Molecular differentiation and distribution of *Cyclaneusma minus* morphotypes from *Pinus radiata* in New Zealand

REPORT INFORMATION SHEET

PROJECT TITLE	DIFFERENTIATION AND DISTRIBUTION OF *CYCLANEUSMA* SPECIES FROM *PINUS RADIATA* IN NEW ZEALAND
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CAREER DEVELOPMENT

This summer project has sparked an interest in me to pursue postgraduate studies after I finish my undergraduate degree. I found microbiology and mycology interesting at university and the opportunity to work in the field over summer was very valuable. I found the laboratory work interesting and helpful to put into practise some of what I had learnt at University. Working alongside accomplished scientists at Scion was a valuable experience that I will never forget and one which I believe will help me in my future career.

EXECUTIVE SUMMARY

Objective

The aim of this project was to determine the prevalence and geographic distribution of two *Cyclaneusma minus* morphotypes “simile” and “verum” using molecular methods.

Key Results

In this study, DNA from 76 Cyclaneusma isolates in the Scion Forest Health Reference Laboratory culture collection were tested. The most prevalent morphotype from the isolates tested was *Cyclaneusma minus* “simile” and this morphotype had the greatest geographical distribution, being found throughout New Zealand. The *Cyclaneusma minus* morphotype-specific primers were effective at only amplifying the DNA of the respective *C. minus* types when tested for specificity against other fungal and *Pinus radiata* DNA. In addition to testing isolates, infected pine needles were also tested by PCR for detection of the “simile” and “verum” morphotypes. This showed that 11% of the samples were “verum” and 39% of the samples contained the “simile” morphotype. It was also found that primers for amplifying the beta-tubulin and elongation-factor genes could differentiate the morphotypes based on amplicon size.

Further Work

In view of the questions raised by some of the data presented in this report, possible further research could be conducted based on the following;

- Increase the sample size for molecular analysis and compare to previous work that characterised the morphology with the same strains.
- Determine if more than two types of *Cyclaneusma minus* are present in New Zealand. Detailed analysis of the genetic diversity would be beneficial. Inclusion of the *C. minus* type strain and also *Cyclaneusma niveum* would also provide more information on the New Zealand populations.
- Statistical analysis of results with reference to the geographical origin of individual strains – including information on micro-climate which has a substantial effect.

Abstract

Cyclaneusma needle-cast (CNC) is a disease involving the ascomycetous fungus *Cyclaneusma minus*. The disease occurs world-wide but is of particular significance in New Zealand where it infects *Pinus radiata* plantations. There are two morphotypes of *C. minus*, termed *C. minus* 'simile' and *C. minus* 'verum'. *C. minus* 'simile' is the most common morphological type and is frequently found in the North Island. The disease is a problem in New Zealand because it detrimentally effects the growth of pine plantations. The estimated financial cost caused by the disease is about $38 million per year. The DNA analysis carried out in this study distinguishes the morphological types, *C. minus* 'simile' and *C. minus* 'verum' based on three genomic regions. The genomic regions targeted include the internal transcribed spacer (ITS), beta-tubulin and elongation factor genes. Two sets of PCR primers were used for analysis of the ITS region. The first set of primers could identify a 180 bp size difference in the ITS regions for the verum and simile morphotypes. The second set amplified DNA from either the verum or simile morphotypes, allowing morphotype-specific detection. Of the samples studied in this paper, *C. minus* simile was more common throughout New Zealand including regions where CNC is known to be more prevalent. The *C. minus* morphotype-specific primers successfully amplified the desired DNA when tested against *Cyclaneusma* spp. isolated as endophytes and directly from *Cyclanuesma*-infected *P. radiata* needles. The beta-tubulin and elongation factor primers used also differentiated the morphotypes based on amplicon size.

Introduction

Cyclaneusma needle-cast (CNC) is a serious disease of many *Pinus* species worldwide. The disease is caused by the ascomycetous fungus *Cyclaneusma minus* which infects the needles. The fungus and subsequently the disease have undergone name changes like many fungi do, this is well documented elsewhere (DiCosmo et al., 1983). Presently there are two *Cyclaneusma* species, *C. minus* and *C. niveum,* in New Zealand and *C. minus* is the only species involved with CNC on *Pinus radiata* (Dick et al., 2009). Within *C. minus* two morphological types have been defined, the morphotypes are termed *C. minus* 'simile' and *C. minus* 'verum'. The two main features that separate the morphological types are the length of apothecia (fruiting bodies) (*C. niveum* apothecia are longer than those of *C. minus*) and the shape and length of pycnidoispores (bacilliform, 6.0-9.5 mm in *C. minus*; sickle-shaped, 12-16 mm in *C. niveum*) (Bulman et al., 2008). Recent research using molecular methods has shown that the two morphotypes are likely to be separate species (Glen et al., 2011).

Fungal endophytes live in the tissues of plants without causing any effects. *Cyclaneusma* is present in needles of healthy and susceptible trees as a fungal endophyte then in some cases it shifts to being a fungal pathogen causing CNC (Podger & Wardlaw, 1990).

