Programme: Bioprotection for Foliar Diseases and Disorders of Radiata Pine

Task 2.8: Confirm taxonomic identifications of the most effective *Trichoderma* isolates based on sequence data

Task 2.9: Evaluate the use of UP-PCR to distinguish between *Trichoderma* strains used in seed treatments

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EXECUTIVE SUMMARY

The main aim of the *Bioprotection for Foliar Diseases and Disorders of Radiata Pine* research programme is to establish long term symbiotic relationships between *Pinus radiata* and endophytic *Trichoderma* species which have been shown to induce systemic resistance and provide protection from foliar diseases. A large number of *Trichoderma* strains have been isolated and screened for the ability to promote growth and supress disease in laboratory and nursery trials. Some of the best isolates have been applied as seed treatments and are currently being evaluated for field performance in plantation trial sites around the country.

Modern research with *Trichoderma* and other biocontrol fungi is reliant on analysis of DNA sequence data for unambiguous identification of isolates to species level. Application of other molecular techniques such as DNA fingerprinting methods may allow differentiation of biocontrol stains from natural fungal populations and obtain information on the long term persistence and activity of *Trichoderma* after field application.

In the research described here we provide robust identifications of the most effective *Trichoderma* isolates based on analysis of DNA sequence data and according to the most recent taxonomic concepts in the genus (Task 2.8). Phylogenetic relationships with other isolates from New Zealand and overseas were also assessed. The utility of a DNA fingerprinting method (UP-PCR) was also evaluated for its ability to discriminate between strains applied as seed treatments in plantation trials (Task 2.9). This research represents an important prequisite step for the further development of specific molecular methods for field monitoring of *Trichoderma* biocontrol strains.

INTRODUCTION

The genus *Trichoderma* has been the subject of extensive taxonomic revision in recent years and currently includes over 200 named species. Due to the recognition of many 'phylogenetic species' which cannot be readily identified using traditional morphological criteria, DNA sequence-based analysis has now become the standard method for *Trichoderma* indentification. Sequence data from the gene encoding for the translation elongation factor 1α (tef1) is generally used for routine identification as analysis of this region is capable of differentiating all known *Trichoderma* species.

The aim of task 2.8 was to identify a number of endophytic *Trichoderma* strains that were isolated from surface sterilised plant roots and have shown good results in bioassays for growth promotion or disease suppression in laboratory and nursery trials. PCR, sequencing, and phylogenetic analysis of tef1 region was used to identify isolates to species level and examine their relationships with other NZ and overseas isolates

Trichoderma species are ubiquitous in the environment, and efforts to determine the field persistence of applied biocontrol strains require the development of specific molecular markers capable of distinguishing the strains of interest from background *Trichoderma* populations that are normally present in field sites. Universally primed PCR (UP-PCR) is a highly reproducible DNA fingerprinting method that has been successfully used to generate unique strain-specific profiles to allow field monitoring of *Trichoderma* and other fungal biocontrol agents. Amplification products from UP-PCR can also be used to develop strain-specific primers for direct detection of fungal from environmental samples such as roots and soil. Task 2.9 aimed to evaluate this method for discriminating between a group of *Trichoderma* isolates currently being tested in plantation trials.

METHODS

DNA extraction

For DNA extraction a loopful of conidia from pure cultures of each of 17 isolates was spread with a glass spreader onto a plate of 1% malt extract agar overlaid with sterile colourless cellophane. Plates were incubated for two days to allow a thin layer of mycelium to cover the plate. Approximately 50 mg of mycelium was harvested with a flamed scalpel into a sterile 1.5 ml Eppendorf tube and frozen at -20°C. Genomic DNA was extracted from mycelium using a Geneaid Genomic DNA Mini Kit (plant) and stored at -20°C.

PCR, sequencing,and phylogenetic analysis

For each isolate an approximately 900 bp fragment from the 5' end of the tef1α gene was amplified using the primer pair tef71f (C AAA ATG GGT AAG GAG GAS AAG AC) and tef997R (CA GTA CCG GCR GCR ATR ATS AG) (Shoukouhi and Bissett 2009). All PCR amplifications were performed in a total reaction volume of 20 µl including 0.4 µM of each primer, 200 uM dNTPs, 2. µl 10X reaction buffer, 2.5 mM MgCl2, 2 µl template DNA and 1U Taq Polymerase.

