European *Fraxinus* species Introduced into New Zealand Retain Many of their Native Endophytic Fungi

MATTHEW W.P. POWER[1,2,3], ANNA J.M. HOPKINS*[1,4], JIE CHEN[1], STINA B.K. BENGTSSON[1], RIMVYS VASAITIS[1] AND MICHELLE R. CLEARY[5]

[1] Swedish University of Agricultural Sciences, Department of Forest Mycology and Plant Pathology, Almas allé 5, 75007 Uppsala, Sweden

[2] Scion (New Zealand Forest Research Institute), Private Bag 3020, Rotorua 3046, New Zealand

[3] Current Address: Department of Environment and Agriculture, Curtin University, Kent Street, Bentley, WA 6102, Australia

[4] Centre for Ecosystem Management, School of Science, Edith Cowan University, 270 Joondalup Drive, Joondalup, WA 6027, Australia

[5] Swedish University of Agricultural Sciences, Southern Swedish Forest Research Centre, Sundsvägen 3, 23053 Alnarp, Sweden.

**Corresponding author: a.hopkins@ecu.edu.au, tel. +61 8 6304 5718*

Power, M.W.P., Hopkins, A.J.M., Chen, J., Bengtsson, S.B.K., Vasaitis, R. and Cleary, M.R. 2017. European *Fraxinus* species Introduced into New Zealand Retain Many of their Native Endophytic Fungi. *Baltic Forestry* 23(1): 74-81.

Abstract

Fraxinus species were introduced in to New Zealand as amenity trees as early as the mid-1850s. As a likely consequence of this early introduction and of their geographic isolation, *Fraxinus* species in New Zealand have not yet been subjected to the devastating impacts of ash decline caused by *Hymenoscyphus fraxinus*. This study used isolations, PCR and cloning methods to examine the endophytic fungi associated with *Fraxinus excelsior* and *F. angustifolia* on the north island of New Zealand.

Keywords: *Fraxinus excelsior*, *Fraxinus angustifolia*, endophytic communities

Introduction

Endophytic fungi, that is those species growing within plants for all or at least a part of their life cycle without causing any obvious symptoms, are a critical component of the plant ecosystem. Endophytes are known to confer benefits to their host, for example, by increasing host tolerance to stress (Rodriguez and Redman 2007), reducing herbivory through the production of toxic alkaloids (Wilkinson et al. 2000), and via antagonistic effects that reduce infection of plant tissues by pathogens (Arnold et al. 2003). International trade and transportation of tree species for use in forestry, horticulture and as amenity trees in parks and landscapes has led to the potential redistribution of endophytes as cryptic hitchhikers within their inhabited host which may lead to new possibilities for endophyte-host interactions. In theory, a plant introduced into a new environment will either remain colonised by its associated fungi from within its native range (the cointroduction hypothesis) or become colonized by fungi from its new environment

(the host-jumping hypothesis) (Shipunov et al. 2008), or both. Although the ecological implications of either of these events are not well known, the plasticity of endophytic interactions could promote parasitism if hosts are physiologically stressed and/or newly acquired fungi could increase host competitiveness (Brown and MacAskill 2005). In extreme cases, fungal assemblages associated with introduced species have caused extensive damage to native tree species following successful host jumps, e.g. *Neonectria fuckeliana* introduced to New Zealand affecting *Pinus radiata* (Dick and Crane 2009) and *Hymenoscyphus fraxineus* introduced to Europe affecting native *Fraxinus* species (Kowalski 2006; Gross et al. 2014). Thus, the endophyte communities of introduced forest species are of fundamental importance to forest ecosystem health.

Since the early-mid 1990s European ash (*Fraxinus excelsior*) has experienced widespread population decline across Europe (Juodvalkis and Vasiliauskas 2002, Timmerman 2011). The causal agent of this decline has been identified as *Hymenoscyphus fraxineus* (Baral et al. 2014); syn. *H. pseudoalbidus*; anamorph *Chalara fraxinea* T. Kowalski (Kowalski 2006, Queloz *et al*. 2010). Typical symptoms include small necrotic spots on leaves, necrotic lesions on rachises and bark, discoloration of wood, and eventual dieback of twigs, branches and crown (Cleary et al. 2013; Bakys et al. 2009a,b). Trees of all age classes are affected. *Hymenoscyphus fraxineus* in Europe causes symptoms on native *Fraxinus*, namely *F. excelsior*, *F. angustifolia,* and *F. ornus* (Kirisits and Schwanda 2015, Kirisits et al. 2010), but also North American *Fraxinus* species have been affected to some degree (Drenkhan and Hanso 2010; McKinney et al. 2014). Asian *Fraxinus* species planted in Europe and in their native origin of East Asia exhibit only minor or no dieback damage to the crown of trees (Drenkhan and Hanso 2010; McKinney et al. 2014; Cleary et al. 2016).

