Commercial in Confidence

Client Report No. 38904

Investigation of the relationship between Sphaeropsis sapinea and Nectria fuckeliana

Matthew Power and Tod Ramsfield

THE JOINT FORCES OF CSIRO & SCION

ensis

Commercial in Confidence

Client Report No. 3890438904

Investigation of the relationship between Sphaeropsis sapinea and Nectria fuckeliana

Matthew Power and Tod Ramsfield

Date: **October 2005**
Client: **Forest Health Research Collaborative**
Contract No:

CSIRO SCION

THE JOINT FORCES OF CSIRO & SCION

CLIENT REPORT No: 3890438904

EXECUTIVE SUMMARY

Objective

This study attempted to determine if initial infection of *Pinus radiata* by *Sphaeropsis sapinea* predisposes the tree to infection by *Nectria fuckeliana*.

Key Results

Specific polymerase chain reactions (PCR) were utilised to identify *S. sapinea* and *N. fuckeliana* in infected tissue. Using these reactions, it was possible to detect *S. sapinea* and *N. fuckeliana* in infected trees.

Wood samples from trees exhibiting flute canker symptoms showed the presence of *N. fuckeliana* when using the specific reactions, but did not always show the presence of *S. sapinea*. These results indicate that *N. fuckeliana* does not need the presence of *S. sapinea* to infect the tree, implying that *N. fuckeliana* is a primary invader.

Further Work

This project allowed optimisation of DNA extraction from *N. fuckeliana* infected *P. radiata*, and the optimised method will be used to further study the epidemiology of this fungus.

ensis

CLIENT REPORT No: 3890438904

TABLE OF CONTENTS

Information for Ensis abstracting:

Contract number	
Client Report No.	38904
Products investigated	DNA marker for *Sphaeropsis sapinea* and *Nectria fuckeliana*
Wood species worked on	*Pinus radiata*
Other materials used	Fungal strains
Location	Tokoiti Forest

INTRODUCTION

Flute Canker is a disease that affects the conifer *Pinus radiata*, and the disease has been linked to the fungus *Nectria fuckeliana*. The early stages of flute canker infection of *P. radiata* resemble those of diplodia whorl canker caused by *Sphaeropsis sapinea* (Bulman and Dick, 2004).

Specific PCR tests for both *S. sapinea* and *N. fuckeliana* have been previously developed.

Flowers *et al* (2003) developed a specific nested *S. sapinea* PCR reaction for detection of latent infections in Austrian pine (*Pinus nigra*) tissues. This method was found to detect *S. sapinea* in bud and bark tissues, and also worked on cultures of the fungus. This test was able to amplify products from both the A and B morphotypes of *S. sapinea* and is expected to be able to amplify morphotype C as this has an identical ITS sequence to morphotype A.

Langrell (2004) developed a similar specific nested PCR reaction for the detection of *N. fuckeliana* from Norway spruce (*Picea abies*) bark extracts.

At present, pruning branches of conifers is a common practice to grow knot free timber; however, if pruning is conducted at the wrong time of the year, there is a risk of infection by *S. sapinea* (Flowers *et al*, 2001).

Nectria fuckeliana

It is hypothesised that *N. fuckeliana* enters the tree through pruning stubs, moving up and down from the entry point killing cambium cells, leading to stem malformation and a flattened area of wood or fluting (Wang and Thode, 2004).

Nectria fuckeliana is described as a secondary pathogen with a host range including conifer species such as *Abies*, *Larix*, *Pinus*, and *Picea*. *Nectria fuckeliana* has a circumboreal distribution, being found in Europe in countries

such as Austria, France Germany, Scotland and Switzerland. It is also found in Canada and the United States (Brayford *et al*, 2004). *Nectria fuckeliana* is also widely distributed in the Otago-Southland region of New Zealand (Wang and Thode, 2004).

Sphaeropsis sapinea

Sphaeropsis sapinea is primarily a wood pathogen that attacks suberised and non-suberised tissue under damp conditions. *Sphaeropsis sapinea* is found throughout New Zealand (Ridley and Dick, 2001).