PCR conditions followed a procedure described by Hoyos-Carvajal *et al*. (2009). DNA was initially denatured for 6 min at 95°C, followed by 4 cycles each of 1 min at 95°C, 90 s at 70°C, 90 s at 72°C, followed by 26 cycles with the annealing temperature decreasing from 68°C to 55°C (a gradient of 0.5°C reduction per cycle), followed by 12 cycles with the annealing temperature at 55°C and a final extension of 7 min at 72°C. PCR products were visualised by ethidium bromide staining following electrophoresis of 5 µl of each product in 1% agarose gels. PCR products were cleaned using a commercial cleanup kit (Zymo Clean & Concentrator-5) and sequenced in both forward and reverse directions using the internal sequencing primers tef85f (AG GAC AAG ACT CAC ATC AAC G) and tef954r (AGT ACC AGT GAT CAT GTT CTT G) (Shoukouhi and Bissett 2009) at the Bio-Protection DNA Sequencing Facility (Lincoln University).

Consensus sequences were assembled from forward and reverse sequencing chromatograms using ChromasPro v. 1.7.6. Following BLAST searching to identify species afilliations, sequences were incorporated in datasets with representative sequences from NZ and overseas *Trichoderma* collections (including type specimens where available). Sequence alignments were performed using MUSCLE (Edgar 2004) implemented in MEGA 6 (Tamura *et al.* 2013). Phylogenetic analyses were conducted in MEGA 6 using the neighbour-joining method (Kimura 2-parameter) with branch support from bootstrap analysis (1000 replicates).

UP-PCR

Thirteen isolates which are currently being examined in plantation trials were analysed. UP-PCR amplifications were performed in a total reaction volume of 25 µl including 0.4 µM of primer L45 primer, 200 uM dNTPs, 2.5 µl 10X reaction buffer, 2.5 mM MgCl2, 2 µl template DNA and 1U Taq Polymerase. Amplification conditions were: 5 min at 94°C, five cycles of 50 s at 94°C, 2 min at 51°C and 1 min at 72°C, 35 cycles of 50 s at 94°C, 90 s at 51°C and 1 min at 72°C, and a final extension step of 72°C for 10 min. The UP-PCR products were separated by electrophoresis on 1.5% agarose at 100V for 3 h.

RESULTS

Tef1 analysis

Amplication products from all isolates using the tef71f and tef997r primers were approximately 900 basepairs (bp) in length (e.g. Fig. 1 below). Consensus sequences of approximately 770 bp were assembled following sequencing with internal primers tef85f and tef954r.

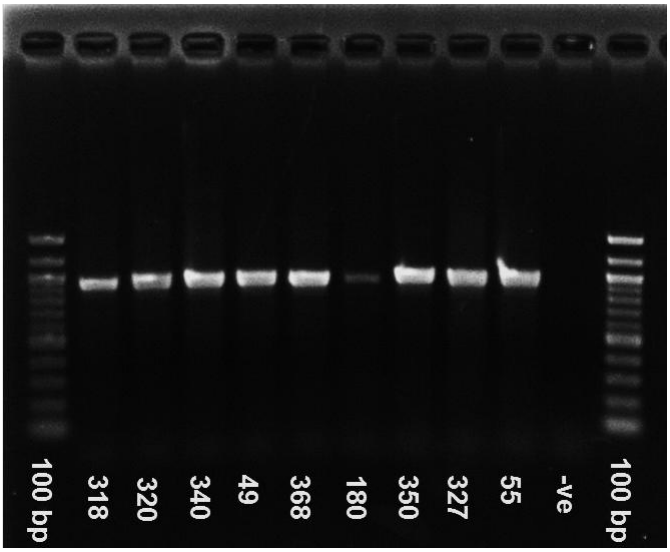


Figure 1. 900 bp fragments amplified by primers trf71f and tef997r.

Table 1.. Identification of isolates following phylogenetic analyses of the tef1 gene.