Fraxinus excelsior and *Fraxinus angustifolia* were introduced to New Zealand by European colonists in the mid-1800s. Since then, both species have established naturally, are commonly found throughout the country, and frequently planted as amenity trees (Allan Herbarium, 2000). No ash species are native to New Zealand. *Hymenoscyphus fraxineus* has not been identified on either *F. excelsior* or *F. angustifolia* in New Zealand, and neither has its non-pathogenic European relative *H. albidus*. The geographic separation of *F. excelsior* and *F. angustifolia* from its native range in Europe provides a unique opportunity to examine endophytic fungal communities in an introduced environment and compare with those documented within its native range to decipher the degree at which communities are influenced by their native or novel associates following establishment. The objective of this study was to: 1) describe the fungal communities associated with *F. excelsior* and *F. angustifolia* in New Zealand and 2) compare community structure to that documented on the same host species in other European countries.

Methods

Study sites and sampling

Field samples were collected from *F. excelsior* and *F. angustifolia* trees at two urban locations in Rotorua, New Zealand (-38.160S, 176.263E; -38.085S, 176.216E). *Fraxinus excelsior* samples were taken from three trees approximately 60-70-years-old that appeared to be healthy. From each tree, three branches were sampled from the lower part of the crown. Samples were collected in August 2009 (during winter) from three different tissue types: bud, bark and wood. *Fraxinus angustifolia* samples were collected in November 2009 (during spring) from rachises of three healthy 20-year-old trees. Samples from all three trees were pooled.

Isolation of fungi in pure culture

Fungal isolations from *F. excelsior* samples were made from all three tissue types (buds, bark and wood). Tissue was surface sterilised with 70% EtOH for 1 min., 3% NaClO for 5 min, repeated 70% EtOH for 1 min, followed by ddH_2O for 1 min, and small sections of tissue (3 x 3 mm) were plated onto 1% malt-extract and incubated at 20°C. Fungal outgrowth was observed for up to 8 weeks thereafter and subcultures of all filamentous fungi was performed to obtain clean isolates. Mycelial tissue was harvested from a select number of representative plates and stored at -20℃ in preparation for DNA extraction. No isolations were performed on *F. angustifolia* samples.

Isolation of DNA, amplification and sequencing

DNA was extracted from both the mycelia of pure cultures and directly from tissue of *F. excelsior* samples. For *F. angustifolia* samples, DNA was extracted directly from tissues. All samples were freeze dried and ground to powder with a Precellys® 24 tissue homogenizer (Bertin Technologies). DNA was extracted using 3% CTAB method described in Cleary et al. (2013), and then purified using the JET quick kit (GENOMED GmbH). All DNA samples were quantified using the NanoDrop (NanoDrop Technologies) and diluted to 1 ng/µL.

PCR amplification of all DNA mycelial samples obtained from pure cultures was performed using primers ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990). The thermal cycling condition was initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30s, annealing at 55°C for 30s, and 72°C for 30s, followed by a final extension at 72°C for 7 min.

Amplifications were performed using the Veriti Thermal Cycler (Applied Biosystems) in 40 µL reactions

containing the following final concentrations: 1 ng/μL template DNA, 0.03 U Dream Taq Polymerase (Fermentas), 200 μM of dNTPs, 2.75 mM of $MgCl_2$, and 0.2 μM of each primer in 1x Buffer. PCR products were visualised on 1% Agarose gels using Gel Green dye (Biotium).

For tissue samples, DNA extraction and PCR was performed similarly to that described above. PCR products were then cloned using the TOPO TA Cloning kit with pCR®2.1-TOPO vector and One Shot TOP10 chemically competent *E. coli* (Invitrogen). Only those samples exhibiting clear bands on the gel were selected for cloning. Bacterial colonies were added directly to a PCR cocktail as described above, but with primers M13 Forward (GTAAAACGACGGCCAG) and M13 Reverse (CAGGAAACAGCTATGAC) (Griffin and Griffin 1993).

All PCR products were purified using AMPure (Agencourt) and sent to Macrogen (Seoul, Korea) for Sanger sequencing on the ABI3730XL in both forward and reverse directions. Sequences were manually aligned and edited using Seqman (DNASTAR Lasergene 8) and Geneious version 5.4.6 (Biomatters Ltd.), respectively. Fungal taxa were determined by comparing sequences with reference databases using BLASTN at GenBank (NCBI), also considering geographic origin of the closest BLAST-sequence match. The ITS sequence homology for delimiting fungal taxa was set at between 98 and 100% for species level, and between 94 – 97% for genus level (Glen et al. 2001; Bakys et al. 2009). Principal component analysis (PCA) was performed on all samples using a presence/absence matrix with Canoco version 4.5 (Plant Research International).

Results

Fungal isolates from F. excelsior:

A total of 36 twig samples were collected from branches of *F. excelsior* trees which yielded 30 bud samples, 34 bark samples and 35 wood samples. Sixty-eight isolates were obtained from these samples, but only 36 isolates were used for sequence identification (the remaining 32 were omitted from further analysis due to unforeseen contamination). Of those 36 samples, 19 originated from bud samples, 15 from bark and two from wood. In total, 23 fungal taxa were detected; ten of which were identified to the species level based on sequence similarities with GenBank entries, 12 were identified to the genus level and one remained unidentified. The fungal taxa belonged to eight orders of Ascomycetes: Eurotiales, Capnodiales, Glomerellales, Hypocreales, Pleosporales, Botryosphaeriales, Diaporthales, Xylariales and an unknown Ascomycete, and to four orders of Basidiomycetes: Ustilaginales, Sporidiales, Cantharellales, Tremellales, and an unknown Microbotryomycetes (Table 1). The most frequently detected fungal taxa were *Phoma* sp. (18%), but this only occurred in the bud tissue.