Pruning wounds are the most common point of infection. The cambium above and below the pruned stub is killed, resulting in depressed areas. Pruning wounds are likely to become infected when the tree is under stress, therefore it is recommended that pruning in hot/dry conditions or removal of 50% or more of the crown is to be avoided.

Objective

This work was conducted to study the hypothesis that infection by *S. sapinea* predisposes the tree to infection by *N. fuckeliana* using DNA based methods.

MATERIALS AND METHODS

Growing the Isolates

Ten *S. sapinea* isolates and two *N. fuckeliana* isolates were sub-cultured onto Potato-Dextrose agar (PDA) plates (Table 1). Sub-culturing was carried out by removing a 1cm x 0.5cm square of the isolate from a stock plate and placing the square onto a new PDA plate, covered with a sterilised cellophane circle. Plates were incubated at 20°C for around three weeks before enough tissue had been grown to allow DNA extraction to be carried out.

Sterilised cellophane circles (BioRad Gel air cellophane support) were placed onto the PDA media before addition of the sub-cultured isolate. Circles had been sterilised in 95% ethanol, 10% bleach and sterile water for 4 minutes

each. Three consecutive water washes were carried out. The cellophane was added to allow easy removal of the fungal tissue for DNA extraction.

Table 1: *S. sapinea* and *N. fuckeliana* isolates used.

Isolate	Species	Location
15.19	*S. sapinea*	Athol Nursery, Tokoroa
895	*S. sapinea*	Wellington Botanical Gardens
897	*S. sapinea*	Omahuta Forest, Northland
912	*S. sapinea*	Mount Maunganui Golf Course
929	*S. sapinea*	Gwavas Forest
941	*S. sapinea*	Aupouri Forest
948	*S. sapinea*	Mc Leans Island
956	*S. sapinea*	Berwick Forest
958	*S. sapinea*	Tairua Forest
967	*S. sapinea*	Warwick Forest
980	*N. fuckeliana*	Tokoiti Forest
8343 / 2	*N. fuckeliana*	Rayonier

DNA Extraction

DNA was isolated from the fungal mycelium of both *S. sapinea* and *N. fuckeliana* using the FastDNA® kit and the FastPrep® Instrument (Qbiogene, Inc., CA). A scalpel blade was used to scrape a volume of approximately 200-500µl mycelium from the plate. Mycelium tissue was placed in a FastDNA (Qbiogene, Inc., CA) tube and the standard fungal extraction method was carried out as per the manufacturer's instructions.

DNA extraction from wood was carried out by cutting shavings from the wood and placing the shavings in a FastDNA® tube. Shavings were cut from wood that appeared to be healthy as well as wood that appeared to be diseased. DNA was also extracted from bark. The standard FastDNA® kit plant extraction method was carried out as per the manufacturer's instructions. Homogenisation of the wood tissue was accomplished by processing the

tissue three times for 20 seconds at speed five in the FastPrep® Instrument with an extra 1/4 inch ceramic sphere added to the tube to aid homogenisation. Each 20 s homogenisation was followed by a two minute incubation on ice. With later *N. fuckeliana* infected wood samples, DNA extraction was modified by initially grinding the wood sample in liquid nitrogen. A volume of powder equivalent to 200µl was added to the FastDNA tube and the standard FastDNA® plant extraction method was carried out.

To amplify DNA directly from the ascospores, perithecia were removed from the wood using sterile forceps. Four to five perithecia were added to 20µl sterile water and crushed and then 3µl of this water was used as the template in the *N. fuckeliana* PCR reaction. The profile and the thermal cycler programme for the reaction were the same as those mentioned below for the second round of the *N. fuckeliana* specific reaction, apart from an increased initial extension time of five minutes. The *N. fuckeliana* specific primers were used in the first round of the reaction, with no second round reaction carried out. PCR was attempted on both immature and mature perithecia.

DNA Quantification

The concentration of extracted DNA was determined on the Hoefer DyNA Quant 200 flourometer (Amersham Pharmacia Biotech). DNA was quantified as per the manufacturer's instructions using the Low Range buffer. The flourometer was calibrated with 100ng/µl calf thymus DNA. Following quantification, the concentration was adjusted to 1ng/µl.

