Microbial community structure and assembly processes in Mediterranean ecosystems: a case study of *Fynbos* (South Africa)

By

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Abstract

The Mediterranean heathland (Fynbos) is world renowned for its levels of plant diversity, endemism, and structure, but also for its mild Pleistocene and poor soils. Majority of microbial studies from the Fynbos have only examined soil sampled from one or two Fynbos vegetation types. In this study, soils were sampled from five Fynbos vegetation types to examine the overall microbial community structure, diversity, and assembly processes. Using 454 Pyrosequencing platform (18S rRNA gene marker for soil nematodes) and. the Illumina HiSeq platform (16S rRNA gene marker for bacteria and ITS 1 region for fungi). A Non-metric dimensional scaling (NMDS) drawn from the environmental Euclidian distance showed that soils from the different Fynbos vegetation types were significantly different. Firstly, this study looked at the nematode community structure, diversity, and which assembly processes were important. The detected phylogenetic signal showed that nematodes in the Fynbos were ecologically coherent (tend to co-occur more than by chance when sampled at random), and the community based on the Bray-Curtis matrix revealed that both SO_4^- and K^+ were delimiting the community structure in the Fynbos. The most abundant feeding group of nematode was the bacteria feeding (BF), however, only the abundance of plant feeding group (PF) was influenced by NH_4 .Nearest taxon index (NTI) revealed that the community was phylogenetically clustered and that ses.MNTD showed that at the local scale deterministic processes are important in assembling the nematode community. Both UniFrac and MNTD matrix were highly influenced by geographical distance and NH_4 respectively. We conclude that in the Fynbos there is niche overlap of nematodes, the phylogenetic community structure also corroborates this, and deterministic processes are important in delimiting the community assembly. A regression analysis showed that OTU richness was significantly influenced by K concentration in Fynbos soil. A look at the fungal community revealed that unlike the nematode community, the NMDS was clustered significantly by

Fynbos vegetation type. A Mantel and partial Mantel tests performed on the Bray-Curtis matrix showed that the fungal community in the fynbos was significantly influenced and delimited by both environment and elevation. Furthermore; elevation, N, pH, and NH_4^+ were shown to be significantly delimiting the community structure. To further capture the community composition, the most dominant phyla were *Ascomycota, Basidiomycota, and Zygomycota*, with only *Ascomycote* and *Zygomycota* showing significant difference across the sites. Conversely, the EcM community in the Fynbos also to an extend clustered by Fynbos vegetation type, however pairwise comparison showed that some Fynbos vegetation were indistinguishable overall there was a significant difference. In the Fynbos the environment is important in delimiting fungal community structure and abundance. This corroborates previous studies on fungi and our study further sheds more light on importance of fungi in ecological process that occur in the soil.

The eukaryote community in Fynbos seems to show some predictability in their community structure and diversity. However, the prokaryote community revealed unique patterns. Firstly, this study looked at what were the diversity patterns before unrevealing the assembly processes. A NMDS of the bacterial community drawn from Bray Curtis distance did not cluster by Fynbos soils. Although, there was no clustering of the bacterial community, Ca was the only edaphic variable that influenced community structure, this is in accordance with previous studies that showed that divalent cations dictate bacterial community structure in Fynbos soils. Mantel and partial Mantel test revealed that both environment and geographic distance influenced bacterial community structure. However, NMDS of the bacterial community drawn from Bray Curtis distance did not cluster by Fynbos soils type. Ca was the only edaphic variable that influenced community structure at the OTU level .Regression analysis revealed that both silt and clay content together with Ca were highly correlated with OTU richness and 16S rRNA gene copy numbers. Of the three most dominant bacterial phyla,

only the relative abundance of *Acidobacteria* differed significantly across all Fynbos soil types, whilst, *Actinobacteria* and *Proteobacteria* did not. Multiple regression on matrices showed that relative abundance of *Bacteroidetes, Chloroflexi, Gemmatimonadetes,* and *Protebacteria* were significantly correlated with total organic Carbon (TOC), silt and clay, SO_4 and Ca respectively. Interestingly, at class level members of *Acidobacteria and Gemmatimonadetes* relative abundance was influenced by Ca and silt and clay. Overall, this study identifies the edaphic variables that influence and shape the bacterial community at different taxonomic levels in the Fynbos. Next, this study looked at community assembly processes that were delimiting the bacterial community in the Fynbos. It was shown by the UniFrac analysis that the community clustered strongly by vegetation types, suggesting a history of evolutionary specialisation into certain vegetation types. The standardised beta mean nearest taxon distance (ses. βMNTD) index, showed no association with vegetation type. However, the overall phylogenetic signal indicates distantly related OTUs tend to co-occur and only a small proportion of closely related OTUs were ecologically coherent. Both NTI (nearest taxon index) and ses.βMNTD significantly deviated from the null models, indicating that deterministic processes governed phylogenetic community structure assembly. Furthermore, ses.βMNTD was significantly higher than null expectations, indicating that over-dispersion in phylogenetic beta diversity is explicable by the differences in environmental conditions across the sites. The community was over-dispersed, attributable to the differences in environmental conditions across sites, even though only weak correlation soil texture could be proved.

Overall, our study indicates that microbial ecology of the Fynbos system is similar to other ecological systems, where both deterministic and stochastic factors govern community structure and assembly.

Keywords: Fynbos, Mediterranean ecosystem, Microbial community, Community assembly processes, Next-Generation Sequencing, Ribosomal RNA gene, ITS region

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ABBREVIATIONS

OTU: Operational Taxonomic Unit

PCR: Polymerase chain reaction

NGS: Next generation sequencing

SRA: Short read archive

rRNA: Ribosomal ribonucleic acid

NMDS: Non-metric multidimensional scaling

PCA: Princincipal coordinate analysis

DNA: Deoxyribonucleic acid

MNTD: Mean nearest taxon distance

ses: Standardised effect size

NTI: Nearest taxon index

PERMANOVA: Permutational multivariate analysis of variance

RDA: Redundancy analysis

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Figure 20 Non-Metric Dimensional Scaling from a) UnFrac matrix which was significantly clustered ($p<0.05$) and b) ses .βMNTD

Figure 21.Frequency estimates for distribution of Nearest Taxon Index (NTI, mean=3.01) and ses.Beta.MNTD (mean=2.95).Each observation is the number of null model standard deviations the observed value is from the mean of its associated null distribution. Ses.Beta.MNTD values <-2 indicate less than expected turnonver; values >+2 indicate greater than expected turnover. Both mean of NTI and Ses.Beta.MNTD were significantly different from expected value of zero for random data ($P<0.01$, t-test)

Figure 22. Regression analysis show that phylogenetic turnover (ses. βMNTD,) was positively correlated to soil texture (clay and silt).

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CHAPTER 1. Mediterranean Soil Microbial Diversity and Recent trends in Microbial Ecology: An introduction

1.1 High levels of endemism and biodiversity

Mediterranean ecosystems are unique with their characteristic climate regimes of mild wet winters, and, hot and dry summers; only occurring in five regions of the world: Cape region of South Africa, the Mediterranean Basin, State of California in the United States of America, central Chile and South to South-western Australia. These regions have conspicuously unusual high levels of plant diversity and endemism that has rendered these regions as 'biodiversity hotspots', with the Cape region of South Africa housing the smallest plant kind floristic kingdom (Cowling 1992; Cuttelod et al. 2009; Mucina and Rutherford 2006; Ojeda et al. 2001).

