Fungal Community Survey of Fraxinus excelsior in New Zealand

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Popular summary:

The European Ash tree (*Fraxinus excelsior*) is widely grown throughout Europe. As a large deciduous tree species, it grows a tall, domed crown and has an attractive tree shape, so it is considered as a popular amenity tree species. European Ash is planted as an important forestry species in some European countries, and also often used for furniture making, due to its excellent wood quality. Ash species were introduced into New Zealand upon colonization in the 1800s.

Recently, ash trees throughout Europe have been observed to become damaged or die due to a severe disease known as ash dieback, caused by the fungus *Chalara fraxinea.* We are concerned about what will happen to the introduced ash tree in New Zealand. To our knowledge, there have been no studies on the fungi that inhabit ash trees in New Zealand. It is unknown which fungal species were present in ash at the time of the introduction to New Zealand, or which New Zealand fungi colonized ash tree after the introduction. Currently, ash dieback is not believed to be present in New Zealand.

The aim of this project was to determine the possible fungal communities on ash trees in New Zealand. We collected bark, bud and wood from three healthy ash trees, and used DNA-based methods to identify the fungi that inhabited these trees. We compared our study with a similar Swedish study to find differences and similarities in the fungi present on New Zealand and European ash trees. In total, we found 90 different fungal species. Of these species found, seven fungi could be species that came to New Zealand with the introduced ash tree. We also found one fungus that could possibly be said to have come from New Zealand. The pathogen causing ash dieback, *Chalara fraxinea*, was not detected.

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Abstract

Fraxinus excelsior (European Ash) is an important broad-leaved tree species in New Zealand; it was introduced to New Zealand when European people colonized the country around 1840. To our knowledge, no systematic survey about the fungal community of ash trees has been carried out in New Zealand, therefore it is unknown which fungal species have been present from the time of the introduction of the ash tree to New Zealand. Since ash dieback in Europe is causing large losses to European forests, people are interested in what happens on ash in New Zealand. DNA-based identification methods were used to examine fungal communities in three trees from the North Island of New Zealand. Fungal isolations, PCR and sequencing methods were used. Ninety fungal taxa were identified in the community survey study, of which 35 species were recorded on bark, 43 species were recorded on wood and 46 species were recorded on bud. More species were detected by direct DNA extraction (78) than by fungal isolation (23) in this experiment. Species composition grouped to bark, wood and bud was analyzed by principal component analysis (PCA). Very little difference in fungal community between tissue types was detected. PCA revealed no specific relationship between the detected fungal communities and the different trees in our survey. Compared with a similar Swedish study, the diversity of detected fungal taxa was high overall. Seven species were identified which could have come to New Zealand with the introduced ash tree; these were *Colletotrichum acutatum, Phoma exigua, Neofabraea alba, Venturia fraxini, Fusarium lateritium, Pilidium concavum and Fusarium oxysporum*. We detected only one species which could potentially have come from New Zealand; *Neofusicoccum parvum. Chalara fraxinea* (ash dieback) was not detected in the New Zealand samples.

Introduction:

Fraxinus excelsior (European Ash or common ash) is the most widely distributed ash species in Europe (Boshier, 2005). As a large deciduous tree species, it can grow to 20–35 m (maximum to 46 m) tall with DBH (diameter at breast height) up to 2 m (maximum to 3.5 m) diameter (Pliûra and Heuertz, 2003). It grows a tall, domed crown and has deciduous leaves, and it is considered as a popular amenity tree species. The wood is white or pinkish white pale color, and wood quality is strong, stretchy and easily bent. Due to these qualities, it is often used for furniture making even though the grain is coarse (Boshier, 2005). The European ash tree requires a fertile soil and prefers pH-neutral soil, generally growing well where pH is above 5-6 (Dobrowolska *et al.*, 2008). *Fraxinus excelsior* is also an important fast growing timber species, because it is relatively pest free compared with many other broadleaf tree species (Evans, 1984). *Fraxinus excelsior* is planted as an important short rotation silviculture species in Sweden; it has outstanding regeneration success and productivity (Gotmark *et al.*, 2005).

Fraxinus excelsior was introduced to New Zealand when European people colonized the country around 1840. Because of its elegant tree shape and beautiful deciduous leaves, it has been planted as an amenity tree in some cities of New Zealand.

The disease known as ash dieback caused by *Chalara fraxinea* (teleomorph: *Hymenoscyphus pseudo-albidus*) (Kowalski and Holdenrieder, 2009b) is damaging *F. excelsior* in Europe. It has caused serious forest health problems in Poland and Lithuania since 1996 (Lygis *et al.*, 2005). Now it has been found in many countries in eastern, central and northern Europe (Andersson *et al.*, 2010) (Figure 1), but it has not been reported in south hemisphere yet. It is fast spreading and affects all ages of ash on various site types (forest, open landscape, nurseries and urban plantings) in Europe (Schumacher *et al.*, 2010). Ash dieback causes wilting of leaves, bark necrosis and cankers on young shoots and stem (Dobrowolska *et al.*, 2011). The disease leads to death within a few years if the tree is young, but it is often observed that the process is slower if the tree is old (Schumacher *et al.*, 2010). Kowalski and Holdenrieder (2009a) found that *Chalara fraxinea* occurred frequently together with *Alternaria alternata, Cytospora sp., Coniothyrium olivaceum, Diplodia mutila, Fusarium spp. and Phomopsis spp.*. Skovsgaard *et al.* (2010) found that the occurrence of ash dieback might depend on the genetics of ash tree and site conditions, they also found that *C. fraxinea* is a primary disease and the disease is associated with *Armillaria gallica*. Because the potentially economic and ecological impacts of ash dieback are huge, the European Plant Protection Organization (EPPO) has put this pathogen on its pathogen alert list (van Opstal, 2011).

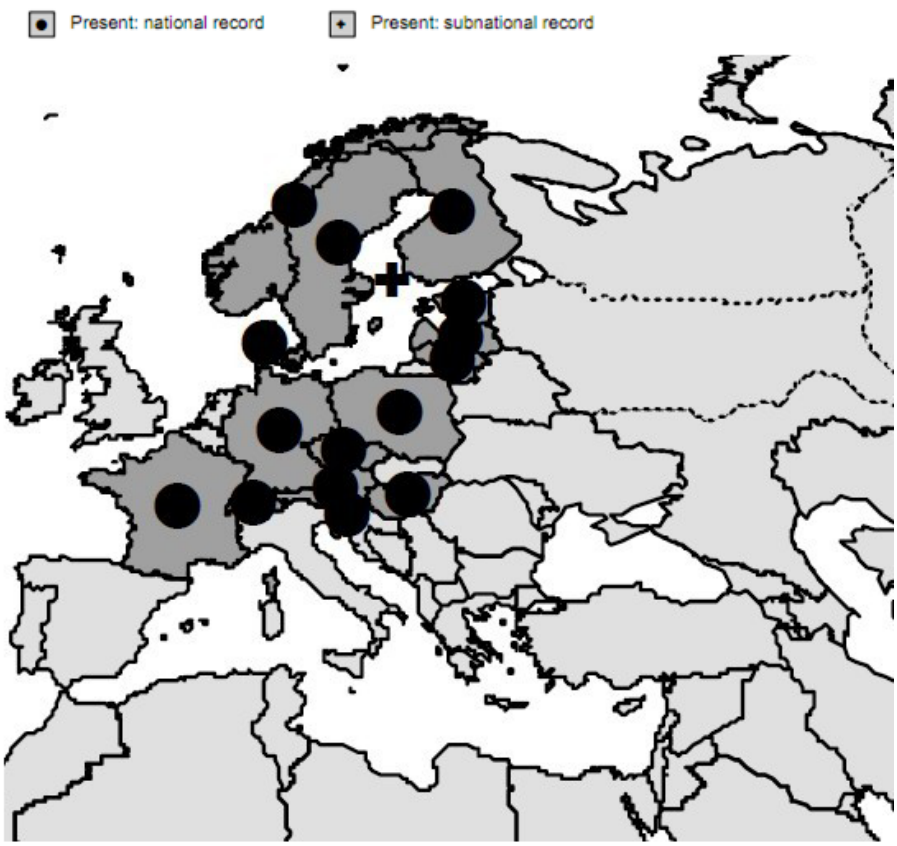

Figure 1. Distribution map of *Chalara fraxinea.*

The figure shows the geographical distribution of *Chalara fraxinea* in Europe (Austria, Czech Republic, Denmark, Estonia, Finland, France, Germany, Hungary, Latvia, Lithuania, Norway, Poland, Slovenia, Sweden, Switzerland) (Kowalski, 2009)