PCR Amplification

The specific reactions were both nested PCR tests that utilised the internal transcribed spacer region (ITS) of the ribosomal DNA (Flowers *et al*, 2003; Langrell, 2004).

The first round PCR reaction for both the *S. sapinea* and *N. fuckeliana* specific reactions was carried out by amplifying ITS PCR products using either

the ITS-5 / ITS-4 primer pair, or the ITS-1 / ITS-4 primer pair. Both primer pairs produced similar results. PCR amplification was carried out in a 15µl reaction containing 2.5ng of template DNA, 0.05pmol/µl of both forward and reverse primers, 0.45 U Taq DNA polymerase (Roche), 1 x reaction buffer (Roche), 1.5mM $MgCl_2$, 0.2mM each of dATP, dGTP, dCTP, dTTP (Roche). The PCR conditions: One cycle at 94°C for 3 min, 13 cycles of 94°C for 35 s, 55°C for 55 s, 72°C for 45 s, 13 cycles of 94°C for 35 s, 55°C for 55 s, 72°C for 2 min, 13 cycles of 94°C for 35 s, 55°C for 55 s, 72°C for 3 min, one cycle of 72°C for 7 min, and a 4°C hold cycle.

The profile of the nested *S. sapinea* specific reaction using the S.sapFOR3 / S.sapREV3 primer pair is similar to the ITS PCR reaction profile mentioned above, apart from using 1/1000 dilutions of first round ITS PCR products as the DNA template, and increasing the $MgCl_2$ concentration to 5mM $MgCl_2$. Products were amplified using the following profile: One cycle at 94°C for 3 min, 30 cycles at 94°C for 30 s, 69°C for 15 s, 72°C for 30 s, one cycle at 72°C for 7 min, and a 4°C hold cycle.

The profile of the nested *N. fuckeliana* specific reaction using the Cct1 / Cct2 primer pair is the same as the ITS PCR profile described above apart from using 1/50 dilutions of first round ITS PCR products as the DNA template. Products were amplified using the following profile: One cycle at 94°C for 3 min, 30 cycles at 94°C for 1 min, 62°C for 1 min, 72°C for 1 min, one cycle at 72°C for 10 min, and a 4°C hold cycle.

Uninfected *P. radiata* DNA was used as a negative control to ensure primers did not cross react with host DNA.

PCR products were electrophoresed on 1% agarose gels and stained using ethidium bromide. Products were visualised under UV light.

Comparison of S. sapinea and N. fuckeliana sequences

S. sapinea and *N. fuckeliana* isolates used in previous studies were identified from the literature and sequences from these isolates were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). Isolate sequences were compared using CLUSTAL X Multiple Sequence Alignment Program (http://bioinformatics.ubc.ca/resources/tools/index.php?name=clustalx).

RESULTS

All tables in the Results section follow the same format:

- A PCR result of x/y indicates x positive result from y tests.
- Ssap = *Sphaeropsis sapinea* infection
- Nfuc = *Nectria fuckeliana* infection
- FB = *Nectria fuckeliana* fruiting bodies

Cultures

DNA was extracted from ten isolates of *S. sapinea* and two isolates of *N. fuckeliana*. When the DNA was amplified with the specific primer pairs, the results were as expected; *S. sapinea* specific primers amplified *S. sapinea* DNA only, and *N. fuckeliana* specific primers amplified *N. fuckeliana* DNA only.

S. sapinea infected wood

DNA was extracted from wood infected with *S. sapinea* collected from Hokitika Cemetery. The extracted DNA showed positive results when run with *S. sapinea* specific primers but no PCR products were produced when run with *N. fuckeliana* specific primers.

