A coastal sand dune in New Zealand reveals high arbuscular mycorrhizal fungal diversity

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Abstract

Arbuscular mycorrhizal fungi (AMF) are components of coastal dune ecosystems around the globe where they provide a range of benefits to plants. The diversity and structure of AMF communities within these ecosystems, however, are poorly known. This study presents the first report of an AMF community in a New Zealand dune. Root samples were collected from the grass *Spinifex sericeus* R. Br., which dominates dunes of the North Island of New Zealand, along a 90m transect at Anawhata Beach. Spores were also collected from here. The AMF community within the roots was surveyed using 454 sequencing of the SSU gene region. Spores were identified by their morphology, and sequenced. The 22 operational taxonomic units recognised following the high throughput sequencing formed a phylogenetically diverse community, including at least 7 genera across the Glomerales and Diversisporales, with an additional genus detected by the spore investigation. Some root and spore derived sequences generated close BLAST matches to AMF from distant countries while others represent previously unknown biodiversity. Spore morphology also suggests undescribed AMF are present. High diversity was found within the genera *Rhizophagus* and *Racocetra*, and within a clade with no matches to a described genus. Spatial heterogeneity was observed, with taxonomic composition changing over distances of only 30m.

Keywords: Arbuscular mycorrhizal fungi, *Spinifex sericeus*, AMF diversity, dune, high throughput sequencing

Introduction

Plants growing in coastal foredunes have adapted to a highly stressful environment. Generally, nutrient levels are low while high winds deliver salt spray and sand, intermittently burying foliage (Maun 2004). Complete habitat obliteration can occur during storms (Nordstrom & Jackson 2013), while sunny days can mean intense light exposure and heat (Hesp 1991). This is in addition to more typical plant stresses resulting from drought (Maun 2009) and pathogens (de Rooij-van der Goes et al. 1995).

Arbuscular mycorrhizal fungi (AMF) have been shown to help plants tolerate stresses including substrate salination (Estrada et al. 2013; Yamato et al. 2008), root parasites (Little & Maun 1996), foliar pathogens (Campos-Soriano et al. 2012) and aridity (Augé 2001). They are important providers of nutrients, particularly phosphorus (Smith et al. 2011). As such, coastal foredunes have been popular sites for AMF studies and a large proportion of described AMF have been found in dunes (Koske et al. 2004). At least one foredune plant is known to be obligately mycorrhizal (Tadych & Blaszkowski 1999) and Koske et al. (2004) suggest it is likely that studies of plants in the context of the natural, stressful environment of the foredune, rather than in the greenhouse, would find further evidence of heavy reliance on these mutualists. Even foredune environments depauperate in plant species can host diverse AMF communities (Rodríguez-Echeverría & Freitas 2006).

Coastal zones are under anthropogenic pressure worldwide (Martinez et al. 2013) and New Zealand presents no exception. Its coastlines have been degraded extensively over the past century (Hilton et al. 2000) and areas of coastal dunes which remain relatively intact are important refuges for endangered species (Hilton 2006). Restoration efforts, often driven by local community groups, typically begin with the introduction of native plants, alongside the removal of invasive flora (Miller & Paul 2007). However, it cannot be assumed that introducing plants to dunes will automatically or quickly lead to the establishment of a diverse, fully functional microbial community appropriate to the new plant community (Greipsson & El-Mayas 2000; Kurtboke & Bellgard 2011).

New Zealand has only three native plants able to establish in mobile foredunes (Hilton 2006) and only one of these, *Spinifex sericeus,* is not currently of conservation concern (de Lange et al. 2013). This grass, also native to Australia and New Caledonia (Mallett & Orchard 2002), is commonly used for initial dune stabilisation in New Zealand restoration projects (Bergin 2011). No molecular study characterising the AMF species associated with this plant, or with any New Zealand dune plant, has been undertaken previously.

High throughput sequencing technologies are increasingly being used to survey AMF communities, and to improve knowledge of their diversity (Hart et al. 2015; Davison et al. 2015). These surveys suggest there are species not yet formally described and enable consideration of the distribution of AMF known only from gene sequences (Davison et al. 2015; Kivlin et al. 2011; Öpik et al. 2014). They also enable the AMF community existing in roots (rather than just present in the soil as spores) to be examined without the need to use 'bait' plants in greenhouses, an approach which may exclude some species (Hazard et al. 2013; Jansa et al. 2002; Sýkorová et al. 2007). This was previously problematic due to difficulties characterising AMF communities via structures produced in roots using microscopy alone (Alkan et al. 2006; Merryweather & Fitter 1998). High throughput sequencing technologies are also encouraging the examination of AMF diversity at different spatial scales (Lekberg et al. 2012; Öpik

et al. 2013; Wehner et al. 2014). Variation in community composition has been shown to occur at local scales of less than 30 centimetres (Mummey & Rillig 2008) up to 50 metres (Davison et al. 2012).

However, species can be missed by molecular screening from root extracted DNA, possibly due to low levels of root occupation at the time of sampling or technical issues such as PCR bias. Sampling from both occupied roots and spores is likely to provide a more complete picture of the AMF community at a site (Varela-Cervero et al. 2015). Rodríguez-Echeverría & Freitas (2006) extracted *Scutellospora persica (= Racocetra persica)* spores from sand collected from *Ammophila arenaria* monocultures but failed to detect this species using a molecular analysis on DNA extracted from roots. The small sub unit (SSU) rDNA region is favoured for studies of AMF distribution and diversity (Öpik et al. 2013) but can underestimate as well as inflate diversity detected from examination of spore morphology (Walker et al. 2007).

In this study, we used 454 pyrosequencing of the SSU rDNA gene to undertake the first analysis of AMF colonising *S. sericeus* in New Zealand. Root samples were collected along a 90 m transect placed across a natural dune. Spores were obtained from the same dune for morphological description. DNA sequences from these spores were compared to those obtained from the high throughput sequencing.

Materials and Methods

Study site

Root samples were collected from Anawhata Beach in the Waitakere Ranges in the west of Auckland, New Zealand during August 2012 (late winter in New Zealand). Sand for spore extraction was collected during June 2015. Adjacent to a nature reserve (Waitakere Ranges Regional Park), this beach is surrounded by steep, forested hillsides. Sands in this region are mineral rich and largely volcanic in origin (Hamill & Ballance 1985). The mean annual rainfall for West Auckland (Henderson North) is 1420 mm, the mean annual low is 10^{o}C and the mean annual high is 20^{o}C (NIWA 2014). *Spinifex sericeus* heavily dominates the foredunes at Anawhata (Fig. 1), and was accompanied only occasionally across the sampled area by *Ficinia nodosa* (Rottb.) Goetgh., Muasya et D.A.Simpson*, Leontodon saxatilis* Lam.*, Senecio skirrhodon* DC. and *Lachnagrostis billarderei* (R.Br) Trin.

Sample collection

A 90 m long transect was placed along a flat, shelf like dune at the north end of the beach (starting point 174^{o} 27.184’ E, 36^{o} 55.103’ S). The transect ran parallel to the shore (south to north) and was an average of 6 metres back from the dune crest. Three root samples of *S. sericeus* were collected at each 30 meter interval along the transect (0m, 30m, 60m, and 90m) using a soil corer of length 30 centimetres and diameter 10.5 centimetres, giving a total of 12 samples. Within each cluster of three samples, each sample was taken approximately 40 centimetres from the other two at the corners of a triangle. Roots were removed by hand from the sand obtained by the corer, determined morphologically to be from *S. sericeus*, and stored at 4^{o}C for three days. They were then cleaned by rinsing and sonication before being vacuum dried at 36^{o}C in a ThermoSavant SPD IIIV SpeedVac®. Sand was combined from the samples within each cluster, and each combined sand sample was then analysed for organic carbon and total nitrogen (Leco test), phosphorous (Olsen-available) and pH in $CaCl_2$ at the Environmental Chemistry Laboratory, Landcare Research, Palmerston North (methods follow Blakemore et al. 1987). Spores were obtained by first collecting one litre of sand from twelve

points using the same sampling methodology at the same location. This sand was stored at 4°C for a maximum of three weeks before spores were extracted.