Apothecia, the fruiting bodies of ascomycetes, are readily found on fallen needles infected with *Cyclaneusma* but not on needles that remain attached to the tree (Podger & Wardlaw, 1990). The ascospores, inside the apothecias, are mainly released following periods of rainfall. The spores can be found all year round and enter needles through the stomata when the correct environmental conditions (principally moisture levels) occur (Gadgil et al., 1977; DiCosmo et al., 1983). The

main infection period occurs in April and the severity of the disease is related to the weather during the infection period (Bulman, 2009).
Recent observations have shown that a mild infection of part of a stand or the whole of the stand is more commonly observed than individually infected trees (McDougal et al., 2012). Also it has been proven that *C. minus* is pathogenic to older plants, but seedlings remain resistant (Bulman et al., 2008).
The disease causes the needles to turn a mottled yellow-brown colour which in turn causes infected trees to appear yellowed (Gadgil, 2001). The main symptom of CNC is a premature casting of needles occurring in two peak periods. The most severe casting event happens in spring followed by a smaller cast in autumn (Gadgil, 2001). It is often difficult to distinguish the symptoms of CNC from other causes of physical damage and casting.
CNC is a problematic disease because it reduces growth of pines destined for trade in the forestry industry (Bulman & van der Pas, 2001).The disease is of particular economic importance in New Zealand as the estimated financial loss amounts to $38 million per annum (Bulman, 2009). Studies of possible control methods have shown that chemical control is not an economically viable option, however the cultivation of resistant strains may be a suitable means of control (Dungey et al., 2006).
In New Zealand *C. minus* occurs in a wide variety of climatic conditions due to the vast distribution of the disease (Podger & Wardlaw, 1990). An extensive study was carried out of more than 70 000ha of *Pinus radiata* forests by Bulman (1988) to determine the distribution of CNC in New Zealand. It was discovered that Northland, Gisborne, Bay of Plenty, and Taupo regions had the highest severity of the disease, and the lowest disease incidence was in Canterbury and Nelson (Bulman, 2001b). Stands aged between 11 to 20 years old have the highest severity of the disease, while stands under 5 years old and over 25 years old have the lowest measured severity of the disease (Bulman, 2001b).
In other countries, particularly the in the Northern Hemisphere, the fungus has been recorded only as endophytic or saprophytic (Drenkhan & Hanso, 2009), although the detrimental effect of *Cyclaneusma minus* on Scots pine (*Pinus sylvestris*) is well documented (Kowalski, 1982; Kowalski, 1993; Merrill & Wenner, 1996).
Detection of CNC can be carried out through assessment of morphological characteristics and molecular analyses. However there is little research on *Cyclaneusma*, especially using molecular techniques.
In this study Polymerase chain reaction (PCR) was used to determine the prevalence and distribution of the two *Cyclaneusma minus* morphotypes. PCR is an analysis used to amplify specific regions of the DNA that can then be compared between samples, either by size of the amplicon analysed by gel electrophoresis or by DNA sequence analysis of the resulting amplicon.
The internal transcribed spacer (ITS) region is a convenient target region for molecular identification of fungi as it can be readily amplified with ‘universal primers’ that are complementary to sequences within the rRNA genes (Gardes & Bruns, 1993).The ITS primers utilize the conserved regions of the 18S, 5.8S, and 28S rRNA genes to amplify the non-coding regions (introns) between them (White et al., 1990). The ITS region was targeted in the first PCR experiment.
Morphotype-specific primers were used in the second PCR experiment targeting the ITS region because the ITS region of the two morphotypes are significantly different (Glen & Ramsfield, 2009).

The beta-tubulin and elongation factor genes are commonly used for phylogenetic analysis due to their conserved DNA sequences. 12 samples were selected for phylogenetic analysis. These samples were selected based on their geographical location in New Zealand and to act as representatives of the local populations. There is a possibility that there are further morphotypes of *C. minus* (Glen et al., 2011; R. Ganley, unpublished).
As the most promising method for control, at this stage, appears to be cultivation of resistant *P. radiata* genotypes, it is necessary to understand the genetic diversity within the genus *Cyclaneusma* in order to cultivate and successfully trial the best genotypes for resistance to Cyclaneusma needle-cast disease.

Materials and Methods

Isolates

The *Cyclaneusma* isolates used in this study were collected from different locations in New Zealand from 1969 to 2011. All *Cyclaneusma* strains were grown on 3% malt agar at 20°C for approximately 14-21 days. All cultures were maintained by the Forest Health Reference laboratory (Scion, New Zealand).

DNA extraction

Cyclaneusma cultures were grown on 3% malt agar with cellophane and DNA was extracted using the FastDNA kit (MP Biomedicals, OH, USA) by scraping mycelium off the cellophane into Lysing Matrix A tubes. DNA was extracted according the manufacturers' instructions, using cell lysis solution CLS-Y. DNA integrity was checked by agarose gel electrophoresis on 0.8% agarose gel, run at 100V for 35mins. Gels were visualised by UV transillumination. DNA was kept at -20°C for long term storage.