Table 2: Sample descriptions and results of specific PCR reactions for *S. sapinea* infected wood from Hokitika Cemetery

Sample Name	Tissue Type	Symptoms	Liquid Nitrogen Grinding	*Nectria fuckeliana* PCR Results	*Sphaeropsis sapinea* PCR Results
8722-1	Branch	Ssap	No	0/2	0/2
8722-2	Branch	Ssap	No	0/2	2/2
8722-3	Branch	Ssap	No	0/2	2/2

DNA was extracted from additional *S. sapinea* infected wood collected from Mangatu Forest. One sample was extracted from wood directly under the bark, and one sample extracted from the heartwood of the sample. This DNA showed positive results for both samples when run with *S. sapinea* specific primers, and both samples showed negative results when run with *N. fuckeliana* specific primers.

Table 3: Sample descriptions and results of specific PCR reactions for *S. sapinea* infected wood from Mangatu Forest

Sample Name	Tissue Type	Symptoms	Liquid Nitrogen Grinding	*Nectria fuckeliana* PCR Results	*Sphaeropsis sapinea* PCR Results
A0072300 Bark	Branch	Ssap	No	0/2	2/2
A0072300 Wood	Branch	Ssap	No	0/2	2/2

Wood Cores and Wood Disks from Dunedin

DNA was extracted from wood cores that were taken from both non-symptomatic trees and trees infected with *N. fuckeliana* growing in Otago. Results were primarily as expected for non-symptomatic and infected trees, however some trees that did not appear to be infected by *N. fuckeliana* produced products when run with *N. fuckeliana* specific primers. This DNA was then run with *S. sapinea* specific primers, and all results were negative with the exception of core 6 which had a weak positive reaction.

Re-testing of the wood core DNA with both of the specific reactions produced results differing from the original reactions. Further re-testing produced continually inconsistent results. An extended method of DNA extraction using liquid nitrogen as mentioned previously appears to have solved this problem. Results from DNA extracted from wood that was first ground in liquid nitrogen were consistent (refer to Table 4).

Table 4: Sample descriptions and results of specific PCR reactions for Dunedin wood cores

Sample Name	Tissue Type	Symptoms	Liquid Nitrogen Grinding	*Nectria fuckeliana* PCR Results	*Sphaeropsis sapinea* PCR Results
Dun 1	Core	No FB	No	1/6	0/2
Dun 2	Core	No FB	No	6/6	0/2
Dun 3	Core	Top of flute	No	3/6	1/2
Dun 4	Core	Beside FB	No	4/6	0/2
Dun 5	Core		No	6/6	1/2
Dun 6	Core	Healthy	No	2/6	1/2
Dun 7	Core	Healthy	No	6/6	0/2
Dun 8	Core	Healthy	No	0/6	0/2
Dun 9	Core	Beside FB	No	6/6	0/2
Dun 10	Core	Healthy un-pruned	No	2/6	0/2
Dun 11	Core	Pruned 1 year ago	No	2/6	0/2
Dun 12	Core	Pruned	No	0/6	0/2
Dun 13	Core		No	6/6	0/2
Dun 14	Core	Nfuc present – not in flute	No	6/6	0/2
Dun 15	Core	Above Nfuc	No	6/6	0/2
Dun 16	Core		No	4/6	0/2
Dun 17	Core		No	4/6	0/2
Core 1	Core	Fluting present	Yes	2/2	0/2
Core 2	Core	Fluting present	Yes	2/2	0/2
Core 3	Core	Fluting present – FB on stub	Yes	0/2	0/2
Core 4	Core	Fluting present	Yes	2/2	0/2
Core 5	Core	Fluting present – no FB present	Yes	0/2	0/2
Core 6	Core	Fluting present	Yes	2/2	0/2
Core 7	Core	Fluting present	Yes	0/2	0/2
Core 8	Core	Fluting present – above stub	Yes	2/2	0/2
Core 9	Core	Fluting present – through stub	Yes	2/2	0/2

Sample Name	Tissue Type	Symptoms	Liquid Nitrogen Grinding	*Nectria fuckeliana* PCR Results	*Sphaeropsis sapinea* PCR Results
Core 10	Core	Fluting present – through stub	Yes	2/2	0/2
Core 11	Core	Fluting present – through stub	Yes	2/2	0/2
Core 12	Core	Fluting present – above stub	Yes	0/2	0/2
Core 13	Core	Fluting present – above stub	Yes	2/2	0/2
Core 14	Core	Fluting present – above stub	Yes	2/2	0/2
Core 15	Core	Fluting present – above stub	Yes	2/2	0/2