DNA extraction and high throughput sequencing: roots

Dried roots were crushed using a ball mill MM301 (Retsch, Dusseldorf, Germany). DNA was extracted from approximately 20mg of powdered material per sample using a Qiagen DNeasy plant mini kit. The initial incubation step was prolonged to one hour and carried out at 65°C with a 1400rpm shake for 15 seconds every three minutes, otherwise the standard protocol was followed. The SSU region was amplified using primers NS31 (Simon et al. 1992) and AML2 (Lee et al. 2008) and an emulsion PCR adaptor. One of 11 different MID tags was annealed to the DNA (the failure of one of the 12 MID tags available meant results were obtained for only eleven samples, the third sample from the second cluster being omitted). Each 25µL PCR reaction contained: (1) either 0.25µL of FastStart High Fidelity Taq polymerase and 0.2mM dNTP, or 0.2 µL of FastStart Taq polymerase and 1mM dNTP (Roche, Basel, Switzerland), (2) 0.4µM of each primer, (3) 2.5µL of the supplied 10X buffer, (4) 1µL of BSA and (5) 1µL of extracted DNA as a template. PCR conditions were: initial heating to 94°C (3 min), 35 cycles at 94°C for 30s, 58°C for 1min and 72°C for 1min; and 72°C for 10min on a GenAmp® PCR system 9700 (Applied Biosystems, USA). Cleaning and purification of amplicons was performed according to the Roche Amplicon Library Preparation Method Manual. The Quant-iT™ PicoGreen® dsDNA Assay kit (Life Technologies Corp., Carlsbad, California) was used to quantify the amplicons; their length was measured using the High Sensitivity DNA Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California). Amplicons were pooled using equimolar quantities and sequenced using the Roche GS Junior Titanium Series sequencing platform at EcoGene (Landcare Research, Auckland, New Zealand). Sequence data has been submitted to the European Nucleotide Archive (accession #PRJEB10954).

DNA extraction, sequencing and morphology: spores

One cup of sand from each well mixed sample was immersed in two litres of water containing 1ml of Tween 80, then passed through 75 µm and 425 µm sieves. The wash from the 75 µm sieve was collected on filter papers while the 425 µm sieve was directly inspected under the microscope. Spores were sorted into morphotypes based on their macroscopic appearance through a dissecting microscope and representatives of each were selected for single spore sequencing, which was undertaken using the Sigma-Aldrich Extract-N-Amp Plant PCR kit. Individual spores were photographed, then crushed in 10 µL of extraction solution, then heated for 10 minutes at 95°c, before 5 µL of dilution solution was added. PCR reactions were carried out with 2 µL of DNA in 5 µL of REDExtract-N-Amp PCR Ready Mix with 0.5 µM each of the primers, being again NS31 (Simon et al. 1992) and AML2 (Lee et al. 2008). PCR products were purified using the Qiagen MinElute® 96 UF PCR purification kit. The thermocycler protocol was as described above. Sequences from both directions were obtained using an Applied Biosystems 3500xL Avant Genetic analyzer using BigDye v.3.1 chemistry. They were assembled and analysed using Geneious v8.0.5 (Kearse et al. 2012). The sequences have been submitted to the ENA and their accession numbers are provided in Online Resource 1. For morphological descriptions, spores from each morphotype were examined in water through a dissecting microscope, and in polyvinyl alcohol lactoglycerol (PVLG) (Omar et al. 1979) and PVLG with Melzer's reagent, 5:1 v/v (PVLG/Melzer's) (Walker et al. 1993) through a compound microscope.

Bioinformatics

QIIME v1.7.0 (Caporaso et al. 2010) was used to split the sequences into forward and reverse directions and remove sequences that did not meet the following quality control criteria: barcode errors, an average quality score of less than 25, six or more ambiguous bases, and homopolymer runs longer than eight base pairs. Based on quality histograms generated using QIIME v1.7.0 (Caporaso et al. 2010), sequences were trimmed to 359 bp in length and any sequences shorter than this were removed in mothur v1.26.0 (Schloss et al. 2009). The Uparse pipeline introduced by Edgar (2013), including dereplication, singleton removal, and operational taxonomic unit (OTU) clustering with chimera filtering, with the final step of mapping OTUs back on to the complete quality-controlled data set, was then followed independently for sequences from each direction. Non-AMF OTUs were identified by BLAST searches against the GenBank database, assigned to phyla provided a minimum continuous query cover value of 80% was attained, and removed from the dataset. All AMF OTUs have been submitted to the ENA, for details of their accession numbers, see Online Resource 2.

To match the AMF OTUs derived from each direction, a de novo assembly was performed in Geneious v7.0.6 (Biomatters Ltd 2011). A minimum overlap identity of 99% was selected, otherwise default settings were used. Where contigs had paired OTUs that differed by one base pair, the contig was amended to match the OTU with the most sequences assigned to it. Where the OTUs making up a contig differed by a single insertion in one of the OTUs, the contig sequence was edited to include this. Online Resource 2 details which OTUs assembled into which contigs.

BLAST searches against GenBank were conducted for each contig, for each OTU that did not assemble into a contig, and for each spore sequence. The top listed sequence with the highest overall scores obtained from a published study was downloaded from each search. BLAST was also used against the Maarj*AM* database (Öpik et al. 2010), to search for records with higher scoring matches. Outgroup species *Mortierella verticillata* (GenBank #AF157145.1) and *Umbelopsis ramanniana* (GenBank #X89435.1) which are in sister clades to the Glomeromycota (Lin et al. 2014; Tisserant et al. 2013) were downloaded from GenBank. To facilitate the phylogenetic placement of the contigs/OTUs, the spore sequences, and the GenBank sequences, the sequences from Fig. 2 in Krüger et al. (2012) were obtained as a reference dataset and, along with the contigs/OTUs, were aligned using MAFFT v7.017 (Katoh et al. 2002) in Geneious. Ambiguous regions were manually edited. A maximum likelihood tree was built in Geneious using PhyML (Guindon & Gascuel, 2003) with the GTR substitution model, to select sequences from the Krüger et al. (2012) Fig. 2 for the final trees. The closest match to each contig/OTU and spore sequence was retained. Half of the remaining sequences were randomly removed from clades containing sequences from our study, while clades without these were reduced to a maximum of three sequences, to simplify the final trees while retaining the phylogenetic diversity represented in the Krüger et al. (2012) tree. A *Glomus perpusillum* sequence (GenBank #FJ164242.1) was downloaded to facilitate identification of a clade without reference sequences. An *Acaulospora scrobiculata* sequence (GenBank #KF412623.1) was obtained, as a spore was morphologically determined to belong to this species, which was not represented in the Krüger et al. (2012) set or by a BLAST match named to species level. The reduced Krüger et al. (2012) set of sequences was aligned as above, with the sequences generated in this study and those downloaded from GenBank, ambiguous regions were manually edited, and terminal gaps were changed to missing data. The CIPRES portal (Miller et al. 2010) was used to build a Bayesian inference (BI) tree using MrBayes v3.2.6, 10 million generations were run, otherwise default settings were used. Tracer v.1.6 (Rambaut et al.

