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The arbuscular mycorrhizal fungi colonising roots and root nodules of New Zealand kauri *Agathis australis*

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ABSTRACT

As the only endemic member in New Zealand of the ancient conifer family, Araucariaceae, *Agathis australis* is an ideal species to study putatively long-evolved mycorrhizal symbioses. However, little is known about *A. australis* root and nodular arbuscular mycorrhizal fungi (AMF), and how mycorrhizal colonisation occurs. We used light, scanning and transmission electron microscopy to characterise colonisation, and 454-sequencing to identify the AMF associated with *A. australis* roots and nodules. We interpreted the results in terms of the edaphic characteristics of the *A. australis*-influenced ecosystem. Representatives of five families of Glomeromycota were identified via high-throughput pyrosequencing. Imaging studies showed that there is abundant, but not ubiquitous, colonisation of nodules, which suggests that nodules are mostly colonised by horizontal transmission. Roots were also found to harbour AMF. This study is the first to demonstrate the multiple Glomeromycota lineages associated with *A. australis* including some that may not have been previously detected.

Introduction

Araucariaceae is an ancient, southern conifer plant family that was widespread globally in the Jurassic Period (Setoguchi *et al.* 1998; Hill & Brodribb 1999; Escapa & Catalano 2013) and remains part of the modern floras of Australia, South America, Melanesia, Malesia, Norfolk Island, and New Zealand (Farjon & Filer 2013). *Agathis australis* (D.Don) Lindl. ex Loudon (kauri; Araucariaceae) is the only *Agathis* species endemic to New Zealand and is basal to and distantly related to the other extant *Agathis* species from Malesia, Melanesia, and Australia (Stöckler *et al.* 2002; Biffin *et al.* 2010; Escapa & Catalano 2013). *Agathis australis* is recognised as one of the largest and longest-living conifers

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anywhere in the world; trunk diameters can commonly exceed 3 m in large specimens, tree heights average 30–50 m, and maximum achievable age is estimated at 1500–1700 y (Steward & Beveridge 2010). *Agathis australis* often form an emergent layer above the forest canopy. These trees have cultural significance for New Zealand Māori, and the largest living tree, *Tane Mahuta*, is a national icon and close to 5 m in diameter.

The genus *Agathis* has been present in New Zealand since the late Oligocene to early Miocene (Lee *et al.* 2007). The only extant species (*A. australis*) is now naturally confined to forests of the northern North Island, New Zealand (Ogden *et al.* 1992). Estimated to once have covered 1.0 to 1.5 million hectares in this area prior to exploitation by logging after European colonisation in the 19th century (Halkett & Sale 1986; Steward & Beveridge 2010), only approximately 7500 ha of old-growth *A. australis* forest remains (Steward & Beveridge 2010). While earlier reductions were due to logging and fire, the extent of *A. australis* is declining in modern times due to 'kauri dieback', a disease caused by *Phytophthora agathidicida* B.S. Weir, Beever, Pennycook & Bellgard (Weir *et al.* 2015). *Phytophthora agathidicida* infects roots; the disease causes yellowing leaves, thinning canopy, dead branches, and lesions, and can lead to tree death (Than *et al.* 2013). Efforts to control the disease and its spread have failed so far, and the future of this tree, a species that exerts enormous influence on surrounding forest composition and structure (Wyse *et al.* 2014), is uncertain.

Agathis australis can grow on low nutrient to infertile soils and will reduce the soil nutrient composition even further (Steward & Beveridge 2010). The protracted decomposition rate of leaf litter leads to accumulations of up to 3 m and a corresponding decrease in soil pH (Steward & Beveridge 2010). As *A. australis* mature, their above-ground appearance changes from narrow, columnar, and strongly monopodial crowns (termed 'rickers' and lasting to 150–200 y in natural settings) to wide, spreading crowns based on several large branches once the trunks have reached 50 cm diameter (mature, over 200 y). Additionally, their root architecture shifts from a well-developed tap root system with lateral roots and fine, feeding roots spread throughout the litter layer to widely spreading, lateral roots with deep peg roots as well as fine roots within the litter and humus layers of the forest (Ecroyd 1982; Steward & Beveridge 2010).

Members of Araucariaceae and another southern hemisphere conifer family, Podocarpaceae, are heterorhizic with indeterminate long roots and spherical determinate short roots (nodules); these nodules contain arbuscular mycorrhizal (AM) fungi (Khan & Valder 1972; Cairney 2000; Russell *et al.* 2002; Dickie & Holdaway 2010; Schwendemann *et al.* 2011). Baylis *et al.* (1963) concluded that the nodules could be considered as an adaptation to mycorrhizal fungi, similar in function to the short roots of pines. AM fungi are very widely distributed in nature, colonising the roots of over 80 % of plant families, including many trees (Bainard *et al.* 2011). Consequences of colonisation vary and include enhanced phosphorus nutrition (Morrison & English 1967), heavy metal tolerance (Leyval *et al.* 1997) and protection from plant pathogens (Vigo *et al.* 2000). AM fungi could thus have important functions in *A. australis* in relation to nutrition acquisition and protection against root-rot pathogens.

To date there have been few studies of *A. australis* root systems (Baylis *et al.* 1963; English 1965; Baylis 1969) and the identity of the AM fungus has never been confirmed. Currently, *A. australis* has been purported to host only one AMF species. McNabb (1958) and English (1965) both regarded this species as *Rhizophagus*. However, subsequently Morrison & English (1967) while investigating the function of *A. australis* root nodules colonised by arbuscular mycorrhizae, characterised the fungus as *Endogone* using the system described by Mosse (1963). *Endogone* has subsequently been amended and all the soil-borne fungi that form arbuscules in mutualistic associations with plants have been transferred to Glomales (Morton & Benny 1990). An investigation of *Araucaria araucana* (Araucariaceae) reported colonisation by 27 AMF species identified by spore morphology (Moreira *et al.* 2007), which are distributed in five families (Krüger *et al.* 2012). A molecular study of *Podocarpus falcatus* (Podocarpaceae) found 20 AMF species in three families (Wubet *et al.* 2006). Work on the mycorrhizae associated with the root nodules of New Zealand Podocarpaceae (Russell *et al.* 2002) showed that the nodules from *Prumnopitys ferruginea*, *Prumnopitys taxifolia*, and *Dacrycarpus dacrydioides* contained five AM fungi belonging to the families Archaeosporaceae and Glomeraceae. Based on these previous studies, we hypothesized that *A. australis* was also likely to have more than the one AMF species associated with it; however, there has been no similar molecular or in-depth imaging studies of the fungi colonising *A. australis* roots to substantiate this conjecture.