Ash dieback was first discovered in southern Sweden in 2002 but it was found to have spread widely by 2004 (Bakys *et al.*, 2009a). Bakys *et al.* (2009b) conducted a survey of ash decline in Sweden. They sampled 20 – 30 years old *F. excelsior* showing symptoms of crown decline from four locations in Sweden, and grouped all the samples into four categories: visually healthy, showing initial necroses, showing advanced necroses, and shoots with dead tops. Fungi were isolated and DNA extraction, PCR amplification and DNA sequencing were applied for all the mycelia morphotypes. They detected 56 fungal taxa; the most frequently isolated fungi were *Chalara fraxinea*, *Gibberella avenacea*, *Alternaria alternata*, *Epicoccum nigrum*, *Botryosphaeria stevensii*, *Valsa* sp., *Lewia* sp., *Aureobasidium pullulans* and *Phomopsis* sp. Their study is considered as a comparison with our study, because it sampled ash in its native range.

Considering the severe ash disease in Europe which causes major losses, we are concerned about what will happen to the introduced ash tree in New Zealand. To our knowledge, no systematic survey about the fungi of ash tree has been carried out in New Zealand. It is unknown which fungal species were present in ash at the time of the introduction to New Zealand, or which indigenous New Zealand fungi may have colonized the ash tree after introduction.

The aims of this project were to:

1. Observe the fungal communities on ash trees in New Zealand.
2. Compare with Swedish fungal communities to learn which fungal species were introduced with ash into New Zealand.
3. Look for indigenous New Zealand fungal species that can be found in the introduced ash.

To achieve these aims, we used DNA-based identification methods. Fungal isolations and direct DNA extraction methods were compared. Fungal isolations by culturing are considered as routine techniques; however they are limited at times. It can be difficult to obtain a pure culture in the laboratory (Pace, 1997). The reasons are: firstly, fast-growing species may outcompete slow-growing species in conventional culturing methods (Straatsma *et al.*, 2001). Secondly, It is easily overlooked by culturing method if fungi don't produce recognizable structures in culture making them difficult to identify (O'Brien *et al.*, 2005). DNA based methods can be used to increase species detection.

The polymerase chain reaction (PCR) is considered to a widely used technique for fungal identification (White *et al.*, 1990; Gardes and Bruns, 1996). The internal transcribed spacer (ITS) region is frequently highly variable among morphologically distinct fungal species; it has been successfully used in fungal molecular characterization research (White *et al.*, 1990; Gardes and Bruns, 1993; Mohamed *et al.*, 2010).

Cloning can separate a single fragment of DNA from a mixture of many fragments. Firstly the fragment of DNA to be cloned is inserted into a vector to produce a recombinant DNA molecule. Secondly, the vector itself is inserted into a host cell (usually a bacterium), where the vector and the DNA fragment, multiply producing numerous identical copies within the host cell. Thirdly, copies of recombinant DNA molecule are passed to the progeny when the host cell divides and further vector replication happens. In the end, a colony of identical host cells is produced after numerous of cell divisions (Brown, 2010). After the cloning procedure, bacterial colonies are analyzed for inserts via PCR using M13 primers. Sequence analysis is then carried out.

Materials and methods:

Study sites and sampling

Field samples were collected from Te Papa Tipu Innovation Park in Rotorua, New Zealand in August 2009 (winter time) (Figure 2). The trees were 60-70 years old, and appeared healthy but had no leaves due to winter season. Three branches were collected from the lower part of three trees respectively. There were 10, 11 and 15 twigs collected from each branch respectively. Samples were grouped into bud, bark and wood from each twig and cut to small pieces. There were 30 bud samples, 34 bark samples and 35 wood samples in total.

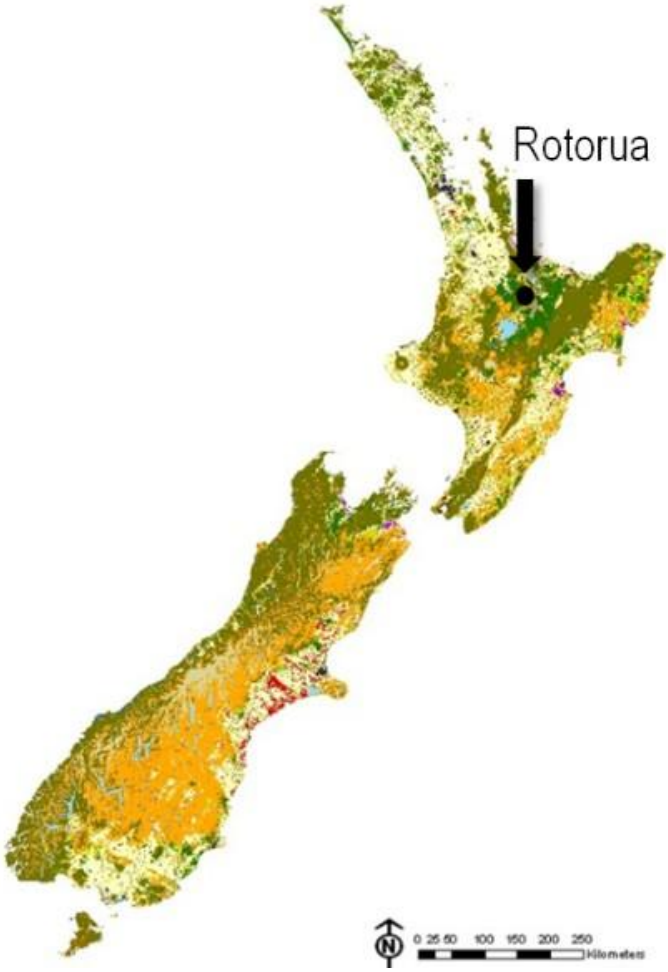

Figure 2. ● Shows the sample collection location: Te Papa Tipu Innovation Park in Rotorua, New Zealand.

As Figure 3 shows, each plant field sample was separated in two parts. One part was cultured for fungal isolates and the other for direct DNA extraction. Both fungal isolations and direct DNA extractions were treated with DNA extraction and ITS PCR. Cloning and M13 PCR were applied only for direct DNA extractions. Sequencing and analysis were applied for both methods.