2014) was used to confirm that the likelihoods of the trees in each chain had converged. CIPRES was also used to build a maximum likelihood (ML) tree with RAxML-HPC2 v8.1.24 (Stamatakis 2014) with the default settings. A maximum parsimony tree was built in PAUP v4.1. (Swofford 2003). The three trees were compared for congruency. The MrBayes tree was visualised and manipulated using FigTree v1.4.2 (Rambaut 2007) then annotated with Microsoft PowerPoint.

Statistical methods

The matrix of number of 454 reads of each OTU per sample was standardised by calculating the relative abundance of each OTU in each sample. These data were then square root transformed to down weight the influence of abundant OTUs and take into account rarer OTUs. Differences in fungal community composition among root samples were then examined using non-metric multidimensional scaling (nMDS) as implemented in PRIMER v6 (Clarke & Warwick 2001) using the Bray Curtis distance measure. To test whether the sampling effort was sufficient to capture the total AMF community across the transect, a species accumulation curve with 999 permutations was generated using PRIMER v6.

Results

A total of 52,885 reads were retained from the high throughput sequencing following quality control. The NS31 primer generated 18,593 of these (median length pre-quality control 550 bases) and the AML2 primer 34,292 (median length pre-quality control 553 bases). Several OTUs, 8 from the NS31 primer and 10 from the AML2 primer, were determined to be non-AMF. Collectively, they contained 341 sequences which were removed from the dataset. Their BLAST matches came from a range of organisms including basidiomycetes, nematodes and plants (Online Resource 3). The mean number of remaining reads per sample was 4,777 (SD = 1,613). The AMF sequences from the NS31 primer sorted into 20 OTUs and those from the AML2 primer sorted into 16 OTUs. When assembled together, these generated 14 contigs. Each of these contigs contained only two OTUs which overlapped by between 193 and 270 bases. For simplification, these contigs, while labelled as such on the tree and in the Online Resources, are referred to henceforth in the text as OTUs, collectively with the OTUs which did not assemble into contigs. This gives a total of 22 unique OTUs. Each sample had a similar number of OTUs, with a mean of 14 OTUs (SD = 1.4). The most dominant OTU contained 57% of the sequences, with the ten most frequent OTUs containing 96% of the sequences (Online Resource 2). Published BLAST matches with 89% identity or better were made for all OTUs (Online Resource 4). There were two instances in which two OTUs (NS31OTU 111 and AML2OTU 123, Contig 5 and NS31OTU 118) had the same BLAST match. No better matches for any sequences were found on the Maarj*AM* database (Öpik et al. 2010).

The main topology of the MP, BI and ML trees was the same (not shown). Placing the OTUs in a phylogeny with the representative Krüger et al. (2012) sequences, shows the AMF detected at Anawhata to have broad phylogenetic spread (Fig. 2). The generic and family level clades of our tree closely match those of Krüger et al. (2012). At least 7 genera are represented. The genera *Rhizophagus* (Glomeraceae) and *Racocetra* (Gigasporaceae) contain a high diversity of OTUs. Six OTUs nested within known genera were isolated on long branches with no close matches from BLAST or to species in the Krüger et al. (2012) study. One poorly supported clade (labelled Glomerales incertae sedis in Fig. 2), also containing several OTUs, matched no known genus and in the SILVA SSU database (http://www.arb-silva.de/browser/ssu) the sequences in this clade were classified only down to the

level of Order, as Glomerales. Whilst the *Glomus perpusillum* sequence sat within this clade when first included in a maximum likelihood tree, the final trees excluded it.

Eleven spore morphotypes were recognised, some of which were identifiable to the species level (Fig. 3, Online Resource 1), and six unique spore sequences were recovered (Fig. 2, Online Resource 1). Three morphotypes generated the same SSU sequence, which places them in *Racocetra*, and which does not match closely to any OTUs derived from roots or to a GenBank sequence. These all had big, colourful spores measuring approximately 200 – 300 µm in diameter or more. Two of these, one morphologically the same as *Racocetra coralloidea* (Fig. 3F-H) and a *Racocetra* sp. resembling *R. verrucosa* (Fig. 3I-K), had well defined morphological features. The morphological identity of the third remains uncertain as it tentatively resembles both *Racocetra fulgida* and *Cetraspora pellucida,* with hyaline to white or slightly yellowish coloration (Fig. 3L-M). It was identified only to the level of family (Gigasporaceae). Spores identified morphologically as *Gigaspora rosea* (Fig. 3D-E) add the *Gigaspora* genus to the diversity recovered from Anawhata. The DNA sequence from this morphological group was typical of *Gigaspora* but somewhat different to sequences accepted as *G. rosea* by Krüger et al. (2012). A smaller brown spore morphotype identified both genetically and morphologically as *Diversispora* (Fig. 3C) also represents diversity not recovered from roots.

Two spore morphotypes produced sequences which match closely with sequences derived from roots. One of these morphologically resembled *Diversispora globifera*, having relatively big reddish brown spores (size about 160-220 µm) covered to a varying degree by a hyphal peridium (Fig. 3B). However the sequence generated by this morphotype places it in *Redeckera*, matched to Contig 4. Another, the *Acaulospora* morphotype (Fig. 3A), which matches contig 2, morphologically resembles *A. scrobiculata,* but its sequence is genetically distinct from that downloaded from GenBank and confirmed to be from this species by de Novais et al. (2014).

The spore derived sequence placed in Glomerales incertae sedis likely came from a contaminant. It was generated from a spore morphologically identified as belonging to the *Racocetra coralloidea* morphotype, and sequences produced by other spores in this group sit within the Diversisporales. Spores resembling *Rhizophagus fasciculatus* of the Glomeraceae family were sporadically recovered but we were not able to obtain sequences from them. These spores showed a strong Melzer reaction (Fig. 3N-O). Three other types of Glomeraceae spores (images not shown), *R. aggregatus*, a *Glomus* or *Rhizophagus* sp. with very tiny spores in loose clusters and a dark brown, tentatively *Glomus* sp., were also detected.

While only a small number of samples were collected, the asymptotic nature of the species accumulation curve suggests that high throughput sampling effort was sufficient to capture the AMF community across the transect (Fig. 4). The nMDS plot shows OTU composition differs by sample area, with the samples grouping together in a manner which reflects the way they were collected (Fig. 5). The five samples from the two clusters in the middle of the dune group together while those from either end of the dune occupy separate multidimensional space. All nitrogen and phosphorus readings were very low, while organic carbon and pH levels showed small but measureable levels of variation (Table 1).

Discussion

A phylogenetically diverse range of AMF occupy *S. sericeus* roots at Anawhata, New Zealand. While only one plant species dominates the dune, 22 AMF OTUs were detected. Similarly complex communities have been reported from foredune environments in other studies. For example, spores of 32 AMF morphotypes were isolated from dunes dominated by *A. arenaria* in Denmark, suggesting this grass is also able to associate with a variety of AMF (Blaszkowski & Czerniawska 2011). As well,

cloning and sequencing the SSU region revealed 69 different AMF sequences from *A. arenaria* roots from two sites in Portugal (Rodríguez-Echeverría & Freitas 2006), and 17 AMF 'phylotypes' were recorded from a single dune in Japan, which hosted a more complex plant community (Yamato et al. 2012).

Additional diversity on the dune was recovered from the spore sequencing and morphology. Root sampling during a different season might recover these AMF within *S sericeus roots*, as the within root composition of some AMF communities has been shown to fluctuate throughout the year (Bainard et al. 2014; Dumbrell et al. 2011). They may be present in *S sericeus roots* but have been missed by the high throughput sequencing. Alternatively, some spores may have been generated from AMF communities occupying alternative host plants, either on the dune or in the surrounding hillsides which are steep and heavily vegetated. Morphological examination of spores also suggests relying entirely on the SSU region may underestimate diversity, with three morphotypes generating the same *Racocetra* sequence. The SSU region sequenced is not evenly variable across all AMF and has been previously noted to be particularly conserved within the Gigasporaceae (Öpik et al. 2013).

