS1 and ITS2 trees revealed 3·4% of the total trees were common to both data sets. The average distance (i.e. measure of topological difference) between pruned ITS1 and ITS2 trees was 3·8 (Max = 8, min = 2). When the distances among trees generated from the ITS1 data set were calculated, 33·7% of the comparisons were found to differ by a distance greater than 3·8 (Max distance = 8). Similarly, 29·7% of the ITS2 tree comparisons were found to differ by a distance greater than 3·8 (Max = 6). Since the average distance between pruned ITS1 and ITS2 trees fell within the range of the natural variation observed within each data set, and the maximum distances within each data set did not exceed that of ITS1 and ITS2 tree comparisons, the differences in the topologies of the pruned ITS1 and ITS2 trees were not significant and the hypothesis that these differences are due to sampling error can not be rejected.

Tree length increased by a distance value of seven when pruned ITS1 sequence data were made to fit the topology of the trees generated from the pruned ITS2 data set. Only a two step increase was, however, required for pruned ITS2 data to fit the topology of the trees generated from the pruned ITS1 data set.

DISCUSSION

In the present study, sequence data from the D2 region of the 28S rRNA gene, the 5.8S rRNA gene and the two ITS regions of the ribosomal gene cluster were assessed for their usefulness in distinguishing among isolates of *Trichoderma*. These data were also assessed for their phylogenetic robustness in predicting the relations of isolates within the genus.

Sequence data from the D2 region differentiated the 50 *Trichoderma* isolates into groups that were mostly consistent