The aim of our research was therefore to determine the AMF species associated with *A. australis* and to examine what, if any, influence they may have in enabling the survival of *A. australis* in nutrient poor soils. We conducted thorough soil analyses to characterise the environment in which *A. australis* grows. We used light, scanning and transmission electron microscopy, and high-throughput 454 sequencing technologies in conjunction with bioinformatics and phylogenetic analyses to characterise colonisation and the diversity of AMF in the roots and root nodules of *A. australis*.

Materials and methods

Study sites and sampling

An initial survey in February 2011 from the Cascades Track, Waitakere Ranges, New Zealand (WGS-84 174.523808E -36.891137S) was undertaken to gather material to test methods of clearing and staining roots. This site was revisited in February 2012 to obtain additional roots for microscopy for this study. Thirty seven root samples were taken in total from two rickers (~150 y old) and one mature tree (~300 y old); numbers of fine roots were variable per sample as these roots are indeterminate, but each sample contained approximately 3 g of fine roots. No tap roots were surveyed in this study. Fine roots (henceforth to be referred to as roots) were collected at 10–15 cm intervals up to depths of 70 cm, but one tree was sampled to 206 cm. These root samples were stored at 10 °C prior to light microscopy. Roots were also collected from the Waitakere Ranges, from near the

Huia Dam (WGS-84 174.572821E -36.992550S) and in Destruction Gully (~WGS-84 174.534205E -37.028533S). Roots were collected over two days in May 2012 from 12 trees and on one day in July 2012 from nine trees (all rickers). All 21 root samples were obtained from within 1 m of the trunk of the tree and from the top 30 cm of soil, and each sample consisted of approximately 2 g of root material. These roots were frozen after collection as they were used primarily for DNA extraction, but they were also processed for light microscopy. Due to the initial failure in adequate staining and resin-infiltration for electron microscopy, five additional root samples consisting of approximately 5 g of roots were collected from near the Huia Dam in November 2012 from five ricker trees and kept on ice until fixation with glutaraldehyde. Before further processing for DNA extraction or microscopy, the roots were cleaned of coarse debris via brushing and sonication.

Soil analyses

Soil samples were also collected from the locations mentioned above. Pits were dug using hand tools and samples recovered at 10–15 cm intervals (see Suppl. Tables 1 and 2 for exact intervals) to match pedogenic horizons, but where such horizons were substantial (i.e., over 15 cm), samples were taken at prescribed intervals. Usually one kilogram of soil was taken at each sample point. The soil samples were placed in thin-walled polyethylene closed bags and stored at 10 °C. Soils were analysed for pH and electrical conductivity (Gavlak *et al.* 1994), assessed for texture by hand (Thien 1979), and examined for colour using Munsell Charts. Further analyses (e.g., to assess extractable phosphorus, carbon-nitrogen ratios, etc.) were carried out at the Landcare Research Environmental Chemistry Laboratory at Palmerston North, New Zealand; details of the analytical procedures can be found at: http://www.landcareresearch.co.nz/services/laboratories/eclab/eclabtest_list.asp (also see (Blakemore *et al.* 1987).

Light microscopy

Cleaned roots and nodules were cleared to remove dark pigments and stained for examination of AM fungi. Roots were cleared by covering them with 30 % hydrogen peroxide and 5–7 drops of 10 % potassium hydroxide (KOH) for 1 h. Roots were then washed with water, covered with 10 % KOH and heated to a simmer (<90 °C) for 1 h. After this roots were washed with water again and soaked in 0.1 N hydrogen chloride (HCl) for up to 15 min. After a further wash, roots were stained with aniline blue (in lactic acid glycerol) and destained with lactic acid glycerol. Roots were then mounted in clear lactic acid glycerol. Some roots were also cleared using 10 % KOH and heat, followed by immersion in 5 % HCl, before staining with trypan blue (in lactic acid and glycerol) with destaining performed using lactic acid and glycerol. At least one slide from each sample (48 total) was examined and consisted of five to seven fine roots that were three to five cm long with multiple nodules. Observations were made with a Nikon Eclipse 80i microscope (Nikon Instruments Inc., Melville, NY) and images were obtained with a Nikon DS-Fi1 camera. Additionally, roots fixed for transmission electron microscopy were also imaged using light microscopy. Approximately 200 nm sections were cut and stained with 1 % methylene blue, 1 % Azure II, and 1 % borax (all w/v) before imaging.

Scanning electron microscopy

Cleaned roots were fixed in 2.5 % glutaraldehyde in 0.1 M Sörensens buffer pH 7 overnight at 4 °C. Roots were then rinsed three times for 20 min in the same buffer at room temperature and then incubated for 90 min in 1 % (w/v) osmium tetroxide in a 0.05 M buffer. This was followed by dehydration in a graded ethanol series. Selected nodules were bisected with a razor blade under ethanol in a glass petri dish and transferred into a porous capsule for critical point drying. After release from the chamber, the nodules were arranged cut face uppermost on adhesive tabs that were attached to SEM stubs. The stubs were stored in a desiccator, then coated with platinum in a sputter coater, and viewed with an FEI XL30 SEM (FEI, Hillsboro, Oregon).

