FOREST OWNERS ASSOCIATION

Programme: **Bioprotection for foliar diseases and disorders of radiata pine**

Strain-specific primers for LU633 and LU584 validated (Milestone 2.4: 01/07/14-31/12/14)

Authors:
Shahjahan Kabir, Kieran Mellow and Rosie Bradshaw

Research Provider: Massey University

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EXECUTIVE SUMMARY

Trichoderma fungi show great promise for improving health of forest trees. The *Bioprotection for foliar disorders and disorders of radiata pine* research programme aims to identify and test the bio-protection ability of specific strains of *Trichoderma* species.

An important question about deployment of specific strains of *Trichoderma* is whether they are able to persist and become established in the soil and/or on roots in the forest, potentially providing long-term protection. Because *Trichoderma spp.* are ubiquitous, to answer this question it is necessary to develop molecular tools that can identify specific species and strains shown to have a bio-protective effect amongst the *Trichoderma* population.

In this project we validated strain-specific primers for *Trichoderma atroviride* strains LU633 and LU584 that have already been deployed as part of 'mix A' in forest trials. Specifically:

(a) PCR conditions (annealing temperatures) for the LU633 and LU584 primers were optmised.
(b) The primers were tested against other *Trichoderma spp.* and other fungal genera and shown to be specific for *T. atroviride* strains LU633 and LU584 for which they were designed.
(c) A single base sequence error was discovered in the LU633-specific region to which one of the primers had originally been designed. This mismatch did not affect specificity for LU633 but a new primer designed to the correct sequence showed better amplification.

The work completed in this task showed that the regions selected for LU633 and LU584 primers are suitable for further development of a quantitative assay specific for these two key *T. atroviride* bio-protection strains (task 2.5). The methods used can also be applied to develop tools for identification of additional specific strains identified by other team members in the research programme. Once the quantitative assay has been developed it will be used to test the durability of *T. atroviride* strains in both controlled and forest conditions.

INTRODUCTION

Trichoderma atroviride strains LU633 and LU584 are constituents of *Trichoderma* 'Mix A' that has been widely used as a bioprotectant in forest trials, and these strains have also performed well in some foliar bioprotection assays under controlled conditions. One important question about use of these bioprotectant treatments is whether the specific *Trichoderma* strains persist in the rhizosphere or on the roots, or if they are gradually lost over time. Because *T. atroviride* is a common soil organism, it is necessary to use strain-specific primers to determine if the bioprotectant strains have persisted.

Development of strain specific PCR primers for *T. atroviride* LU633 and LU584 commenced in 2011 by Dr Rebecca McDougal as part of an AGMARDT-funded project at Massey University. In collaboration with Assoc. Prof Murray Cox she identified unique regions in the genome sequences of these strains that were suitable for primer development, and carried out initial validation tests, but was unable to complete the work.

The aim of Task 2.4 was to complete validation of *T. atroviride* strain specific primers for LU584 and LU633, as well as a *T. atroviride* species-specific primer, using conventional PCR. Although Dr McDougal had achieved amplification of LU584 and LU633 with her probes, the PCR conditions needed optimising to determine the best annealing temperature. Dr McDougal had checked the sequence of the LU584 region to which the strain-specific primers were designed, but not the LU633 region, thus it was important to check this before proceeding. Dr McDougal had used the LU584/633 primers with a few other fungal species to ensure specificity for *T. atroviride* strains, but further species and strains needed to be tested.

Specific objectives:

- PCR annealing temperatures optimized.
- Primer specificity checked using other fungi.
- LU633-specific PCRs sequenced.