PCR Amplification

PCR reactions were performed using the KAPA2G Robust HotStart Readymix (2X) (KAPA Biosystems), according to the manufacturer's instructions. Each 25µl PCR reaction contained 6.75µl of PCR grade water, 12.5µl 2X KAPA2G Robust HotStart ReadyMix, 1.25µl of each primer (10µM), 1.25µl DMSO, 2µL DNA. The cycling conditions consisted of an initial denaturation step of 95ºC for 3 min, then 35 cycles of 95ºC (15 sec), annealing at primer-specific temperatures (see Table 1) for (15 sec), and 72ºC (15 sec), and a final extension step of 72ºC (10 min). Gel electrophoresis was performed with 1% (wt/vol) agarose in TBE and visualized by UV transillumination after staining with Redsafe Nucleic Acid Staining Solution (iNtRON Biotechnology). Four PCR experiments were conducted targeting the ITS, *C. minus* specific ITS, beta-tubulin, and elongation factor genes (Table 1).

TABLE 1 - List of primers used in this study for PCR amplifications

Gene region	Primer set	Sequence (5'-3')	TA* (°C)	Author
ITS	ITS1-F	CCTGGTCATTTAGAGGAAGTAA	55	(Gardes and Bruns, 1993)
	ITS4	TCCTCCGCTTATTGATATGC		
Cm 'simile' specific ITS	Cm 'simile' F	CCGGGCCTTATGGTCCGC	60	(Ramsfield, 2009)
	Cm 'simile' R	CAGGCGCCAGCCCAGCG		
Cm 'verum' specific ITS	Cm 'verum' F	CCGGGCCTTCGGGCCTAC	60	(Ramsfield, 2009)
	Cm 'verum' R	CAGGCACCAACCCAGGC		
Beta-tubulin	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	55	(Glass & Donaldson, 1995)
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC		
Elongation factor	EF1-728F	CATCGAGAAGTTCGAGAAGG	55	(Carbone & Kohn, 1999)
	EF1-986R	TACTTGAAGGAACCCTTACC		

*Note: TA= Annealing temperature in PCR reaction

Specificity testing of Cyclaneusma minus *specific primers*

The *C. minus* morphotype-specific primers designed by Glen and Ramsfield (2009) were tested in a PCR reaction involving other species that may be present in a pine forest, either in the needles as endophytes or as pathogens. This gives an indication of the specificity of the primers (Appendix 4).

Detection of Cyclaneusma spp. in P. radiata needles

Needles were collected from two sites on Long Mile road (Rotorua) for direct testing for the presence of *C. minus* using the morphotype-specific primers. Each sample contained two fascicles from one tree which were chosen based on the appearance of the characteristic symptoms of CNC (yellow mottling and in some cases being easily detached). From the first sample site, 7 samples were collected and from the second site a further 11 samples were collected. The needles were frozen over night at -20°C then freeze dried for 24-48h. The needles were then placed in a mortar with liquid nitrogen and crushed using a pestle. For each sample sterile equipment was used to prevent contamination. The DNA was then extracted using a modified method (ref Stoger et al 2004) utilising the Extract-N-Amp PCR ReadyMix kit. RedExtract-N-Amp^{TM} (Sigma-Aldrich) extraction buffer (400µl) was added to the powdered needles and the tubes were vortexed for 20s, and then incubated at 90°C for 30min. The RedExtract-N-Amp^{TM} dilution buffer was added (400µl) and the tube was vortexed again for 20s. The extracts were then diluted 1:10 (in a 1:1 ration of extraction:dilution buffers as the diluent), and 5µl of the diluted extracts were used per PCR reaction (Stöger & Ruppitsch, 2004). PCR was performed using the RedExtract-N-Amp^{TM} PCR ReadyMix, according to the manufacturer's instructions. A second round of PCR using the KAPA2G Robust HotStart Readymix (2X) was performed using the first round as template due to the possibility of fungal DNA titre being low.

Experiment with a secondary Cyclaneusma *collection at Scion*

From a collection of *Cyclaneusma* species stored as agar plugs, 34 samples were chosen to add to this study's sample population. To extract the DNA, two plugs were collected and 100µl of Tris:EDTA buffer (10:1) was added. The plugs were then crushed using a micropestle and frozen over night at -20°C. They were incubated on a heat block the following day for 10min at 80°C then centrifuged at 16, 100 × g for 5min. The DNA was purified using the ChargeSwitch gDNA Plant Kit (Invitrogen) according to the manufacturer's instructions. An ITS PCR experiment using 8.35µl of the purified DNA was conducted using the KAPA2G Robust HotStart Readymix (2X) kit (KAPA Biosystems), according to the

manufacturer's instructions. Bovine serum albumin (BSA) was added to manage the inhibiting components in the PCR solution.

Results and Discussion

Distribution of Cyclaneusma minus *simile and verum*

The morphotypes of *C. minus* were defined based on morphological characteristics, colony colour and texture, production of pigment, margin definition and formation of fruiting structures in previous studies (Bulman et al., 2008). Colony morphologies were recorded and compared to those previously published (Dick et al., 2001). It was observed that the colony morphologies are quite variable and often difficult to define. The morphological characteristics can depend largely on the age of the culture and the type of medium, among other factors (Dick et al., 2001). Dick et al (2001) found that the morphotype *C. minus* simile is the most common type in the North Island which is consistent with the results of this study (Fig 1 & Appendix 1). However in their study *C. minus* verum was the most common type identified overall which does not correlate with our results (Appendix 1). CNC is most prevalent in the North Island, particularly in Northland, Auckland, and the central North Island. In Northland, Taupo, and Auckland the majority of samples from this study were of the simile morphotype (Fig 1 & Table 3).