Transmission electron microscopy

Nodules were kept wet and dissected from roots with a scalpel. In most cases the nodule was partially sliced through to facilitate access for dehydration and resin infiltration. Nodules and roots were fixed in 4 % fresh paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M Sörensens phosphate buffer pH 7 (24 h at 4 °C). Samples were post-fixed in 1 % osmium tetroxide in 0.1 M Sörensen's phosphate buffer. This was followed by dehydration in a graded ethanol series after which the samples were placed in 100 % acetone. Samples were initially infiltrated with 1:1 812 epoxy resin:acetone and then with 100 % epoxy resin (two changes over four days, with slow agitation on a rotating platform). Samples were embedded in fresh resin in flat moulds and cured at 60 °C for 48 h. Ultrathin sections (80 nm) were cut and placed on copper 200 mesh grids. Sections were stained with uranyl acetate and lead citrate. Sections were viewed with a Philips CM12 (Philips Electron Optics, Eindhoven, Netherlands) and images were obtained with a Gatan Bioscan (Gatan Inc., Pleasanton, California).

DNA extraction and pyrosequencing

At least 1 g of cleaned roots and nodules per sample were frozen in liquid nitrogen and crushed using a mortar and pestle. The roots were further crushed using a Ball Mill MM301 (Retsch, Düsseldorf, Germany). Approximately 0.25 g of powdered root material per sample was extracted using the PowerSoil DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, California). The small subunit (SSU) region of rDNA was amplified and tagged. DNA was amplified, using primers NS31 (Simon *et al.* 1992) and AML2 (Lee *et al.* 2008), with an emulsion PCR adaptor and one of 12 different MID tags. PCR reactions were conducted in 25 μL reactions and contained 1.25 units of FastStart High Fidelity or FastStart Taq polymerase (Roche, Basel, Switzerland), 0.4 μM of each primer, 0.2 mM of dNTP, 2.5 μL of 10× buffer and 1 μL of extracted genomic DNA as a template. PCR conditions were as follows: initial

heating to 94 °C (3 min), 35 cycles of denaturation at 94 °C (30 s), annealing at 58 °C (1 min) and extension at 72 °C (1 min), followed by a 10-min extension at 72 °C. Amplicons were cleaned and purified using the Agencourt AMPure XP-PCR purification system (Beckman Coulter, Brea, California). Amplicons were quantified using the Quant-iT PicoGreen dsDNA Assay kit (Life Technologies Corp., Carlsbad, California). Amplicon length was measured and trace amounts of primer-dimers were detected using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California). Equimolar concentrations of amplicons were pooled and sequenced using the Roche GS Junior Titanium Series sequencing platform at EcoGene (Landcare Research, Auckland, New Zealand). One run was conducted on the samples collected in May and another run was conducted on the samples collected in July.

Bioinformatics

The sequences from the two runs were combined. These sequences were split into forward and reverse directions using QIIME v 1.7.0 (Caporaso *et al.* 2010) and those which did not meet the following quality control criteria were removed: any barcode errors, an average quality score of less than 25, more than six ambiguous bases, homopolymer runs longer than eight base pairs. Quality histograms were generated in QIIME. Following examination of these, mothur v 1.26.0 (Schloss *et al.* 2009) was used to trim all reads to 323 base pairs. Sequences from each direction were then processed through the UPARSE pipeline developed by Edgar (2013) including dereplication, singleton removal, and molecular operational taxonomic unit (OTU) clustering at 97% sequence similarity with chimera filtering, with the final step being the mapping of OTUs back on to the complete quality-controlled dataset. BLAST searches against the NCBI database were used to identify non-AMF OTUs, which were removed from the dataset. A maximum likelihood (ML) tree was constructed in GARLI v 2.0 (Zwickl 2006) with reference sequences from major lineages of Glomeromycota (Krüger *et al.* 2012) to evaluate whether OTUs not conclusively identified as AMF via BLAST should be included in the final dataset; two OTUs thus identified were removed from further analyses.

A *de novo* assembly was performed in Geneious v 7.0.6 (http://www.geneious.com (Drummond *et al.* 2012; Kearse *et al.* 2012), to determine whether any of the uni-directional OTUs paired up. A minimum overlap identity of 100 % was selected, otherwise default settings were used. The consensus sequences from the resulting contigs, along with the unmatched OTUs, were used to generate phylogenetic trees. All of the contigs and OTUs that were included in the phylogenetic tree were queried via BLAST against the NCBI database to assess if they matched any AMF sequences (including and excluding ones from uncultured fungi).

Phylogenetic analyses

The contigs and OTUs were aligned with a subset of SSU reference sequences from Krüger *et al.* (2012). *Mortierella verticillata* (GenBank# AF157145) and *Umbelopsis ramanniana* (as *Umbelopsis rammanianus* GenBank# X89435) were used as outgroups (Tisserant *et al.* 2013; Lin *et al.* 2014). A further alignment was performed using MUSCLE v 3.8.31 (Edgar 2004) in Mesquite v 2.75 (Maddison & Maddison 2013) and ambiguous regions were manually edited. To select taxa from the Krüger *et al.* (2012) study to use in the final trees, a maximum likelihood tree was built in GARLI using the default settings, including two search replications. The sister sequence with the shortest distance to each contig/OTU was retained and approximately one third of the remaining sequences were randomly excluded from the dataset to simplify the final tree, ensuring that all the clades from the Krüger *et al.* (2012) study were retained (Suppl. Table 3). The final dataset, consisting of 125 sequences, was aligned again in MUSCLE as described above and ambiguous regions manually edited (Suppl. File 1).

A Bayesian inference (BI) phylogeny was built using MrBayes v 3.2.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) in the CIPRES portal (Miller *et al.* 2010) with 10 million generations, otherwise default settings were used. Tracer v 1.6 (Rambaut *et al.* 2013) was used to assess that the likelihoods of the trees in each chain had converged. CIPRES was also used to build a ML phylogeny with RA×ML HPC-2 (Stamatakis 2014), with 1000 bootstrap repetitions and otherwise using the default settings. The GTR + I + G model of evolution was used for the Bayesian and ML analyses as assessed by ModelTest v 3.7 (Posada & Crandall 1998). Bootstrap values were then visualised by building a consensus tree in PAUP v 4.1 (Swofford 2003). Bootstrap values of 70 % or higher and posterior probability values of 90 % or higher are reported. The trees were compared for congruency.