METHODS

T. atroviride LU584 and LU633 strains were obtained from Lincoln University. PCR primer sequences are shown in Table 1. DNA was extracted from fungal mycelium of LU584 and LU633, grown on PDA, using a CTAB method (Appendix 1). PCR reactions were set up in 25 μl volumes containing 2.5 μl 10× Buffer, 1.5 mM $MgCl_2$, 0.4 μM of each primer, 1.25 U of FIREPol® DNA Polymerase (Solis BioDyne, Tartu, Estonia) and 1 μl of genomic DNA (approx. 50 ng). The cycling conditions were 95°C for 4 min, then 30 cycles of 94°C (1 min), primer-specific annealing temp (1 min) and 72°C (1 min), and a final extension step of 72°C (10 min). PCR annealing temperatures for all pairs of primers were tested by gradient PCR with 55°C-61°C for all primer pairs except RM7 and RM8 for which 52°C-61°C was used.

Table 1. PCR primers for *T. atroviride* LU584 and LU633 conventional PCR.

Target strain	Primer name	Primer Sequence	Amplicon size (bp)	Annealing temperature (°C) [3]
LU584	RM1 RM2	CACTTCCAACTTATTTTCTGCC CCGTACATGATGCCTCACA	95	58
T. atroviride (species)[1]	RM3 RM4	TATTAACTGGTTCCATCGTTGG TGTGTCCTGGTCGACGTTG	154	58
LU633	RM5 RM6	GCAAGTTGGATACAGTTGGCT CCAGTAACTAGAATCCCATG[4]	72	58
T. atroviride (species)[2]	RM7 RM8	TACTCATTGGACGTTCAAC ATGCACCATTGTGCGAGTC	116	55

[1] This amplicon flanks region containing LU584 SNPs, [2] This amplicon flanks region containing LU633 SNPs
[3]Annealing temperatures as initially used by Rebecca McDougal.
[4]Note this primer sequence was subsequently changed to correct a sequence error (see page 6).

To check the DNA sequences on which the strain-specific primers were designed, PCR was used to amplify LU584 and LU633 DNA using primer pairs RM3/RM4 and RM7/RM8. These primer pairs are shown in Table 1 as *T. atroviride* specific and amplify both LU584 and LU633. They flank the strain-specific PCR primer regions (see Figure 2) such that RM3/RM4 flank the LU584-specific RM1/RM2 region and RM7/RM8 flank the LU633-specific RM5/RM6 region. PCR products were column purified (QIAGEN, Limburg, Netherlands) then sequenced in both directions using the same primers used for amplification.

Primer specificity was checked in PCR reactions with genomic DNA from a range of fungal isolates as shown in Tables 2 and 3, and primer pairs shown in Table 1. To ensure that lack of amplification was due to lack of primer binding and not to poor DNA quality, PCR amplification of each fungal isolate was also carried out using universal fungal primers ITS1F (5' CTTGGTCATTTAGAGGAAGTAA 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al., 1990) as a positive control.

RESULTS AND DISCUSSION

PCR annealing temperatures optimised

The results of the optimisation assay using a range of annealing temperatures (Figure 1) showed that, in every case except for RM5/RM6, the annealing temperatures originally used by Rebecca McDougal (as shown in Table 1) are optimal. Amplification of RM5/RM6 was weak at the original temperature of 58°C and a lower temperature of 55-56°C improved PCR product levels. However it was also noted that amplification of LU633 with its strain-specific primers RM5/RM6 was weaker than that of LU584 with its strain-specific primers RM1/RM2. The RM5/RM6 primer combination also consistently produced a smaller-sized band in the negative control due to primer-dimers in which primers anneal to each other and amplify a small non-specific product.

For the *T. atroviride* species-specific primers, RM3/RM4 consistently gave better amplification than RM7/RM8 in this and other repeats of this experiments, leading to a preference for use of RM3/RM4 for detection of *T. atroviride* at species level.