Table 3 – Distribution of samples among the New Zealand Biological Regions

Code	Biological region	n	simile	verum
AK	Auckland	11	11	0
BP	Bay of Plenty	8	3	5
CL	Coromandel	1	0	1
DN	Dunedin	5	2	3
FD	Fiordland	3	1	2
GB	Gisborne	3	3	0
HB	Hawke's Bay	3	1	2
MB	Marlborough	1	1	0
ND	Northland	4	3	1
NN	Nelson	5	5	0
OL	Otago Lakes	2	1	1
SC	South Canterbury	1	0	1
SL	Southland	4	3	2
TO	Taupo	11	9	2
TK	Taranaki	1	1	0
WA	Wairarapa	1	1	0
WD	Westland	2	2	0
WN	Wellington	8	7	2
WO	Waikato	1	1	0

Note: Only Biological regions from which samples were collected were used in this study

TABLE 4 - Distribution of *C. minus* morphotypes within New Zealand based on molecular analyses of all samples used in this study

Distribution	*C. minus* simile	*C. minus* verum
North island	75%	25%
South island	67%	33%
Total (NZ)	66%	34%

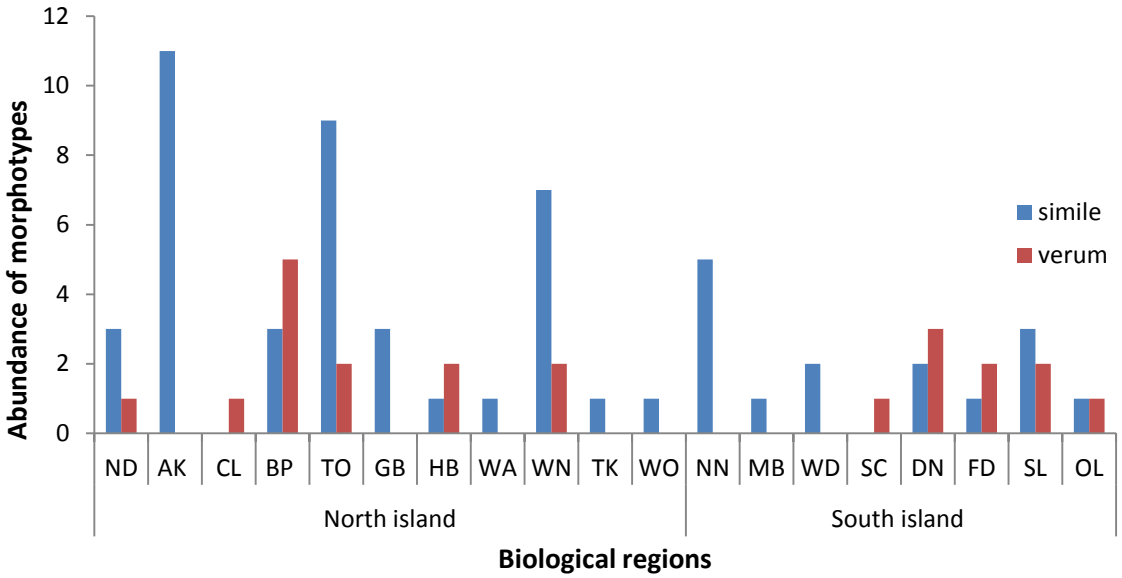

FIG. 1 – Geographical distribution of *C. minus* morphotypes in this study.

Characterisation of additional Cyclaneusma *isolates (uncultured)*

Isolates were selected from a secondary collection of *Cyclaneusma* spp. stored at 4 °C as mycelium on agar plugs, in water vials. These cultures were tested by direct PCR from the plugs as opposed to culturing the isolates first, due to time constraints with the project timeline (*Cyclaneusma minus* takes 14-21 days to grow in culture). Out of the 34 samples, 14 gave a positive result with ITS PCR reactions (Appendix 1).

FIG. 2 – Cultural form of *C. minus* 'simile' (Dick et al., 2001).

Morphological analysis of Cyclaneusma *cultures*

In the study by Dick et al. (2001) specimens were identified based on morphological characteristics alone. As a non-experienced pathologist I think using morphology analysis alone would have been unwise for this study, and by using molecular techniques it allowed for a reliable analysis of the types present. This is because with no experience identifying fungi cultures on the basis on morphological differences, the results of the study could well have been compromised through incorrect identification.

The cultures shown in Figures 2 and 3 are an example of the expected morphology of each type. However it was observed that the appearance of the cultures varied greatly and some even appeared to be a mixture of the two types. Morphological analysis alone could be more subjective than molecular analysis. An obvious potential problem of using only morphology analysis to define a specimen is misidentification, which is a possible occurrence with *Cyclaneusma* cultures. The

FIG. 3 – Cultural form of *C. minus* 'verum' (Dick et al., 2001).

morphology of *Cyclaneusma* is influenced by many factors including culture type, media for growth and time which may affect the reliability of morphology analysis to distinguish the morphotypes.