The coexistence of ecologically equivalent plant species in these regions has been of great interest to ecologists, and, identifying what ecological drivers maintain this coexistence has been a major challenge. Fire and geological stability have been shown to be drivers that maintain these high levels of biodiversity and endemism, paradoxically, factors that are most common thought to be correlated with biodiversity in these ecosystems; regional topography and climate heterogeneity are poor predications of diversity (Allsopp 2014; Cuttelod et al. 2009). Natural disturbance have been accredited with playing a crucial role in maintaining biodiversity in these Mediterranean ecosystems.

Although these regions share similar environmental characteristics, they have different disturbance regimes. All five Mediterranean regions experience drought during the summer months, however, the northern regions (California, Mediterranean Basin and Chile) experience far more severe drought and prolonged drought than the southern regions (Cape region of South African and South to South-western Australia)(Colwell and Huq 1999; Cowling et al. 2003; Mucina and Rutherford 2006). The southern regions lie on stable and

ancient landscapes, which have highly leached soils that are relatively nutrient poor, and they experience far more frequent fires (10-15 years) (Allsopp 2014; Mucina and Rutherford 2006). The combination of the periodic droughts, geologically stable and nutrient poor soils and fire frequency have apparently kept the plant extinction rates low. These factors have also promoted a higher community turnover and diversification in the southern region, furthermore, they have evolved species-rich landscapes in topographically homogenous areas. Natural selection has allowed fine-scale discrimination of habitats and niches under stable and predictable frequent fires, and, this makes the southern regions far more diverse than their northern counterparts (Cowling et al. 2009; Etienne et al. 2006; K. E. Marais et al. 2014; Ojeda et al. 2001).

2 Microbial diversity and analysis

2.1 Initial sequence processing and quality control

Initial sequence processing and sequence quality began with raw sequence files (fastaq and sff formats) obtained from HiSeq and 454 pyrosequencing respectively, and metadata (which contain primer/barcode information as well as the sample identification code). The pyrosequencing samples are initially de-mutliplexed according to the unique primer information and subsequent removal of the adaptors/linker/primer sequences. For both formats, low quality sequence (i.e. maximum homopolymer at 9bp, minimum ambiguous base of 1, and, minimum quality score of 25) are trimmed off. These steps ensure minimum level of nucleotide degeneracy and sequencing errors when sorting out barcode and primer sequences (i.e. to allow one mismatch in barcode and two mismatches in primer sequences).

2.2 Sequence alignment, pre-clustering, chimera removal and classification

The sequences are then aligned against a reference alignment database (SILVA with 51,000 column), using a combination of k-mer and pair-wise alignment in Mothur (Schloss et al. 2009). The aligned sequences are then de-noised with ‘pre.cluster’ command, which applies a pseudo-single linkages algorithm with the goal of removing sequences that are linked to sequencing errors (Huse et al. 2010). Putative chimeric sequences (generated during the PCR which are artificial recombinants between two or more parental sequnences) are detected and removed using the Chimera Uchime algorithm(Edgar et al. 2011) imbedded in the Mothur program. Thereafter, the aligned sequences are classified against a reference taxonomy file using the naïve Bayesian algorithm. Both SILVA (eukaryote) and EzTaxon-e database

provides a representative sequence information (type sequence) as well as strain sequence information from validly published species (Chun et al. 2007; O. S. Kim et al. 2012). Then, closely related sequences are clustered together based on phylogeny and pair-wise sequence similarity. This provides more taxonomically meaningful information than similarity-based OTU clustering. After the sequences are aligned and all the erroneous sequences are removed, a distance matrix is calculated and clustering process generates OTUs at different cutoff levels.

2.3 OTU, Phylotype and phylogenetic analysis

Once an OTU table and species/sample matrix are generated, a myriad of diversity analysis can be performed using various diversity indices. To compare levels of diversity between samples, a standardised number is sequences is used. The aligned sequences then can be used to build a phylogenetic tree, which then can be used to infer phylogenetic diversity, extend of over-dispersion or clustering of lineages, a community distance matrix of the samples, and, to infer community assembly processes. Using the OTU community matrix and the matrix generated from the phylogeny has given rise to the field of phylogenetic community ecology (Webb et al. 2002). The analysis of sequences and further analysis is depicted in Figure 1.

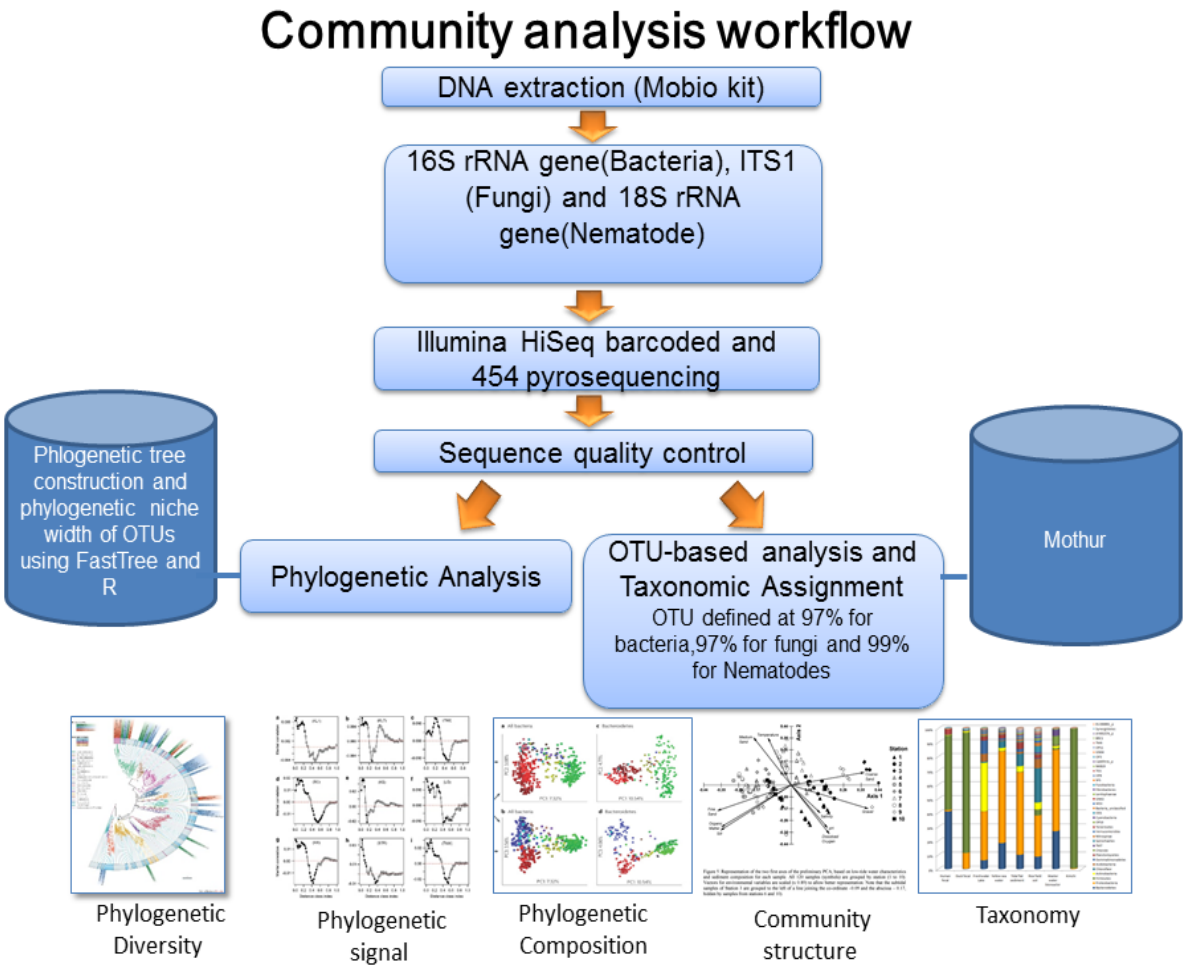

Figure 2 A community analysis procedure and protocol using 454-pyrosequencing, ITS1 region, 16S rRNA and 18s rRNA data.