DNA was extracted from various points along a wood core (Core 15 – pruned stub trial above *N. fuckeliana* #1) infected with *N. fuckeliana*. All points along the wood core displayed positive results for *N. fuckeliana* when put through a specific *N. fuckeliana* PCR reaction. This same DNA displayed negative results for all points along the core when run in an *S. sapinea* specific PCR reaction. This represented a cross section of the tree and *N. fuckeliana* was found to be present all the way to the centre of the tree.

Table 5: Sample descriptions and results of specific PCR reactions for Wood core #15

Sample Name	Tissue Type	Symptoms	Liquid Nitrogen Grinding	*Nectria fuckeliana* PCR Results	*Sphaeropsis sapinea* PCR Results
Dun 15-1	Core	Above Nfuc	No	1/1	0/1
Dun 15-2	Core	Above Nfuc	No	1/1	0/1
Dun 15-3	Core	Above Nfuc	No	1/1	0/1
Dun 15-4	Core	Above Nfuc	No	1/1	0/1

DNA was also extracted from infected wood discs. Two samples per disc were taken – one from an area of disc that was diseased, and an area of wood that appeared healthy. Samples did not produce expected results for both specific PCR reactions in all cases.

Table 6: Sample descriptions and results of specific PCR reactions for wood disks

Sample Name	**Tissue Type**	**Symptoms**	**Liquid Nitrogen Grinding**	***Nectria fuckeliana* PCR Results**	***Sphaeropsis sapinea* PCR Results**
1-un-1	Wood Disk	Non-symptomatic Wood	No	0/2	1/1
1-un-2	Wood Disk	Non-symptomatic Wood	No	1/1	1/1
1-in-1	Wood Disk	Stained Wood – Ssap	No	4/4	3/3
1-in-2	Wood Disk	Stained Wood – Ssap	No	1/1	1/1
2-un-1	Wood Disk	Non-symptomatic Wood	No	1/1	1/1
2-un-2	Wood Disk	Non-symptomatic Wood	No	1/1	1/1
2-in-1	Wood Disk	Stained Wood – Ssap	No	1/1	1/1
2-in-2	Wood Disk	Stained Wood – Ssap	No	3/3	3/3
3-1	Wood Disk	Stained Wood – Ssap	No	1/1	1/1
3-2	Wood Disk	Stained Wood – Ssap	No	1/1	1/1
3-3	Wood Disk	Stained Wood – Ssap	No	0/1	0/1
3-4	Wood Disk	Stained Wood – Ssap	No	1/1	1/1
M1/1-1	Wood Disk	Stained Wood – Nfuc	No	1/1	0/1

Sample Name	Tissue Type	Symptoms	Liquid Nitrogen Grinding	*Nectria fuckeliana* PCR Results	*Sphaeropsis sapinea* PCR Results
M1/1-2	Wood Disk	Stained Wood – Nfuc	No	1/1	0/1
G1/2-i	Wood Disk	Stained Wood – Nfuc	No	1/1	0/1
G1/2-c	Wood Disk	Non-symptomatic Wood	No	1/1	1/1
G1/7-i	Wood Disk	Stained Wood – Nfuc	No	1/1	0/1
G1/7-c	Wood Disk	Non-symptomatic Wood	No	1/1	1/1
G1/8-i	Wood Disk	Stained Wood – Nfuc	No	1/1	0/1
G1/8-c	Wood Disk	Non-symptomatic Wood	No	1/1	0/1
G1/9-i	Wood Disk	Stained Wood – Nfuc	No	1/1	0/1
G1/9-c	Wood Disk	Non-symptomatic Wood	No	1/1	1/1
PS20/5-i	Wood Disk	Stained Wood – Nfuc	No	1/1	0/2
PS20/5-c	Wood Disk	Non-symptomatic Wood	No	1/1	0/2
PS20/6-i	Wood Disk	Stained Wood – Nfuc	No	1/1	0/2
PS20/6-c	Wood Disk	Unsymptomatic Wood	No	1/1	2/2

Infected Bark response to specific fungal primers

DNA extracted from *N. fuckeliana* infected bark was amplified using the ITS-4 / ITS-5 reaction and the *N. fuckeliana* specific reaction, indicated the presence of *N. fuckeliana* in the bark.