Isolate	*Identification*
FCC49	**sp. nov. "792"**
FCC55	***Trichoderma harzianum***
FCC161	***Trichoderma harzianum***
FCC180	***Trichoderma crassum***
FCC275	***Trichoderma atroviride***
FCC318	***Trichoderma. atrobrunneum***
FCC319	***Trichoderma atrobrunneum***
FCC320	***Trichoderma atrobrunneum***
FCC322	***Trichoderma koningiopsis***
FCC327	***Trichoderma harzianum***
FCC333	***Trichoderma harzianum***
FCC340	***Trichoderma harzianum***
FCC350	***Trichoderma koningiopsis***
FCC360	***Trichoderma atroviride***
FCC362	***Trichoderma crassum***
FCC368	***Trichoderma atrobrunneum***
FCC392	***Trichoderma polysporum***

The greatest number of FCC isolates grouped in a clade centred around *T. harzianum* (Fig. 2*)*. Several isolates with identical tef1 sequences (FCC 55, 161, 327, 333, 340) clustered with other NZ and overseas isolates of *T. harzianum* according to the strict definition of this species following a recent taxonomic revision (Chaverri *et al.* 2015). Four isolates (FCC 318, 319, 320, 368) and another NZ isolate (LU688) grouped closely with the newly described species *Trichoderma atrobrunneum.* However, the NZ isolates of *T. atrobrunneum* form a distinct separate clade which may represent an additional species*.*

Two isolates (FCC 322, 350) were identified as *T. kongiopsis (*Fig. 3a*)*. FCC 350 was shown to have an identical sequence to previous isolations of this species from NZ while FCC 322 grouped more closely with overseas representatives.

The two isolates identified as *T. atroviride* in this study (FCC275, 360) each fall into distinct clades previously identified within this species in earlier taxonomic work at the Bio-Protection Centre (Fig. 3b.) These clades will be formally recognised as separate species in an upcoming publication by by Centre researchers.

FCC 49 corresponds with another new species identified at Bio-Protection Centre, currently known as “species 792” (Fig. 4a.) and thought to represent a taxon endemic to NZ. All collections of this species have been made from surface sterilised roots suggesting that the species may be restricted to this habitat.

Two isolates with identical sequences were identified as *T. crassum* (Fig. 4b) grouping more closely with an overseas representative than with previous NZ collections of this species.

A single isolate (FCC 392) was determined as *T. polysporum* (Fig. 5), and this identification is also supported by the distinctive white conidia produced by this strain in agar cultures. Significant phylogenetic diversity was found following analysis of available sequences for this species suggesting that the group should probably be revised and split into further species.