The majority of OTUs were not closely matched by spore sequences. Studies undertaken on dunes using spore based methods alone typically find fewer than 6 species within single sites (Błaszkowski & Czerniawska 2011). Purely spore based approaches can fail to detect some AMF as spore production can be seasonal (Greipsson & El-Mayas 2000), highly heterogeneous spatially (Friese & Koske 1991), dependent on environmental conditions and possibly entirely absent in some species (Sanders 2004).

The phylogenetic tree and spore morphology suggests undescribed AMF, some of which have not yet been found elsewhere, are contributing to the diversity of the AMF community at Anawhata (Fig. 2). Several OTUs are placed on long branches and lack close BLAST matches. Given the paucity of AMF records from Australasia (Davison et al. 2015, Öpik et al. 2010, Öpik et al. 2013), this is not surprising. These AMF may be unique to New Zealand, or they may be simply undetected elsewhere as AMF diversity is poorly known in many regions of the world (Öpik et al. 2013). Of particular interest is the Glomerales incertae sedis clade consisting entirely of OTUs from this study, a spore sequence, and BLAST matches to environmental samples. This clade was resolved on all trees examined and suggests an entire lineage occupying the dune that either awaits formal taxonomic description, or that comprises known AMF not yet sampled genetically. The spore morphotype descriptions, which in some instances place the spores in alternative genera to that determined by sequencing, reveal the taxonomic work still needed on these fungi, particularly those from undersampled regions like New Zealand.

Recent work has shown that many AMF are both widely dispersed and found in a variety of environments (Davison et al. 2015). Data from GenBank matches reveals that some AMF at Anawhata occur in other parts of the world, or at least have close relatives elsewhere. Some of these matches are from quite different habitats. BLAST matches with identity results of 99% were made

for eleven OTUs and all six spore sequences (Online Resource 4). Coastal dunes from Japan (AB326003.1), Poland (FR686956.1) and Brazil (GU385897.1) hosted three of these matches while a further match was made with an Acaulosporaceae sequence from a lakeside dune in Michigan, USA (JN252439.1). Another Anawhata OTU clusters on the phylogenetic tree with a *Glomus perpusillum* sequence (FJ164242.1). *G. perpusillum* was described from collections from a coastal dune in Italy. Błaszkowski et al. (2009) suggested it is likely to be rare, at least in the northern hemisphere but in

addition to grouping with our sequence, it closely matches a GenBank sequence sourced from a maize field in China (KC797122.1). Environmental flexibility is also shown by the match from the Brazilian dune - this AMF has also been detected in agricultural fields. Other matches were found with sequences from a tropical mountain rainforest in Ecuador (JX297087.1, JX297074.1) although these were with Anawhata spores, which may not be from AMF occupying *S sericeus* roots but rather have dispersed to the dune from neighbouring forest.

According to OTU sequence frequency, the community at Anawhata is dominated by Glomeraceae. The majority of reads within known genera represent *Rhizophagus* or *Glomus* species, with the most dominant taxon being placed within the genus *Rhizophagus*. *Rhizophagus* spp. (often recorded as *Glomus*, Schüßler & Walker 2010) were dominant in other molecular surveys of dunes (Rodríguez-Echeverría & Freitas 2006; Yamato et al. 2008; Yamato et al. 2012) as well as those based on spore quantification (Blaszkowski & Czerniawska 2011), and this genus is likely to be a significant dune occupant worldwide.

Three of the OTUs within the Glomeraceae collectively contain 80% of the sequence data. Extreme dominance by a minority of AMF is commonly seen in AMF communities analysed by high throughput sequencing and these are commonly Glomeraceae (Dumbrell et al. 2010). Growth in roots rather than proliferation in the surrounding substrate is characteristic of this family (Hart & Reader 2002). This could at least partly explain the frequency with which this family dominates molecular analyses targeting extracts from roots, although similar results have been reported from analyses from soil samples (Alguacil et al. 2011; Hijri et al. 2006). Results using sequence frequency as a measure of relative abundance must be interpreted with caution, however, as the degree to which this accurately reflects biomass in roots is not known. Molecular techniques from DNA extraction through to PCR and sequencing can favour some species and even some 454 barcodes over others, resulting in the inflation of some sequence numbers (Amend et al. 2010; Bellemain et al. 2010; Berry et al. 2011; Lindahl et al. 2013).

Although the three most frequently detected OTUs were present in all samples, spatial heterogeneity is a further characteristic of most OTUS within the AMF community at Anawhata. The nMDS plot reveals the species assemblages at each end of the transect are different from each other and different from the community in the middle of the sampling area. A clumped distribution, with most species not being evenly distributed across samples, is not unusual for AMF and was also observed by Maherali & Klironomos (2012) and Davison et al. (2012). These authors suggest differences in host plant species and soil quality may provide some explanation but this is not likely to be the case at Anawhata. We detected little variation in major soil nutrients or pH levels within the transect, so soil chemical differences do not appear to be driving community change in our study. Factors such as exposure to salt spray and substrate moisture may differ along the transect, and these may be controls over AMF community composition (Sun et al. 2013; Yamato et al. 2012). It is also possible the pattern is a result of under sampling within each cluster; more sampling is needed to tease out the true degree of difference between the communities in each section of the dune. However, the spatial clumping seen does show that care needs to be taken when designing field surveys to examine controls over AMF community structure such as host plant species.

This study demonstrates that the Anawhata foredune examined supports a diverse range of AMF even though it is dominated by only one host plant species, *S sericeus*. The work represents the first step in

characterising the AMF associated with this plant in New Zealand. These fungi may be important for maintaining plant vigour, and for dune restoration efforts. They include a mix of AMF found in other parts of the world together with others which have not been detected previously in sequencing-based studies. Better characterising the previously undetected AMF by collecting further morphological and genetic data would add to knowledge of global AMF diversity. Undertaking further studies to determine their distribution in New Zealand and elsewhere in the Southern Hemisphere would be worthwhile. More intense sampling is also needed within individual dunes to elucidate the precise spatial scale over which community changes occur.

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Figures and tables

Fig. 1 Dune at Anawhata, New Zealand, from which *Spinifex sericeus* roots, and spores of arbuscular mycorrhizal fungi, were collected.