Results

Soil analyses

The soils at the three locations were similar in that they were highly leached, with similar parent materials, and with podzolization, which is the translocation of sesquioxides in soils (Buol *et al.* 2003), being an overall soil process. The soils all had accumulations, and mostly substantial accumulations, of organic materials on the surfaces (Suppl. Tables 1 and 2). The mineral soil at the surface had much organic material incorporated such that the gradation from the surface O-horizon materials or 'duff' to the mineral soil was gradual. Ultimately, the subsoil was dominated by clays derived from volcanic ash, which comprised a half metre to a metre of the solum. Under this were concretions of iron having red and green colours, many of which hardened irreversibly on drying. The pH of soil samples were acidic, some readings were as low as 3.2, and nutrients were limiting in most horizons (Suppl. Tables 1 and 2). The carbon to nitrogen ratio was 20 to 1 or higher, which indicates carbon in the system is not easily decomposable. Extractable P was also very low (<five mg kg^{-1}). The very low pH strongly suggested that nutrient cations (calcium, magnesium, potassium) were low. Data collected from the Cascades Track in 2012 (Suppl. Table 2) show that these bases were low and base saturation of clays was less than 50 %.

Using information from the soil extractions and analysis made in 2011 from the Cascades Track, which was repeated

in 2012 (Suppl. Table 2), carbon to nitrogen to phosphorus (C:N:P) ratios were constructed based on total analysis. For these three analyses, data were taken from mostly materials near the surface (decomposed litter, e.g., Oe-horizon material) to a depth of 19 cm into the mineral soil. Total organic carbon averaged 41.8 % (±15.8 %), total nitrogen 1.4 % (±0.4 %), and total phosphorus 0.052 % (±11.3 %). Therefore, C:N:P ratios averaged approximately 25 000:60:1 on a molar basis (5000:60:1–32 000:60:1) demonstrating that the soils under *Agathis australis* are severely limited by nitrogen and phosphorus. Available N and P were also very low in these soils. Nitrate N ranged from 1.2 to 7.1 mg kg^{-1} and ammonium N from 7 to 66 mg kg^{-1}. Available phosphate P ranged from less than 1 to 6 mg kg^{-1}.

Nodules and roots

The fine feeding nodulated roots are loosely scattered throughout the litter and humus layers of the forest and are easy to sample. Due to the growth pattern of the fine roots no attempt was made to gauge their lengths. The nodules (Fig 1) are not larger than 1 mm in diameter and are irregularly spaced along the root. Several root sections had strings of nodules attached together (Fig 1) representing consecutive years of growth as young nodules form at the apex of old ones. Younger nodules are less pigmented than the older ones (Fig 1). Nodules also appear to be loosely connected to the fine roots as it was easy to dislodge them during the cleaning process. Before cleaning, roots were covered with external hyphae and extremely sticky clay, and a concerted effort was needed to brush or wash this debris off the roots. Nodulated roots lacking pigmentation were found at depths up to 2 m.

Microscopy

It was challenging to clear and stain the surface roots due to the recalcitrant tannins present in the roots. No attempt was made to measure the percentage of total mycorrhizal colonisation of the roots as clearing was inconsistent; however, of the roots and nodules that were cleared satisfactorily, over 95 % of the cortical cells were colonised. Light microscopy demonstrated that the nodules can be extensively colonised by structures associated with AM fungi (Fig 2–6). Arbuscules (Figs 2 and 6), vesicles (Fig 4), and loosely-coiled, thin-walled pelotons (Fig 5) were observed illustrating that *Agathis australis* are colonised by arbuscular mycorrhizae. Staining of fungal cells demonstrated that the nodules are extensively colonised by arbuscules in the central cortex but that the first two rows of cells adjacent to the epidermis (outer cortex) have only scattered fungal hyphae (Fig 2). The central cortical cells contain little cytoplasm and can have new or degenerating arbuscules. Tannins along the epidermis do not appear to prevent nodule colonisation by external hyphae (Fig 2). Although most of the nodules were colonised by AM fungi, there were some that were uninfected, especially the young nodules. The fungal infection was present in the *A. australis* roots (Fig 6) but appears to be not as pervasive when compared with the nodules (Figs 2, 3 and 4). Evidence of nodule infection (arbuscules and pelotons) was seen at all depths, even up to 2 m deep. Spores were rarely observed either in the *A. australis* litter or soil; however, they were occasionally seen in association with nodules (not shown). The fungal hyphae ranged in diameter and were thick- or thin-walled. Hyphae were primarily aseptate, but occasional septa were observed.

Similarly, it was challenging to fix, resin-infiltrate, and embed the *A. australis* nodules and roots for electron microscopy. Only nodules that were cut in half prior to fixation had cell contents that were well-preserved. Infiltration times were doubled to ensure adequate penetration by the resin prior to curing. The scanning electron microscope (SEM) clearly showed structures associated with arbuscular mycorrhizae such as trunk hyphae (Figs 7 and 8) and arbuscules (Fig 8). The dynamic nature of arbuscules was apparent as generating and degenerating structures were observed in adjacent cells (Fig 8). Transmission electron microscopy (TEM) demonstrated the presence of fungal cells within the root cells (Fig 9). Colonised plant cells are mostly packed with fungal hyphae with little room for plant cytoplasm. The fungal hyphae could be thick- or thin-walled with numerous vacuoles or just one large vacuole (Figs 9–11). The hyphae were multinucleate (Figs 9 and 12). The hyphae were able to penetrate the tannins in the roots and nodules (Figs 10 and 11). Pelotons were also observed via TEM with multiple vacuoles and nuclei (Fig 12). Mitochondria were observed in TEMs (Fig 12). Septa were not apparent, except in one instance (Suppl. Fig 1), which is most likely from a species of Ascomycota.