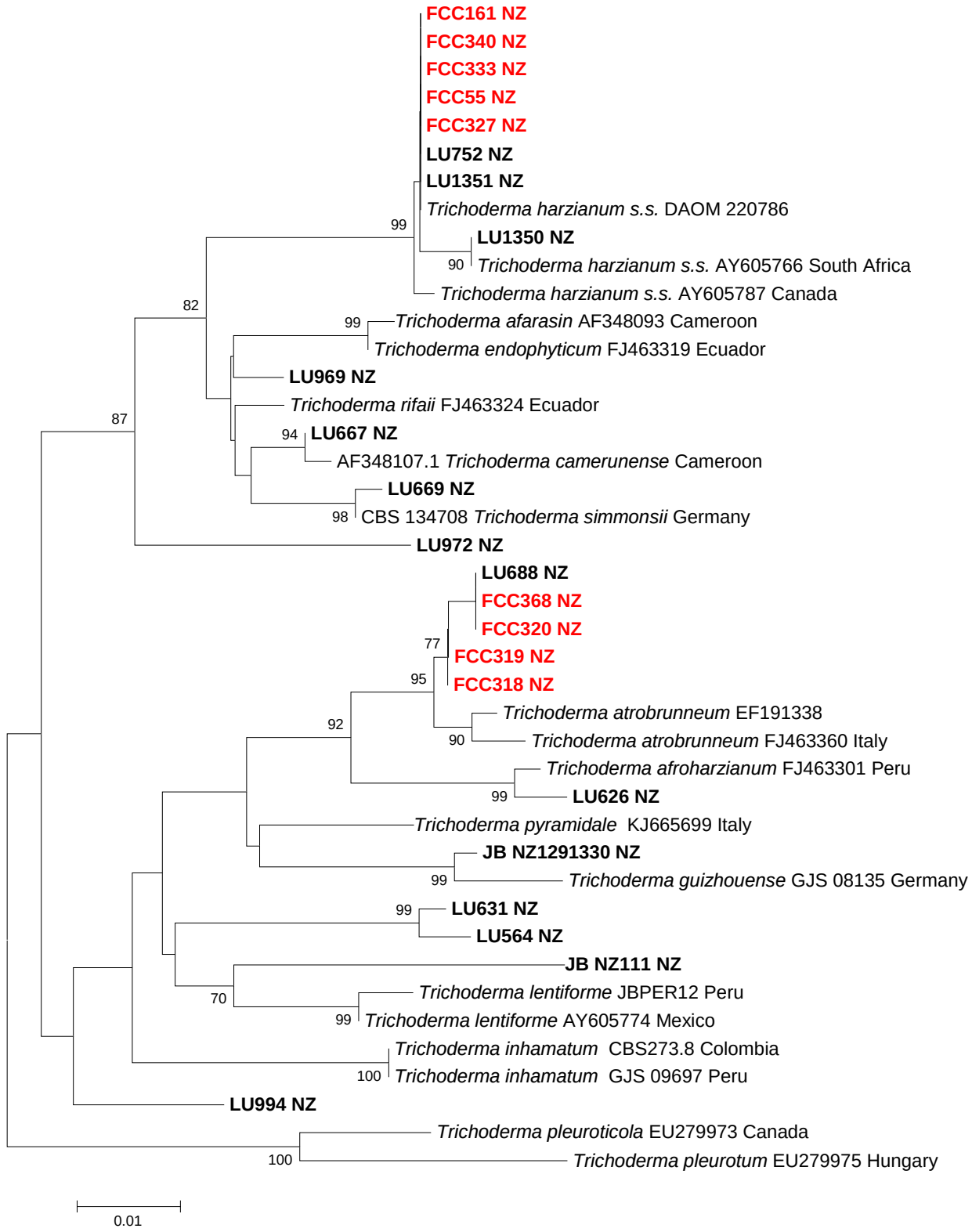


Figure 2. Neighbour-joining analysis of sequences from isolates in the *Trichoderma harzianum* clade. Sequences obtained during this study are in red with other NZ sequences in bold. Bootstrap values >70% are indicated at branches.