Tissue samples from F. excelsior

PCR products from 15 of 30 bud samples, 15 of 34 bark samples, and 16 of 35 wood samples were selected for cloning, and was successful on all with the exception of two bark samples. Seventy-eight fungal taxa were identified of which 21 were identified to species level, 23 were identified to genus level, and 34 remained unidentified (Table 2). These fungal taxa represented by 11 orders of Ascomycetes: Helotiales, Dothideales, Hypocreales, Botryosphaeriales, Capnodiales, Glomerellales, Pleosporales, Eurotiales, Chaetothyriales, Diaporthales, Saccharomycetales, and two unknown ascomycetes, and seven orders of Basidiomycetes: Agaricostilbales, Tremellales, Exobasidiales, Malasseziales, Polyporales, Sporidales, Boletales, and one unknown basidiomycete.

Tissue samples of F. angustifolia

Twenty fungal taxa were identified from rachises of *F. angustifolia*, of which eight were identified to species level, eight to a genus level and four remained unidentified. The fungal taxa represented three orders of Ascomycetes: Dothideales, Diaporthales, Pleosporales, and three orders of Basidiomycetes: Tremellales, Erythrobasidiales, Sporidales, and four unknowns.

Comparison of community composition

To look for similarities in *Fraxinus* fungal communities between New Zealand and Europe, the identified fungal taxa on *F. excelsior* and *F. angustifolia* were compared to the published literature of Przybyl (2002a,b) Lygis et al. (2006), Bakys et al (2009a,b), Davydenko et al. (2013), Scholtysik et al. (2013), Hauptman et al. (2013), and Kowalski and Czekaj (2010), all which report associated fungal taxa to either diseased or healthy *F. excelsior* trees. Of the fungal taxa detected in New Zealand *Fraxinus* samples, several taxa were similar to that reported in *F. excelsior* in Europe (Tables 1-3). At the species level, familiar fungi included *Aureobasidium pullulans*, *Colletotrichum acutatum*, *Epicoccum nigrum*, *Fusarium lateritium*, *F. oxysporum*, *Neofabraea alba*, *Penicillium canescens*, *Phoma exigua*, *P. exigua* var. *exigua*, and *Venturia fraxini*. Of all the fungal taxa detected in New Zealand *Fraxinus* samples, only one species was uniquely associated to *F. angustifolia*: *Erythrobasidium hasegawianum*. It is not known how these communities may compare to those located on native New Zealand trees, though some evidence suggest that *Epicoccum nigrum*, *Phoma* sp., and *Pleosparaceae* sp. may be more cosmopolitan in nature (Ganley 2008; Johnston et al. 2012).

Table 1. Identification of fungal isolates from *F. excelsior* samples in New Zealand

Putative fungal taxon	Closest BLAST match	Number of base-pairs matched	% Match	Origin of closest match	Tissue type	Presence on *Fraxinus* in Europe[a]
Aspergillus versicolor	*Aspergillus versicolor*	567/570	99%	Lithuania	Bud	
Ascomycota sp.	*Ascomycota sp.*	505/511	98%	China	Bark	
Ceratobasidium sp.	*Ceratobasidium sp.*	539/541	99%	Sweden	Bark	
Cladosporium phaenocomae	*Cladosporium phaenocomae*	566/566	100%	Netherlands	Bud	
Colletotrichum acutatum	*Colletotrichum acutatum*	583/583	100%	Germany	Bud	+
Davidiella sp.	*Davidiella sp.*	559/559	100%	Czech Republic	Bark	
Epicoccum nigrum	*Epicoccum nigrum*	549/549	100%	Czech Republic	Bud	+
Fusarium lateritium	*Fusarium lateritium*	567/567	100%	Germany	Bud	+
Lophiostoma corticola	*Lophiostoma corticola*	553/553	100%	New Zealand	Bark , Bud	+
Microbotryomycetes sp.	*Microbotryomycetes sp.*	655/692	94%	USA	Bark	
Neofusicoccum parvum	*Neofusicoccum parvum*	589/589	100%	Sweden	Wood	
Penicillium sp.	*Penicillium sp.*	604/604	100%	China	Bark, Bud, Wood	+
Phaeosphaeria sp.	*Phaeosphaeria sp.*	432/439	98%	New Zealand	Bud	
Phoma exigua	*Phoma exigua*	542/542	100%	Germany	Bud	+
Phoma sp.	*Phoma sp.*	540/542	99%	USA	Bud	+
Phomopsis sp.	*Phomopsis sp.*	585/589	99%	USA	Bark	+
Pleosporales sp.	*Pleosporales sp.*	497/519	95%	USA	Bark, Bud	
Rhodosporidium babjevae	*Rhodosporidium babjevae*	594/594	100%	USA	Bark	
Rhodotorula bacarum	*Rhodotorula bacarum*	656/667	98%	Japan	Bark	
Rhodotorula sp.	*Rhodotorula sp.*	575/581	98%	Finland	Bark, Bud	
Tremellales sp.	*Tremellales sp.*	511/515	99%	USA	Bark	
Xylariaceae sp.	*Xylariaceae sp.*	525/545	96%	USA	Bud	+

[a] compared to that reported by Przybyl (2002a,b), Lygis et al. (2006), Bakys et al. 2009a,b; Davydenko et al. 2013; Scholtysik et al. 2013; Hauptman et al. 2013; Kowalski and Czekaj 2010. '**+**' indicates similar presence of the fungal organism on both *Fraxinus* species in New Zealand and Europe.