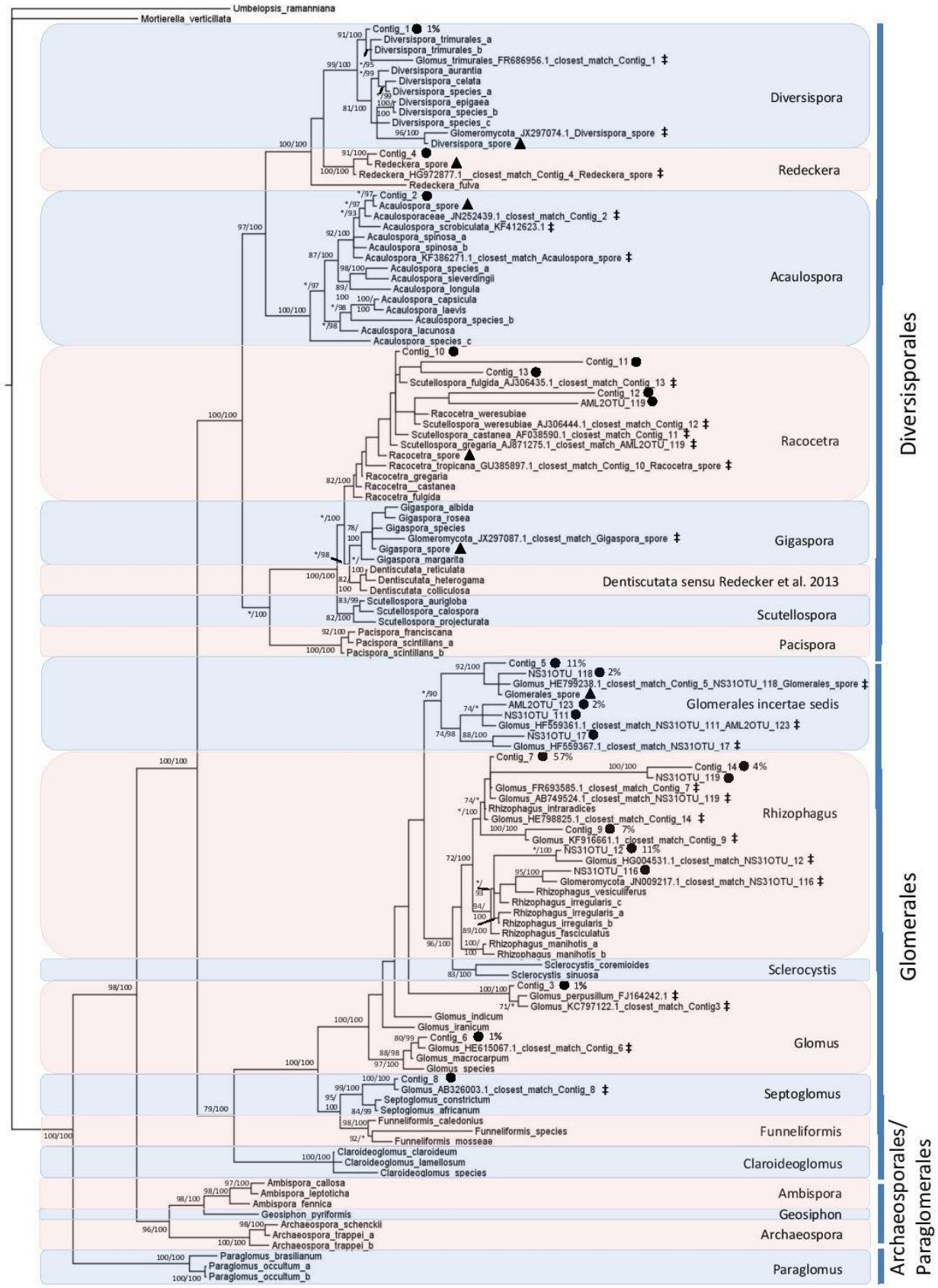
Umbelopsis_ramanniana
Mortierella_verticillata
Contig_1 1%
Diversispora_trimurales_a
Diversispora_trimurales_b
Glomus_trimurales_FR686956.1_closest_match_Contig_1 ‡
Diversispora_aurantia
Diversispora_celata
Diversispora_species_a
Diversispora_epigaea
Diversispora_species_b
Diversispora_species_c
Glomeromycota_JX297074.1_Diversispora_spore ‡
Diversispora_spore
Diversispora
Contig_4
Redeckera_spore
Redeckera_HG972877.1__closest_match_Contig_4_Redeckera_spore ‡
Redeckera_fulva
Redeckera
Contig_2
Acaulospora_spore
Acaulosporaceae_JN252439.1_closest_match_Contig_2 ‡
Acaulospora_scrobiculata_KF412623.1 ‡
Acaulospora_spinosa_a
Acaulospora_spinosa_b
Acaulospora_KF386271.1_closest_match_Acaulospora_spore ‡
Acaulospora_species_a
Acaulospora_sieverdingii
Acaulospora_longula
Acaulospora_capsicula
Acaulospora_laevis
Acaulospora_species_b
Acaulospora_lacunosa
Acaulospora_species_c
Acaulospora
Contig_10
Contig_11
Contig_13
Scutellospora_fulgida_AJ306435.1_closest_match_Contig_13 ‡
Contig_12
AML2OTU_119
Racocetra_weresubiae
Scutellospora_weresubiae_AJ306444.1_closest_match_Contig_12 ‡
Scutellospora_castanea_AF038590.1_closest_match_Contig_11 ‡
Scutellospora_gregaria_AJ871275.1_closest_match_AML2OTU_119 ‡
Racocetra_spore
Racocetra_tropicana_GU385897.1_closest_match_Contig_10_Racocetra_spore ‡
Racocetra_gregaria
Racocetra_castanea
Racocetra_fulgida
Racocetra
Gigaspora_albida
Gigaspora_rosea
Gigaspora_species
Glomeromycota_JX297087.1_closest_match_Gigaspora_spore ‡
Gigaspora_spore
Gigaspora_margarita
Gigaspora
Dentiscutata_reticulata
Dentiscutata_heterogama
Dentiscutata_colliculosa
Dentiscutata sensu Redecker et al. 2013
Scutellospora_auriglobа
Scutellospora_calospora
Scutellospora_projecturata
Scutellospora
Pacispora_franciscana
Pacispora_scintillans_a
Pacispora_scintillans_b
Pacispora
Contig_5 11%
NS31OTU_118 2%
Glomus_HE798238.1_closest_match_Contig_5_NS31OTU_118_Glomerales_spore ‡
Glomerales_spore
AML2OTU_123 2%
NS31OTU_111
Glomus_HF559361.1_closest_match_NS31OTU_111_AML2OTU_123 ‡
NS31OTU_17
Glomus_HF559367.1_closest_match_NS31OTU_17 ‡
Glomerales incertae sedis
Contig_7 57%
Contig_14 4%
NS31OTU_119
Glomus_FR693585.1_closest_match_Contig_7 ‡
Glomus_AB749524.1_closest_match_NS31OTU_119 ‡
Rhizophagus_intraradices
Glomus_HE798825.1_closest_match_Contig_14 ‡
Contig_9 7%
Glomus_KF916661.1_closest_match_Contig_9 ‡
NS31OTU_12 11%
Glomus_HG004531.1_closest_match_NS31OTU_12 ‡
NS31OTU_116
Glomeromycota_JN009217.1_closest_match_NS31OTU_116 ‡
Rhizophagus_vesiculiferus
Rhizophagus_irregularis_c
Rhizophagus_irregularis_a
Rhizophagus_irregularis_b
Rhizophagus_fasciculatus
Rhizophagus_manihotis_a
Rhizophagus_manihotis_b
Rhizophagus
Sclerocystis_coremioides
Sclerocystis_sinuosa
Sclerocystis
Contig_3 1%
Glomus_perpusillum_FJ164242.1 ‡
Glomus_KC797122.1_closest_match_Contig3 ‡
Glomus_indicum
Glomus_iranicum
Contig_6 1%
Glomus_HE615067.1_closest_match_Contig_6 ‡
Glomus_macrocarpum
Glomus_species
Glomus
Contig_8
Glomus_AB326003.1_closest_match_Contig_8 ‡
Septoglomus_constrictum
Septoglomus_africanum
Septoglomus
Funneliformis_caledonius
Funneliformis_species
Funneliformis_mosseae
Funneliformis
Claroideoglomus_claroideum
Claroideoglomus_lamellosum
Claroideoglomus_species
Claroideoglomus
Ambispora_callosa
Ambispora_leptoticha
Ambispora_fennica
Ambispora
Geosiphon_pyriformis
Geosiphon
Archaeospora_schenckii
Archaeospora_trappei_a
Archaeospora_trappei_b
Archaeospora
Paraglomus_brasilianum
Paraglomus_occultum_a
Paraglomus_occultum_b
Paraglomus
Diversisporales
Glomerales
Archaeosporales/
Paraglomerales

Fig. 2 BI tree based on partial SSU sequences, illustrating the phylogenetic diversity of the AMF detected in a New Zealand sand dune. Bootstrap values greater than 70/posterior probability values greater than 90 are indicated above the branches. Most sequences are from Krüger et al. (2012); those with a GenBank accession number in the label were downloaded from GenBank (indicated with a ‡) and represent the closest BLAST matches to our New Zealand sequences or a species not provided by the reference sequences, those labelled with a Contig or OTU number are the sequences obtained from the New Zealand sand dune (indicated with a ●). The frequency (%) at which the ten most common New Zealand contigs or OTUs were detected is shown after their name. The taxonomic names used for the labels on the Krüger et al. (2012) sequences are derived from those provided by the authors, apart from those in the Dentiscutata clade which was named following Redecker et al. (2013). The GenBank record names are used for the BLAST matches. Genus-level clades are indicated, the generic names for the clades (excluding the Dentiscutata clade) matching those accepted by Krüger et al. (2012) and also matching the generic names accepted for the sequences within those clades by the SILVA SSU database (http://www.arb-silva.de/browser/ssu).