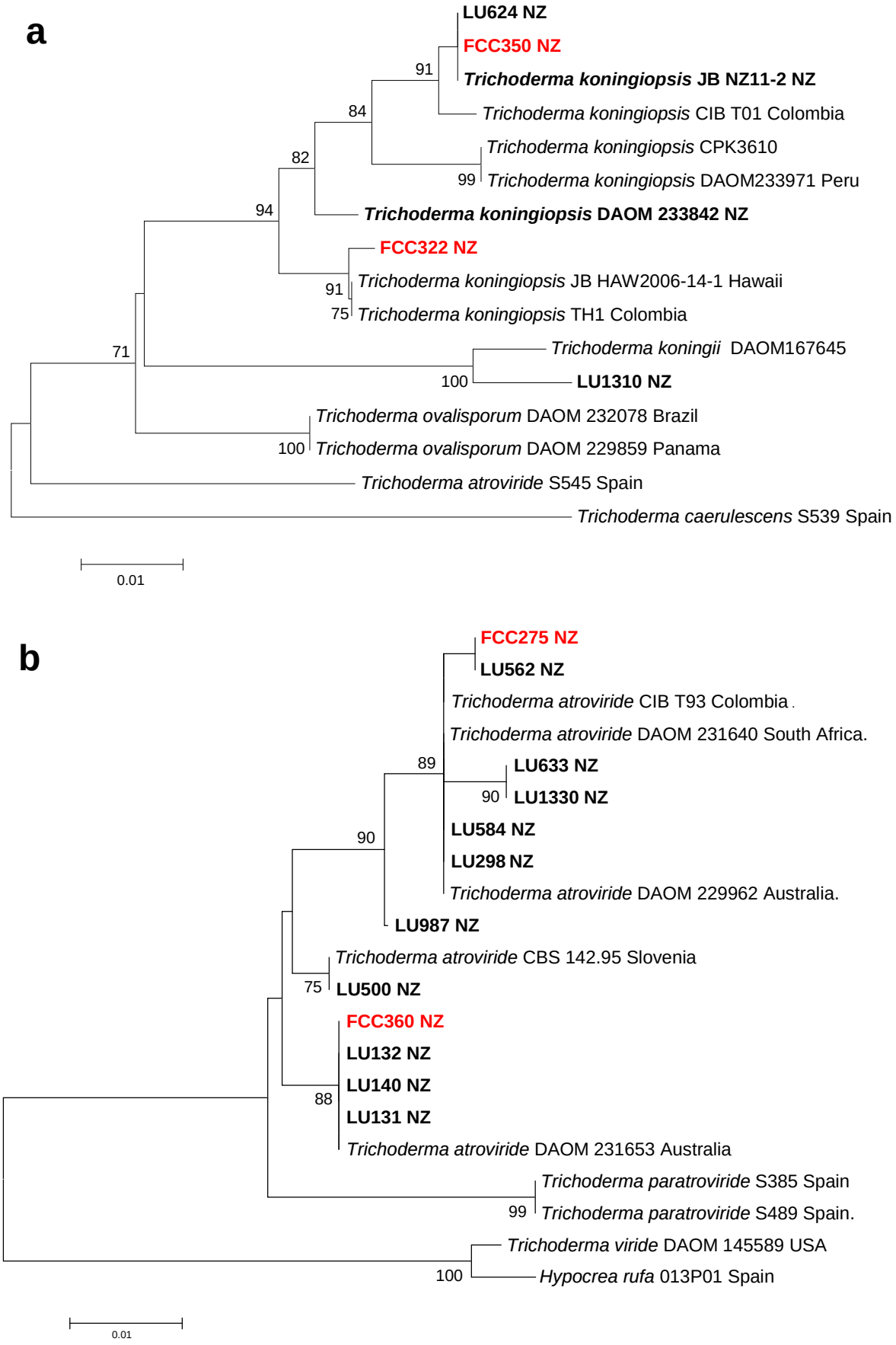