Principal Component Analysis of fungal community for trees and different tissue types

The fungal species community composition from *F. excelsior* samples was analysed by principal component analysis (PCA) and grouped by tissue type (bark, wood and bud). There was no clear delineation of fungal community among sampled trees and no differentiation in fungal community structure between the different tissue types sampled (Figure 1).

Discussion

Ninety fungal taxa were detected in this study; an extremely high richness of fungi given the small number of samples taken from just six ash trees in New Zealand. Of these, it was possible to give species or generic identities to more than half. Species able to be identified included those previously known to be endophytes (e.g. *Bionectria ochroleuca*; Promputtha et al. 2005), ubiquitous filamentous fungi such as *Penicillium* spp. as well as yeasts such as *Cryptococcus favescens*, *Rhodotorula bacarum* and *Saccharomyces cerevisiae* already known to inhabit plants.

The well-known pathogen of ash in Europe, *Hymenoscyphus fraxinus* and its non-pathogenic relative *Hymenoscyphus albidus* were not found in this study. *Hymenoscyphus fraxinus* has spread very fast since it was first recorded in Poland and Lithuania (Juodvalkis and Vasiliauskas, 2002, Przybył 2002a), but it has not yet been recorded as an introduced, invasive species outside of Europe. Though other *Hymenoscyphus* species can be found naturally associated with native *Nothofagus* species in New Zealand (Johnston et al. 2012), this study provides no evidence of any *Hymenoscyphus* species associated with exotic *Fraxinus* in New Zealand although the use of species-specific primers (e.g. Johansson et al. 2010) and more extensive sampling of ash from a wider geographic distribution would be more conclusive. The only pathogen of ash previously recorded in New Zealand, *Hysterographium fraxini*, was not found (Cannon 1999). This is not unexpected as *H. fraxini* is primarily found on the south island of New Zealand (http://www.nzffa.org.nz/farm-forestry-model/the-essentials/forest-health-pests-and-diseases/diseases/Hysterographium-fraxini), not the north where the samples were taken for the present study.

Several of the fungi found in healthy ash tissue in this study are known and are recognised as pathogens of ash in Europe and North America. These include *Neofabraea alba*, *Fusarium lateritium*, *Phoma exigua* and

Table 2. Identification of fungi directly from *F. excelsior* tissue samples in New Zealand via direct DNA extraction and PCR cloning

Putative fungal taxon	Closest BLAST match	Number of base-pairs matched	% Match	Origin of closest match	Tissue type	Presence on *Fraxinus* in Europe[a]
Articulospora sp.	*Articulospora proliferate*	512/542	94%	Canada	Bud	
Ascomycete sp.	*Ascomycete sp.*	606/608	99%	Brazil	Wood	
Ascomycota sp.	*Ascomycota sp.*	530/545	98%	China	Bark, Bud, Wood	
Aureobasidium pullulans	*Aureobasidium pullulans*	616/616	100%	Spain	Bud	
Basidiomycota sp.	*Basidiomycota sp.*	617/639	97%	Czech Republic	Bark, Bud, Wood	
Bensingtonia yuccicola	*Bensingtonia yuccicola*	679/683	99%	USA	Wood	
Bionectria ochroleuca	*Bionectria ochroleuca*	608/608	100%	Czech Republic	Wood	
Botryosphaeria parva	*Botryosphaeria parva*	594/597	99%	Brazil	Wood	
Cladosporium sp.	*Cladosporium sp.*	591/591	100%	China	Wood	
Colletotrichum acutatum	*Colletotrichum acutatum*	618/620	99%	Germany	Bud	+
Cryptococcus flavescens	*Cryptococcus flavescens*	568/568	100%	Austria	Bud, Wood	
Cryptococcus sp.	*Cryptococcus sp.*	523/524	98%	USA	Bud	+
Dioszegia sp.	*Dioszegia sp.*	467/479	97%	USA	Bud	
Epicoccum sp.	*Epicoccum sp.*	566/583	97%	USA	Bark, Bud, Wood	+
Exobasidium arescens	*Exobasidium arescens*	605/609	99%	Germany	Wood	
Exobasidium sp.	*Exobasidium rhododendri*	591/615	96%	England	Bud	
Fusarium lateritium	*Fusarium lateritium*	595/600	99%	Germany	Bud, Wood	+
Fusarium oxysporum	*Fusarium oxysporum*	580/582	99%	Czech Republic	Bark, Bud, Wood	+
Fusarium sp.	*Fusarium sp.*	590/595	99%	Czech Republic	Bark, Bud, Wood	
Herpotrichia parasitica	*Herpotrichia parasitica*	515/532	98%	USA	Wood	
Herpotrichia sp.	*Herpotrichia parasitica*	515/532	96%	USA	Bark	
Kabatina sp.	*Kabatina thujae*	595/631	94%	USA	Bud	
Malassezia restricta	*Malassezia restricta*	767/773	99%	Belgium	Wood	
Malassezia sp.	*Malassezia globosa*	767/789	97%	Germany	Wood	
Neofabraea alba	*Neofabraea alba*	578/582	99%	Netherlands	Bark	+
Neofusicoccum parvum	*Neofusicoccum parvum*	596/597	99%	Sweden	Wood	
Penicillium brevicompactum	*Penicillium brevicompactum*	619/619	100%	Japan	Wood	
Penicillium canescens	*Penicillium canescens*	622/622	100%	Brazil	Bud	
Penicillium sp.	*Penicillium sp.*	620/621	99%	China	Bud, Wood	+
Penicillium spinulosum	*Penicillium spinulosum*	616/616	100%	Czech Republic	Wood	
Phaeomoniella sp.	*Phaeomoniella sp.*	513/520	98%	Korea	Bud	
Phialophora sp.	*Phialophora europaea*	580/613	95%	Switzerland	Wood	+
Phoma exigua	*Phoma exigua*	576/579	99%	Germany	Bud	+
Phoma sp.	*Phoma sp.*	577/579	99%	USA	Bud	+
Phomopsis sp.	*Phomopsis sp.*	592/599	98%	USA	Bark, Wood	+
Pilidium concavum	*Pilidium concavum*	485/486	99%	USA	Bark	
Pleosporales sp.	*Pleosporales sp.*	682/689	98%	China	Bark, Bud, Wood	
Polyporus tuberaster	*Polyporus tuberaster*	647/647	100%	USA	Bark, Bud, Wood	
Rhodotorula sp.	*Rhodotorula sp.*	637/641	99%	Germany	Bud	
Saccharomyces cerevisiae	*Saccharomyces cerevisiae*	877/891	98%	France	Bark	
Suillus granulatus	*Suillus granulatus*	706/715	98%	China	Wood	
Suillus sp.	*Suillus tomentosus*	715/732	97%	Canada	Bud, Wood	
Tremellales sp.	*Tremellales sp.*	561/565	99%	USA	Bud, Wood	
Venturia fraxini	*Venturia fraxini*	585/586	99%	Netherlands	Wood	+