Table 7: Sample descriptions and results of specific PCR reactions for bark samples

Sample Name	Tissue Type	Symptoms	Liquid Nitrogen Grinding	*Nectria fuckeliana* PCR Results	*Sphaeropsis sapinea* PCR Results
Bark 1	Bark	FB present	No	4/4	0/3
Bark 2	Bark	FB present	No	4/4	0/3
Bark 3	Bark	FB present	No	4/4	0/1
Bark 4	Bark	FB present	No	4/4	1/1

Amplification from fruiting bodies

All crushed perithecial samples taken produced products when amplified using the *N. fuckeliana* specific reaction. The immature samples produced faint bands, while the mature samples produced bright bands, reflecting the relative abundance of ascospores within the perithecia.

Table 8: Sample descriptions and results of specific PCR reactions for perithecial samples

Sample Name	Tissue Type	Symptoms	Liquid Nitrogen Grinding	*Nectria fuckeliana* PCR Results	*Sphaeropsis sapinea* PCR Results
Immature #1	Perithecia	FB	No	1/1	Not Tested
Immature #2	Perithecia	FB	No	1/1	Not Tested
Mature #1	Perithecia	FB	No	1/1	Not Tested
Mature #2	Perithecia	FB	No	1/1	Not Tested

Comparison of S. sapinea and N. fuckeliana sequences

Comparison of the *S. sapinea* and *N. fuckeliana* sequences showed differences between the sequences where the specific primers were designed. This indicates that the second round PCR primer pairs should only amplify DNA from the fungus for which they were designed.

DISCUSSION

S. sapinea specific PCR reaction

The *S. sapinea* specific PCR reaction needed to be optimised from the literature to produce single bands when viewing upon agarose gels. Magnesium concentration was increased from 1.5mM to 5mM as was annealing temperature (65°C $\rightarrow$ 69°C). PCR products obtained from the initial ITS reaction were diluted to 1/1000 instead of 1/50 as stated in literature to be used as the template for the *S. sapinea* reaction. These changes were conducted to remove an extra band of size 450 – 500bp that was showing on agarose gels.

Specific Amplification of S. sapinea and N. fuckeliana using DNA extracted from cultures or mycelium

S. sapinea and *N. fuckeliana* specific PCR reactions were able to differentiate between *S. sapinea* and *N. fuckeliana* isolates and infected tissue using diluted PCR products from both ITS-1F / ITS-4 and ITS-4 / ITS-5 reactions.

ITS reactions amplified all isolates and indicated the presence of fungal DNA. Both ITS reactions produced similar results. This indicates that the different ITS reactions work equally well for both *S. sapinea* and *N. fuckeliana*.

Specific Amplification of S. sapinea and N. fuckeliana using DNA extracted from infected tissue

Amplification of Dunedin wood core DNA with the *N. fuckeliana* specific reaction produced inconsistent results for a number of reactions. These inconsistent results are due to the low levels of DNA extracted from the core. The modified DNA extraction method from wood tissue involves an extra grinding step which produces a fine powder which is then used in the standard FastDNA (Qbiogene, Inc., CA) method. This step is more efficient in producing higher yields of DNA from the extraction, therefore consistent results are able to be produced. Multiple banding using this new method may need to be rectified.

Nested PCR

The *S. sapinea* and *N. fuckeliana* specific PCR reactions are both nested reactions involving two PCR reactions each. This method of amplification is highly susceptible to cross contamination. Because of this, careful laboratory technique must be used when carrying out these reactions to avoid false results.