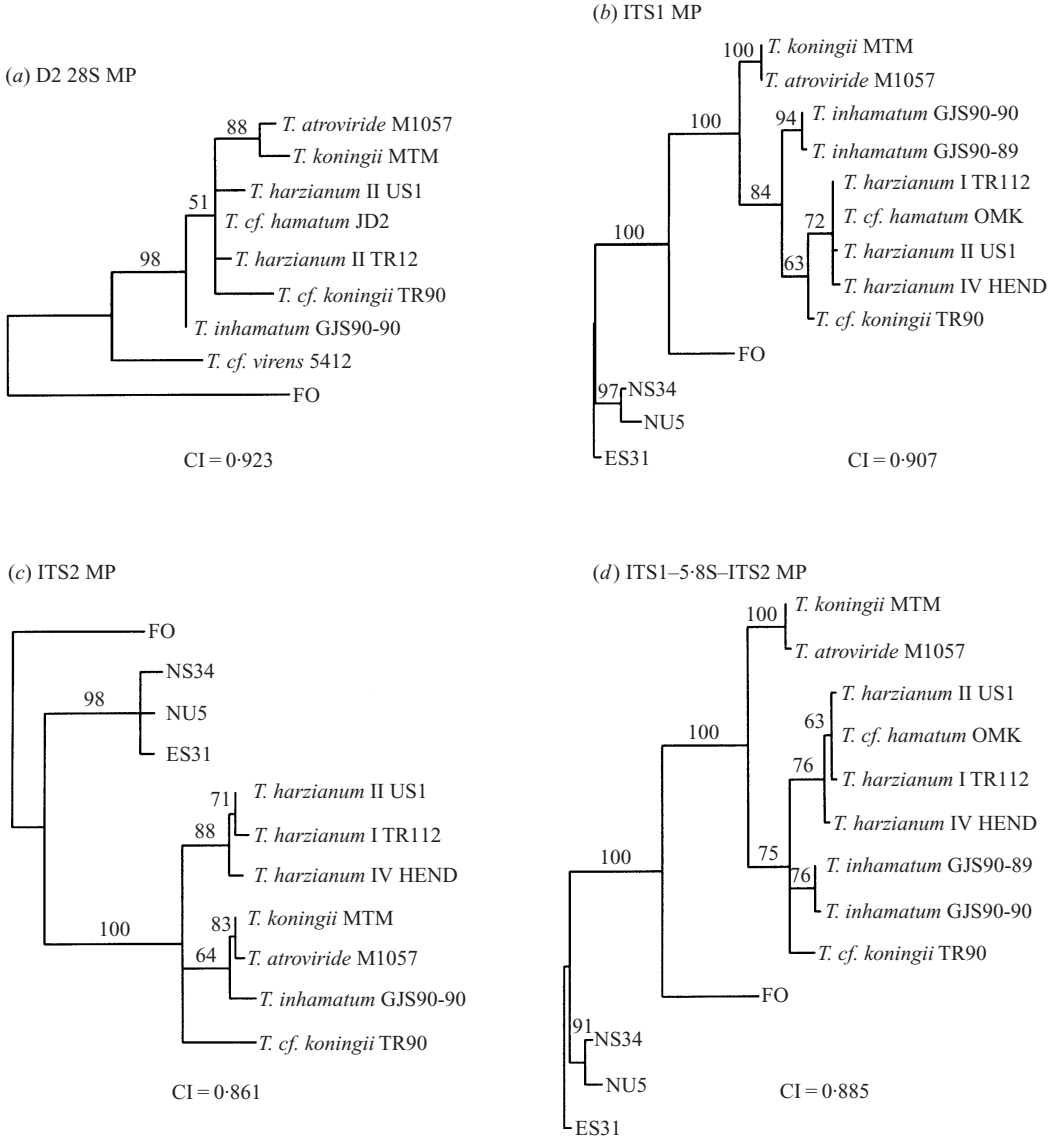

Fig. 3. Most parsimonious (MP) estimates of *Trichoderma* isolate phylogeny (*a*) for sequence data from the D2 region, with *Fusarium oxysporum* (FO) specified as the outgroup, and for sequence data from the (*b*) ITS1, (*c*) ITS2 and (*d*) ITS1, 5.8S and ITS2 combined regions, with *Neotyphodium uncinatum* (NU5), *Neotyphodium* sp. (NS34), *Epichloë* sp. (ES31) and *F. oxysporum* (FO) specified as the outgroups. Values above branches indicate the degree of bootstrap support expressed as percentage of 1000 bootstrapped trees in which the corresponding clades are present. Branches for which there are no bootstrap values occur in less than 50% of the bootstrapped trees. CI = consistency index.

with morphology, grouping them at a level between genus and species. These D2 groupings did not, however, correlate to the sections proposed by Bissett (1991*a*), since the different sub-groups of *T. harzianum* were distributed between two sequence types. The few exceptions to consistency between the D2 sequence and morphological groupings were where the *T. atroviride* isolates were separated into two different sequence Types (Types 1 and 3), the separation of six *T. cf. hamatum* isolates into two types (five in Type 2 and one in Type 3), and where the *T. harzianum* II isolates were separated into two types (seven had Type 3 sequence and one had unique sequence). Muthumeenakshi *et al*. (1994) and Kuhls *et al*. (1997) also found sequence data provided more consistent and reliable groupings of *Trichoderma* species than morphological identifications. Such findings emphasize the difficulties experienced in using morphological and cultural characters alone to differentiate isolates of this genus.

The low level of variation among isolates in the D2 sequence suggests this region was not suitable for determining phylogenetic relationships within or among species of *Trichoderma*. Similarly, Muthumeenakshi *et al*. (1994) found insufficient variability in the D2 region to distinguish among morphologically distinct isolates of the species *T. harzianum*.

Of the four regions of ribosomal DNA tested (D2, ITS1, 5.8S and ITS2), sequence data from the ITS1 region contained the most informative data enabling the greatest differentiation among the *Trichoderma* isolates.

ITS sequences of our strains were compared with those from the studies of Muthumeenakshi *et al*. (1994, 1998) and Ospina-Giraldo *et al*. (1998) to determine whether the isolates