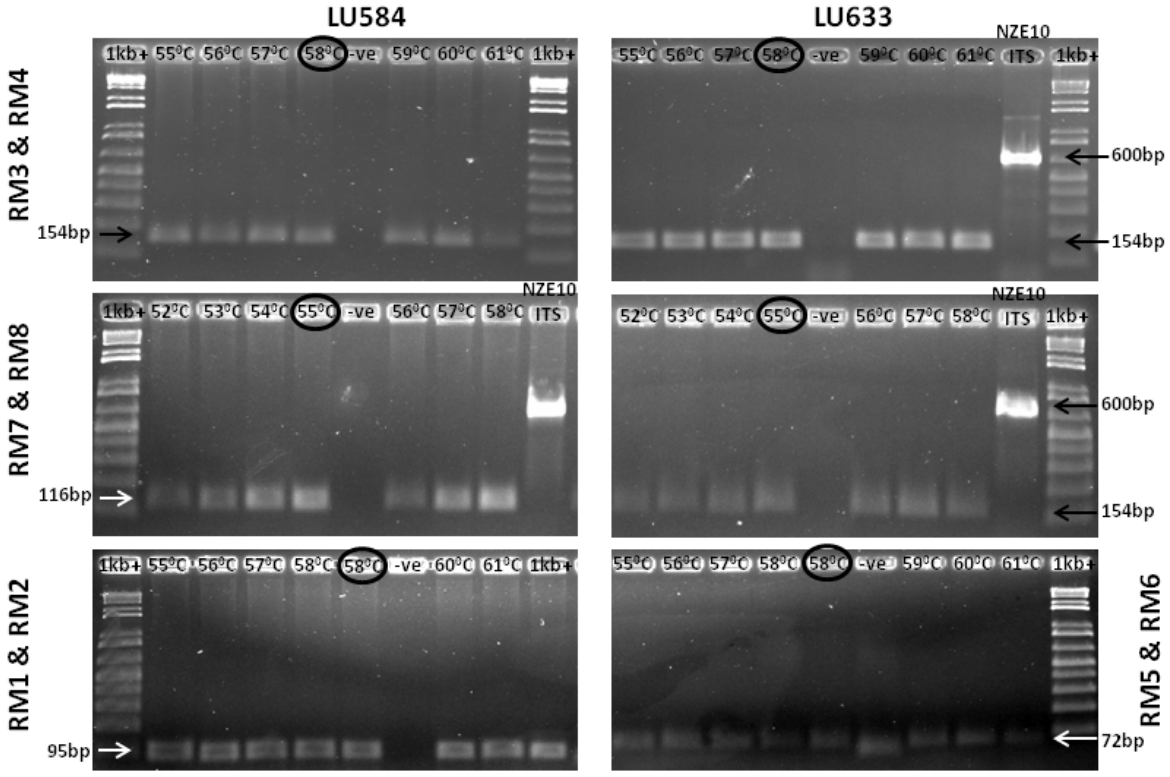


Figure 1. Gradient PCR for optimising annealing temperature.
The left and right columns show agarose gel photographs with PCR products obtained using LU584 and LU633 gDNA template respectively. PCR results using *T. atroviride*-specific primer pairs RM3/RM4 and RM7/RM8 with both templates are shown in the top and middle rows. The bottom row shows strain-specific PCR of LU584 with RM1/RM2 primers (left) and LU633 with RM5/RM6 primers (right). Numbers above each lane indicate annealing temperature used in PCR; circled values are the original temperatures used by R. McDougal as shown in Table 1. NZE10 ITS is a positive PCR control. Primer dimers seen in the negative control were also seen in repeats of this experiment (results not shown). Expected sizes of PCR products are indicated.

Primer specificity checked using other fungi.

PCR with primer pairs shown in Table 1 was carried out using genomic DNA extracted from a range of *T. atroviride* strains, other Trichoderma species and other fungal genera. As shown in Table 2, the primers performed as expected. The *T. atroviride* -specific primer pairs RM3/RM4 and RM7/RM8 amplified all *T. atroviride* strains. The strain-specific primer pairs RM1/RM2 and RM5/RM6 only amplified LU584 and LU633 respectively, as expected.

Table 2: Amplification of *Trichoderma* strains and species with LU584- and LU633-strain specific and T. atroviride-specific primers.