2.4 Objectives of this study

The Mediterranean ecosystem is recognised as one of the biological hotspots; with its high levels of plant species diversity and endemism, the system is characterised by nutrient poor soils and mild Pleistocene climate (Wintle et al. 2011). The *Fynbos* and *Marquis* are the major vegetation type of the Mediterranean ecosystem in South Africa and Israel respectively. Many of the plant species of the system are obligate seeders and rely on fire for regeneration, climate change is likely to increase the frequency of fires in the system (Allsopp 2014; Cowling 1992; Keith et al. 2008; Wintle et al. 2011). The *Fynbos* and *Marquis* have been widely studied, and the causes of the very high plant diversity have been widely discussed (Cowling 1992; Cowling et al. 2003; K. E. Marais et al. 2014; Mucina and Rutherford 2006; Ojeda et al. 2001). However, little is known about *Fynbos* and *Marquis* microbial community structure, diversity and processes. Some efforts have been made to try and fill this knowledge gap (Bachar et al. 2010; D'Ascoli et al. 2005; J. Fourie et al. 2011; Slabbert et al. 2009; Slabbert et al. 2010b; Stafford et al. 2005). The few studies that exist in these areas have only looked at a specific aspect of soil microbiota; bacteria, archaea, fungi or metazoan. As invaluable are these studies are they do not provide a clear and concise description of the status of soil biota in these systems. Here we investigate the community structure of the soil biota that play an ecologically imperative role in soil biochemistry and processes (bacteria, fungi and nematode) and the roles of niche-based and neutral processes in delimiting these communities. Using the 16S rRNA gene, ITS1 region and 18S rRNA gene datasets that was compiled from the Cape region of South Africa and Mediterranean Basin (Israel). The present study will provide an in-depth understanding of microbial ecology in these two ecosystems, but, also shed more light on what ecological processes are important in assembling microbial communities in Mediterranean ecosystems. This study will fundamentally answer the following questions:

- What is the nature of the microbial community in Mediterranean soils, what environmental variable influence the structure, and, diversity of these microbial communities? Are there any environmental variable that are linked to the differences in microbial community structure in these both Mediterranean regions?
- What is the phylogenetic nature of changes in community structure of microbes in space and locality in Mediterranean soils? Are there distinct phylogenetic signals that reveal the true nature of the microbial communities?
- What ecological community assembly processes act to assemble communities, and, to what extend are deterministic or neutral processes important in Mediterranean ecosystems.

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CHAPTER 2. Sampling, DNA extractions, and soil Analysis

Sampling and site description

Samples were collected from pristine sampling sites within the Table Mountain National Park and Cape Nature Conservation network that are not easily accessible to the public, with the exception of one site which was located within the City of Cape Town Metropolitan area The soil samples were taken from four Fynbos vegetation types: 1) Alluvial (S 33° 58’ 39.65”,E 18° 56’ 39.39”) from its position at the foot mountains and associated with high elevations, it is much wetter with courser sediments and consequently higher levels of leaching. Soils are duplex and dystrophic plinthic catenas and grey rigid sands. Typically referred to as an Entisol, Alluvial soils have slight development where their properties are determined by the parent material (Manning and Goldblatt 2012; Wilding et al. 1983). 2) Limestone (S 34° 37’ 56.19”, E 19° 34’ 41.15”) is found in low hills with plains, with shallow alkaline bedrock, and grey rigid sands on limestone formations. 3) Sand (S 33° 57’ 14.26”, E 18° 29’ 05.30”) is the largest unit sampled found on Quaternary and Tertiary sands of aeolin origin. The soils are generally acidic with deep grey regic sands that are often white (Manning and Goldblatt 2012). 4) Shale (S 33° 56’ 54.99”, E 18° 27’ 19.0002”) with gentle to steep slopes, the soils are mostly clay and clayey loams derived from shale. Like Alluvial , the area is linked to more fog precipitation and orographic rain (Manning and Goldblatt 2012). Finally, 5) Sandstone (S 34° 4’ 55”, E 18° 23’ 55”; S 33° 58’ 39.65”, E 18° 56’ 39.39”) which is the bed stone on which the Fynbos evolved.

Mediterranean soils are by definition soils from Mediterranean climate zones- which alternates between cool moist winters and hot dry summers. Samples were taken in early August of 2013, which is in the winter season when the soils were at field capacity. As, pedogenetic processes occur during the rainy winter season when evapotranspiration is at its lowest, when conditions are perfect for effective dissolution and leaching of soluble elements, and migration of clay particles (Verheyen and De la Rosa 2005).

At each sampling site, sets of five samples were taken within a hectare square; four corners, and geometric centre of the hectare square. At each sample point, five subsamples were taken within a $1m^2$ quadrat, taking a scoop of about 100 g of 0-5 cm depth. The five subsamples were pooled together to make one sample for that $1m^2$. Genomic DNA was extracted within hours of sampling (1-3 hours depending on site location) using the MOBIO Power Soil DNA extraction kit (MOBIO Laboratories, Carslbad, Ca, USA) and to serve as controls random empty vials were chosen and were run following manufacturer's instructions. The isolated DNA was stored at -80°C prior to PCR.

DNA extractions and PCR

Nematodes

To assess the entire nematode community in the fynbos, 100g of fresh soil samples was loaded onto the Baermann funnel for 24 hours. The live nematodes that had migrated down were collected into Falcon tube as described in Viglierchio and Schmitt (1983). Furthermore, nematodes that were less active were captured by sugar floatation (40% w/v solution) and centrifugation (Jenkins 1980). DNA was extracted using the MoBio Power Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA), also to serve as a control empty vials were chosen and were run according to manufacturer's instructions. The isolated DNA was stored -80°.

The extracted nematode DNA from both extraction methods were combined prior to PCR amplication. The extracted DNA was amplified using primers NF1 (*C.elgans* 1226-1250 bp position) and 18Sr2b (*C.elgans* 1567-1588 bp position) towards the 3' end of 18S rDNA with PCR described in (Porazinska et al. 2009; Porazinska et al. 2010). PCR amplification was

performed in 50 µl reactions using the following cycle settings: 95 ℃ for 2 min s; 30 cycles of 95 ℃ for 1 min, 50 ℃ for 45 s, 72 ℃ for 3 min, and 72 ℃ for 10 min.