Pyrosequencing and bioinformatics

PCR products were obtained for eight of the 12 samples for the first 454 run and six of the nine samples for the second 454 run. Using the amplicon pipeline, 62 539 reads were obtained for the first run and 47 810 reads were obtained for the second run. One of the samples yielded no usable reads and was removed from further analyses. Following quality control in QIIME on the total dataset from both runs combined, 37 964 reads were retained from the NS31 primer and 41 138 reads were retained from the AML2 primer. Each root sample averaged a total of 6120 sequences. Forward and reverse sequences were analysed separately as the results showed that certain OTUs sequenced only from one direction (Suppl. Table 4). The NS31 primer yielded 32 AMF OTUs, accounting for 46 % of the retained sequences. The AML2 primer yielded 14 AMF OTUs, and one chimeric OTU (present in one sample and removed from further analyses), accounting for 76 % of the retained sequences. Sample 3 contained 26 OTUs (including those used to construct contigs), which is the largest number present in a sample; Sample 12 contained only nine OTUs, which was the least recovered from a sample. Three AML2 OTUs, 11, 16, and 123, were present in all the samples. When assembled together, eight OTUs from each direction paired into contigs (labelled 1–8) (Fig 13) containing regions of overlap of between 124 and 152 base pairs. Distribution of the contigs was as follows: Contig 3 and Contig 6 in all samples, Contig 7 in 11 samples, Contig 2 in eight samples, Contig 1 in four samples, Contig 5 in two samples, and Contig 4 and Contig 8 each in only one sample (Suppl. Table 4).

Phylogenetic analyses

The phylogenetic tree comprised of 85 SSU reference sequences (Krüger *et al.* 2012), two outgroup taxa, 30 AMF OTUs, and eight contigs (Fig 13). Since the forward and reverse sequences were analysed separately, the same species could be represented by different OTUs. The 30 AMF OTUs were distributed as follows: 26 in Glomeraceae, two in Acaulosporaceae, one in Gigasporaceae, and one in Archaeosporaceae. The eight contigs were distributed as follows: three in Glomeraceae (3, 6, 7), two in Acaulosporaceae (2, 8), one in Gigasporaceae (4), one in Archaeosporaceae (5), and one in Diversisporaceae (1). None of the AMF reads were close to taxa within the Claroideoglomeraceae or Pacisporaceae.

Within Archaeosporaceae, one contig and one NS31 OTU are in a clade (99 % MLBS (Maximum Likelihood bootstrap)/ 1.0 BPP (Bayesian Posterior Probability)) that is sister (100 % MLBS/1.0 BPP) to two *Archaeospora* taxa. The placement of Contig 1 within Diversisporaceae is well-supported (100 % MLBS/1.0 BPP) but its position within the clade is not resolved. Within Gigasporaceae NS31 OTU 156, in an unsupported clade (–/.93 BPP) of four *Scutellospora* species, and Contig 4 are supported as part of this family (100 % MLBS/1.0 BPP). Contigs 2 and 8, NS31 OTU 185, and AML2 OTU 152 are distributed within the highly supported Acaulosporaceae (97 % MLBS/.99 BPP). The three contigs within Glomeraceae are recovered in a well-supported (83 % MLBS/1.0 BPP) clade consisting of *Glomus macrocarpum* and an undescribed *Glomus*; this clade also contains two AML2 OTUs and nine NS31 OTUs. The other 12 NS31 OTUs and three AML2 OTUs are distributed throughout Glomeraceae. Four OTUs and one contig had 95 % or less sequence identity (with uncultured fungi included) to any AMF sequence in the NCBI database (Fig 13). When uncultured fungi were excluded from the BLAST searches, five OTUs