[a] compared to that reported by Przybyl (2002a,b), Lygis et al. (2006), Bakys et al. 2009a,b; Davydenko et al. 2013; Scholtysik et al. 2013; Hauptman et al. 2013; Kowalski and Czekaj 2010. '**+**' indicates similar presence of the fungal organism on both *Fraxinus* species in New Zealand and Europe

Table 3. Identification of fungi from *F. angustifolia* tissue samples in New Zealand via direct DNA extraction and PCR cloning, and comparative presence of similar type taxa to *F. excelsior* in Europe

Fungal taxon	Closest BLAST match	% Match	Number of base-pairs matched	Origin of closest match	Presence on *Fraxinus* in Europe[a]
Ascomycota sp.	Ascomycota sp.	97	525/539	USA	
Aureobasidium pullulans	*Aureobasidium pullulans*	100	619/619	Spain	+
Basidiomycete	Uncultured basidiomycete yeast	98	641/653	Germany	
Cryptococcus sp.	Uncultured Cryptococcus clone	99	554/557	Sweden	+
Cryptococcus sp.	Uncultured Cryptococcus clone	99	526/527	Sweden	+
Cryptococcus sp.	*Cryptococcus sp.*	99	521/524	USA	+
Diaporthe eres	*Diaporthe eres*	98	596/608	Lithuania	
Dioszegia sp.	*Dioszegia sp.*	98	470/479	USA	
Dioszegia takashimae	*Dioszegia takashimae*	99	534/542	Germany	
Epicoccum nigrum	*Epicoccum nigrum*	99	578/580	Sweden	+
Erythrobasidium hasegawianum	*Erythrobasidium hasegawianum*	98	605/617	Germany	
Lewia infectoria	*Lewia infectoria*	99	633/637	United Kingdom	
Phaeomoniella sp.	*Phaeomoniella capensis*	89	512/574	New Zealand	
Phaeosphaeria sp.	*Phaeosphaeria sp.*	97	539/639	Spain	+
Phaeosphaeria sp.	*Phaeosphaeria sp.*	96	556/582	USA	+
Phoma exigua var. exigua	*Phoma exigua var. exigua*	99	573/576	Germany	+
Phoma sp.	*Phoma sp.*	99	573/575	Brazil	
Rhodotorula sp.	*Rhodotorula aurantiaca*	100	415/425	USA	
Rhodotorula sp.	Uncultured Rhodotorula clone	98	409/418	Austria	
Tremellomycetes sp.	Uncultured Tremellomycetes	99	558/561	Germany	
Trichosporon sp.	*Trichosporon laibachii*	99	530/531	China	
Unidentified	Uncultured soil fungus clone	95	605/638	USA	
Unidentified	Uncultured fungus clone	99	586/589	USA	
Unidentified	*Sporobolomyces syzygii*	89	571/641	Japan	