Species	Strain	LU584-specific (RM1/RM2)	*T. atroviride*-specific (flanking LU584 region) (RM3/RM4)	LU633-specific (RM5/RM6)	*T. atroviride*-specific (flanking LU633 region) (RM7/RM8)
T. atroviride	LU584	+	+	-	+
T. atroviride	LU633	-	+	+	+
T. atroviride	LU132	-	+	-	+
T. atroviride	LU140	-	+	-	+
T. atroviride	LU517	-	+	-	+
T. atroviride	LU519	-	+	-	+
T. atroviride	LU525	-	+	-	+
T. hamatum	LU592	-	-	-	-
T. harzianum	LU612	-	-	-	-
D. septosporum	NZE10	-	-	-	-
P. radiata	-	-	-	-	-

A further specificity test was carried out using additional target species (including forest isolates obtained from Scion) and a new LU633 new primer RM6K (see next section). In each case there was amplification with the positive (ITS) control but not with the specific primers (Table 3).

Table 3: Further specificity testing of LU584- and new LU633-strain specific primers

Species (and strain)	LU584-specific (RM1/RM2)	LU633-specific (RM5/RM6K)	Positive control (ITS1F/ITS4)
T. atroviride LU584	+	-	+
T. atroviride LU633	-	+	+
T. atroviride LU519	-	-	+
T. atroviride LU525	-	-	+
Strasseria geniculata	-	-	+
Passalora arachidicola	-	-	+
Sphaeropsis sapinea	-	-	+
Cyclaneusma minus	-	-	+
Sclerotinia sclerotiorum	-	-	+
Mycosphaerella cryptica	-	-	+
Pestalotiopsis sp.	-	-	+
Laccaria proxima	-	-	+
Dothistroma septosporum NZE10	-	-	+
Pinus radiata[1]	-	-	+

[1]Positive control was CAD primers (Chettri et al 2012).

LU633-specific PCRs sequenced

PCR amplification and sequencing of the region of LU633 on which the strain-specific primers were designed revealed an error in the region originally used to design primer RM6:

original sequence: 5' ..CATGGGATTCTAGTTACTGG.. 3'
corrected sequence: 5' ..CATGCGATTCTAGTTACTGG.. 3'

A fresh isolate of LU633 was obtained from Dr. T. Reglinski; sequence analysis of the correspondng region from this strain revealed the same error. Thus it is unlikely a mutation had occured at this position in the Massey University stock of LU633 since the work of Dr. R. McDougal, and suggested the original sequence was incorrect. The presence of this sequence error was consistent with the weak amplification of LU633 with its strain-specific primers RM5/RM6 noted from Figure 1, as well as poor amplification in real-time PCR (milestone 2.5 report, due 30 June 2015).

Thus a new primer, RM6K, was designed as shown below (reverse complement of above as RM6 is a reverse primer). In addition to correcting the sequence error the position of the primer was moved by one nucleotide to decrease matches between LU584 and LU633 at the 3' end where specificity is most important (see Figure 3). PCR amplification with the new RM6K primer confirmed it is specific for LU633 (Figure 2) and showed stronger amplification than the specific LU584 primers/template combination. However some primer-dimer artefacts were still seen.

original primer: RM6 5' CCAGTAACTAGAATCCCATG 3'
new primer RM6K 5' GCCAGTAACTAGAATCGCAT 3'