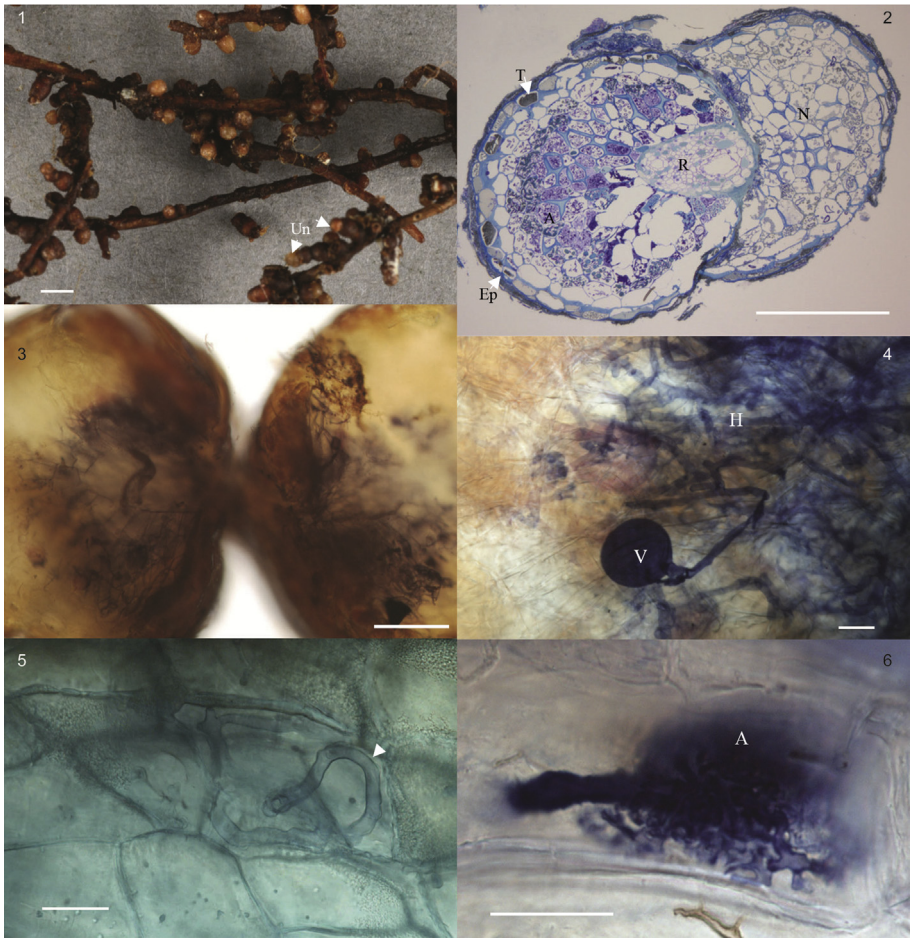

Fig 1 – Light microscopic images of *A. australis* mycorrhizal roots and nodules. 1. *Agathis australis* fine roots with unpigmented (Un), new nodules developing on pigmented, older nodules. Scale bar: 2 mm. 2. Structure of mycorrhizal nodules in *A. australis*. Two hundred nanometer section of a root and two nodules stained with methylene blue. The nodules (N) are completely surrounded by the epidermis (Ep). The nodules have extensive arbuscules (A) in the central cortex, and a few in the outer cortex, as compared to the fine root (R) section that has no obvious arbuscules. The tannins (T) appear to be present only in the outer cortical cells. Scale bar: 0.5 mm. Image courtesy of Adrian Turner. 3. *A. australis* nodule (split in half) with hyphae clustered towards one end of nodule. Scale bar: 0.25 mm. 4. AMF vesicle (V) associated with nodule. Note uneven staining of mycorrhizal hyphae (H). Scale bar: 20 μm. 5. Loosely coiled peloton (arrow) in cortical cell of nodule. Scale bar: 50 μm. 6. Root squash showing arbuscule (A) (1000×). Scale bar: 25 μm.

and two contigs had 95 % or less sequence identity to any AMF sequence in the NCBI database (Fig 13).

Discussion

Podzolization is a major pedogenic process in *Agathis australis*-associated soils. This is a function of the high rainfall and leaching of soluble, organic-acid, decomposition products from the litter and organic layers produced by *A. australis*. The resulting acidic throughflow of water in these soils contribute to leaching of nitrogen and other nutrients out of the root zones (Jongkind *et al.* 2007). Although ectomycorrhizal fungi have been shown to have a role in weathering and podzolization, AMF associated with *A. australis* have not contributed to the formation of podzols (van Breemen *et al.* 2000) that were found up to 2 m below the surface. Due to the higher soil weight and smaller volume of pore space at these depths the co-occurring nodules were somewhat distorted in shape. The nodules were unpigmented; however, these nodules, unlike the unpigmented nodules closer to the surface, were all colonised. The colonised nodules probably function in water