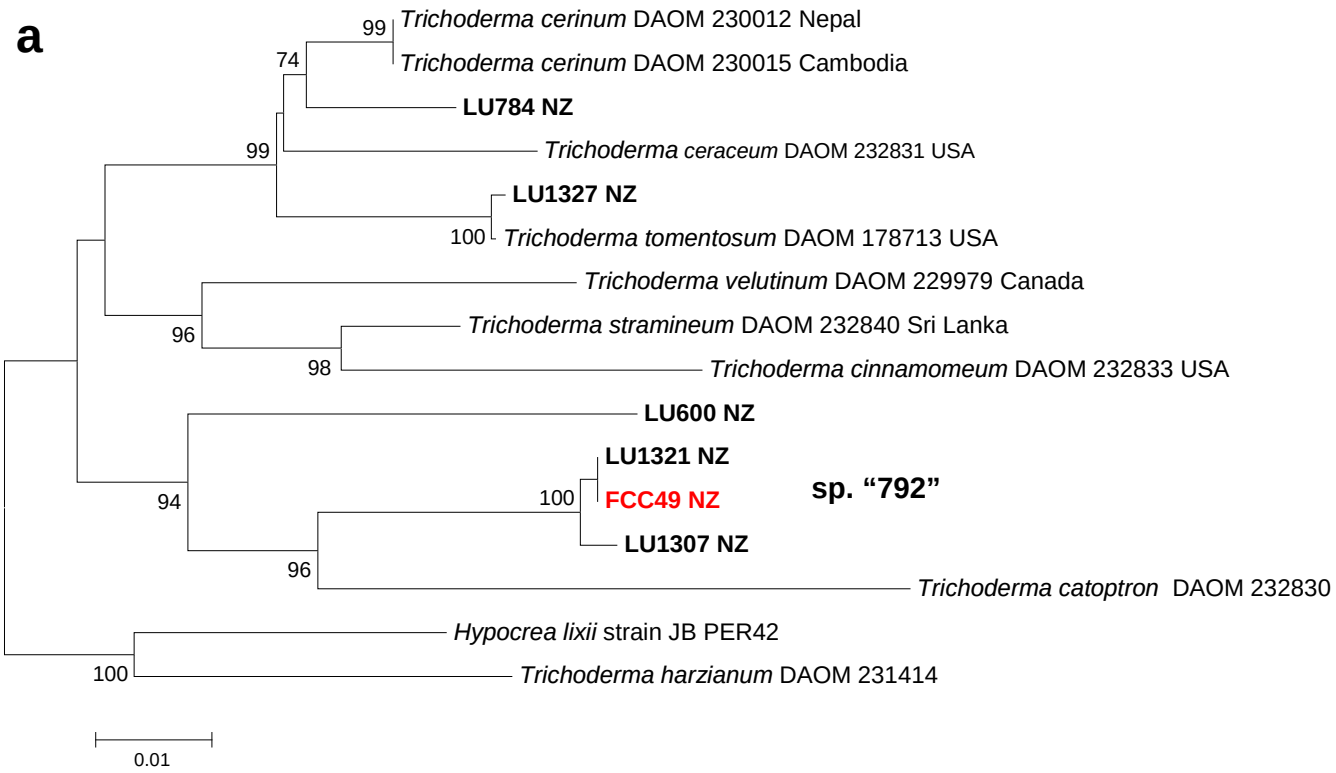