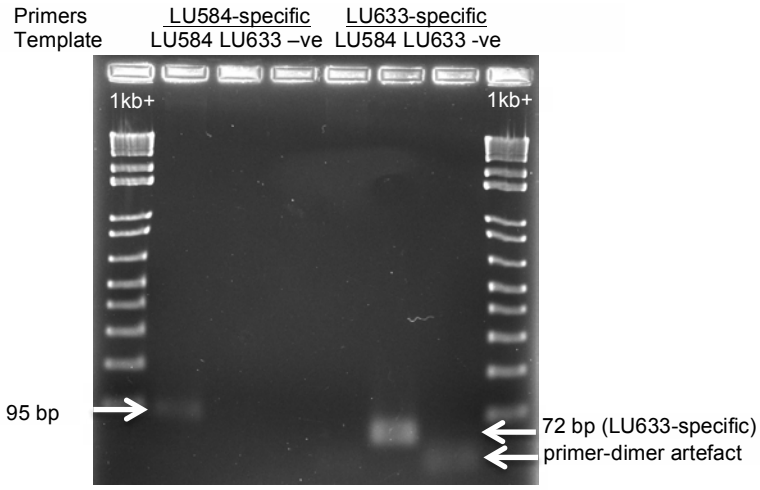
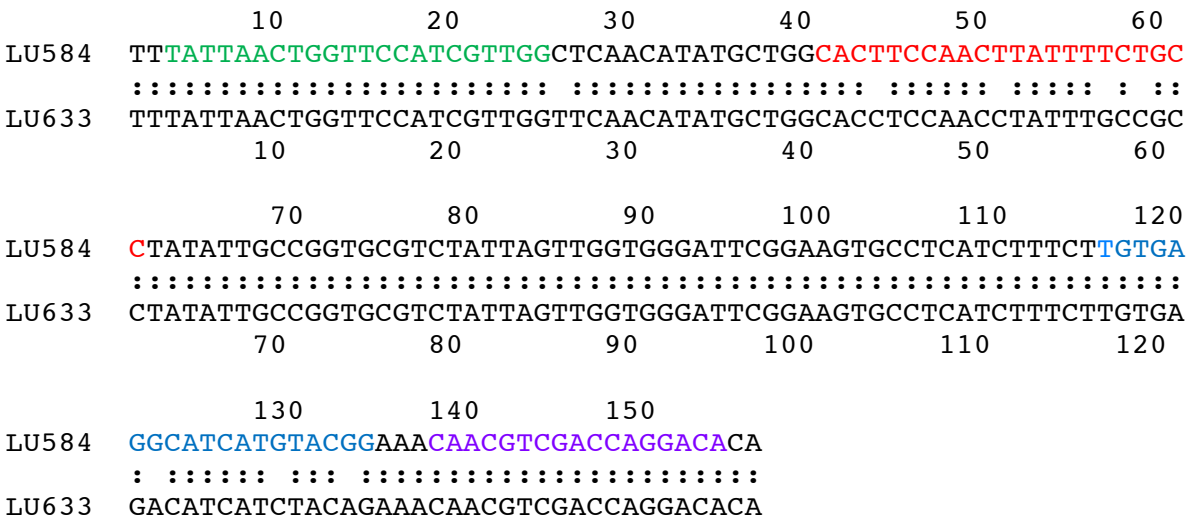
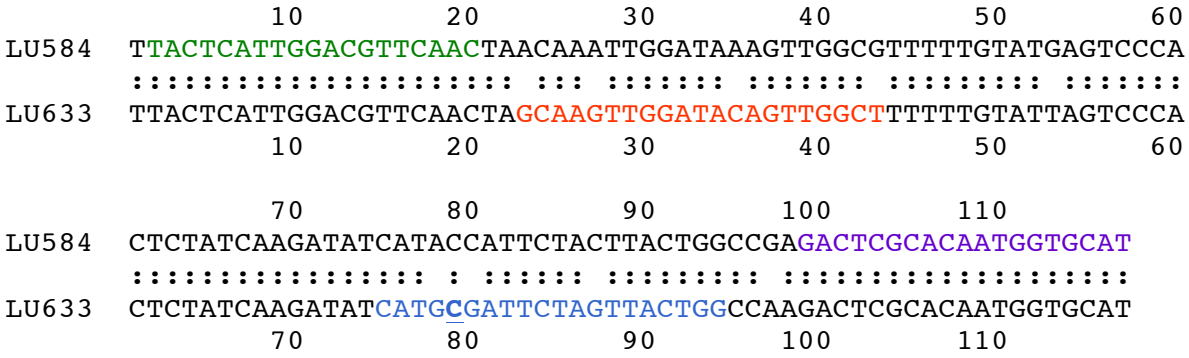


Figure 2. Specificity testing of new LU633 primers RM5 and RM6K.
Strain-specific PCR of LU584 with RM1/RM2 primers (left) and LU633 with RM5/RM6K primers (right). The new RM6K primer was specific for LU633 and gave good amplification (intense band at 72 bp), although primer dimer artefacts of a smaller size were still seen. Expected sizes of PCR products are indicated.