Sequence similarity

Sequence analysis of *S. sapinea* and *N. fuckeliana* sequences showed no similarity between the areas of *S. sapinea* and *N. fuckeliana* sequence that contained the species-specific primers. These results indicate that the primers should bind only to the fungal species which they were designed to amplify. Therefore any results from these primers should correctly differentiate between *S. sapinea* and *N. fuckeliana* template DNA.

RECOMMENDATIONS AND CONCLUSIONS

Both *S. sapinea* and *N. fuckeliana* specific reactions performed well on DNA extracted from cultures. The *S. sapinea* specific reaction also performed well when tested on *S. sapinea* infected tissue. The *N. fuckeliana* specific reaction did not perform as well when tested on *N. fuckeliana* infected tissue. Inconsistent results were observed when using Dunedin core DNA that was collected in Tokoiti Forest. These inconsistencies were solved through liquid nitrogen grinding prior to DNA extraction.

Despite the problems that were encountered with the *N.* fuckeliana DNA amplification from infected wood we found that a tree infected with *N. fuckeliana* is not also consistently infected with *S. sapinea*. This demonstrates that *N. fuckeliana* does not need an initial infection of *S. sapinea* to infect the tree. *Nectria fuckeliana* was also found in wood prior to perithecia production, providing a method by which infection can be confirmed prior to sporulation.

From these results it appears that *N. fuckeliana* can act as a primary invader, infecting the target tree without the need for the presence of another fungus, such as *S. sapinea*.

ACKNOWLEDGEMENTS

Thanks to the Nectria Focus Group for their assistance, and to the FHRC for financial support.

REFERENCES

Bulman, L., Dick, M. 2004: The Nectria Research Programme. *Forest Health News No.147.*

Flowers, J., Nuckles, E., Hartman, J., Vaillancourt, L. 2001: Latent Infection of Austrian and Scots Pine Tissues by *Sphaeropsis sapinea*. *Plant Disease Vol. 85 (10): 1107-1112.*

Flowers, J., Hartman, J., Vaillancourt, L. 2003: Detection of Latent *Sphaeropsis sapinea* Infections in Austrian Pine Tissues Using Nested-Polymerase Chain Reaction. *Phytopathology Vol.93 (12): 1471-1477*

Langrell, S.R.H. 2004: Development of a nested PCR detection procedure for *Nectria fuckeliana* direct from Norway spruce bark extracts. *FEMS Microbiology Letters 242, 185-193*

Ridley, G.S, Dick M.A. 2001: An Introduction to the Diseases of Forest and Amenity Trees in New Zealand. *Forest Research Bulletin 220.*

Wang, W., Thode, D. 2004: Nectria and the implications for radiata in Otago and Southland. *New Zealand Tree Grower.*

Brayford, D., Honda, B.M, Mantiri, F.R and Samuels, G.J. 2004: *Neonectria* and *Cylindrocarpon*: the Nectria mammoidea group and species lacking microconidia. *Mycologia Vol.96, 572-597*

APPENDICES

Appendix A – Fungal-specific PCR results

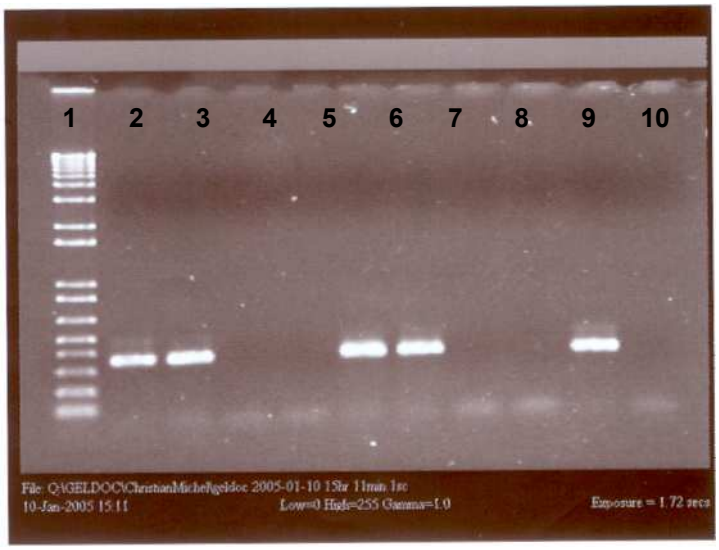

Figure 1: *S. sapinea* specific PCR reaction.
1% agarose gel with 7.5µl ladder. 5µl PCR product and dye loaded. Gel run at 110V for 60 minutes.