Figure 3. Neighbour-joining analyses of sequences from isolates in the **a)** *Trichoderma koningiopsis* and ***b)*** *Trichoderma atroviride* clades. Sequences obtained during this study are labelled in red with other NZ sequences in bold. Bootstrap values >70% are indicated at branches.



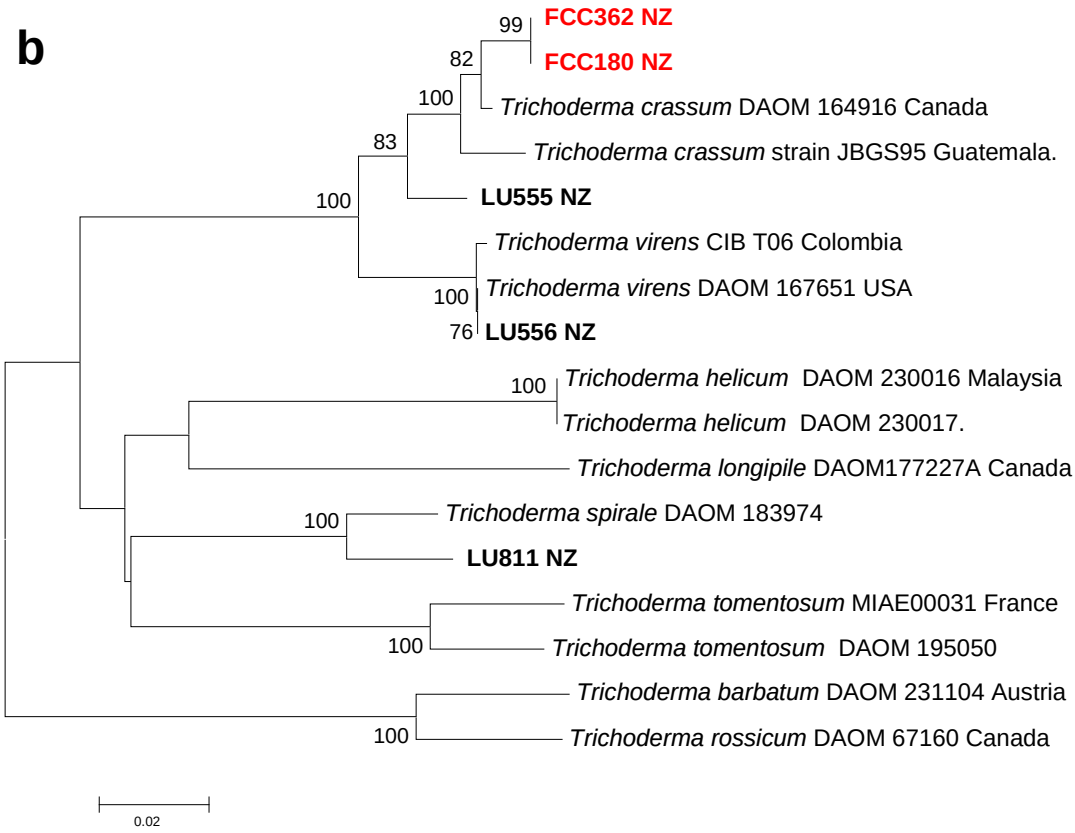


Figure 4. Neighbour-joining analyses of sequences from isolates in the **a)** sp. nov "792" and ***b)*** *Trichoderma crassum* clades. Sequences obtained during this study are labelled in red with other NZ sequences in bold. Bootstrap values >70% are indicated at branches.

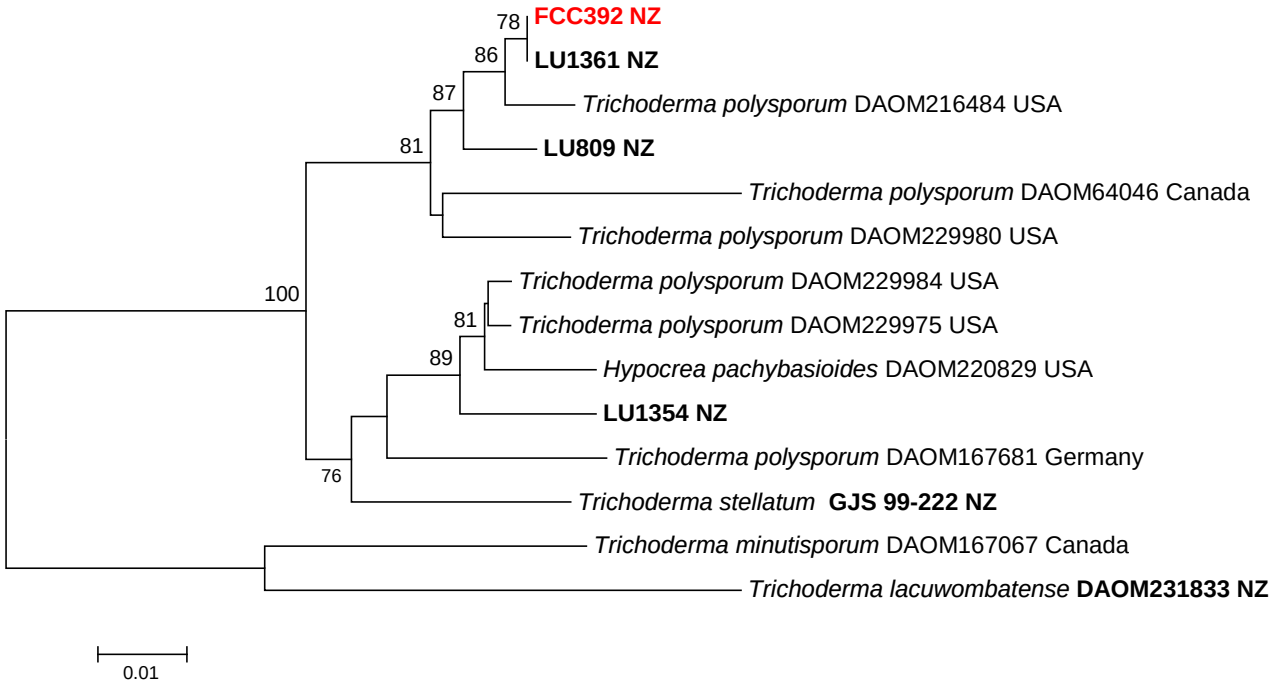


Figure 5. Neighbour-joining analyses of sequences from isolates in the *Trichoderma polysporum* clade. Sequences obtained during this study are labelled in red with other NZ sequences in bold. Bootstrap values >70% are indicated at branches.