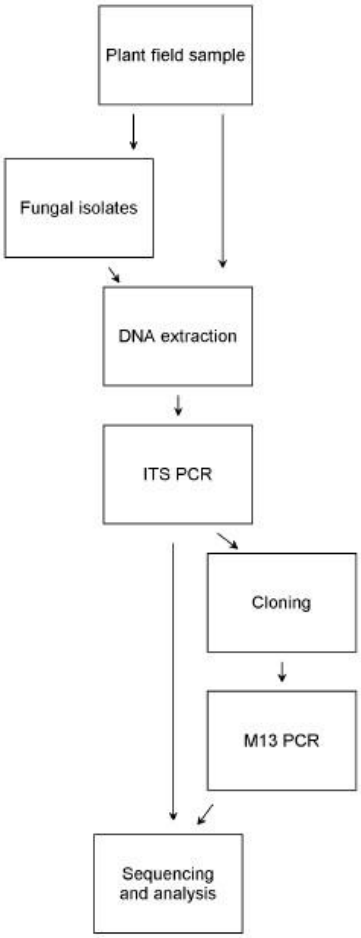

Figure3. The flowchart of experiment methods used for the field samples. The middle procedures show both fungal isolations and direct DNA extraction used; the left-side shows the procedures only used for fungal isolations; the right-side shows the procedures only used for direct DNA extraction.

Fungal isolations

The ash shoot materials were sterilized with 70% ethanol for one minute, then with 3% NaClO for five minutes, repeat sterilizing with 70% ethanol for one minute and dipped into sterile water for one minute. Small pieces of ash (3x3 mm) from all three types of samples (bark and wood samples were taken from the same spot, while buds were taken from another part of the twig) were placed on agar-plates containing 1% malt-extract agar. Fungal outgrowth was observed after one week, two weeks, four weeks and after eight weeks. Pure cultures of all filamentous fungi and yeasts were obtained. Sixty eight isolates were obtained from all samples. The fungi were gently scraped from the medium into 1.5ml centrifuge tubes (Eppendorf) using a sterilized scalpel and stored at -20°C. The fungal isolation step was carried out by Stina Bengtsson prior to commencement of this project.

DNA was extracted using the CTAB method (Velegraki *et al.*, 1999). Forty seven isolations were freeze-dried and ground to fine powder with Precellys 24 Lysis & Homogenization (Bertin Technologies). For each isolation, CTAB–buffer (3%) was used to extract for 1 hour at 65°C, followed by 5 minutes centrifugation. Chloroform was then used for extracting DNA; DNA was deposited by isopropanol for 30 minutes and washed with 70% ethanol. The DNA was purified with JET quick (GENOMED GmbH). All the samples were measured for DNA content and quality

using the NanoDrop (NanoDrop Technologies) and then were diluted to 1 ng/µL.

For the fungal isolations, the primers ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) (White *et al.*, 1990) were used to amplify the ribosomal ITS region. The ITS PCR reaction recipe was: Green buffer (Fermentas) (includes 10u Dream Taq and 4mM $MgCl_2$), 0.4mM dNTPs, 1.5mM $MgCl_2$, 0.4mM Primer ITS1F, 0.4mM Primer ITS4, 2% BSA enhancer (proportion: 10%), 0.05u Dream Taq (Fermentas). The PCR reaction was applied with the steps based on standard protocol: initial denaturation step at 94°C for 5 min; 35 amplification cycles of denaturation at 94°C for 30 s; annealing at 55°C for 30 s and extension at 72°C for 30 s; a final extension step at 72°C for 7 min. The results were examined on a 1% agarose gel using Gelgreen (Biotium). Samples were ignored if displaying multiple bands (Figure 4 a), 41 out of 47 had single bands.

All the samples were sent to Macrogen (Seoul, Korea) to sequence in both the forward and reverse directions. Sequences were aligned and manually edited with Seqman (DNASTAR Lasergene 8). Sequences were run using Blastn in the NCBI database to look for sequence matches that could help to identify them (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ITS sequence homology for delimiting fungal taxon (presumed species) was set at 98–100%, and for delimiting genus level was 94–97% (Glen *et al.*, 2001).

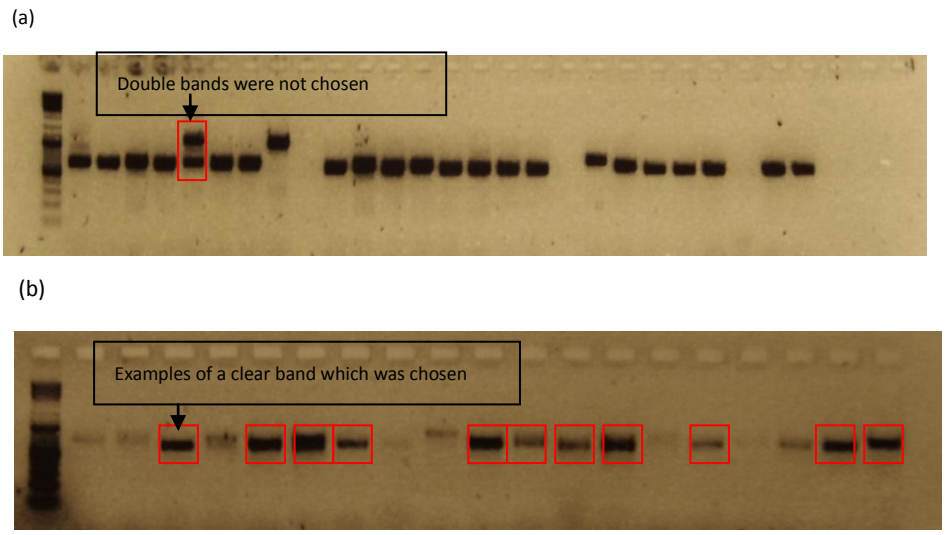

Figure 4. The examples of PCR gel pictures with two different primers. (a)The PCR of fungal isolations by ITS primers: only single band samples were chosen for sequencing. (b) The PCR of direct DNA extractions by M13 primers: only single band samples were chosen for cloning and sequencing.

Direct DNA extraction

For the direct DNA extraction, 34 samples from bark, 35 samples from wood and 30 samples from bud were extracted with the CTAB method as described above except for the wood samples which used the CTAB method with the following changes: equivalent volumes of approximately 300 µL powder of each sample were collected after grinding using Precellys 24 Lysis & Homogenization (Bertin Technologies) with three beads at speed 4.0. The wood samples were ground twice because they were difficult to grind. The amount of CTAB buffer was reduced for

the wood samples to get higher concentrations of DNA. PCR was carried out using primer ITS1-F and ITS4; PCR conditions were the same as described above. Only those samples giving clear bands on the gel after PCR were chosen for cloning.

Forty six samples, consisting of 16 wood samples, 15 bark samples and 15 bud samples with clear bands (Figure 4 b) in ITS PCR were selected for next step where cloning was applied to separate mixed fungal communities. PCR products were cloned with the TOPO TA Cloning Kit with pCR®2·1- TOPO vector and One Shot TOP10 chemically competent *E. coli* (Invitrogen). Lysogeny broth (LB) plates which contained Ampicillin and X-Gal was used to select clones. Eight clones of each sample were picked if they were white or slightly blue and diluted with 200 µL ddH_2O. Small amounts of bacteria were used directly for the PCR, but with primers M13 Forward (GTAAAACGACGGCCAG) and M13 Reverse (CAGGAAACAGCTATGAC) (Griffin and Griffin, 1993). The cycle condition with M13 primers were: initial denaturation step at 94°C for five min; 35 amplification cycles of denaturation at 94 °C for 30 s; annealing at 55°C for 30 s and extension at 72°C for 30 s; a final extension step at 72°C for 7 min. The M13 PCR reaction recipe was: Green buffer(Fermentas) (includes 10U Dream Taq and 4mM $MgCl_2$), 0.4mM dNTPs, 1.5mM $MgCl_2$, 0.4mM Primer M13 F, 0.4mM Primer M13 R, 0.05u Dream Taq. The purification of PCR products with AMPure (Agencourt) was applied to get rid of salts, unincorporated dNTPs and unused primers before sequencing. All samples were sent to Macrogen (Seoul, Korea) for sequencing. Sequencing was carried out in both the forward and reverse direction as described above.