PCR Amplification

The ITS PCR (using primers ITS1F/ITS4) differentiates based on size of the amplicon. The second ITS PCR (using the morphotype-specific primers) differentiates based on nucleotide (DNA sequence) differences in primers. The simile and verum primers, used in the *Cyclaneusma* morphotype-specific PCRs, are morphotype specific, meaning they should only amplify one or the other morphotype. The primers target regions of the gene that have nucleotide differences and when the number of differences is too high they cannot anneal and amplify. Strain NZFS1000 gave no ITS PCR result but gave a positive simile result in the *C. minus* specific PCR experiment. Strain NZFS744 gave a positive verum result in the ITS PCR experiment and both negative simile and negative verum results in the *C. minus* specific PCR experiment. The experiment was redone however the results remained the same with no positive outcomes. Strain NZFS767 gave no results after two ITS PCR experiments and a *C. minus* specific PCR experiment. In the third ITS PCR trial the result was a positive simile which correlates with the results of a second *C. minus* specific PCR experiment. Sometimes amplification in both simile and verum was observed but usually one had a greater PCR yield than the other. The strain NZFS759 gave both a positive simile and positive verum result in the *C. minus* specific PCR experiment. The annealing temperature was increased from 58°C to 60°C, and this yielded only a positive simile result in the subsequent *C. minus* specific PCR experiment. This was similarly observed in the strain NZFS110Ca which showed a positive verum result in a *C. minus* specific PCR experiment as well as a weak detection as a positive simile result (the annealing temperature was at 60°C) (Table 5).

Specificity testing of* Cyclaneusma minus *specific primers

The *C. minus* morphotype-specific primers were tested against a range of fungal and oomycete DNA to check the specificity. The electrophoresis gel showed that with an annealing temperature of 63°C only the *C. minus* simile and verum positive controls amplified, therefore the primers should be effective at amplifying only the DNA of *C. minus* (Appendix 4).

Characterising* Cyclaneusma *endophytes with morphotype-specific primers

In 2008, Dr Rebecca Ganley (Scion, New Zealand) isolated pine endophytes from two trees, one visually healthy and one with CNC symptoms, selected from one stand in a plantation forest and 25 needles were collected from each tree. This was repeated in three different plantations (R. Ganley, unpublished). From the endophytes isolated, many were identified as *Cyclaneusma* spp. In this study an ITS PCR experiment was conducted using 25 samples of *Cyclaneusma* DNA isolated from those endophytes. From the 25 DNA preparations used, only eight samples amplified. All eight samples amplified as a positive 'verum' morphotype. (Appendix 2). It is possible that such a low amplification rate was observed due to the age and purity of the DNA samples.

Detection of* Cyclaneusma *spp. from infected* P. radiata *needles

Low rates of PCR detection of *Cyclaneusma* from pine needle tissue were observed initially. For this reason, two rounds of PCR were performed to increase

the possibility of detection from potentially low fungal DNA titres. In the first round of the PCR testing for the presence of *Cyclaneusma* directly with the morphotype-specific primers, simile was detected from two trees and verum was not detected in any (Appendix 3). This is most likely because there was not enough DNA amplified to view in gel electrophoresis. By repeating the PCR using the first round PCR mix as the template it meant the titre of DNA would be much greater making it possible to view results in a gel (Appendix 3). Nine out of the 18 samples amplified in the second round. *C. minus* simile was detected in seven needle samples and *C. minus* verum was detected in two samples. No samples showed the presence of both morphotypes (Appendix 3).

Beta-tubulin and elongation factor PCR results

It was shown that the 'simile' and 'verum' morphotypes can be distinguished based on size differentiation in the elongation-factor and beta-tubulin gene PCR experiments (Fig 2). The primers Bt1a and Bt1b amplified all of the strains however they were weak compared to the Bt2a and Bt2b primers (Fig 2). Also they did not differentiate the morphotypes based on size whereas the Bt2a and Bt2b primers did, as is shown clearly in Fig 2. The simile morphotypes have longer beta-tublin fragments than the verum therefore they do not migrate as far through the gel (Fig 4).

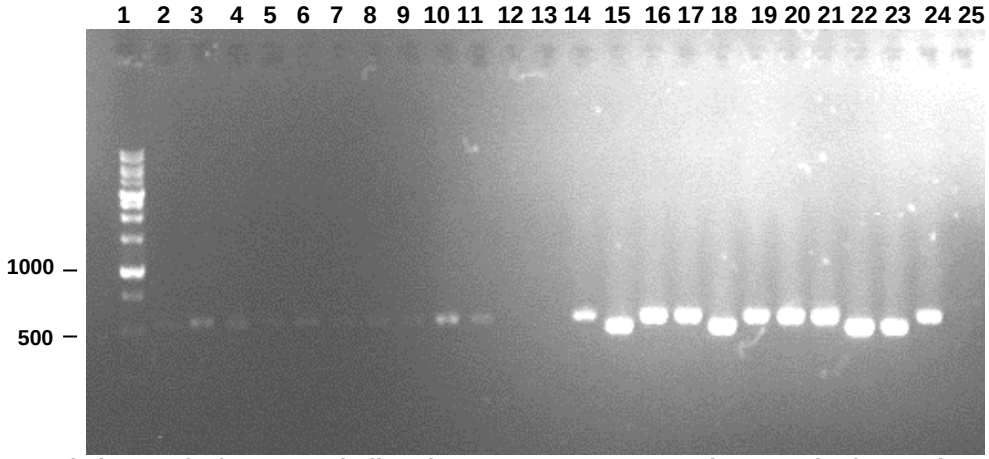

FIG. 4 - **Gel photo 09/01/14 Beta-tubulin primer sets. Lanes 2 – 13 show results from using primers Bt1a and Bt1b.** Lane: 1, 1 kb ladder; 2, 721; 3, 753; 4, 758; 5, 759; 6, 764; 7, 767; 8, 768; 9, 809; 10, 1800; 11, 3276; 12, positive simile control; 13, negative H_2O control. **Lanes 14 - 24 show results from using primers Bt2a and Bt2b.** Lane: 14, 721; 15, 753; 16, 758; 17, 759; 18, 764; 19, 767; 20, 768; 21, 809; 22, 1800; 23, 3276; 24, positive simile control; 25, negative H_2O control.