Targeting a ~400bp region of the 18S rRNA gene. The PCR products were visualised by electrophoresis in 1% agarose gel and were purified using the QIAquick PCR Purification Kit (Qiagen) and quantified using PicoGreen (Invitrogen) spectrofluorometrically (TBS 380, Turner Biosystems, Inc., Sunnyvale, CA, USA). 250 nanograms of purified PCR product of each sample were combined in a single tube and sent for sequencing. The amplified product was pyrosequenced using a 454 GS-FLX Titanium systems (Roche). Control samples showed no presence of genomic DNA when visualised by electrophoresis in 1% agarose gel.

Fungi and Bacteria

Inter transcribed spacer region 1 (ITS1) was amplified using primer pairs ITS1F (5'GAACCWGCGGARGGATCA -3') and ITS2R (5' GCTGCGTTCTTCATGATGC 3') for fungi. PCR reactions were carried as previously reported. QIAquick PCR purification kit(Qiagen) was used to purify the PCR product and PicoGreen (Invitrgen) spectroflourometrically (TBS 380, Turner Biosystems, Inc., Sunnyvale, CA, USA) for quantification. The paired end sequencing was performed at Celemics (Seoul, South Korea) using 2X150bp HiSeq2000 (Illumina) according to manufacturer's instructions. Library and initial quality filtering were performed as previously reported (Zhou et al. 2011).Control samples showed no presence of genomic DNA and when visualised by electrophoresis in 1% agarose gel

Small subunit ribosomal genes were amplified using primers 338F(5'-XXXXXXXX-GTACTCCTACGGGAGGCAGCAG -3') and 553R(5'TTACCGCGG CTGCTGGCAC-3') for the bacterial 16S rRNA gene; where "X" represents the barcode sequence . PCR reactions were carried as previously reported (Zhou et al. 2011). QIAquick PCR purification kit

(Qiagen) was used to purify the PCR product and PicoGreen (Invitrgen) spectroflourometrically (TBS 380, Turner Biosystems, Inc., Sunnyvale, CA, USA) for quantification. The paired end sequencing was performed at Celemics (Seoul, South Korea) using 2X150bp HiSeq2000 (Illumina) according to manufacturer's instructions. Library and initial quality filtering were performed as previously reported (Zhou et al. 2011).Control samples showed no presence of genomic DNA and when visualised by electrophoresis in 1% agarose gel.

The relative abundance of the 16S rRNA gene copy numbers of bacteria were measured by quantitative PCR (qPCR) using primers and PCR conditions as described in (Fierer et al. 2005; Lauber et al. 2008). Standard curves to estimate the 16S rRNA gene abundance were generated using a 10-fold dilution of plasmid containing a full length copy of *E.coli* 16S rRNA gene. The 10µl qPCR mixture contained 5µl of ABgene SYBR Master Mix (ABgene, Rochester, NY, USA), 0.25µl (10pmol μl^{-1}) of both forward and reverse primers, 3.5µl sterile DNA-free water, and 1µl of template DNA (1ng). The reaction was carried out using Eco Real-time PCR system (Illumina, San Diego, CA, USA).

CHAPTER 3. Understanding Drivers of Soil Eukaryote Diversity and Community Assembly Processes in Fynbos Soils

3 Community Structure and Assembly processes of *Nematoda* in a Mediterranean heathland (Fynbos) of South Africa

Introduction

The ubiquitous nature of nematodes makes them an important component of the soil biota, and, they play an integral role in primary production and ecosystem functioning. Recent studies indicate that soil biota may have similar biogeographical patterns as the above-ground biota (Nielsen et al. 2014; Nunan et al. 2003; Wu et al. 2011). Local diversity and assemblage structure of nematodes has been widely documented, however, larger scale patterns and ecological processes of nematode are still poorly understood, especially from the point of view of using modern molecular techniques (Nielsen et al. 2014; Procter 1984; G. Yeates and Boag 2004)

The use of the 18S rRNA gene in documenting metazoan and nematode community richness and structure has in recent years become a useful tool for ecologists (Behnke et al. 2006; Nakacwa et al. 2013; Porazinska et al. 2007; Porazinska et al. 2009; Porazinska et al. 2010; Richards and Bass 2005). The relatively conserved nature of the gene allows for detailed phylogenetic analysis, and in recent years there has been a growing body of work, trying to resolve the discrepancies that exist in the phylogeny of *Nematoda* (Edgecombe et al. 2011; Holterman et al. 2006; van Megen et al. 2009; Zrzavy et al. 1998).

In this study I used partial 18S rRNA sequences to shed more light on the overall diversity, community structure and community assembly processes of phylum *Nematoda* in the fynbos. Previous studies that have compared the effectiveness of using multiple genes and single gene have shown that the use of single 18S rRNA sufficed to reproduce the true phylogeny of the phylum (Holterman et al. 2006; Mallatt and Giribet 2006; Nosenko et al. 2013; van

Megen et al. 2009). Using phylogenies and coupling these with community ecology principles has led to the field of phylogenetic community ecology (Webb et al. 2002). To understand the relative influence of stochastic and deterministic processes, ecologist use null models. In particular null models that couple phylogenetic community composition and randomisations has become common practice (Graham and Fine 2008; Stegen et al. 2012; Binu M Tripathi et al. 2015; Webb 2000; Webb et al. 2002).There are only few studies that have looked at the processes governing phylogenetic community structure assembly in Mediterranean climate system.

Here I investigate the community structure, functional diversity and roles of both deterministic (niche-based) and neutral process play in delimiting the nematode phylogenetic community structure in Fynbos soils. Using an 18S rRNA gene dataset that was compiled from sample sets in five different Fynbos vegetation types, we examined the community structure and phylogeny of nematode communities in these five habitats. The present study aims not only to gain a better understanding of nematology in the Fynbos, but also to discern what underlying ecological processes are important in assembling the community.

We hypothesise that:

1. Each Fynbos vegetation type will harbour distinct nematode communities and have evolved distinct nematode lineages, and the phylogenetic signal (likelihood of related species resembling one another ecologically one another more than they are chosen at random from a phylogenetic tree (Blomberg and Garland 2002)) from the Fynbos will indicate that closely related Operational Taxonomic Unit (OTUs) occupy similar niches, due to conservatism in their ability to adapt and exploit the environment.

2. Nematode community structure will be suited to each Fynbos vegetation type and nematode functional diversity will be greatly influenced by the edaphic variables in each vegetation type.
3. Nematode community assembly in the Fynbos will be influenced by deterministic rather than the neutral processes, just is the case for other microbes (Ofiteru et al. 2010; Stegen et al. 2013; Binu M Tripathi et al. 2015; Wang et al. 2013b)

Statistical analysis

The sequence data obtained was processed following the 454 SOP in Mothur (Schloss et al. 2009). Sequences were aligned using Mothur (default settings: kmer searching with 8mers and the Needleman–Wunsch pairwise alignment method). The sequences were then aligned against the SILVA 115 eukaryotic database, and further filtered to remove gaps. The sequences were then pre-clustered using the Mothur implementation of pseudo-single linkage pre-clustering algorithm 176 from Huse and colleagues(Huse et al. 2010). Putative chimeric sequences were detected and removed via the Chimera Uchime algorithm contained within Mothur (Edgar et al. 2011) in *de novo* mode, which first splits sequences into groups and then checks each sequence within a group using the more abundant groups as reference. .All trimmed quality sequences were classified using SILVA-ARB (database containing aligned 18S rRNA sequences; http://arb-silva.de/download/arb-files/ , September 23, 2013) and using SILVA SSU database for alignment.