Statistics

Principal component analysis (PCA) is considered to offer a deeper insight into the data structure and help to conclude when the results are not instantly obvious (Zitko, 1994). Based on presence/absence data, PCA was carried out with the default setting using Canoco version 4.5 (Plant Research International).

Meta-analysis is the analysis of a collection of analytic results to integrate the findings (Dersimonian and Laird, 1986). It is a good method to estimate the magnitude of the effect and analyse the sources of variation (Koricheva *et al.*, 2009). A survey of ash decline in Sweden (Bakys *et al.*, 2009a; Bakys *et al.*, 2009b) and our ash community study in New Zealand were compared using a meta-analytical approach to achieve our aims.

Results:

Fungal community as defined by fungal isolation

Table 1 shows the Blasting result and best matching of fungal isolation when we run using Blastn in the NCBI database. The judgment of sequence match quality depends on the E-Value of the blasting (0 is the best), max identity and query coverage (100% is the highest). Twenty sequences had good sequence match quality; two of them had medium sequence match quality because of lower max identity or lower query coverage. For two of them (*Lophiostoma corticola* and *Phaeosphaeria sp.*) the origin of the closest match is New Zealand.

Table 1 Blasting information and best match of fungal isolation from F. excelsior, New Zealand

*Final species	*Closest blast match	Number of bases matched	Max identity	*Origin of closest match	Sequence match quality
Aspergillus versicolor	*Aspergillus versicolor*	567/570	99%	Lithuania	Good
Cladosporium phaenocomae	*Cladosporium phaenocomae*	566/566	100%	Netherlands	Good
Colletotrichum acutatum	*Colletotrichum acutatum*	583/583	100%	Germany	Good
Epicoccum nigrum	*Epicoccum nigrum*	549/549	100%	Czech Republic	Good
Fusarium lateritium	*Fusarium lateritium*	567/567	100%	Germany	Good
Lophiostoma corticola	*Lophiostoma corticola*	553/553	100%	New Zealand	Good
Neofusicoccum parvum	*Neofusicoccum parvum*	589/589	100%	Sweden	Good
Phoma exigua	*Phoma exigua*	542/542	100%	Germany	Good
Ascomycota sp.	*Ascomycota sp.*	505/511	98%	China	Good
Davidiella sp.	*Davidiella sp.*	559/559	100%	Czech Republic	Good
Penicillium sp.	*Penicillium sp.*	604/604	100%	China	Good
Phaeosphaeria sp.	*Phaeosphaeria sp.*	432/439	98%	New Zealand	Good
Phoma sp.	*Phoma sp.*	540/542	99%	USA	Good
Phomopsis sp.	*Phomopsis sp.*	585/589	99%	USA	Good
Pleosporales sp.	*Pleosporales sp.*	497/519	95%	USA	Good
Xylariaceae sp.	*Xylariaceae sp.*	525/545	96%	USA	Medium
Rhodosporidium babjevae	*Rhodosporidium babjevae*	594/594	100%	USA	Good
Rhodotorula bacarum	*Rhodotorula bacarum*	656/667	98%	Japan	Good
Ceratobasidium sp.	*Ceratobasidium sp.*	539/541	99%	Sweden	Good
Microbotryomycetes sp.	*Microbotryomycetes sp.*	655/692	94%	USA	Medium
Rhodotorula sp.	*Rhodotorula sp.*	575/581	98%	Finland	Good
Tremellales sp.	*Tremellales sp.*	511/515	99%	USA	Good

* "Final species" means the final determined species when we blast in NCBI database, "Closest blast match" means the best match in NCBA database, "Origin of closest match" means which country the best match comes from.

Table 2 shows the frequencies of detection of fungal isolates from ash trees in New Zealand. Sixty-eight samples were isolated, but only 36 (53%) were used in the final analysis, 32 (47%) got contaminated because of mites. Twenty-three fungal taxa were found based on all samples by searching on NCBI database, of which 16 of the species were *Ascomycota*, and six species were *Basidiomycota*. In total, ten (44%) were identified to species level, 12 (52%) were identified to genus level and one (4%) was still unidentified. The most frequently isolated fungus was *Phoma* (18%). It was notable that only two fungal isolates were from wood sample, they were *Neofusicoccum parvum* and *Penicillium sp.*

Table 2 Frequencies of detection of fungal isolation from F. excelsior, New Zealand

	Fungal taxon	Analysed tissues from		
		Bark	Wood	Bud
Ascomycota	*Aspergillus versicolor*	-	-	1
	Cladosporium phaenocomae	-	-	1
	Colletotrichum acutatum	-	-	1
	Epicoccum nigrum	-	-	1
	Fusarium lateritium	-	-	1
	Lophiostoma corticola	2	-	1
	Neofusicoccum parvum	-	1	-
	Phoma exigua	-	-	4
	Ascomycota sp.	-	-	1
	Davidiella sp.	1	-	-
	Penicillium sp.	1	1	1
	Phaeosphaeria sp.	-	-	1
	Phoma sp.	-	-	3
	Phomopsis sp.	1	-	-
	Pleosporales sp.	2	-	1
	Xylariaceae sp.	-	-	1
Basidiomycota	*Rhodosporidium babjevae*	1	-	-
	Rhodotorula bacarum	1	-	-
	Ceratobasidium sp.	1	-	-
	Microbotryomycetes sp.	2	-	-
	Rhodotorula sp.	1	-	1
	Tremellales sp.	1	-	-
	unidentified sp.	1	-	-
	Total	15	2	19

Fungal community as defined by direct DNA extraction

Table 3 shows the Blasting information and best matching of direct DNA extractions. The judgment of sequence match quality depends on the E-Value of the blasting (0 is the best), max identity and query coverage (100% is the highest). Thirty-two of blasting results had good sequence match quality and 12 of them had medium sequence match quality because of lower max identity or lower query coverage. Most of the origins of closest match are from Europe, there are also some from other areas such as Asia and USA.

Table 3 Blasting information and best match of direct DNA extractions from F. excelsior, New Zealand

*Final species	Closest blast match	Number of bases matched	Max identity	Origin of closest match	Sequence match quality
Aureobasidium pullulans	*Aureobasidium pullulans*	616/616	100%	Spain	Good
Bionectria ochroleuca	*Bionectria ochroleuca*	608/608	100%	Czech Republic	Good
Botryosphaeria parva	*Botryosphaeria parva*	594/597	99%	Brazil	Good
Colletotrichum acutatum	*Colletotrichum acutatum*	618/620	99%	Germany	Good
Fusarium lateritium	*Fusarium lateritium*	595/600	99%	Germany	Good
Fusarium oxysporum	*Fusarium oxysporum*	580/582	99%	Czech Republic	Good
Herpotrichia parasitica	*Herpotrichia parasitica*	515/532	98%	USA	Good
Neofabraea alba	*Neofabraea alba*	578/582	99%	Netherlands	Good
Neofusicoccum parvum	*Neofusicoccum parvum*	596/597	99%	Sweden	Good
Penicillium brevicompactum	*Penicillium brevicompactum*	619/619	100%	Japan	Good
Penicillium canescens	*Penicillium canescens*	622/622	100%	Brazil	Good
Penicillium spinulosum	*Penicillium spinulosum*	616/616	100%	Czech Republic	Good
Phoma exigua	*Phoma exigua*	576/579	99%	Germany	Good
Pilidium concavum	*Pilidium concavum*	485/486	99%	USA	Good
Saccharomyces cerevisiae	*Saccharomyces cerevisiae*	877/891	98%	France	Good
Venturia fraxini	*Venturia fraxini*	585/586	99%	Netherlands	Good
Ascomycete sp.	*Ascomycete sp.*	606/608	99%	Brazil	Good
Ascomycota sp.	*Ascomycota sp.*	530/545	98%	China	Medium
Cladosporium sp.	*Cladosporium sp.*	591/591	100%	China	Good
Epicoccum sp.	*Epicoccum sp.*	566/583	97%	USA	Medium
Fusarium sp.	*Fusarium sp.*	590/595	99%	Czech Republic	Good