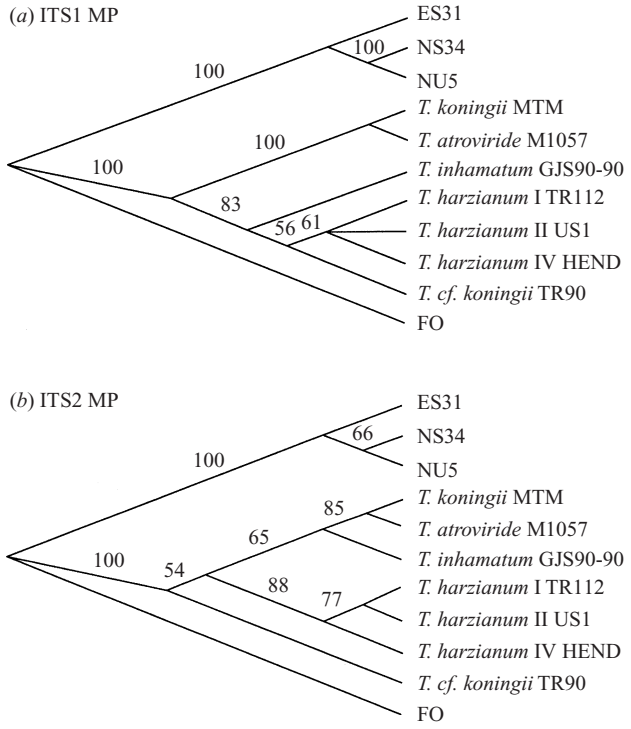

Fig. 4. Most parsimonious (MP) trees for sequence from the (*a*) ITS1 and (*b*) ITS2 data sets. The ITS1 data set has had sequence for the taxa OMK and GJS90-89 removed so that both ITS1 and ITS2 data sets have identical taxa. Both trees represent the 50% majority rule consensus tree from a bootstrapped (20 replications) heuristic search using the TBR branch swapping and random taxon addition (20 replications) options. The trees differ in their placement of the *T. inhamatum* isolate GJS90-90.

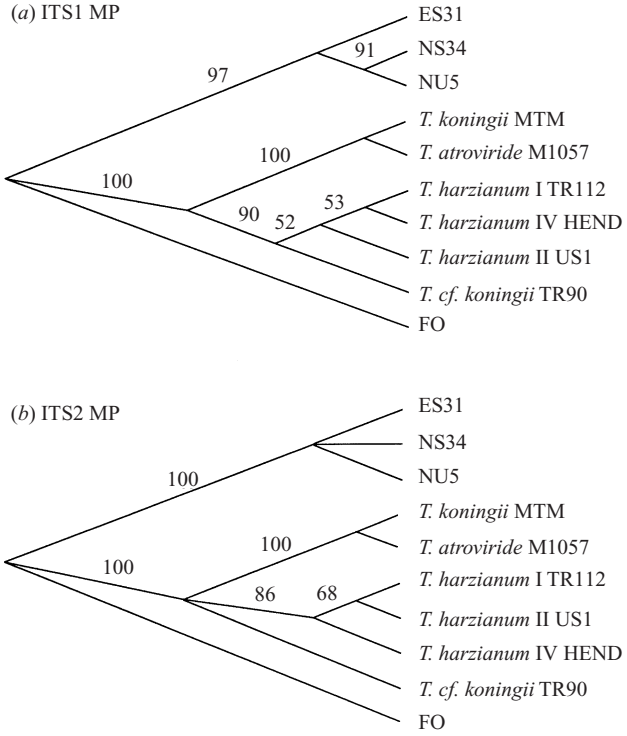

Fig. 5. Most parsimonious (MP) trees for sequence from the (*a*) ITS1 and (*b*) ITS2 data sets. In addition to the removal of OMK and GJS90-89 sequence from the ITS1 data set, both ITS1 and ITS2 data sets have had GJS90-90 sequence removed. Both trees represent the 50% majority rule consensus tree from a bootstrapped (20 replications) heuristic search using the TBR branch swapping and random taxon addition (20 replications) options.

that exhibited good biological control activity were similar to those that were aggressive pathogens of commercial mushrooms in the British Isles and North America. Their studies found isolates that were aggressive colonizers of commercially grown mushrooms possessed either Th2 or Th4 type ITS1 sequence and that less aggressive isolates possessed either Th1 or Th3 type sequences. None of the strains tested in the present study possessed the aggressive type sequences, Th2 and Th4. In fact, the majority of these isolates shared greater sequence similarity to isolates of the less aggressive groups, Th1 and Th3. In particular, the two *T. harzianum* I isolates TR112 and TR108 shared 100% sequence similarity to the Th1 isolates.