All statistical analyses were performed using the R program (R Development Core Team, 2007) and both non-metric multidimensional scaling (NMDS) and analysis of similarity using (PERMANOVA) with 999 permutations (M. J. Anderson 2001) were performed using R

'Vegan' package Dixon (2003) and Oksanen et al. (2013). Further analysis were conducted using the Picante v1.4 (Kembel et al. 2010) package in R. The number of reads was standardised for cross-comparison between samples. Diversity indices such as Faith's phylogenetic diversity (PD) and OTU richness were estimated on the basis of sequence similarity. A Principal Component Analysis (PCA) was created from calculating the Euclidian distance of environmental variables to visualise the difference in soil physical and chemical properties of the each fynbos type.

Phylogenetic signal

A maximum likelihood tree was inferred from all aligned sequences using Fast Tree 2 *(Price et al. 2010)* under the GTR substitution model as previously assessed by (Nosenko et al. 2013) . A maximum likelihood tree was inferred from all aligned sequences using Fast Tree 2 under the Jukes and Cantor model (Price et al. 2010). Using the resultant tree, Mantel correlograms were used to evaluate the phylogenetic signal in the 18S rRNA gene across a range of phylogenetic depths and significance was drawn from 999 permutations (Diniz et al. 2010). Phylogenetic distances were portioned into classes (0.02 units) and the correlation coefficient was relating OTU phylogenetic distance to environmental-optimum distance (Diniz et al. 2010). An environmental-optimum for each OTU was calculated for each environmental variable as in Stegen et al. (2012), Wang et al. (2013b) and Binu M Tripathi et al. (2015). Between OTU optimum difference was calculated as Euclidean distances using optima for all environmental variables. Phylogenetic diversity Faith's PD (Faith 1992) was also calculated using the phylogenetic tree, this index calculates the branch length of the community in each sample using the phylogeny.

Community composition and functional diversity

To visualise the nematode community composition, a non-metric multidimensional scaling (NMDS) and analysis of similarity using (PERMANOVA) with 999 permutations (M. J. Anderson 2001) were performed using R 'Vegan' package. The community was visualised using the un-weighted UniFrac (Lozupone and Knight 2005),the ses.MNTD and Bray-Curtis matrix(Bray and Curtis 1957). A Canonical Correspondence Analysis of nematode community OTU matrix was used to visualise which environment were important in delimiting the community structure.

Classified nematode families were binned into their respective functional feeding guilds using (Bongers and Bongers 1998; H. Fourie et al. 2001; Jordaan et al. 1992; M. Marais and Swart 2013; Ntidi et al. 2012; G. W. Yeates et al. 1993). The relative abundance of the different feeding guilds were visualised and then correlated with environmental variables.

Community turnover and assembly processes

Community turnover was quantified using the UniFrac and the mean nearest taxon distance (βMNTD) (Fine and Kembel 2011; Stegen et al. 2012). βMNTD is the mean phylogenetic distance to closest relative in a paired community of taxa (Fine and Kembel 2011) and is sensitive to the changes of lineages close to the phylogenetic tips. Ses. MNTD was computed as the number of standard deviations that observed βMNTD depart from the mean null distribution (999 null iterations) based on random shuffling of OTU labels across the tips of the phylogeny (Fine and Kembel 2011; Stegen et al. 2012; Stegen et al. 2013; Wang et al. 2013b). This randomisation hold constant observed species richness, occupancy, and turnover. Thus, it provides an expected level of βMNTD given observed species richness, occupancy, and turnover.

To understand and discern which assembly processes were important in delimiting nematode community, Mantel and partial Mantel tests were performed using the community matrices controlling for environment, geographical and elevation distance to determine their relative roles in explaining the phylogenetic community composition in the Fynbos (Wang et al. 2013a; Wang et al. 2013b). A multiple regression on matrices (MRM) (UniFrac, ses. MNTD and OTU matrices) was performed on the (environmental, geographical and elevation) distance matrices (Legendre et al. 1994). Furthermore, a multiple regression on matrix using individual environmental variables made it possible to assess the relative importance of each variable, reverse selection (in which all the individual environmental matrices were regressed against the phylogenetic matrices, and those environmental matrices that were non-significant were the removed.) was performed.

Finally, to infer the phylogenetic community composition, we used the nearest taxon index (NTI) to characterise the community composition within each sample point (Webb *et al.*, 2002). For each community NTI that is greater than two indicates phylogenetic clustering; whilst NTI less than two indicates phylogenetic over-dispersion. A mean NTI of all samples that is greater than 0 indicates clustering and NTI with a mean less that 0 indicates overall over-dispersion.

Deposited 454 read accession numbers

The 18s rRNA data has been submitted to MG-RUST (Meyer et al. 2008)under the accession numbers from 4641277.3-4641300.3

Results

From the 23 samples, at the 99% sequence similarity we obtained from 34,584 high quality sequences, and, after standardising the sequences between samples there were 1008 nematode OTUs identified.

A PERMANOVA of the edaphic variable from each of the five Fynbos vegetation sites indicated that the all the soils had distinct physical chemical properties from one another and they clustered significantly by Fynbos vegetation type ($p<0.05$). The difference in edaphic variable was visualised on a PCA plot (Figure 2). Both Alluvial and Shale soils were the most nutrient rich when compared to soils from other Fynbos vegetation types, and, also had the highest soil texture. Interestingly, soils from Sand Fynbos vegetation type had the highest concentration of Ammonium (NH_4^+). Limestone and Sandstone soils had similar lower pH and sand (%).