[a] compared to that reported by Przybyl (2002a,b), Lygis et al. (2006), Bakys et al. 2009a,b; Davydenko et al. 2013; Scholtysik et al. 2013; Hauptman et al. 2013; Kowalski and Czekaj 2010. '**+**' indicates similar presence of the fungal organism on both *Fraxinus* species in New Zealand and Europe

Venturia fraxini. *Neofabraea alba* was detected in healthy bark samples in the present study and is known to cause Coin Canker of Ash in Northeastern North America (Angeles et al. 2006). *Fusarium lateritium* is one of the most common fungi on diseased ash (Kowalski and Czekaj 2010), often isolated from dead buds and necrotic stems in European ash (Pukacki and Przybyl 2005). *Fusarium lateritium* was obtained from bud samples in the present study. *Phoma exigua* is the causal agent of ash seedling canker, a disease reported from nurseries in Belgium where it caused severe losses (Schmitz et al. 2006). This fungus has also been reported from ash seedlings in France and Great Britain causing little or no symptoms (Schmitz et al. 2006). *Venturia fraxini*, the causal agent of Fraxinus leaf blotch (Anselmi 2001) was detect from wood samples in the present study. Several other, more generalist pathogens, previously identified as causing disease in other woody plants or shrubs were also found in the present study. These include *Colletotriochum actuatum,* which causes disease on a range of crops and fruit trees (Freeman 2008), The presence of all of these pathogens in healthy plant tissue is not unexpected; pathogens, especially those causing disease in woody tissue, often exhibit a latent endophytic phase which reverts to a pathogenic state when environmental conditions become suitable (e.g. Brown and MacAskill 2005; Crane et al. 2009).

Of the 90 species detected in this study, almost one third had been previously reported from ash species in Europe. Many of these are fungi with a worldwide distribution such as *Fusarium lateritium*, *Fusarium oxysporum* and *Phoma exigua,* however several are known predominantly from Europe and have not previously been recorded in New Zealand. It is not possible to determine from this study whether the specific strains observed arrived in New Zealand with the introduction of ash or colonised ash once it was established in New Zealand. This is also the case for the majority of the other species observed only on ash in this study (and not previously known from European studies), as these species are generally widely distributed throughout the world.

The presence of several common European taxa in European *Fraxinus* established in New Zealand lends support to the cointroduction hypothesis, that is, that these species were cryptic hitchhikers on ash plants when they

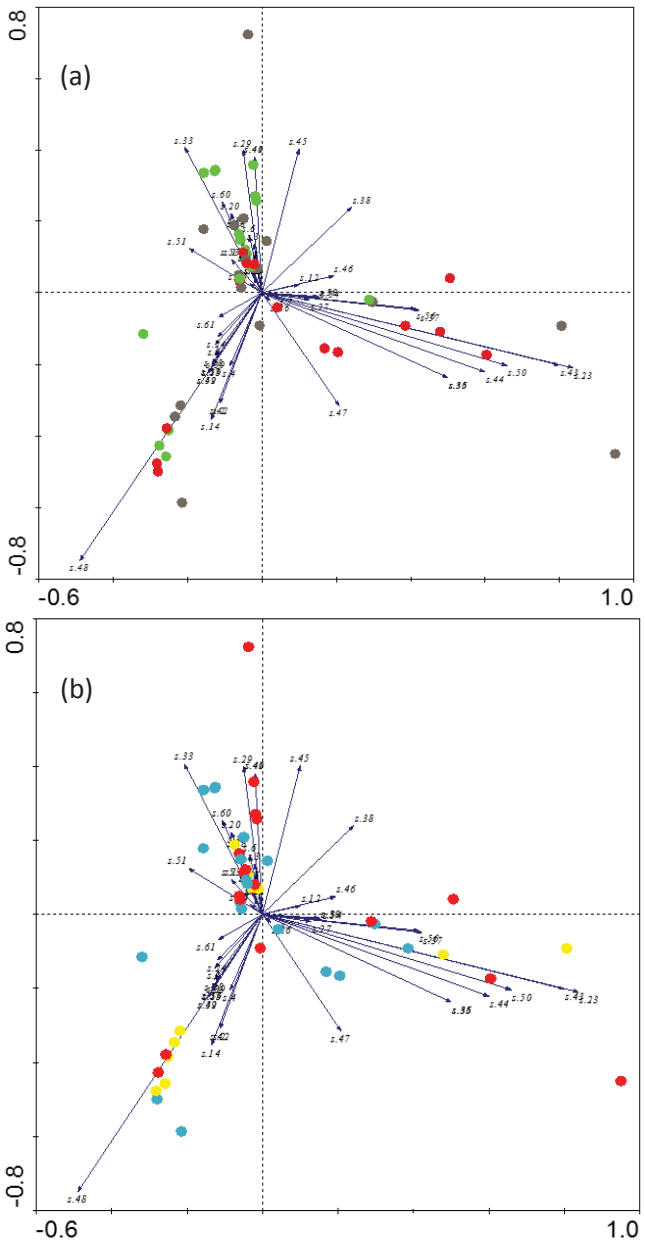

Figure 1. PCA ordination of *Fraxinus excelsior* fungal community. Arrows denote fungal species driving the interactions. Coloured dots denote (a) samples from buds (green), bark (red) and wood (grey), and (b) different trees

were first introduced. In contrast, few fungal species were identified that could be definitively described as being exclusively from New Zealand (and thus also in support of the host-jumping hypothesis) (Shipunov et al. 2008). The high number of known pathogens associated with ash in this study, many of which could have been cointroduced, highlights the importance of the live plant pathway for the movement of potential pathogens around the world.