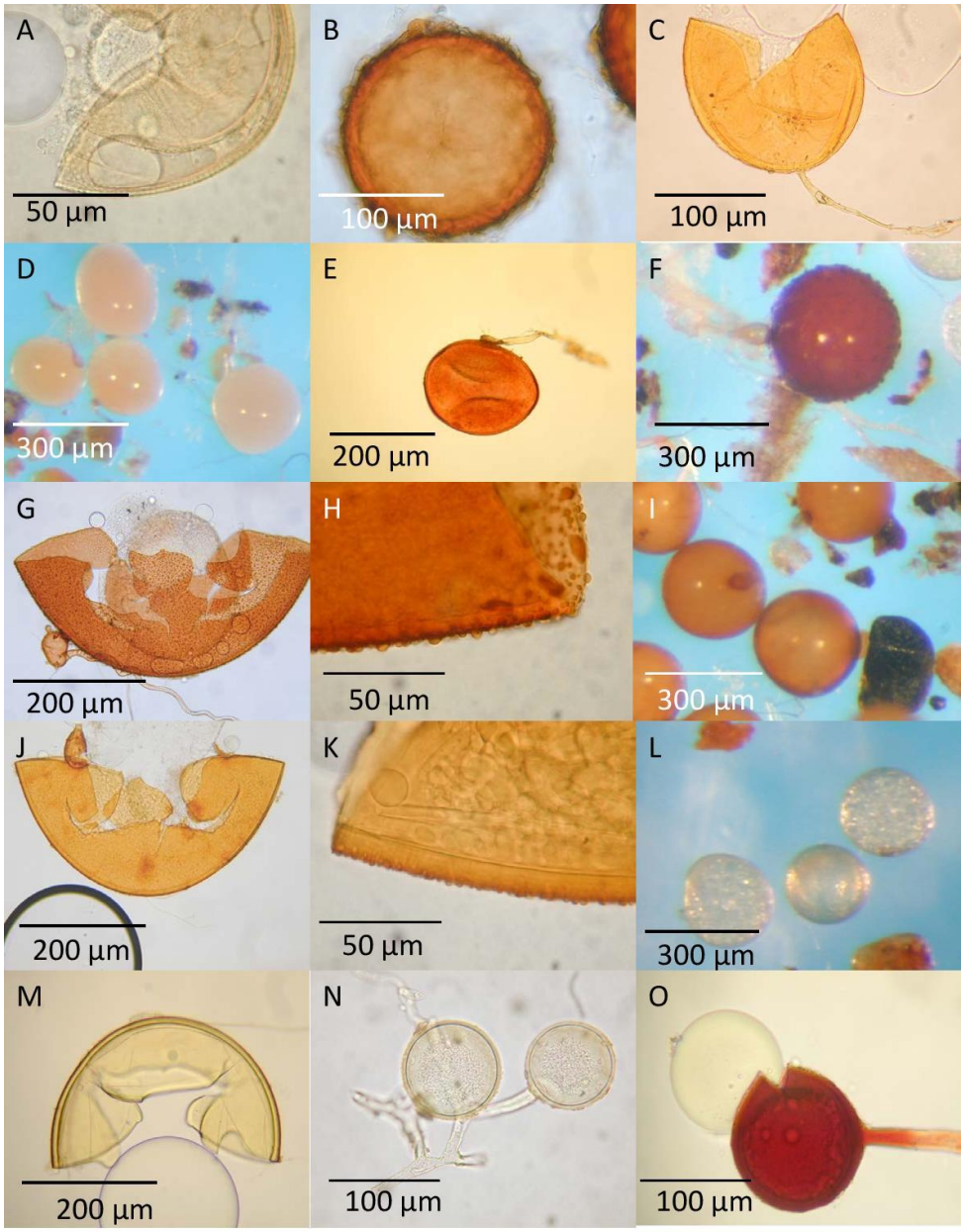

Fig. 3 Glomeromycotan spores from the rhizosphere of *Spinifex sericeus*. A. Crushed *Acaulospora* sp. spore in PVLG showing depressions on the surface and several wall layers. B. Intact spore resembling *Diversispora globifera* in PVLG showing the hyphal peridium. C. Crushed spore in PVLG of a *Diversispora* sp. D-E. *Gigaspora rosea*. D. Pink spores in water. E. Intact spore showing a reaction in PVLG + Melzer. F-H. *Racocetra coralloidea*. F. Spore with ornamented surface in water. G. Crushed spore in PVLG showing the wall structure. H. Crushed spore in PVLG showing large warts at the surface. I-K. *Racocetra* sp. I. Spores in water. J. Crushed spore in PVLG. K. Crushed spore in PVLG, spore showing fine warts at the surface and the inner wall layer. L-M. *Racocetra* sp. (or *Cetraspora* sp.). L. Hyaline spores in water. M. Crushed slightly yellow spore showing two wall layers. N-O. *Rhizophagus fasciculatum* like spore. N. Intact spores in PVLG. O. Slightly crushed spores showing a strong red reaction in PVLG + Melzer.

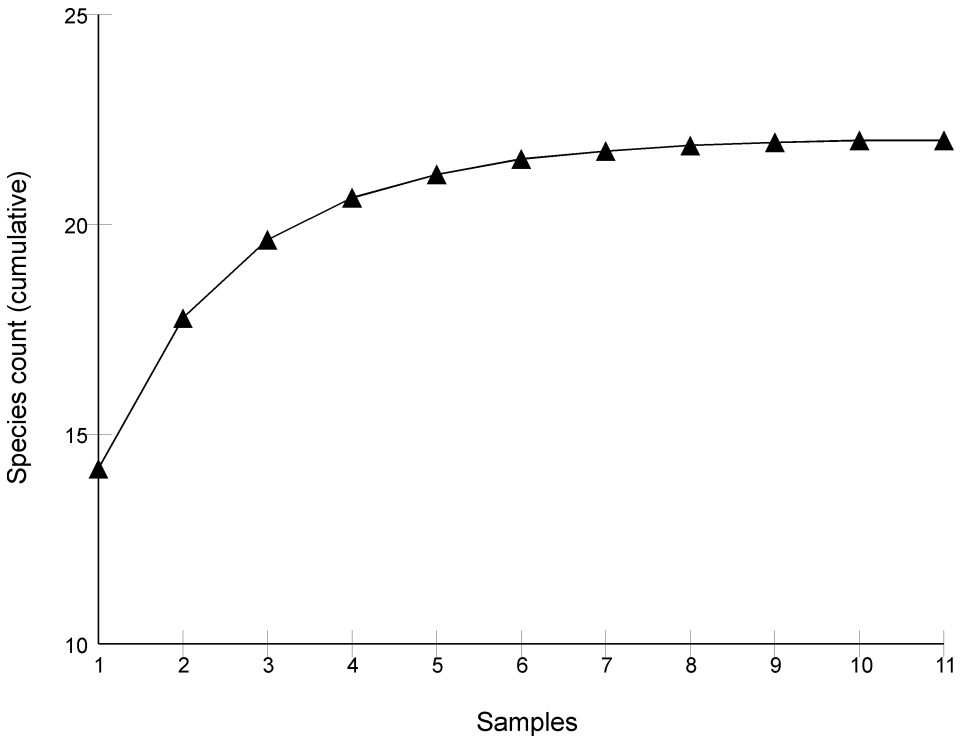

Fig. 4 Species accumulation curve based on operational taxonomic units representing arbuscular mycorrhizal fungi occupying 11 root samples from *Spinifex sericeus.* The samples were collected from a dune at Anawhata, New Zealand.