Final species	Closest blast match	Number of bases matched	Max identity	Origin of closest match	Sequence match quality
Herpotrichia sp.	*Herpotrichia parasitica*	515/532	96%	USA	Medium
Kabatina sp.	*Kabatina thujae*	595/631	94%	USA	Medium
Penicillium sp.	*Penicillium sp.*	620/621	99%	China	Good
Phaeomoniella sp.	*Phaeomoniella sp.*	513/520	98%	Korea	Medium
Phialophora sp.	*Phialophora europaea*	580/613	95%	Switzerland	Medium
Phoma sp.	*Phoma sp.*	577/579	99%	USA	Good
Phomopsis sp.	*Phomopsis sp.*	592/599	98%	USA	Good
Pleosporales sp.	*Pleosporales sp.*	682/689	98%	China	Good
Bensingtonia yuccicola	*Bensingtonia yuccicola*	679/683	99%	USA	Good
Cryptococcus flavescens	*Cryptococcus flavescens*	568/568	100%	Austria	Good
Exobasidium arescens	*Exobasidium arescens*	605/609	99%	Germany	Good
Malassezia restricta	*Malassezia restricta*	767/773	99%	Belgium	Good
Polyporus tuberaster	*Polyporus tuberaster*	647/647	100%	USA	Good
Suillus granulatus	*Suillus granulatus*	706/715	98%	China	Good
Articulospora sp.	*Articulospora proliferate*	512/542	94%	Canada	Medium
Basidiomycota sp.	*Basidiomycota sp.*	617/639	97%	Czech Republic	Medium
Cryptococcus sp.	*Cryptococcus sp.*	523/524	98%	USA	Good
Dioszegia sp.	*Dioszegia sp.*	467/479	97%	USA	Medium
Exobasidium sp.	*Exobasidium rhododendri*	591/615	96%	England	Medium
Malassezia sp.	*Malassezia globosa*	767/789	97%	Germany	Medium
Rhodotorula sp.	*Rhodotorula sp.*	637/641	99%	Germany	Good
Suillus sp.	*Suillus tomentosus*	715/732	97%	Canada	Medium
Tremellales sp.	*Tremellales sp.*	561/565	99%	USA	Good

* “Final species” means the final determined species when we blast in NCBI database, “Closest blast match” means the best match in NCBA database, “Origin of closest match” means which country the best match comes from.

Table 4 shows the frequencies of detection of species from direct DNA extractions in ash trees in New Zealand. Forty-six field samples, comprising 16 wood samples, 15 bark samples and 15 bud samples, were chosen to clone and 44 of these (96%) (16 wood samples, 13 bark samples and 15 bud samples) were successfully cloned. Seventy-eight fungal taxa were found by searching on the NCBI database. Of these 29 species were *Ascomycota*, and 15 species were *Basidiomycota*. Twenty-one (27%) were identified to species level, 23 (29%) were identified to genus level and 34 (46%) was still unidentified. The most frequently cloned species was *Fusarium oxysporum* (4.2%).

Table 4 Frequencies of detection of direct DNA extractions from F. excelsior, New Zealand

	Fungal taxon	Analysed tissues from		
		Bark	Wood	Bud
Ascomycota	*Aureobasidium pullulans*	-	-	2
	Bionectria ochroleuca	-	1	-
	Botryosphaeria parva	-	1	-
	Colletotrichum acutatum	-	-	1
	Fusarium lateritium	-	3	2
	Fusarium oxysporum	5	2	1
	Herpotrichia parasitica	1	1	-
	Neofabraea alba	2	-	-
	Neofusicoccum parvum	-	2	-
	Penicillium brevicompactum	-	1	-
	Penicillium canescens	-	-	1
	Penicillium spinulosum	-	1	-
	Phoma exigua	-	-	1
	Pilidium concavum	1	-	-
	Saccharomyces cerevisiae	1	-	-
	Venturia fraxini	-	1	-
	Ascomycete sp.	-	1	-
	Ascomycota sp.	5	4	4
	Cladosporium sp.	-	3	-
	Epicoccum sp.	1	2	2
	Fusarium sp.	5	3	1
	Herpotrichia sp.	1	-	-
	Kabatina sp.	-	-	1
	Penicillium sp.	-	3	1
	Phaeomoniella sp.	-	-	1
	Phialophora sp.	-	1	-
	Phoma sp.	-	-	3
	Phomopsis sp.	1	3	-
	Pleosporales sp.	3	1	1
Basidiomycota	*Bensingtonia yuccicola*	-	1	-
	Cryptococcus flavescens	-	2	4
	Exobasidium arescens	-	1	-
	Malassezia restricta	-	3	-
	Polyporus tuberaster	2	1	-
	Suillus granulatus	-	1	-
	Articulospora sp.	-	1	-
	Basidiomycota sp.	1	1	3
	Cryptococcus sp.	-	-	3
	Dioszegia sp.	-	-	1
	Exobasidium sp.	-	-	2
	Malassezia sp.	-	2	-
	Rhodotorula sp.	-	-	1

Fungal taxon	Analysed tissues from		
	Bark	Wood	Bud
Suillus sp.	2	1	1
Tremellales sp.	-	1	2
unidentified sp. 35-3	-	-	1
unidentified sp. 84.35	1	-	1
unidentified sp. 75-5	-	1	-
unidentified sp. 34	-	-	1
unidentified sp. 35	-	1	1
unidentified sp. 36	-	-	2
unidentified sp. 39	-	1	-
unidentified sp. 40	-	-	2
unidentified sp. 65-8	-	-	1
unidentified sp. 44	1	1	-
unidentified sp. 48-7	-	1	-
unidentified sp. 97-10	1	-	-
unidentified sp. 65	-	-	1
unidentified sp. 66	-	-	1
unidentified sp. 75-3	-	1	-
unidentified sp. 76	1	2	-
unidentified sp. 77	1	1	-
unidentified sp. 80	1	2	-
unidentified sp. 83-2	1	-	-
unidentified sp. 96	1	-	2
unidentified sp. 97	-	1	-
unidentified sp. 98	1	-	-
unidentified sp. 101	-	-	1
unidentified sp. 102	-	-	1
unidentified sp. 103	3	3	2
unidentified sp. 104	-	-	1
unidentified sp. 13-6	-	-	1
unidentified sp. 112	1	-	-
unidentified sp. 114	-	1	-
unidentified sp. 119	9	6	4
unidentified sp. 39-1	-	-	1
unidentified sp. 35-3	-	-	1
unidentified sp. 36-10	-	1	-
Total	54	72	64

Comparison between different sample types and methods

In all, 90 fungal taxa were identified in the community survey study, of which 35 species were recorded on bark, 43 species were recorded on wood and 46 species were recorded on bud. Eight species were detected from all three types of samples: *Polyporus tuberaster, Fusarium oxysporum, Phomopsis sp., Davidiella sp., Herpotrichia sp., Cryptococcus sp* and two unidentified species (Figure 5a).