A) *T. atroviride* specific RM3 and RM4; LU584 specific RM1 and RM2



B) *T. atroviride* specific RM7 and RM8; LU633 specific RM5 and RM6

Figure 2. Sequences of regions of *T. atroviride* LU584 and LU633 used to design strain-specific primers.
Regions used for primer design for LU584 (top) and LU633 (bottom) are shown. In each case the outer-most primers (RM3/RM4 and RM7/RM8 shown in green and purple) are species-specific and amplify both LU584 and LU633. The inner primers (RM1/RM2 and RM5/RM6 shown in red and blue) are specific to the LU584 or LU633 strains. Colons in the alignments indicate identical nucleotides in the two strains; the primers are designed to regions where the LU584 and LU633 sequences are not identical to each other. The position of the nucleotide error within the RM6 primer is underlined. This diagram shows the new corrected nucleotide (C), as opposed to the original incorrect nucleotide (G).

CONCLUSION

Validation of PCR primers for *T. atroviride* strains LU633 and LU584 was performed that will underpin development of a system to test the durability of *Trichoderma* bioinoculants in the forest. Annealing temperatures in the PCR reaction were optimised and specificity for LU633 and LU584 strains determined using a range of genomic DNA from *Trichoderma spp.* and other fungi.

An error was discovered in the sequence originally used to design LM6, one of the PCR primers specific for LU633. This error helped to explain the poorer PCR amplification obtained with the LU633 specific primers. A new correct primer (LM6K) was designed that showed better amplification, although primer-dimer artefacts were seen in negative control PCR reactions with both LM6K as well as with LM6 primers. Whilst primer-dimer artefacts can be distinguished from real PCR products by size, it is not ideal to have them in a system in which we are likely to be looking for very low concentrations of biocontrol strains.

Following on from the work described in this report, initial results with quantitative real-time PCR have been obtained (task 2.5 report, due 30 June 2015). We are developing a probe-based quantitative system based on the identified LU584 and LU633-specific regions verified in task 2.4. The use of a specific probe in addition to the specific primers will make the detection system more sensitive and specific and will also remove any potential problems with primer-dimer formation.

ACKNOWLEDGEMENTS

The efforts of Dr Rebecca McDougal and Assoc. Prof. Murray Cox in intial development of the LU633 and LU584 specific PCR primers are acknowledged. Dr Tony Reglinski is thanked for provision of a fresh isolate of LU633.

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APPENDICES

Appendix 1: CTAB DNA extraction method

Method based on Stewart and Via (1993).

Samples (*Trichoderma* LU584, LU633 or pine roots) were ground with liquid nitrogen in a 2 ml micro centrifuge tube for DNA extraction. To this was added 750 µl pre-heated (65^{o}C) DNA extraction buffer (containing 2% CTAB, 1.42 M NaCl, 20 mM EDTA, 100 mM Tris HCl pH 8.0, polyvinylpyrrolidone 40 (Sigma Chemical Co., St. Louis, MO)). The samples were vortexed for 2 min and incubated in a water bath for 30 min at 65^{o}C with occasional mixing during this period. Then an equal volume of freshly prepared phenol:chlorophorm:IAA (25:24:1) was added and briefly vortexed. The sample was then centrifuged for 5 min at 13,000 g and the supernatant transferred to a new tube and followed by another chlorophorm:IAA (24:1) wash. Then two-thirds volume of isopropanol was added to the extracted supernatant, mixed gently and incubated at room temperature for 5 min at -20^{o} C for 30 min. The tube was then centrifuged for 5 min at 13,000 g to obtain a DNA pellet. After discarding supernatant the DNA pellet was washed with 70% alcohol and dried for 30 min at room temperature followed by elution in 20 µl elution buffer (Buffer AE, www.qiagen.com). In addition 2 µl RNase (10 mg/ml) was added to the eluted solution and was kept at 55^{o}C for 30 min.