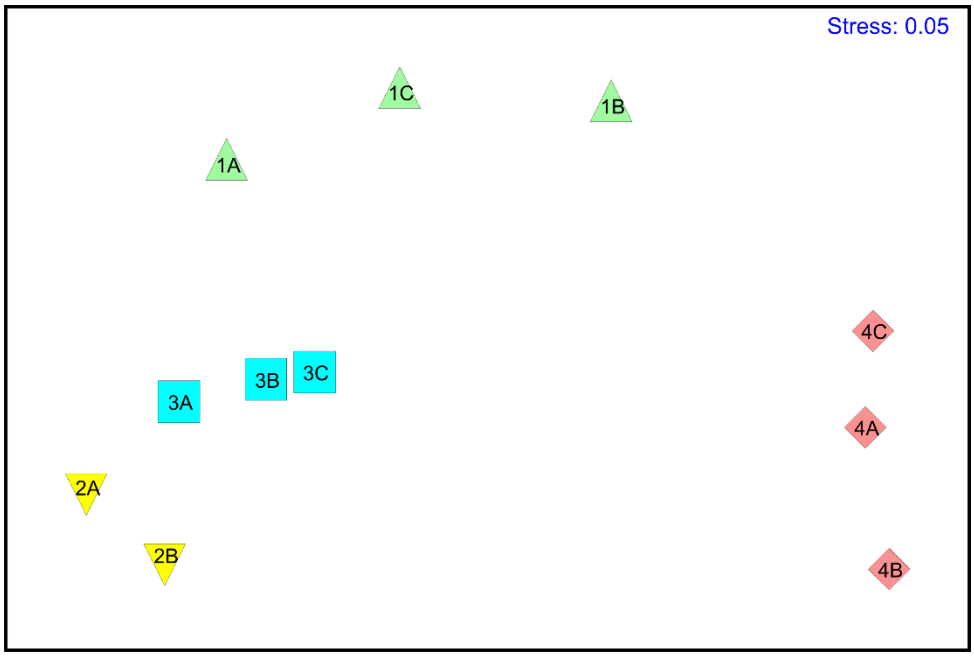

Fig. 5 Spatial patterning of the arbuscular mycorrhizal fungal communities in *Spinifex sericeus* roots along a 90 metre dune section at Anawhata in New Zealand, based on non-metric multidimensional scaling ordination of 11 samples collected at four points. Letters inside the shapes represent individual samples while the numbers and shapes represent sampling points. The final stress value for this solution was 0.05.

Table 1 Substrate chemical characteristics (pH, organic C, total N, and Olsen P) of locations regularly spaced at 30m intervals along a 90m transect on a dune at Anawhata beach.

Transect position	pH	Organic C (%)	Total N (%)	Olsen P (mg/kg)
0 m	6.56	0.04	<0.05	<2
30 m	6.72	0.04	<0.05	<2
60 m	6.92	0.05	<0.05	<2
90 m	7.05	0.06	<0.05	<2

Online resource captions (resources to follow):

Online Resource 1 Table showing spore morphotype names, Fig. 3 reference, genus (and Fig. 2 name) indicated by the SSU sequence and ENA accession number for spores recovered from a dune at Anawhata, New Zealand.

Online Resource 2 Table showing the contigs and OTUs representing arbuscular mycorrhizal fungi derived from sequences generated by pyrosequencing DNA extracted from *Spinifex sericeus* roots collected from a dune at Anawhata, New Zealand with the number of sequences in each contig (derived from combining two matching operational taxonomic units or OTUs) and uncombined OTU (NS31 OTUs from forward reads, AML2 OTUs from reverse reads) by sample. The total number of contigs and OTUs per sample, and the relative frequency of these across all samples combined, is also shown, along with the ENA accession numbers for each OTU.

Online Resource 3 Table showing BLAST matches from GenBank, where at least 80% continuous query cover could be obtained, for operational taxonomic units (OTUs) which do not represent arbuscular mycorrhizal fungi. Samples which generated these came from the roots of *Spinifex sericeus* plants growing along a dune at Anawhata, New Zealand. The identity result for the match, as shown on GenBank, is provided, as well as the phylum of the match. The number of sequences per OTU by sample, and total number of OTUs per sample is also given.

Online Resource 4 Table showing published BLAST matches from GenBank for all contigs (consisting of combined matching operational taxonomic units or OTUs), uncombined OTUs (NS31 OTUs from forward reads, AML2 OTUs from reverse reads), and spore sequences representing the arbuscular mycorrhizal fungal community sampled along a dune at Anawhata, New Zealand. The closest match to a GenBank record from a published study is provided, as well as the identity result, and the accepted taxonomic name (to the level of genus) based on the Fig. 2 phylogeny.

Spore morphotype name	Fig. 3 reference	Spore sequence/Fig. 2 name (genus)	ENA accession #
Acaulospora scrobiculata	A	Acaulospora spore	LN890627
Diversispora globifera	B	Redeckera spore	LN890623
Diversispora sp.	C	Diversispora spore	LN890624
Gigaspora rosea	D, E	Gigaspora spore	LN890626
Racocetra coralloidea	F, G, H	Racocetra spore	LN890625
Racocetra verrucosa	I,J,K	Racocetra spore	LN890625
Gigasporaceae sp.	L,M	Racocetra spore	LN890625
Rhizophagus fasciculatum	N,O	N/A	N/A
N/A	N/A	Glomerales spore	LN890622
Rhizophagus aggregatus	N/A	N/A	N/A
Glomus/Rhizophagus sp.	N/A	N/A	N/A
Glomus sp.	N/A	N/A	N/A