Phylogenetic analysis of New Zealand strains of C. minus

A phylogenetic tree using sequences from a different region of the beta-tubulin gene (using primers Bt1a and Bt2b) was produced by Prihatini et al. (unpublished). In the study by Prihatini et al. strains NZFS725 and NZFS764 were also positioned in the *C. minus* 'verum' clade and strains NZFS759 and NZFS758 here also positioned in the *C. minus* 'simile' clade (Fig 5).

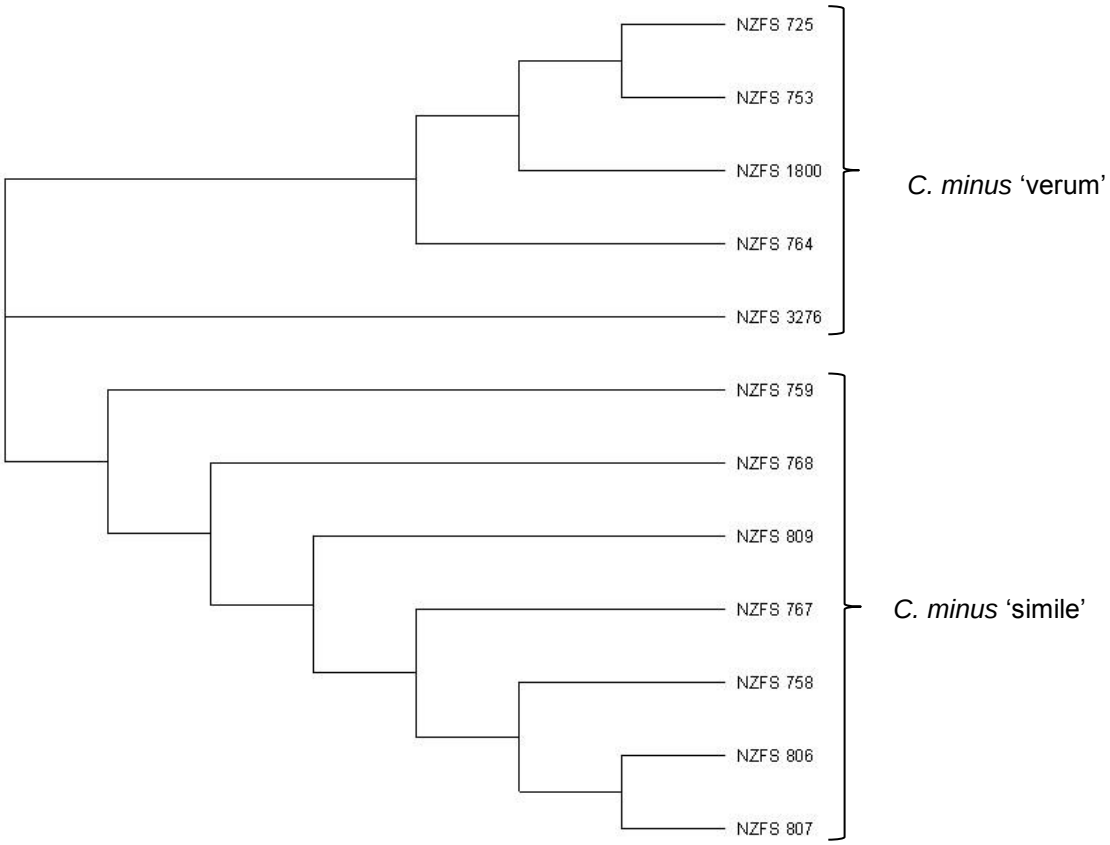

FIG 5 – Neighbour joining Phylogenetic tree of beta-tubulin nucleotide alignment sequences. This tree was made using Bt2a/2b DNA sequences of New Zealand (NZFS) isolates (this study).