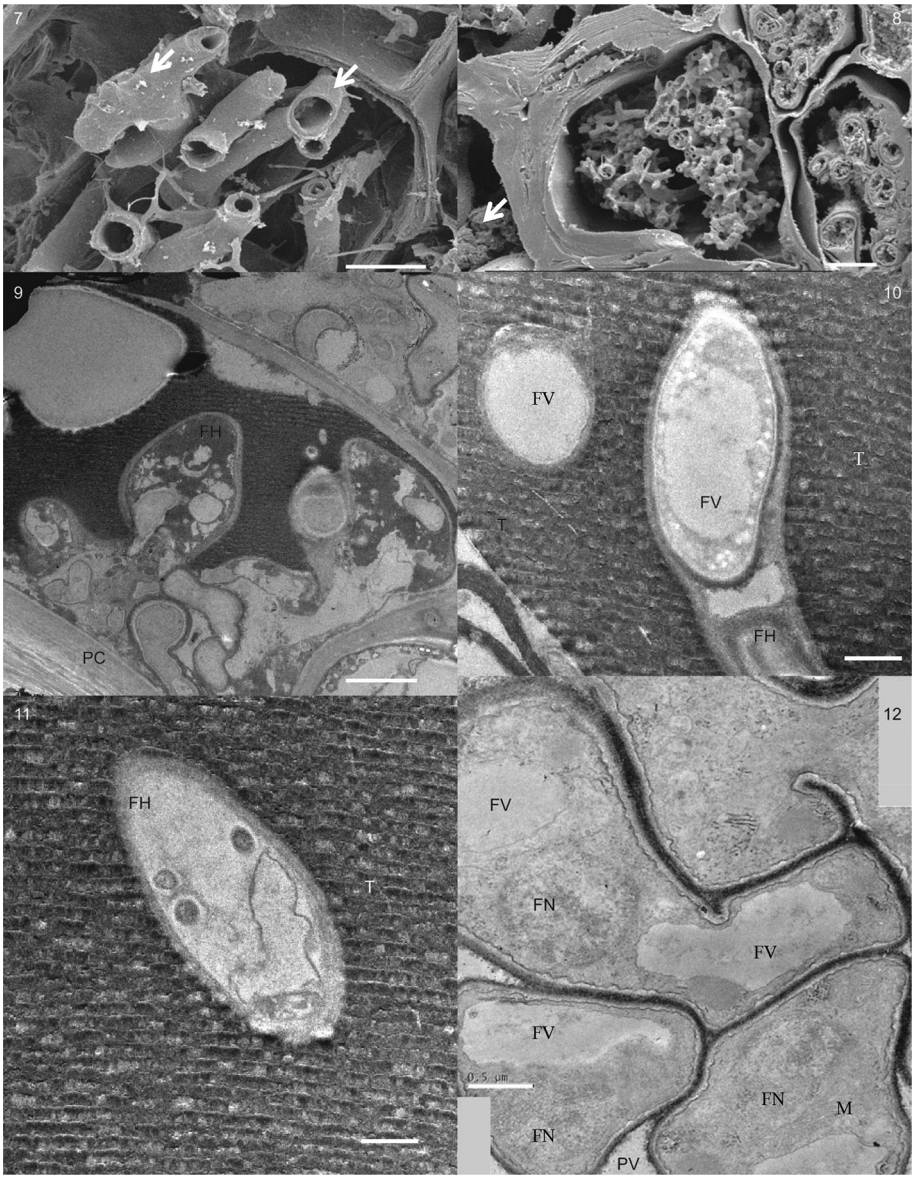

Fig 7 – Electron micrographs of *A. australis* nodules. 7. Scanning electron micrograph (SEM) of trunk hyphae (arrows) in cortical cell. Scale bar: 10 μm. Image courtesy of Adrian Turner. 8. SEM of arbuscule. Note collapsing arbuscule in neighbouring cell (arrow). Image courtesy of Adrian Turner. Scale bar: 10 μm. 9. Transmission electron micrograph (TEM) of fungal hyphae (FH) within the plant cell (PC) within a nodule (3200×). Plant cell consists of mostly fungal hyphae and tannins with scant cytoplasm. Scale bar: 2 μm. 10. TEM of fungal hypha (FH) within tannins (T) of root cell. Fungal vacuoles (FV) are observed. Image courtesy of Adrian Turner. Scale bar: 0.5 μm. 11. TEM of fungal hypha (FH). Tannins (T) do not prevent presence of fungal hyphae within root cell. Scale bar: 0.5 μm. 12. TEM of putative peloton cross section within plant cell vacuole (PV) (6500×). Fungal vacuoles (FV), fungal nuclei (FN), and mitochondria (M) are observed. Scale bar: 0.5 μm.

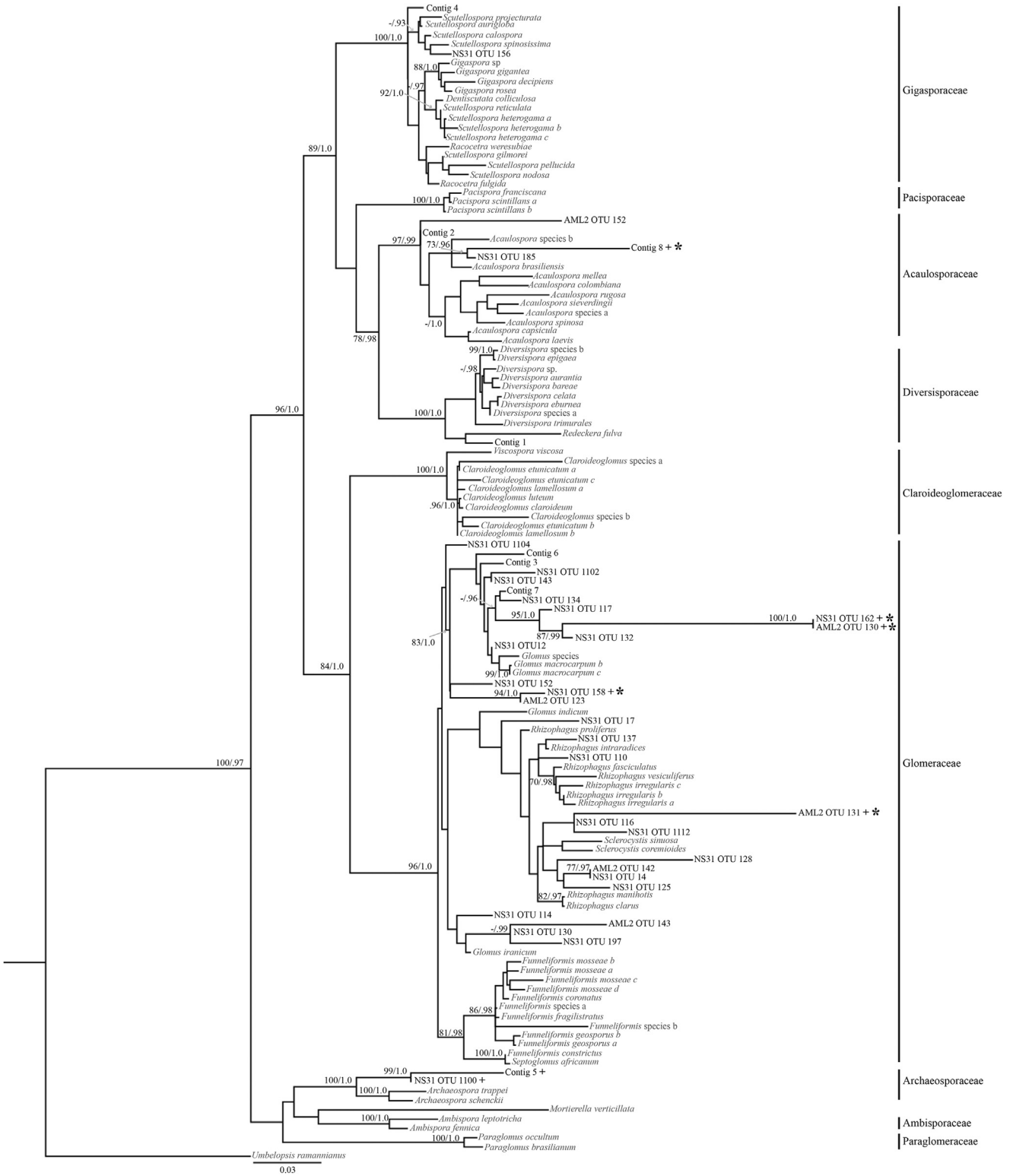

Fig 13 – Maximum likelihood phylogeny of Glomeromycota constructed in GARLI. Glomeromycota families are delineated. Sequences referenced were obtained from Krüger *et al.* 2012 (also see Suppl. Table 3). OTUs are labelled with numbers and the primer name, and contigs are labelled 1–8. Contigs and OTUs that had ≤95 % sequence identity to all AMF sequences from the NCBI database are marked with * and with + if they had ≤95 % sequence identity to known AMF sequences from the NCBI database. Bootstrap values higher than 70 % are reported and are followed by posterior probabilities higher than 90 % (.90).

uptake as the soils between one and a half to 2 m below the surface consist of up to 30 % water and there doesn't appear to be any nutrients at those depths (Suppl. Table 2).