Using the two different methods, 23 species were detected in fungal isolates, and 78 species of which were detected by direct DNA extractions. Eleven species were recorded by both methods (Figure 5b), they were: *Ascomycota sp., Penicillium sp., Phaeomoniella sp., Phomopsis sp., Pleosporales sp., Rhodotorula sp., Tremellales sp., Colletotrichum acutatum, Fusarium lateritium, Neofusicoccum parvum, Phoma exigua.*

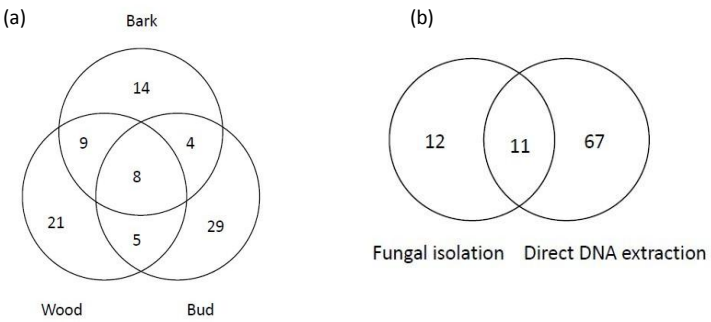

Figure 5. Comparison of number of fungal species recorded from (a) bark, wood, or bud; from (b) fungal isolation and direct DNA extraction. Figure (a) show the number of species recorded exclusively from one, two or all three sample types. Figure (b) show the number of species documented with either method or with both methods.

PCA of different sample types and trees

Species composition was analyzed by the principal component analysis (PCA) from a matrix of 90 taxa by 61 samples which were grouped as bark, wood and bud. Very little difference was observed in fungal community between sample types, but some of the bud samples distributed as the cluster together (Figure 6a) which indicated there were some similarity in fungal community structure between these clustered samples.

The arrows indicate the detected fungal species (Figure 6b). The species that drove most of the bud samples to cluster which were: *Penicillium brevicompactum* (s.33)*, Penicillium canescens* (s.29), *Suillus granulatus* (s.51), *Ascomycota sp.* (s.40). Some species influenced some samples to

approximately opposite direction such as: *Fusarium oxysporum* (s.23), *Bensingtonia yuccicola* (s.50), *Botryosphaeria parva* (s.36), *Phialophora sp.* (s.44), *Epicoccum sp.* (s.47). The longest arrow is the fungal species: *Polyporus tuberaster (*s.48).

Species composition was also analyzed by the principal component analysis (PCA) based on all samples which were grouped to different trees when they were collected. No clear patterns can be observed relating to host tree (Figure 6c) which means there was no specific relationship between the detected fungal communities and the different trees.

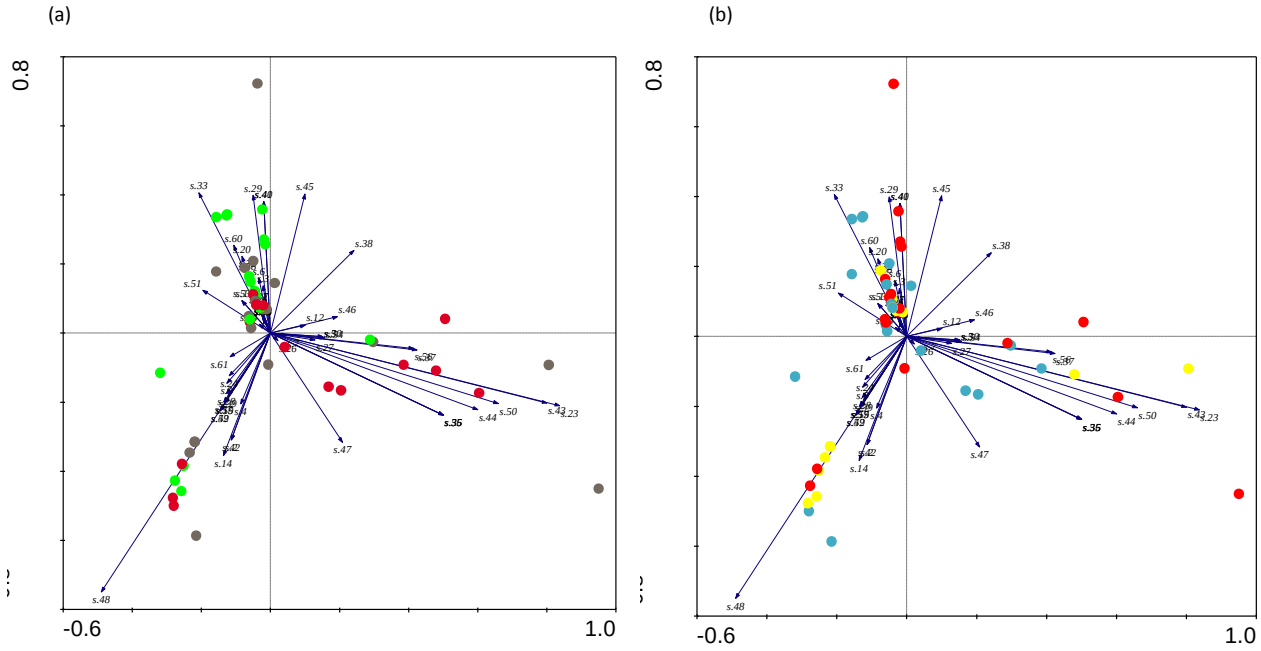

Figure 6. Ordination diagram based on PCA show how the species distribute in different factors. In (a) and (b), the arrows indicate different fungal species. In (a) the different color spots indicate different types of samples: bark is red, bud is green and wood is brown. In (b), the different color spots indicate the samples were collected from different trees. Appendix 1 is the list of species.

Meta-analysis

Compared with the study of Bakys *et al.* (2009b), the diversity of detected fungal taxa was high overall (Table 5). Ninety fungal taxa were detected in the New Zealand samples but 56 fungal taxa in the Swedish study. In our study, we detected 28 fungal taxa to species level and 27 fungal taxa to genus level. In Bakys' study, they detected 34 to species level and 22 to genus level. The number of unidentified species of New Zealand samples (35) was also higher than the number of unidentified species of Swedish samples (4).

Table 5 The comparison of identification results of different fungal taxa between Swedish and New Zealand samples

	Our research	Bakys *et al.* 2009
Identified to Species level	28 (31%)	34 (61%)
Identified to Genus level	27 (30%)	22 (39%)
Number of unidentified samples	35 (39%)	4 (7%)
Total fungal taxa	90 (100%)	56(100%)

Table 6 shows the comparison of identified fungal species between the study of Bakys *et al.* (2009b) and our study. Compared with Swedish study, seven species were found both in the Swedish ash study and the New Zealand ash study. A total of 21 species were only found in the New Zealand ash study.

Table 6 The comparison of identified fungal species between Swedish and New Zealand samples

The fungal taxa found in both studies	The fungal taxa only found in the New Zealand study
Aureobasidium pullulans	*Aspergillus versicolor*
Colletotrichum acutatum	*Bensingtonia yuccicola*
Epicoccum nigrum	*Bionectria ochroleuca*
Fusarium lateritium	***Botryosphaeria parva*** (teleomorph of *N. parvum*)
Neofabraea alba	*Cladosporium phaenocomae*
Phoma exigua	*Cryptococcus flavescens*
Venturia fraxini	*Exobasidium arescens*
	Fusarium oxysporum
	Herpotrichia parasitica
	Lophiostoma corticola
	Malassezia restricta
	Neofusicoccum parvum
	Penicillium brevicompactum
	Penicillium canescens
	Penicillium spinulosum
	Pilidium concavum
	Polyporus tuberaster
	Rhodosporidium babjevae
	Rhodotorula bacarum
	Saccharomyces cerevisiae
	Suillus granulatus

(**Bold**: Has been identified as a pathogen)

Discussion:

Species identification

In Table 5, the diversity of detected fungal taxa in New Zealand study was higher than Swedish study. The reason for the higher diversity of New Zealand samples could be:

1. In our study, we sampled from 60-70 years old trees; but in the Bakys *et al.* (2009b) study, they sampled from young seedlings. It is possible that older trees contain more fungal taxa than young seedlings. For example, in a study of species richness of ectomycorrhizal (EM) fungi in Douglas-fir, a greater richness for species or species groups was observed in old-growth tree stands (Smith *et al.*, 2002). de Jager *et al.* (2001) studied mango phylloplane and found community density and diversity increased gradually with leaf age. In a study of the diversity and abundance of fungi in trembling aspen different stand age, old aspen mixed wood stands had the highest species richness and diversity of woody substrates in all decay stages (Crites and Dale, 1998).