Comparison of ITS1 sequence of the *T. harzianum* isolates (US1, TR112, TR108, G4 and HEND) to that of the proposed 'true' *T. harzianum* neotype strain of Gams & Meyer (1995) (CBS 226·95; EMBL AJ222720), revealed that US1 shared 100% nucleotide sequence similarity with it. The remaining four *T. harzianum* isolates also shared high sequence similarity to the neotype, with Th1 isolates TR112 and TR108 differing in only three nucleotide positions, and the G4 and HEND isolates differing by four. This high similarity in ITS1 sequence lends support for the identities of these isolates as 'true' *T. harzianum*. Further to this, Muthumeenakshi *et al.* (1998) recently proposed that their Th1 isolates corresponded to the neotype *T. harzianum* strain, based on ITS1 sequence similarity.

With respect to the other species investigated, *T. atroviride* M1057, OG3, TTS, M1037, HPP1, TBHPP7, D and D74 all shared 100% ITS sequence similarity with three other *T. atroviride* isolates (GenBank X93948, X9394, Z48817), three isolates referred to as *T. harzianum/atroviride* (Kuhls *et al.*, 1996) and a biocontrol *T. harzianum* strain LC1 (Schlick *et al.*, 1994). Of these, the *T. harzianum* isolates have since been reidentified as *T. atroviride* (W. Gams, pers. comm.). Similarly, Opsina-Giraldo *et al.* (1998) have reported that the *T. harzianum* Th3 group of Muthumeenakshi *et al.* (1994) shared high ITS sequence similarity with isolates of *T. atroviride*. Based on their results, these authors propose Th3 to be *T. atroviride*. The *T. atroviride* strains examined in our study also shared 99% sequence similarity in the ITS1 region to that of the Th3 *T. harzianum* group of Muthumeenakshi *et al.* (1994). Furthermore, the morphology of our strains is consistent with that of *T. atroviride*. It is, therefore, clear that Th3 is *T. atroviride* and should henceforth be referred to as such, and not as a group of *T. harzianum*.

Similarly, *T. koningii* MTM differed by only one nucleotide in the ITS2 region when its ITS sequence was compared to that of the proposed *T. koningii* neotype (CBS 459.96, GenBank Z79628) of Liekfeldt *et al.* (1998).

For the remaining strains used in this study, only some of the identities were supported when ITS sequence was compared with those of the same species in the GenBank

database. These included the *T. harzianum* I, II and IV isolates, TR112, TR108, HPP1, TBHPP7 and US1. In contrast, *T. cf. hamatum* OMK and *T. inhamatum* GJS90-89 and GJS90-90 shared greater sequence similarity to other species. *T. cf. koningii* TR90 was unique in that it not only shared $< 90\%$ ITS sequence to other *T. koningii* isolates in the database but also shared low sequence similarity with other *Trichoderma* spp., suggesting this isolate may be an outlier of *Trichoderma*.

With respect to the analysis of sequence data, numerous studies have been published where fungal phylogenies are predicted using ribosomal sequence data but no indication is given of how robust the data were and, therefore, how reliable the phylogenies were (for example, Bryan, Daniels & Osbourn 1995; Glenn *et al*., 1996; Sreenivasaprasad *et al*., 1996; Kuhls *et al*., 1997). Results of the present study reveal the importance of conducting such tests as, in this instance, different data sets for the same group of isolates produced significantly different phylogenies. A number of steps were undertaken to test the reliability of the phylogenies produced. First, a suitable outgroup was selected. The outgroup had to be adequately removed from the *Trichoderma* ingroup but possess enough phylogenetic signal to place it correctly within the tree. Results show four outgroups were required to root the trees with sufficient confidence.

Secondly, for the same group of isolates, four different data sets were analysed using two different methods to determine if the data sets all produced the same phylogeny. Although the two neighbour joining and parsimony methods produced the same relative groupings of isolates for the different data sets, significant differences in topology were observed between the two ITS data sets. The order of taxa within the ITS2 sequence trees appeared to be in the reverse order to those within the ITS1 trees. When the two data sets were combined and analysed, the ITS1 order dominated over the ITS2 and the resolution of some groups within the tree was poor.