Despite soils containing large reserves of nitrogen – often up to four times the amount found under other forest types – the growth of *A. australis* is often nitrogen limited (Silvester 2000). Although nitrogen use efficiency by *A. australis* is approximately twice that of other trees (Silvester 2000), there is low biologically-available nitrogen. Much of the nitrogen present in this system is due to asymbiotic nitrogen fixation in the leaf litter (Silvester 1978, Silvester 2000). But large amounts of soil nitrogen under *A. australis* are also immobilised as heterocyclic nitrogen in the organic matrix (Verkaik *et al.* 2006). AMF have been shown to uptake complex nitrogenous compounds (Whiteside *et al.* 2012) and in turn transfer the nitrogen to the trees. This could explain why the low level of biologically-available nitrogen has been linked to a proliferation of nodules and to an increased level of AMF infection (English 1965).

There is also low biologically-available phosphorus in the *A. australis* soil system, which is to be expected from these highly leached, acidic soils. Morrison & English (1967) reported an elevated level of phosphorus uptake by mycorrhizal nodules as compared with uncolonised nodules. Such a condition can benefit the host plant. A prevalence of root modifications/symbiotic relationships is strongly associated with ecosystems characterised by nutrient depauperate edaphic substrates especially nitrogen and phosphorus e.g., as in Australia (Bowen 1981).

Agathis australis has numerous determinate short roots (=nodules) on a fine root network. Seedlings of *A. australis* planted in sterile potting soil formed nodules but showed no signs of fungal colonisation after two (pers. obsv.) or three years (Baylis 1969). These observations confirm that although nodules can accommodate AM fungi they form as part of the root system ontogeny; however, the growth of the seedlings stalls after three years without AMF or fertilisation. Dickie & Holdaway (2010) hypothesise that the form of the nodules allows for the maximisation of AMF colonisation with minimum cost to the plant. Regardless of whether the sole purpose of nodules is to house AMF, the association of AMF with root nodules of ancient conifers is long-evolved and stable in form (Schwendemann *et al.* 2011).

In natural settings, AM fungal colonisation occurs approximately five months after the emergence of the new season's nodules (English 1965). Since nodule colonisation by AMF does not occur in conjunction with nodule formation, colonisation probably occurs through horizontal transmission via the soil. One root sample showed hyphal penetration from soil (data not shown). Hyphae were not observed connecting roots and nodules or immature and mature nodules. Vertical transmission within the root system may be possible due to the absence of suberin and the presence of hyphae within the root cortex. New nodules form at the tip of the older nodules and are covered with the outer cell layers of the older nodule. Since all older nodules are colonised, it seems plausible that hyphae from the cortex of older nodules could migrate into immature ones although this was not observed in our study. Podocarp nodules are solely colonised by fungal re-invasion from the soil, but here the endodermis forms a barrier to hyphal entry into the nodule from the root cortex (Russell *et al.* 2002). However, Russell *et al.* (2002) also reported fungal hyphae extending from the cortex of old nodules to the epidermis of developing nodules. Clear images of hyphae from older nodules or roots into newly developing nodules are needed to definitively answer whether vertical transmission of AMF is also responsible for fungal colonisation.

The paucity of spores in the surrounding soil together with the hyphae observed on the nodule/root surfaces suggest that the AM fungi have to be in close association with *A. australis* in order to colonise new nodules as well as new plants. The lack of spores may also be a result of weather conditions or seasonal variation as sampling did not occur in spring. The observation of occasional septate hyphae demonstrates that other fungal species, e.g., Ascomycota, also colonise *A. australis* root systems. Other species of Araucariaceae (e.g., *Araucaria araucana*) are known to be colonised by both AMF as well as dark septate endophytes known to be Ascomycota (Diehl & Fontenla 2010). Further research is needed to characterise the non-AMF root endophytes of *A. australis*.

Our study has demonstrated that there are multiple lineages of Glomeromycota associated with *A. australis* roots and nodules. It is still uncertain whether each nodule harbours several different species of AM fungi or just one species per nodule. The data suggest that AMF communities are patchily distributed in the root samples. For example, AML2 OTU 123 was recovered from each sample whereas AML2 OTU 152 was only recovered from one sample. Additionally the highest number (24) of OTUs plus contigs was recovered from only one sample as compared with the lowest number (8), which suggests that the trees may not have the same number of AMF species or the same species composition. Repeated sampling as well as the characterisation of AMF associated with *A. australis* at distant sites will confirm if each tree has the same species composition.

Conservatively, there are five AMF 'species' within our samples that are not close to previously obtained sequences of Glomeromycota (including environmental sequences) or seven AMF 'species' when compared to sequences from described species of Glomeromycota. These results suggest that there may be species that are uniquely associated with *A. australis*; however, this may be an artefact, reflecting the poor characterisation of AMF diversity in New Zealand similar to the Russell *et al.* (2002) study as analysed in Dickie & Holdaway (2010).

In contrast, the majority of AMF sequences that were recovered in this study are close (greater or equal to 95 % sequence similarity) to known species from diverse habitats and localities (eg., *Trifolium* from U.S.A. or *Melilotus* from Italy). The species are in general typical of AMF communities, i.e., composed mostly of *Glomus* with some representation from other Glomeromycota families. Dickie & Holdaway (2010) state that there was no specificity of AMF to podocarps and that any apparent specificity may be the result of abiotic and biotic properties of the environment. A comparison of AMF associated with other plants in the vicinity as well as *A. australis* at distant sites will confirm if there is any true host specificity.

The trees sampled for this study were all symptom-free in a site where *Phytophthora agathidicida* has been observed.

Additionally, the roots of *P. agathidicida*-symptomatic trees have not been sampled for AMF and it is yet unclear whether *P. agathidicida* displaces the resident AMF in the nodules once it invades *A. australis*. Colonisation by AMF has been shown to reduce necrosis in tomato plants (Vigo *et al.* 2000) and may function in a similar fashion in *A. australis*. Further research is essential to evaluate whether the presence of certain AMF species such as the ones recovered from these healthy trees afford protection against *Phytophthora*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2016.01.015.

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