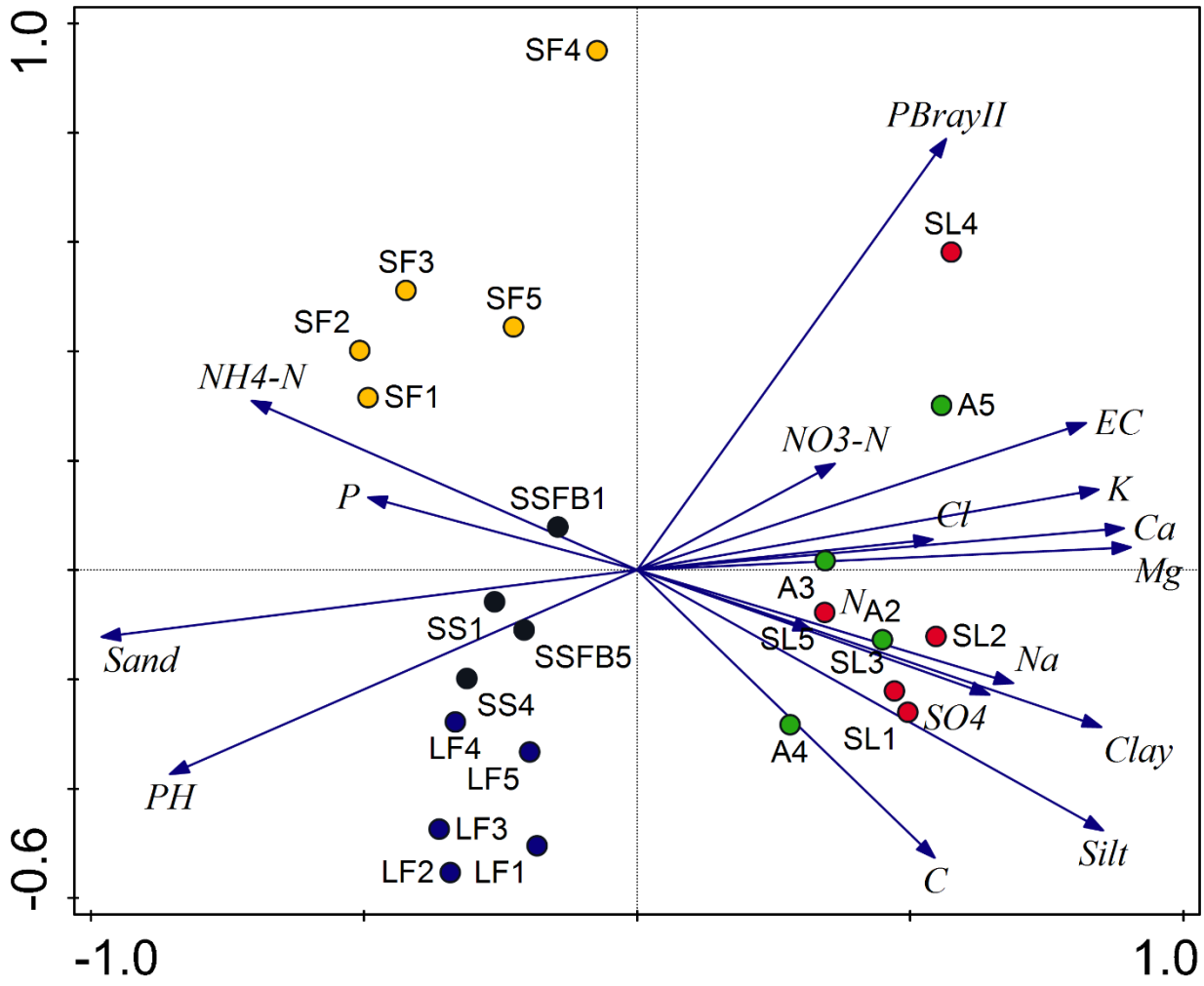

Figure 2. Principal component analysis of environmental variables based on Euclidean distance with “A”= Alluvial Fynbos, “SL”=Shale Fynbos, “LF”=Limestone Fynbos, “SS” and “SSFB”=Sandstone Fynbos, and, “SF”= Sand Fynbos

Phylogenetic signal

The phylogenetic signal using the Mantel correlogram showed significant correlations over short phylogenetic distances ($p<0.05$, Figure 3).The signal indicated that in the five Fynbos vegetation types across short phylogenetic distance the nematodes were ecologically coherent, indicating that closely related OTUs occupy similar niches. At a broad scale phylogenetically closely related nematodes seem to occupy the same or similar niches.

Community composition and functional diversity

The non-metric multidimensional scaling from the UniFrac matrix revealed that samples did not cluster according to Fynbos vegetation type. The community structure based on the Bray-Curtis matrix also revealed that each Fynbos vegetation type did not harbour a distinct nematode community. A community NMDS based on the MNTD matrix further reiterated a similar trend.

A canonical correspondence analysis performed using the community data and the environmental variable revealed that Sulphate (SO_4^-) and Potassium (K^+) significantly influenced the nematode community in the Fynbos (Figure 4, $p<0.05$). The CCA showed that the nematode community in Alluvial Fynbos and Shale Fynbos were positively influenced by (SO_4^-) and (K^+).

The functional diversity (feeding guilds) of nematode in the Fynbos (Figure 5) showed that there bacteria feeding nematodes (BF) were the most abundant group, followed by the omnivore/predator (OP) group with the fungi feeding (FF) group being the least abundant. Interestingly, only the plant feeding (PF) group was statistically different across all the Fynbos vegetation types. Overall, the abundance of BF and PF group were both significantly negatively correlated with NH4+ and only in PF was negatively correlated with Nitrogen in Sand Fynbos (Table 1.)