Lane 1 – 1Kb+ ladder (Invitrogen)
Lane 2 – 929 (S)
Lane 3 – 941 (S)
Lane 4 – 8343/2 (N)
Lane 5 – 980 (N)
Lane 6 – 929 (S)
Lane 7 – 941 (S)
Lane 8 – 8343/2 (N)
Lane 9 – 980 (N)
Lane 10 – 958 (*S. sapinea* positive control)
Lane 11 – H_2O (negative control)

Lanes 2 – 5 had ITS-4/ITS-5 products at 1/1000 dilution for target DNAs, Lanes 6 – 9 had ITS-1F/ITS-4 products at 1/50 dilution for target DNA.
(N) = *N. fuckeliana* isolate (S) = *S. sapinea* isolate

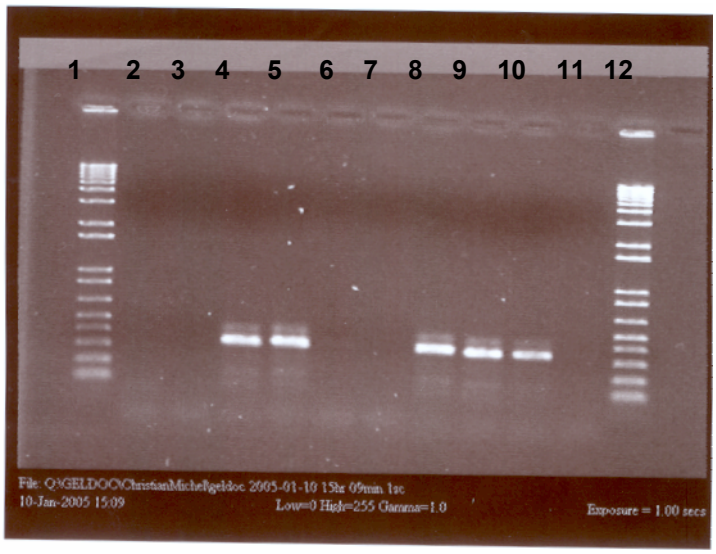

Figure 2: *N. fuckeliana* specific PCR reaction.

1% agarose gel with 7.5µl ladder. 5µl PCR product and dye loaded. Gel run at 110V for 60 minutes.

Lane 1 – 1Kb+ ladder (Invitrogen)
Lane 2 – 929 (S)
Lane 3 – 941 (S)
Lane 4 – 8343/2 (N)
Lane 5 – 980 (N)
Lane 6 – 929 (S)
Lane 7 – 941 (S)
Lane 8 – 8343/2 (N)
Lane 9 – 980 (N)
Lane 10 – 8343/2 (*N. fuckeliana* positive control)
Lane 11 – H_2O (negative control)
Lane 12 – 1Kb+ ladder (Invitrogen)

Lanes 2 – 5 are ITS-4/ITS-5 products at 1/1000 dilution for target DNAs, Lanes 6 – 9 are ITS-1F/ITS-4 products at 1/50 dilution for target DNAs.
(N) = *N. fuckeliana* isolate (S) = *S. sapinea* isolate

Appendix B – Primer Sequences

Primer Name	Primer Sequence (5'-3')
ITS-1	TCCGTAGGTGAACCTGCGG
ITS-4	TCCTCCGCTTATTGATATGC
ITS-5	GGAAGTAAAAGTCGTAACAAGG
S.sapFOR3	GCTTTGGCGGCTCTTTG
S.sapREV3	CTACTACGCTTGAGGGCTGAA
Cct1	ACCCCAAACCCTTATTTCTG
Cct2	ACGGCGTGGCCGCGCCGCTT