2. The sampling techniques were different. Bakys *et al.* (2009b) carried out field sampling in autumn. They collected by symptom categories: necrotic leaves, necrotic leaf stalks, necrotic shoot bark, small bark wounds, large bark wounds with necroses and necrotic bark from stem cankers. We collected field samples in winter when there were no leaves on the tree. We took samples which appeared healthy. The methods they used were more or less the same as ours, but they combined T-RFLP analysis with cloning to enable processing of a large set of samples. It is possible that using different sampling techniques and methods could lead to different results.

3. According to our study, there may be more species present in New Zealand trees. This is unexpected, given that the trees have been introduced into New Zealand. In contrast, Fisher *et al.* (1993) did a comparative study of fungal endophytes in leaves, xylem and bark of Eucalyptus in Australia and England. It is a similar case that Eucalyptus is an introduced tree species from Australia comparing with our study. The sample units were clearly separated according to their geographic origin, they found the incidence of fungal colonization was much higher in the Australian material. Dunstan *et al.* (1998) studied the diversity of ectomycorrhizal fungi on introduced pine in Australia and found the diversity of ECM fungi was very low compared with other Southern Hemisphere countries. Many studies showed

there were more fungal communities on trees in indigenous country compared with the countries where the tree was introduced. Therefore, we did not expect to find more fungal species in our study than the study of Bakys *et al.* (2009b).

In Table 5, there were a large number of species (35) that could not be identified even to genus level. The reasons for the high number of unidentified species could be:

1. The sequence match quality was poor which means the max identity was lower than 94% when we blasted, or species sequences are not currently available on NCBI. It is possible that there are not many fungal species sequenced in New Zealand, or that there have not been many studies of this host tree. There is also the possibility that some of our unidentified species are new species.

2. When we analyzed the sequences, there is more than one result of a different species that aligned closely with our sequence. The analysis made us concerned about the integrity of NCBI database. The sequences which people upload to NCBI may be of poor quality or poorly identified. The combination of molecular techniques and fungal isolates can reduce the risk when we identify species. For the same sample, both fungal isolation and direct DNA extraction should be compared as Figure 7 shows.

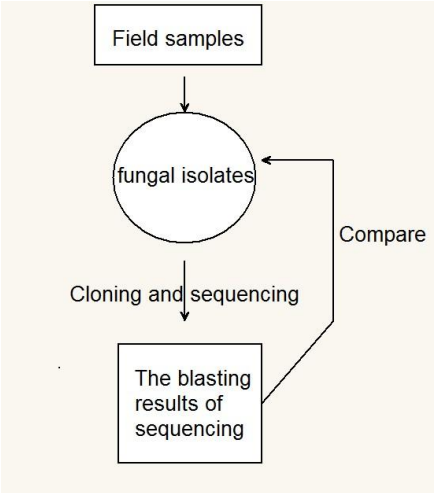

Figure 7. The flowchart of combining molecular techniques and fungal isolates, it used for confirming blasting results. The blasting result should be compared with fungal isolates to make sure of the correct identification.

Two fungal identification methods were compared – direct DNA extraction from plant tissue and fungal isolations. Overall there were 23 taxa detected (one unidentified sp. included) by fungal isolations and 78 fungal taxa (34 unidentified sp. included) were detected by direct DNA extractions. From these results it appears that direct DNA extraction can detect more fungal taxa in this experiment.

The reasons for this could be:

1. In culture, some fungi grow slowly or fungal competition reduces the chance to detect them.
2. In culture, contamination could reduce the chance of successful isolation.

However using isolations allows the chance to confirm sequence matches with cultural characteristics. So it is more useful when working in an area where fungi are not well characterized.

Meta-analysis of detected species

The fungi identified in Table 6 as being identified in both the Swedish and New Zealand studies could potentially have been introduced when the ash tree was introduced into New Zealand. They have also been found worldwide.

Colletotrichum acutatum was detected on all three types of tissues. The pathogen affects a wide range of crops and causes diseases on fruit trees. The symptoms are irregular leaf spot, bud rot, necrosis, and black spot on fruit. It can kill plants in the nurseries and also plants that have been transplanted to the field when the root necrosis and crown rot happen (Freeman, 2008). It is also possible that *C. acutatum* is a potential pathogen to ash tree. *Epicoccum nigrum* was detected in this survey. In Bakys' research, *E. nigrum* presented in shoots of all health categories (Vasiliauskas *et al.*, 2006); it may not cause disease on *F. excelsior*. *Fusarium lateritium* was detected on bud samples in this survey. It is one of the most common fungi on diseased ash trees (Kowalski and Czekaj, 2010). *Fusarium lateritium* was isolated from dead buds and the necrosis occurring on the main stem of European ash tree (Pukacki and Przybyl, 2005). *Neofabraea alba* was detected on bark samples in our survey. It was recorded as "Coin Canker of Ash" in Northeastern North America. The unusual cankers on bark surfaces were round and copper colored (Angeles *et al.*, 2006). It could be an important pathogen to New Zealand ash tree even though it was detected on the healthy looking ash. *Phoma exigua* (causing ash tree seedling canker) was detected on wood samples in our survey. It was reported in Belgian nurseries where it caused severe losses. Similar symptoms had been observed on seedlings of ash tree in France and Great Britain, but it never caused diseases there (Schmitz *et al.*, 2006). It is possible that *P. exigua* is a potential threat to New Zealand ash tree seedlings. *Venturia fraxini* was also detected on wood samples in this survey. It was reported as *Fraxinus* leaf blotch caused by *V. fraxini* In Italy (Anselmi, 2001) and it caused leaf necrosis of street trees in Argentina (Fresa, 1963). It is surprising to find *V. fraxini* in a wood sample.

There were a number of fungi that were only identified in the New Zealand study (Table 6). These

fungi could potentially have become associated with the ash tree after it was introduced to New Zealand. But it is also possible they are species found worldwide, or are species not found by Bakys *et al.* (2009b), but still native to ash.