The third step was to perform congruence studies on the two data sets. Results from these revealed sequence from *T. inhamatum* GJS90-90 and GJS90-89 was corrupting tree topologies. The *T. inhamatum* isolates differed in ITS sequence by just one nucleotide in the ITS1 region. Waalwijk *et al*. (1996) observed a similar discordance between ITS1 and ITS2 sequence based phylogenies generated for species of *Fusarium* and suggest that the differences may be due to the hypothetical co-existence of several ITS types in the ancestral *Fusarium* species. The recent discovery of more than one sequence type in the ITS2 region of individual *Gibberella fujikuroi* isolates lends support to this (O'Donnell, Cigelnik & Nirenberg, 1998).

For our study, however, an alternative explanation might be that the sequence of *T. inhamatum* was corrupting the ITS trees and may be doing so because it could potentially be a hybrid of the two different groups to which it was assigned. By referring to sequence data and comparing the number of nucleotides that GJS90-90 had in common with each group (i.e. the MTM and M1057 group and the TR112, US1 and HEND group), it appears that GJS90-90 shares sequence with the TR112, US1 and HEND group in the ITS1, the 5.8S regions plus the first 76 nucleotides of the ITS2 region. For the remainder of the ITS2 region and the D2 region of the 28S like rRNA gene, which lies downstream from the ITS2 region, there was an almost equal number of shared nucleotides with each group. This overall pattern suggests that a recombination event could have occurred within the ITS2 region of this isolate. In which case, the ITS1 and initial ITS2 regions represent one end of the recombination event and the rest of the ITS2 and the D2 regions represent a region of heteroduplex DNA.

In further support of this explanation, the 700 bp stretch of proposed heteroduplex DNA falls within the average length (i.e. 1·5 kb long) of heteroduplex regions observed in yeast (Borts & Harber, 1987). Results of the present study, therefore, suggest that the tree congruence studies of Rodrigo *et al*. (1993) may provide an alternative method for determining the presence of recombinant DNA and the sites of recombination events in fungal genomes as opposed to the more traditional restriction site polymorphism studies.

By removing *T. inhamatum* sequence from both data sets, it was found that both data sets essentially produced the same topologies. Differences can be attributed to the lack of phylogenetic information within the ITS2 region as indicated by the fact that ITS2 sequence data was close to producing the ITS1 tree topology (i.e. an increase in tree length of only two when ITS2 data was made to fit the ITS1 tree topology). Had any of the original phylogenies been taken at face value, some of the isolate relationships would have been in error, based on an incorrect phylogeny.

Examination of the pruned phylogenetic ITS trees show morphological species were not always clearly defined. This is illustrated by the fact that the species *T. koningii* had strains 'misplaced' in the same clade as isolates identified as a different morphological species. In other words *T. koningii* was paraphyletic and its phylogenetic relationship to other species within the genus was unresolved. Kuhls *et al*. (1997) also found morphological species of *Trichoderma*, including *T. koningii*, to be paraphyletic when the ITS sequence was analysed using the maximum parsimony method. Similarly, analysis of other molecular and biochemical data also support this finding. For example, Stasz *et al*. (1989) found morphological species of *Trichoderma* to be paraphyletic when isoenzyme data from 71 isolates, representing four morphological species, were analysed using the parsimony method. Leuchtmann, Petrini & Samuels (1996) also found UPGMA analysis of isoenzyme data identified paraphyletic *Trichoderma* species.

Although not always consistent with morphology, the grouping of *Trichoderma* isolates based on ITS and D2 sequences was conserved between the two data sets. For instance, isolates sharing a common D2 sequence (TR112, HEND and US1) were also grouped together in both ITS trees. In addition, the close relationship between *T. atroviride* M1057 and *T. koningii* MTM was also conserved. Although the ITS1 sequence gave the best overall estimate of the phylogenetic tree, like the other sequence data, this data could not be used to resolve phylogenetic relationships among isolates sharing the Type 3 D2 sequence. More data would be required to fully resolve these phylogenetic relationships. Analysis of other variable regions within the genome may provide the required level of variability.

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