UP-PCR

UP-PCR banding profiles generated with primer L45 for the isolates currently in plantation trials are shown in fig. 6 .Visual analysis of banding patterns allowed discrimination of isolates at species level but did not separate all isolates identified as *T. harzianum*. Isolates FCC55, 327, and 340 showed indentical banding patterns which corresponds with the identical tef1 sequences observed in these isolates. All other isolates produced unique banding profiles. In some cases (e.g. FCC 275, 319, and 322) profiles were very similar but could be distinguished by the presence or absence of bands at one or two positions.

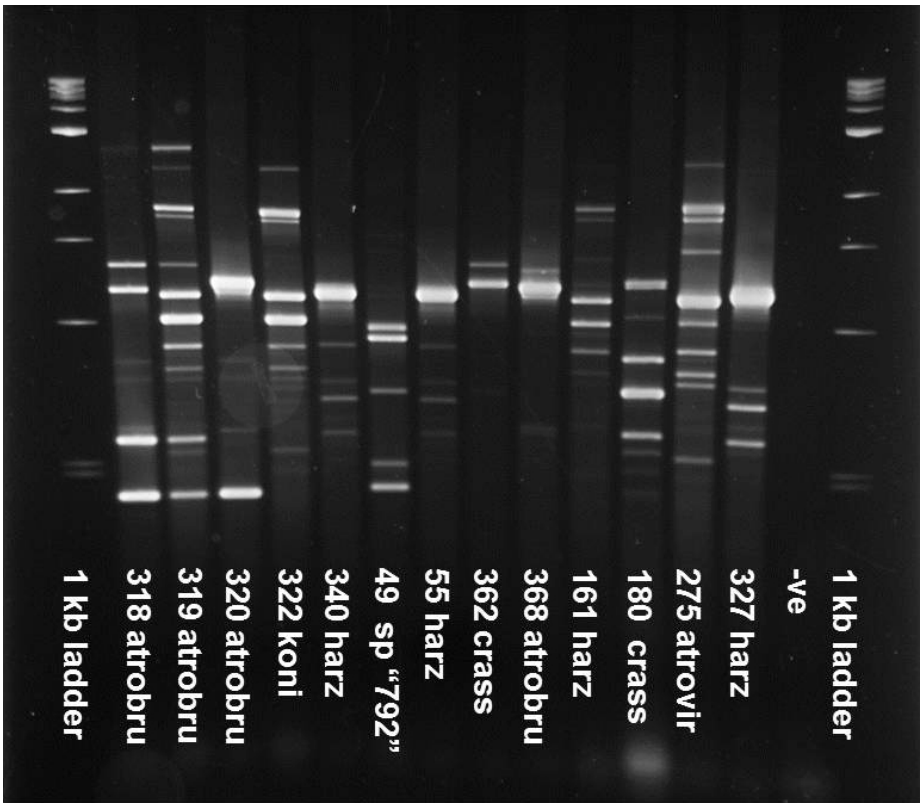


Figure 6. UP-PCR banding profiles generated with primer L45

CONCLUSION

Seventeen *Trichoderma* isolates which have shown the best performance in enhancing *P. radiata* health were identified by sequencing and phylogenetic analysis of the tef1 gene region. Isolates showed considerable diversity and were characterised within seven species, indicating that biocontrol is not necessarily restricted to certain taxa. At least one species ("sp.792") is considered likely to have a specific reference for root habitats. It is possible that isolates identified in this study as *T. atrobrunneum* may also represent a unique root-associated species. Further sequencing of additional genes would resolve whether these isolates should be split into a separate species.

An initial evaluation of the UP-PCR technique indicated that this method may be useful for differentiating between *Trichoderma* strains that form the the basis of current plantation trials. However, UP-PCR profiles should also be generated from members of natural populations of *Trichoderma* present at trial sites. It is also suggested that one or more additional UP-PCR primers are also tested to determine whether these can discriminate between *T. harzianum* isolates that could not be separated in this study.

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