Acknowledgements

The authors wish to acknowledge Scion and the following individuals for their assistance in various aspects of this study: Margaret Dick (Scion), Katarina Ihrmark (SLU), Rena Gadjieva (SLU), and Hanna Millberg (SLU). Funding was provided by the Swedish Foundation for International Cooperation in Research and Higher Education (STINT) and Carl Tryggers Stiftelse för Vetenskaplig Forskning.

References

Allan Herbarium 2000. Ngā Tipu o Aotearoa - New Zealand Plant Names Database. Landcare Research, New Zealand. Available http://nzflora.landcareresearch.co.nz/ (Accessed 2 February 2012).

Angeles, G., Adams, G.C., and Putnam, M.L. 2006. Effect of Neofabraea alba on bark and wood anatomy of *Fraxinus* spp. *International Association of Wood Anatomists Journal* 27: 409-418.

Anselmi, N. 2001. Main diseases of high quality timber broadleaves [Principali malattie degli impianti industriali di latifoglie di pregio. Annali]. Accademia Italiana di Scienze Forestali, pp. 79-99 (in Italian)

Arnold, A.E., Mejia, L.C., Kyllo, D., Rojas, E.I., Maynard, Z., Robbins, N., and Herre E.A. 2003. Fungal endophytes limit pathogen damage in a tropical tree. Proceedings of the National Academy of Sciences of the United States of America, 100: 15649-15654.

Bakys, R., Vasaitis, R., Barklund, P., Ihrmark, K., and Stenlid, J. 2009a. Investigations concerning the role of *Chalara fraxinea* in declining *Fraxinus excelsior*. *Plant Pathology* 58: 284-292.

Bakys, R., Vasaitis, R., Barklund, P., Thomsen, I.M., and Stenlid J. 2009b. Occurrence and pathogenicity of fungi in necrotic and non-symptomatic shoots of declining common ash (*Fraxinus excelsior*) in Sweden. *European Journal of Forest Research* 128: 51-60.

Baral, H.O., Queloz, V., and Hosoya T. 2014. *Hymenoscyphus fraxineus,* the correct scientific name for the fungus causing ash dieback in Europe. *IMA Fungus* 5: 79-80.

Brown, A. V., and Macaskill, G. 2005. Shoot diseases of pine. Forest Research Information Note 69. Forestry Commission, Edinburgh.

Cannon P. F. 1999. *Hysterographium fraxini* By: Source: IMI Descriptions of Fungi and Bacteria 141: 1401-1410.

Cleary, M.R., Daniel, G., and Stenlid, J. 2013 Light and scanning electron microscopy studies of the early infection stages of *Hymenoscyphus pseudoalbidus* on *Fraxinus excelsior*. *Plant Pathology* 62: 1294-1301.

Cleary, M., Nguyen, D., Marčiulynienė, D., Berlin, A., Vasaitis, R., and Stenlid J. 2016. Friend or foe? Biological and ecological traits of the European ash dieback pathogen Hymenoscyphus fraxineus in its native environment. *Scientific Reports* 6: 21895

Crane, P., Hopkins, A.J.M. Dick, M.A, and Bulman, L.S. 2009. Behaviour of *Neonectria fuckeliana* causing a pine canker disease in New Zealand. *Canadian Journal of Forest Research* 39: 2119-2128.

Dick, M.A. and Crane, P.E. 2009. *Neonectria fuckeliana* is pathogenic to *Pinus radiata* in New Zealand. *Australasian Plant Disease Notes* 4: 12–14.

Drenkhan, R, and Hanso, M. 2010. New host species for *Chalara fraxinea*. New Disease Reports 22: 16. [doi: 10.5197/j.2044-0588.2010.022.016].

Freeman, S. 2008. Management, survival strategies, and host range of *Colletotrichum acutatum* on strawberry. *Hortscience* 43: 66-68.

Ganley R. 2008. Density and diversity of fungal endophytes isolated from needles of *Pinus radiata*. SCION. Forest Biosecurity Research Council. Client Report No. 12925. 12 pp.

Griffith, G.S., and Boddy, L. 1988. Fungal communities in attached ash (*Fraxinus excelsior*) twigs. *Transactions of the British Mycological Society* 91: 599-606

Griffith, G.S., and Boddy, L. 1990. Fungal decomposition of attached angiosperm twigs. 1. Decay community-development in ash, beech and oak. *New Phytologist* 116: 407-415.

Glen, M., Tommerup, I.C., Bougher, N.L., and O'Brien, P.A. 2001. Interspecific and intraspecific variation of ectomycorrhizal fungi associated with *Eucalyptus* ecosystems as revealed by ribosomal DNA PCR-RFLP. *Mycological Research* 105: 843-858.

Griffin, H.G. and Griffin A.M. 1993. DNA sequencing. Methods in molecular biology; DNA sequencing protocols. 23: 1-8.