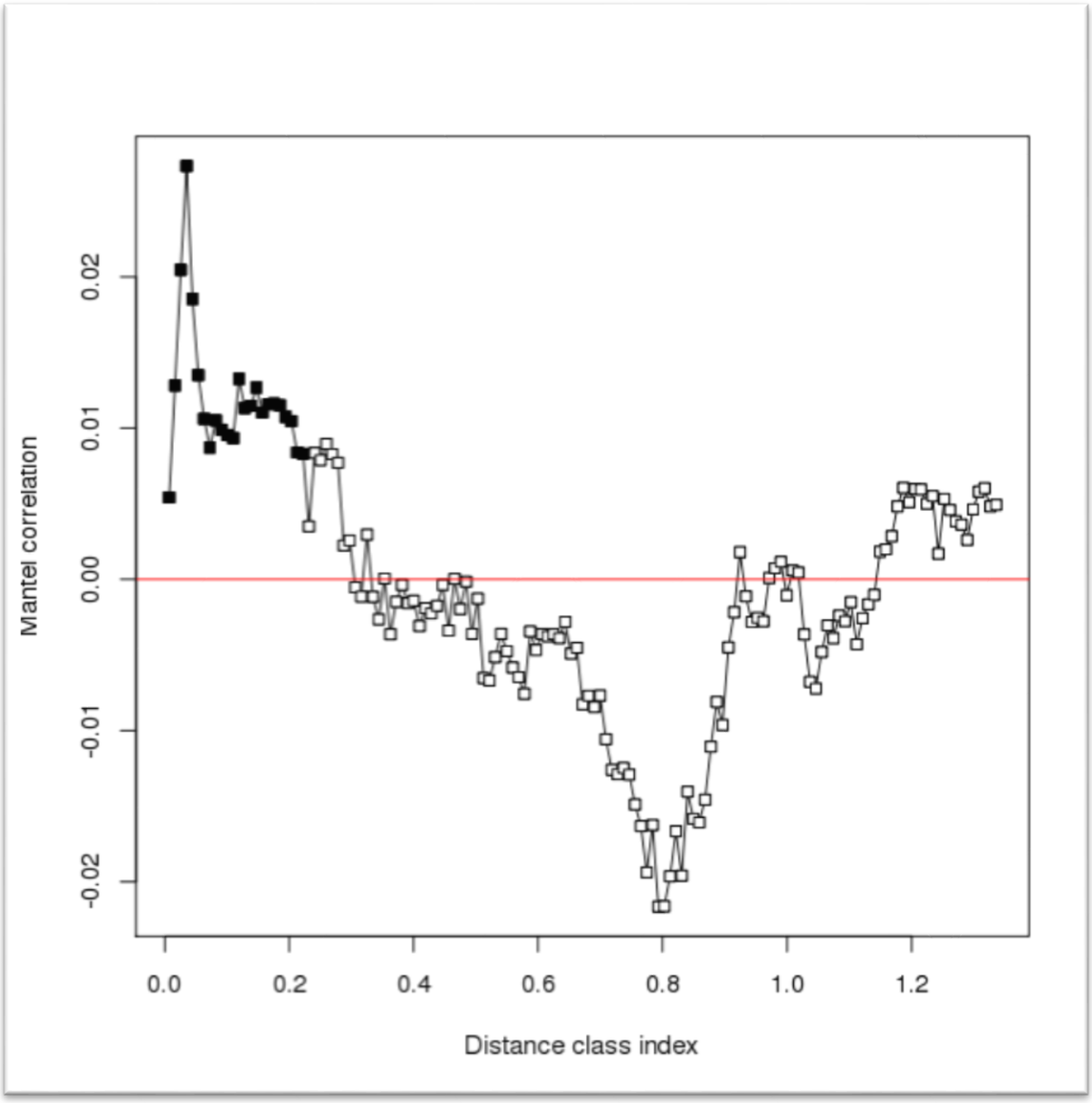

Figure 3. Mantel correlogram between the pairwise distance of OTU niche distance and phylogenetic distance. Significant correlation ($p<0.05$, solid squares) phylogenetic signal in species ecological niches.

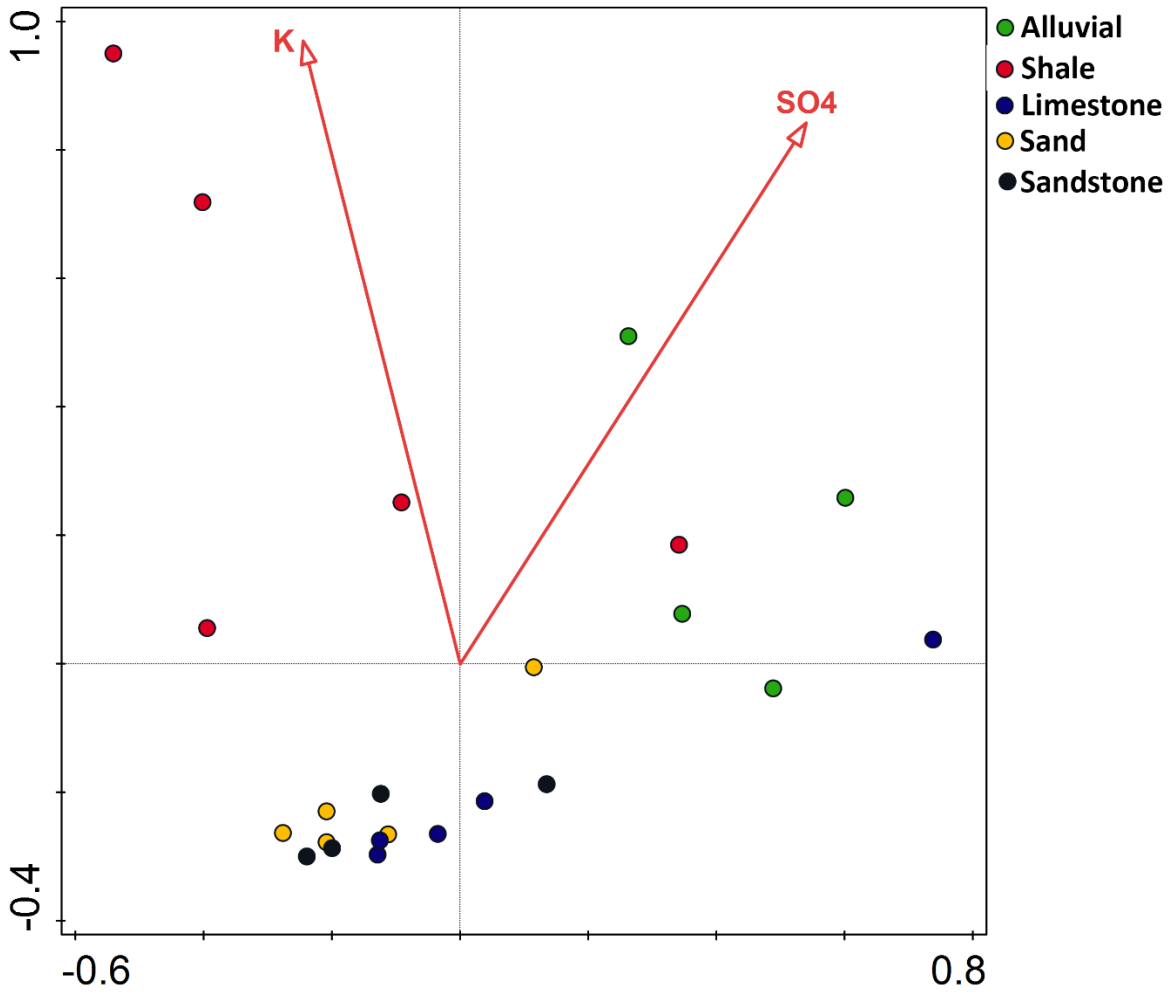

Figure 4 Canonical correspondence analysis of nematode community based on 18S rRNA gene OTUs (99% sequence similarity) with significantly correlated environmental variables