Contig/OTU	ENA Accession #	1A	1B	1C	2A	2B	3A	3B	3C	4A	4B	4C	Total	Frequency
Contig 7 (AML2OTU_11, NS31OTU_11)	LN890586, LN890587	2526	6012	3249	3028	3092	2585	2999	2431	1454	1540	1229	30145	57.37%
Contig 5 (AML2OTU_13, NS31OTU_14)	LN890588, LN890589	241	974	418	1	60	102	345	383	798	1486	1195	6003	11.42%
NS31 12	LN890590	69	930	428	214	147	177	236	153	1252	1342	699	5647	10.75%
Contig 9 (AML2OTU_14, NS31OTU_13)	LN890591, LN890592	258	768	392	348	504	395	421	386	1	0	0	3473	6.61%
Contig 14 (AML2OTU_12, NS31OTU_120)	LN890593, LN890594	0	0	102	348	437	427	424	460	0	0	0	2198	4.18%
NS31 118	LN890595	6	139	60	0	9	6	23	66	184	245	131	869	1.65%
AML2 123	LN890621	13	14	15	130	330	18	48	38	49	88	79	822	1.56%
Contig 6 (AML2OTU_15, NS31OTU_16)	LN890596, LN890597	10	19	581	0	0	0	0	0	0	15	0	625	1.19%
Contig 3 (AML2OTU_117, NS31OTU_18)	LN890598, LN890599	0	1	0	10	1	0	1	3	0	426	0	442	0.84%
Contig 1 (AML2OTU_16, NS31OTU_19)	LN890600, LN890601	13	0	0	1	43	4	0	11	240	41	81	434	0.83%
Contig 10 (AML2OTU_17, NS31OTU_110)	LN890602, LN890603	0	30	133	0	6	27	6	18	21	9	115	365	0.69%
Contig 8 (AML2OTU_112, NS31OTU_114)	LN890604, LN890605	38	189	117	0	0	0	0	0	0	0	0	344	0.65%
NS31 116	LN890606	6	15	6	26	25	75	16	3	9	36	67	284	0.54%
Contig 2 (AML2OTU_18, NS31OTU_112)	LN890607, LN890608	0	0	0	4	0	27	5	40	9	140	0	225	0.43%
Contig 4 (AML2OTU_19, NS31OTU_115)	LN890609, LN890610	16	24	21	5	1	1	7	3	125	5	8	216	0.41%
NS31 111	LN890611	0	0	0	26	150	0	0	0	0	0	0	176	0.33%
NS31 17	LN890612	0	0	0	19	103	0	0	0	0	0	0	122	0.23%
NS31 119	LN890613	0	0	0	5	15	15	9	11	0	0	0	55	0.10%
Contig 13 (AML2OTU_110, NS31OTU_117)	LN890614, LN890615	0	4	30	0	0	4	0	0	2	0	0	40	0.08%
Contig 11 (AML2OTU_116, NS31OTU_125)	LN890616, LN890617	1	0	13	0	0	0	0	1	4	4	1	24	0.05%
Contig 12 (AML2OTU_121, NS31OTU_126)	LN890618, LN890619	0	1	15	0	0	3	0	1	0	0	1	21	0.04%
AML2 119	LN890620	0	2	5	0	0	0	0	0	3	2	2	14	0.03%
Total sequences		3197	9122	5585	4165	4923	3866	4540	4008	4151	5379	3608	52544	
Total contigs/OTUs		12	15	16	14	15	15	13	16	14	14	12	22	

OTU	BLAST match	Match identity	Match accession	Phylum	Sample											
					A1	A2	A3	B1	B2	C1	C2	C3	D1	D2	D3	Total
NS31 15	*Anemarrhena asphodeloides*	99%	JQ283939.1	Anthophyta	0	0	0	0	12	9	2	3	45	18	27	116
NS31 113	*Moniliophthora* sp.	100%	AY916753.1	Basidiomycota	0	0	0	1	27	4	0	1	0	30	6	69
AML2 111	Poaceae sp.	99%	HQ993323.1	Anthophyta	0	0	0	2	1	3	1	2	15	6	2	32
AML2 114	*Prismatolaimus intermedius*	98%	EU040133.1	Nematoda	1	0	0	0	4	0	0	0	10	1	5	21
AML2 124	*Ceratobasidium* sp.	98%	EF154347.1	Basidiomycota	0	0	0	1	7	3	0	0	0	6	0	17
NS31 123	*Prismatolaimus intermedius*	97%	AF036603.1	Nematoda	0	0	0	0	1	0	0	0	7	0	6	14
NS31 122	Alaimus sp.	95%	AJ966514.1	Nematoda	0	0	0	3	0	2	0	0	1	2	2	10
AML2 113	Arthropoda	84%	HM070368.1	Arthropoda	0	0	6	0	0	0	0	0	0	0	3	9
AML2 126	*Tylenchus davainei*	89%	AY284588.1	Nematoda	4	0	0	2	0	0	1	1	0	0	0	8
AML2 122	*Anemarrhena asphodeloides*	99%	JQ283939.1	Anthophyta	0	0	0	0	0	0	1	1	2	1	2	7
AML2 115	*Paramphidelus hortensis*	96%	AY284739.1	Nematoda	0	0	0	2	0	1	0	0	1	1	2	7
AML2 120	Ricinunlei sp.	94%	EF023364.1	Arthropoda	0	0	0	0	6	0	0	0	0	0	0	6
AML2 118	*Tylenchus davainei*	90%	AY284588.1	Nematoda	1	0	0	0	2	2	0	0	0	1	0	6
NS31 121	NA	NA	NA	NA	0	0	6	0	0	0	0	0	0	0	0	6
NS31 127	*Basiria gracilis*	82%	EU130839.1	Nematoda	0	0	0	1	1	3	0	0	0	0	0	5
NS31 124	*Conocybe lactia*	100%	DQ437683.1	Basidiomycota	0	0	0	0	0	1	0	0	0	3	0	4
AML2 125	*Notholca acuminata*	96%	AY218115.1	Rotifera	0	0	0	0	0	0	0	0	2	0	0	2
NS31 128	*Rhabdolaimus* sp.	94%	EF659836.1	Nematoda	0	0	0	0	2	0	0	0	0	0	0	2
				Total sequences	6	0	12	12	63	28	5	8	83	69	55	341
				Total OTUs	3	0	2	7	10	9	4	5	8	10	9	

Contig/OTU/spore	Name (genus) based on Fig. 2	BLAST match accession number	Name provided in GenBank	BLAST match identity score
NS31_12	*Rhizophagus* sp.	HG004531.1	*Glomus* sp.	99%
NS31_17	Glomerales sp.	HF559367.1	*Glomus* sp.	98%
NS31_111, AML2_123	Glomerales sp.	HF559361.1	*Glomus* sp.	99%, 99%
Contig_5, NS31_118, *Glomerales* _ spore	Glomerales sp.	HE799238.1	*Glomus* sp.	98%, 96%, 99%
Contig_3	*Glomus* sp.	KC797122.1	*Glomus* sp.	99%
Contig_8	*Septoglomus* sp.	AB326003.1	*Glomus* sp.	99%
Contig_6	*Glomus* sp.	HE615067.1	*Glomus* sp.	99%
NS31_116	*Rhizophagus* sp.	JN009217.1	Glomeromycota sp.	98%
Contig_14	*Rhizophagus* sp.	HE798825.1	*Glomus* sp.	89%
NS31_119	*Rhizophagus* sp.	AB749524.1	*Glomus* sp.	93%
Contig_7	*Rhizophagus* sp.	FR693585.1	*Glomus* sp.	99%
Contig_9	*Rhizophagus* sp.	KF916661.1	*Glomus* sp.	96%
Contig_4, *Redeckera* _spore	*Redeckera* sp.	HG972877.1	*Redeckera* sp.	99%, 99%
Contig_1	*Diversispora sp.*	FR686956.1	*Glomus trimurales*	99%
Contig_2	*Acaulospora* sp.	JN252439.1	Acaulosporaceae	99%
Contig_12	*Racocetra* sp.	AJ306444.1	*Scutellospora weresubiae*	94%
AML2_119	*Racocetra* sp.	AJ871275.1	*Scutellospora gregaria*	95%
Contig_10, *Racocetra* _spore	*Racocetra* sp.	GU385897.1	*Racocetra tropicana*	99%, 99%
Contig_13	*Racocetra* sp.	AJ306435.1	*Scutellospora fulgida*	95%
Contig_11	*Racocetra* sp.	AF038590.1	*Scutellospora castanea*	94%
Gigaspora _spore	*Gigaspora* sp.	JX297087.1	Glomeromycota sp.	99%
Acaulospora _spore	*Acaulospora* sp.	KF386271.1	*Acaulospora* sp.	99%
Diversispora _spore	*Diversispora* sp.	JX297074.1	Glomeromycota sp.	99%