Gross, A., Holdenrieder, O., Pautasso, M., Queloz, V., and Sieber, T.N. 2014. *Hymenoscyphus pseudoalbidus* the causal agent of European ash dieback. *Molecular Plant Pathology* 15: 5-21.

Juodvalkis, A. and Vasiliauskas, A. 2002. The extent and possible causes of dieback of ash stands in Lithuania. *LŽUU Mokslo Darbai,Biomedicinos Mokslai* 56: 17-22.

Kirisits, T., Matlakova, M., Mottinger-Kroupa, S., Halmschlager, E., and Lakatos, F. 2010. Chalara fraxinea associated with dieback of narrowleafed ash (*Fraxinus angustifolia). Plant Pathology* 59: 411.

Kirisits, T. and Schwanda K. 2015. First definite report of natural infection of *Fraxinus ornus* by *Hymenoscyphus fraxineus. Forest Pathology* 45: 430-432.

Kowalski, T. 2006. *Chalara fraxinea* sp. nov. associated with dieback of ash (*Fraxinus excelsior*) in Poland. *Forest Pathology* 36: 264-270.

Kowalski, T. and Czekaj A. 2010. Disease symptoms and fungi on dying ash trees (*Fraxinus excelsior* L.) in Staszow Forest District stands. *Lesne Prace Badawcze* 71: 357-368.

Johnston, P.R., Johansen, R.B., Williams, A.F.R., Wikie, J.P., and Park, D. 2012. Patterns of fungal diversity in New Zealand Nothofagus forests. *Fungal Biology* 116: 401-412.

Lygis, V., Vasiliauskas, R., Larsson, K.H., and Stenlid J. 2005. Wood-inhabiting fungi in stems of *Fraxinus excelsior* in declining ash stands of northern Lithuania, with particular reference to *Armillaria cepistipes*. *Scandinavian Journal of Forest Research* 20: 337-346.

McKinney, L.V., Neilsen, L.R., Collinge, D.B., Thomsen, I.M., Hansen, J.K., and Kjær, E.D. 2014. The ash dieback crisis: genetic variation in resistance can prove a long-term solution. *Plant Pathology* 63: 485–499.

Pavlic, D., Slippers, B., Coutinho, T.A., and Wingfield M.J. 2007. Botryosphaeriaceae occurring on native *Syzygium cordatum* in South Africa and their potential threat to Eucalyptus. *Plant Pathology* 56: 624-636.

Promputtha, I., Jeewon, R., Lumyong, S., McKenzie, E.H.C. and Hyde, K.D. 2005. Ribosomal DNA fingerprinting in the identification of non sporulating endophytes from *Magnolia liliifera* (Magnoliaceae). *Fungal Diversity* 20: 167-186.

Przybył, K. 2002a. Fungi associated with necrotic apical parts of *Fraxinus excelsior* shoots. *Forest Pathology* 32: 387-394.

Przybył, K. 2002b. Mycobiota of thin roots showing decay of *Fraxinus excelsior* L. young trees. *Dendrobiology* 48: 65-69.

Pukacki, P.M. and Przybyl, K. 2005. Frost injury as a possible inciting factor in bud and shoot necroses of *Fraxinus excelsior* L. *Journal of Phytopathology* 153, 512-516.

Queloz, V., Grunig, C.R., Berndt, R., Kowalski, R., Sieber, T.N., Holdenrieder, O. 2011. Cryptic speciation in *Hymenoscyphus albidus. Forest Pathology* 41: 133-142.

Rodriguez, R. and Redman, R. 2007. More than 400 million years of evolution and plants still can't make it on their own: plant stress tolerance and habitat expansion via fungal symbiosis. Comparative Biochemistry and Physiology – Part A: Molecular and Integrative Physiology 146: 20.

Schmitz, S., Zini, J., Etienne, M., Moreau, J.-M., Chandelier, A., and Cavelier, M. 2006. Effectiveness of thiophanate-methyl, trifloxystrobin and vinclozolin on canker caused by *Phoma exigua* Desm. on ash tree seedlings. *Biotechnologie Agronomie Societe et Environnement* 10: 25-31.

Shipunov, A., Newcombe, G., Raghvendra, A.K. and Anderson C.L. 2008. Hidden diversity of endophytic fungi in an invasive plant. *American Journal of Botany* 95: 1096-1098.

Timmermann, V., Børja, I., Hietala, A.M., Kirisits, T., and Solheim, H. 2011. Ash dieback: pathogen spread and diurnal patterns of ascospore dispersal, with special emphasis on Norway. Bulletin OEPP/EPPO Bulletin [EPPO Workshop on Chalara fraxinea, A Major Threat for Ash Trees in Europe, Oslo, Norway, 30 June-2 July 2010.], 41: 14-20.

White, T.J., Bruns ,T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: a guide to methods and applications. (Innis M.A., Gelfand D.H., Sninsky J.J., White T.J., eds). Academic Press, New York, USA: 315–322.

Wilkinson, H.H., Siegel, M.R., Blankenship, J.D., Mallory, A.C., Bush, L.P. and Schardl C.L. 2000. Contribution of fungal loline alkaloids to protection from aphids in a grass-endophyte mutualism. *Molecular Plant-Microbe Interactions* 13: 1027-1033.