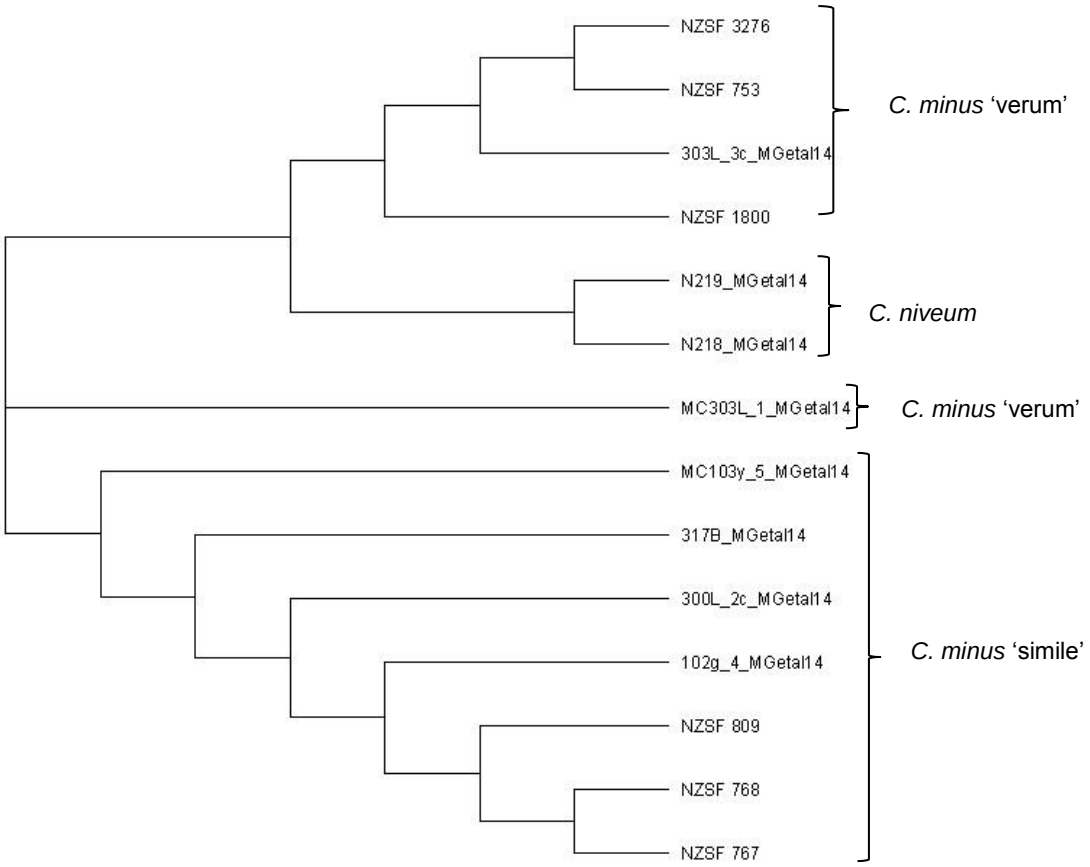

FIG. 6 - Neighbour joining phylogenetic tree generated with elongation factor DNA sequences. This tree was made using DNA sequences of New Zealand (NZFS) isolates (this study) and sequences of New Zealand isolates from another study (Prihatini et al.).

Sequences were obtained from the phylogenetic study of *Cyclaneusma* by Prihatini et al. and used to construct a phylogenetic tree in combination with sequences from isolates in this study. As in the study by Prihatini et al. the *Cyclaneusma niveum* clade was more closely associated with the *C. minus* 'verum' clade. In the study by Prihatini et al., strains 303L/3c and MC303L/1 were grouped in the same clade. Strains 300L/2c, 102g/4, 317B, and MC103y/5 were also grouped into the *C. minus* 'simile' clade (Prihatini et al.). This correlates with the PCR results for strain 102g/4 (same as NZFS758).
In the beta-tubulin phylogeny, NZFS3276 was positioned between the two clades (Fig 5), but in the elongation factor phylogeny it was positioned within the verum clade (Fig 6). PCR results indicated that this isolate belonged to the verum group (Table 4). In the elongation factor phylogeny strain MC300L/1 is positioned separately from the three clades, similar to NZFS3276 in the beta-tubulin phylogeny (Fig 6). In addition, other New Zealand isolates analysed by Prihatini et al. for the elongation factor gene were positioned in the same clades as the phylogenetic tree constructed for this study (Fig 6). Overall our results are in strong agreement with those of Prihatini et al. with respect to grouping of the verum and simile morphotypes.

Conclusions

This work represents a comprehensive molecular study of the prevalence of the known *Cyclaneusma minus* morphological types in New Zealand, with reference to geographical distribution. Knowledge of the diversity and distribution of *C. minus* morphotypes will contribute to enhanced understanding of the complete *C. minus* sp. Also this report provides information that will be important for future studies of *Cyclaneusma* population genetics and diversity.

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Appendix 1

Results of ITS PCR and morphotype-specific 'simile'/'verum' PCR

Culture collection number	ITS PCR simile	ITS PCR verum	Cm specific PCR simile	Cm specific PCR verum	Morphotype based on morphological analyses	Morphotype based on molecular analyses
110	-	+	-	+	simile	verum
110A	-	+	-	+	verum	verum
110B	-	+	-	+	ND	verum
110Ca	-	+	-	+	verum	verum
110Cb	-	+	-	+	verum	verum
110D	+	-	+	-	simile	simile
110E/1	+	-	+	-	simile	simile
110 E/2a	+	-	+	-	simile	simile
110E/2 b	+	-	+	-	ND	simile
110F/1	+	-	+	-	simile	simile
110 F/2	+	-	+	-	simile	simile
110G	-	-	-	-	verum	simile
680	+	-	+	-	simile	simile
681	+	-	+	-	simile	simile
682	+	-	+	-	verum	simile
685	+	-	+	-	verum	simile
686	+	-	+	-	ND	simile
687	+	-	+	-	ND	simile
688	+	-	+	-	simile	simile
721	+	-	+	-	verum	simile
722	-	+	-	+	verum	verum
723	-	+	-	+	simile	verum
724	-	+	-	+	ND	verum
725	-	+	-	+	verum	verum
726	-	+	-	+	simile	verum
727	+	-	+	-	simile	simile
744	-	+	-	-	verum	verum
745	+	-	+	-	ND	simile
746	+	-	+	-	verum	simile
747	+	-	+	-	simile	simile
748	+	-	+	-	simile	simile
749	+	-	+	-	simile	simile
752	-	+	-	+	simile	verum
753	-	+	-	+	simile	verum
758	+	-	+	-	simile	simile
759	+	-	+	-	verum	simile