Neofusicoccum parvum (*Botryosphaeriaceae*) was detected on ash wood samples. It is a species that is poorly understood in terms of its geographic distribution and host range, since the taxonomic history of the *Botryosphaeriaceae* is confused, the previous records are difficult to interpret. *Botryosphaeria parva* is the teleomorph of *N. parvum*. As an important canker pathogen of woody plants, this species can cause tip die-back, stem cankers, cracking of the bark, necrosis and tree mortality (Heath *et al.*, 2011). *Neofusicoccum parvum* has been reported on *Eucalyptus* spp., and has also been proved it is a pathogen to *Eucalyptus* spp. (Pavlic *et al.*, 2007). Eucalyptus is planted as a fast growing species in New Zealand. Ash could also be a host of *N. parvum,* there could be host-jumps following factitious introduction. Host-jump frequently associates to geographically changed hosts. Usually fungi jump from native trees to introduced trees (Slippers et al., 2005). Its teleomorph *Botryosphaeria parva* causes dieback and crown rot of blueberry in New Zealand (Sammonds *et al.*, 2009). It is also recorded as a grapevine trunk disease pathogen with a wide range of decline and dieback symptoms worldwide including New Zealand (Aroca *et al.*, 2006; Urbez-Torres *et al.*, 2006; van Niekerk *et al.*, 2006; Mundy and Manning, 2010). It is also recorded causing dieback on *Rhododendron* sp. (Bertetti *et al.*, 2011) and canker disease on cork oak (Linaldeddu *et al.*, 2007) in Italy. The pathogen may have been present in the tree in a latent phase showing no disease symptoms, and therefore the tree would have appeared in a healthy condition. *Fusarium oxysporum* was detected on all three types of ash tissues in our survey. It has been shown to be able to kill 2-month-old seedlings of *F. excelsior* in growth chamber conditions; it might be responsible for the decay of roots and be involved together with other factors (other pathogenic fungi, droughts, frost) in the dying process of European ash trees (Przyby, 2002). It was also found as a major root pathogen of cranberry in New Zealand (Miller *et al.*, 2006). *Polyporus tuberaster* is a white-rot fungus which can degrade lignin, it grows in Japan, Europe, and North America (Kawabe and Morita, 1994). It was found in our wood sample. *P. tuberaster* is also called “the Canadian Tuckahoe Indian bread stone fungus” (Pegler, 2000). It is recorded that *Pilidium concavum* can cause tan-brown rot in strawberry fruit (Lopes *et al.*, 2010; Debode *et al.*, 2011); the ash tree could also be a host for it although this seems surprising given that strawberries are not closely related to ash.

Bionectria ochroleuca is an endophyte (Promputtha *et al.*, 2005). *Penicillium* spp. is very common to find in plant fungal community survey, although their role is unclear. A study suggests the use of *Penicillium* sp. inoculants to increase the plant growth in alkaline soil (Wakelin *et al.*, 2007). *Cryptococcus flavescens, Bensingtonia yuccicola, Rhodosporidium babjevae, Rhodotorula bacarum, Saccharomyces cerevisiae* are yeasts. It was noticed that *Suillus granulates*, an ectomycorrhizal fungus (Dunstan *et al.*, 1998), also was detected in one of our wood samples. It

was not expected to find in healthy ash wood sample. There is also the possibility that the sample was contaminated.

Of the species discussed above, *Neofusicoccum parvum* could possibly have transferred from *Eucalyptus* to ash in New Zealand.

Critical analysis of our study:

The only recorded ash tree pathogen in New Zealand - *Hysterographium fraxini* was not detected in this survey. *Hysterographium fraxini* is regarded as a weak saprobic pathogen (Cannon, 1999). The sample collection location was in the north of New Zealand, but most of the records of *H. fraxini* are in the south of New Zealand. This also indicates the geographical limitations of our sample collection. A broad geographical sample collection is recommended for future studies. The number of ash trees surveyed was also small, and all trees sampled were described as being in a healthy condition, with no disease symptoms identified.

Because of the limitation of time, we only picked eight clones from each sample. More samples and more picking colonies could increase the number of species detected, it will carry out if we can do it again.

It helps for the identification if we check the fungal isolation of the same sample to confirm the sequence identification as Figure 7 suggests.

Chalara fraxinea was not detected in this survey; however we did not specifically look for this pathogen. *C. fraxinea* has spread very fast in Europe since the first observation in Poland and Lithuania, but it has not been reported to have spread to the southern hemisphere yet. The use of *C. fraxinea* species-specific primers (Johansson *et al.*, 2010) on all samples is planned for all New Zealand samples.

It was found to be difficult to extract DNA from wood samples; we did the DNA extraction twice for the wood samples when we found most of the wood samples had no PCR picture bands. It could be some chemical component may inhibit the PCR reaction. For example, many phenolic compounds of the lignin metabolism and many chemicals used for wood treatment are potential PCR inhibitors (Rachmayanti *et al.*, 2009). We suggest that a longer grinding time be used to make sure wood is grounded to fine powder. The amount of CTAB buffer should also be appropriately reduced to get higher concentration.

Conclusion:

We understand more about the fungal community of ash in New Zealand from this study by fungal isolations and direct DNA extraction methods. There were 90 fungal taxa detected in this survey. The diversity was high overall but there were also many unidentified species. Compared with a study of Swedish fungal communities in ash, our diversity of detected fungal taxa was higher in spite of that we only collected from healthy trees while the Swedish survey also included diseased samples. There are seven species that could be pathogenic species that could have came to New Zealand with the introduced ash tree, for example, *Colletotrichum acutatum, Phoma exigua, Neofabraea alba, Venturia fraxini, Fusarium lateritium, Pilidium concavum, Fusarium oxysporum*. *Chalara fraxinea* (the cause of ash dieback) was not detected in the New Zealand samples; however it was not specifically expected or looked for. We detected only one species which could possibly be said to have come from New Zealand. This was *Neofusicoccum parvum.*

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Appendix 1

Taxa for PCA from different sample types and trees	
species number	species name
S. 1	*Aspergillus versicolor*
S. 12	*Phaeosphaeria sp.*
S. 13	*Xylariaceae sp.*
S. 14	*Phomopsis sp.*
S. 15	*Phoma sp.*
S. 16	*Davidiella sp.*
S. 17	*Rhodosporidium babjevae*
S. 18	*Rhodotorula bacarum*
S. 19	*Microbotryomycetes sp.*
S. 2	*Neofusicoccum parvum*
S. 20	*Rhodotorula sp.*
S. 22	*Ceratobasidium sp.*
S. 23	*Fusarium oxysporum*
S. 24	*Herpotrichia parasitica*
S. 25	*Saccharomyces cerevisiae*
S. 26	*Pilidium concavum*
S. 27	*Neofabraea alba*
S. 29	*Penicillium canescens*
S. 3	*Cladosporium phaenocomae*
S. 30	*Phoma exigua*
S. 31	*Aureobasidium pullulans*
S. 32	*Bionectria ochroleuca*
S. 33	*Penicillium brevicompactum*
S. 34	*Penicillium spinulosum*
S. 35	*Venturia fraxini*
S. 36	*Botryosphaeria parva*
S. 37	*Fusarium sp.*
S. 38	*Penicillium sp.*
S. 40	*Ascomycota sp.*
S. 41	*Kabatina sp.*
S. 42	*Cladosporium sp.*
S. 43	*Ascomycete sp.*
S. 44	*Phialophora sp.*
S. 45	*Herpotrichia sp.*
S. 46	*Phaeomoniella sp.*
S. 47	*Epicoccum sp.*
S. 48	*Polyporus tuberaster*
S. 49	*Cryptococcus flavescens*
S. 5	*Lophiostoma corticola*

S. 50	*Bensingtonia yuccicola*
S. 51	*Suillus granulatus*
S. 52	*Malassezia restricta*
S. 53	*Exobasidium arescens*
S. 54	*Suillus sp.*
S. 55	*Basidiomycota sp.*
S. 56	*Tremellales sp.*
S. 57	*Dioszegia sp.*
S. 58	*Articulospora sp.*
S. 59	*Malassezia sp.*
S. 6	*Fusarium lateritium*
S. 60	*Exobasidium sp.*
S. 61	*Cryptococcus sp.*
S. 7	*Colletotrichum acutatum*
S. 8	*Epicoccum nigrum*
S. 9	*Pleosporales sp.*

*Unidentified species were not in the list