761	+	-	+	-	simile	simile
762	+	-	+	-	ND	simile
763	+	-	+	-	simile	simile
764	-	+	-	+	simile	verum
765	+	-	+	-	ND	simile
766	+	-	+	-	verum	simile
767	-	-	+	-	simile	simile
768		-	+	-	simile	simile
769	+	-	+	-	verum	simile
770	+	-	+	-	simile	simile
771	+	-	+	-	verum	simile
773	+	-	+	-	verum	simile
805	+	-	+	-	simile	simile
806	+	-	+	-	simile	simile
807	+	-	+	-	simile	simile
808	+	-	+	-	simile	simile
809	+	-	+	-	simile	simile
1000	-	-	+	-	verum	simile
1800	-	+	-	+	verum	verum
2745	+	-	+	-	simile	simile
2746	+	-	+	-	simile	simile
3276	-	+	-	+	simile	verum
3277	+	-	+	-	ND	simile
3303	+	-	+	-	simile	simile
3325	-	+	-	+	verum	verum
3617	+	-	+	-	verum	simile
110/5	-	+	ND	ND	ND	verum
112a/1	+	-	ND	ND	ND	simile
114g/1	+	-	ND	ND	ND	simile
117/2	+	-	ND	ND	ND	simile
130/1	+	-	ND	ND	ND	simile
151/7	-	+	ND	ND	verum[1]	verum
154/5	+	-	ND	ND	ND	simile
188	+	-	ND	ND	ND	simile
199/1	+	-	ND	ND	ND	simile
200/1	-	+	ND	ND	ND	verum
202/5	-	+	ND	ND	ND	verum
252B	+	-	ND	ND	simile[1]	simile
296H/1A	+	-	ND	ND	"Mix"[1]	simile
312B	-	+	ND	ND	verum[1]	verum

Note: +; positive detection, -; negative detection, +/-; weak detection, ND; not determined.
[1] Turner and Dick (1999).

Appendix 2

Results of specificity testing of endophytic DNA extracted from *Pinus radiata.*

Name	ITS PCR simile	ITS PCR verum
CR2N 16(2)	-	-
CR4N 7(4)	-	+
CR4 N20-2	-	-
CR5N 8(3)	-	+
CS3N 1Y(5)	-	-
CS3N 5Y(2)	-	-
CS6 N15Y-2	-	-
CS6 N15Y-8	-	-
PR1 N1(1)	-	+
PR1 N2(base)(5)	-	-
PR1 N2(base)(6)	-	+
PR1 N10(3)	-	+
PR1 N16(3)	-	-
PR1 N18(3)	-	-
PR1 N18(4)	-	-
PR3 N7(2)	-	-
PR3 N16(4)	-	-
PR3 N18(3)	-	-
PS2 N1G(9)	-	-

PS2 N9G(1)	-	-
PS2 N9G(6)	-	+
PS2 N9G(22)	-	+
PS2 N15Y(4)	-	-
PS4 N4G(4)	-	-
PS6 N5G(12)	-	+
positive simile control	+	-
positive verum control	-	+
negative control	-	-

Appendix 3

Results of Needles experiment testing for presence of *C. minus* morphotypes

Trial area	Tree ID	Round 1 PCR 'simile'	Round 2 PCR 'simile'	Round 1 PCR 'verum'	Round 2 PCR 'verum'
	875-223	+	+	-	-
	875-293	-	-	-	-
	883-522	-	+	-	-
1	885-9	-	-	-	-
	885-196	+	+	-	-
	885-198	-	-	-	-
	886-915	-	+	-	-
	#1	-	+	-	-
	#2	-	-	-	-
	#3	-	-	-	-
	#4	-	-	-	-
	885-500	-	-	-	+
2	887-7	-	+	-	-
	889-716	-	-	-	+
	889-722	-	-	-	-
	892-217	-	+	-	-
	892-219	-	-	-	-
	892-521	-	-	-	-

Appendix 4

Results of Specificity test of morphotype-specific primers

Specimen	simile (+)	verum (+)
Dothistroma septosporum	-	-
Diplodia pinea	-	-
Peridermium harknessii	-	-
Armillaria novae-zelandia	-	-
Xylariaceae sp.	-	-
Pinus radiata	-	-
Phytophthora pluvialis	-	-
Phytophthora kernoviae	-	-
Phytophthora cactorum	-	-
Phytophthora multivora	-	-
Pythium irregulare	-	-
Positive 'simile' control (NZFS3617)	+	-
Positive 'verum' control (NZFS 764)	-	+
Negative control	-	-