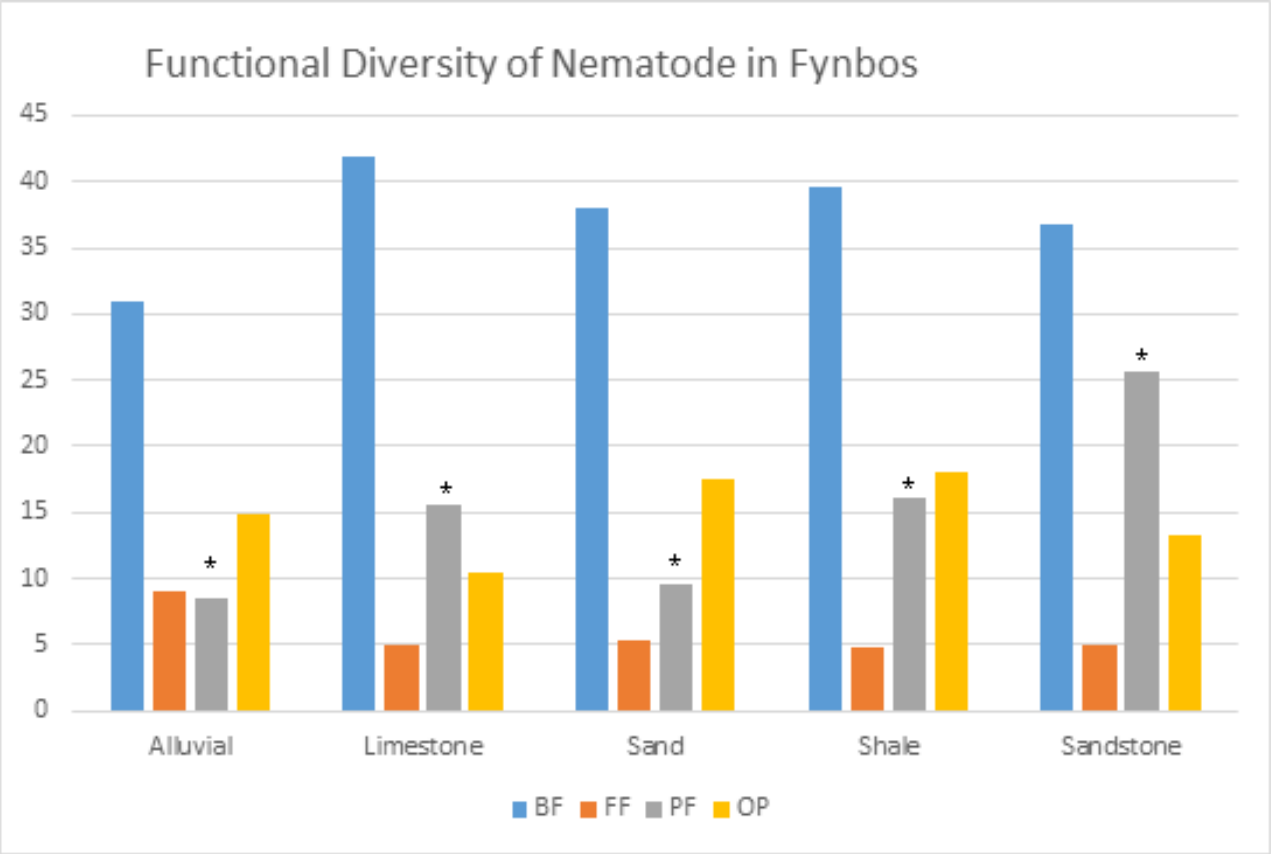

Figure 5. Feeding categories of Nematode within the Fynbos with significant difference in abundance of PF across all sites (where BF= Bacteria Feeding, FF=Fungi Feeding, OP=Omnivorous/Predator and PF=Plant Feeding) (*=$p<0.05$).

Table1. Spearman correlation coefficients between the different feeding groups and environmental variable within the fynbos where(* $p \leq 0.05$, ** $P \leq 0.001$, *** $p \leq 0.0001$ and BF=bacteria feeding, FF=Fungi Feeding, OP=Omnivore/predator and PF= Plant Feeder)

	BF	**FF**	**OP**	**PF**	
NH_4^+	-0.46*			-0.46**	

Community turnover and assembly processes

A T-test revealed that the overall NTI index mean significantly deviated from zero, indicating that the phylogenetic community composition was clustered (t_{23} = 6.9416, $P \ll 0{,}0001$). Although, not all communities exhibited the overwhelming clustering phenomenon, only 5% of the samples did not significantly deviate from zero (Figure 6).

To infer the underlying processes that govern community turnover and assembly, we found that phylogenetic turnover rates in the Fynbos to be higher than expected And, ses.MNTD mean was significantly different from the expected value of zero (T-test, t_{253} = -958.2552, $P \ll 0{,}0001$,) , indicating that at the local scale deterministic processes governed phylogenetic community assembly in the Fynbos.

The Mantel tests showed that only UniFrac was significantly correlated with geographical distance, and, when controlling for the effects of both elevation and environmental distance, geographical distance was also correlated with of UniFrac matrix (Figure 7).

MRM analysis performed on the MNTD matrix with individual environmental matrices indicated that NH_4^+ was the most important identifiable environmental variable that influenced community assembly in the Fynbos (Table 2). A similar analysis using the Bray-Curtis matrix revealed that, Potassium (K), total Nitrogen (N), and, Calcium (Ca^{2+}) were important

(Table 2).

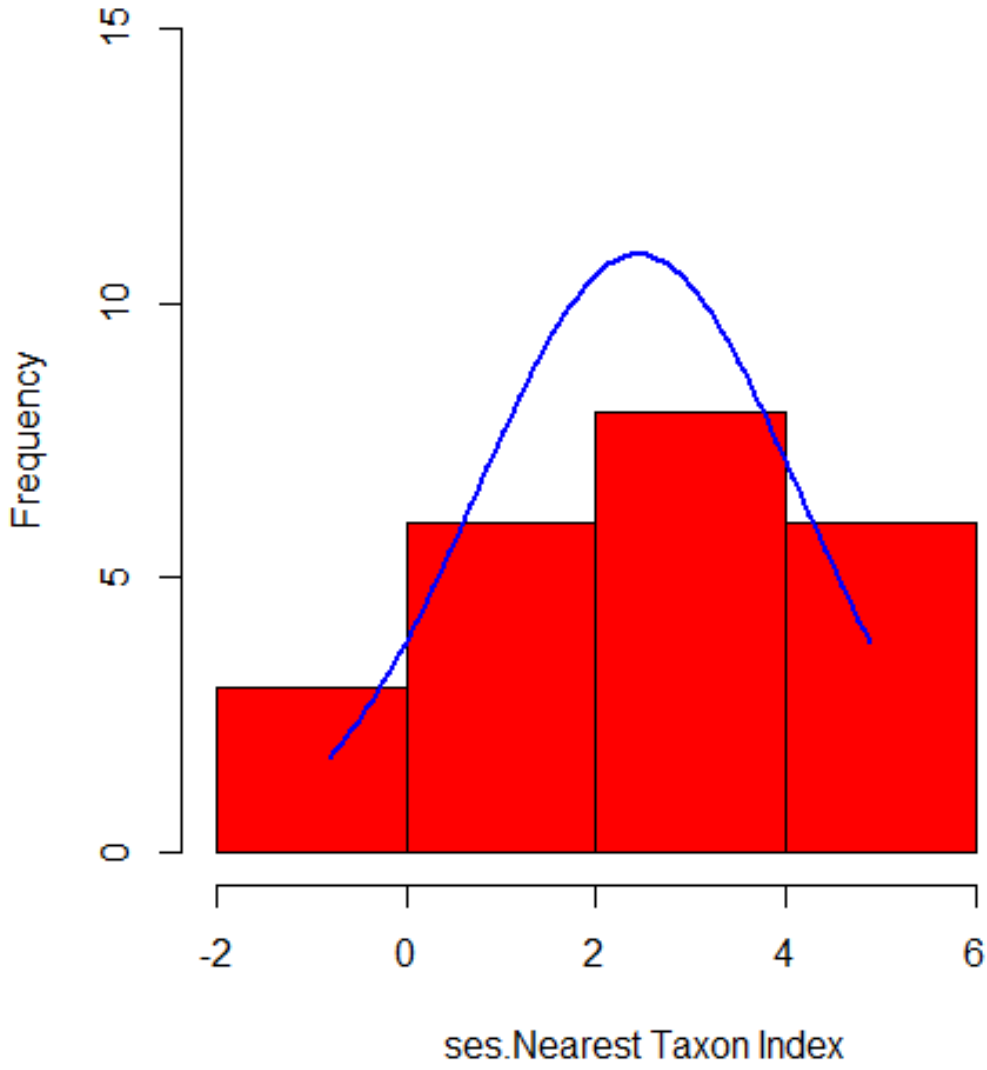

Figure 6.Frequency estimates for distribution of Nearest Taxon Index (NTI, mean=2.43).Each observation is the number of null model standard deviations the observed value is from the mean of its associated null distribution. Ses.NTI values <-2 indicate less than expected turnover; values >+2 indicate greater than expected turnover at the local scale. Ses.NTI significantly different from expected value of zero for random data (P<0.0001, t-test)

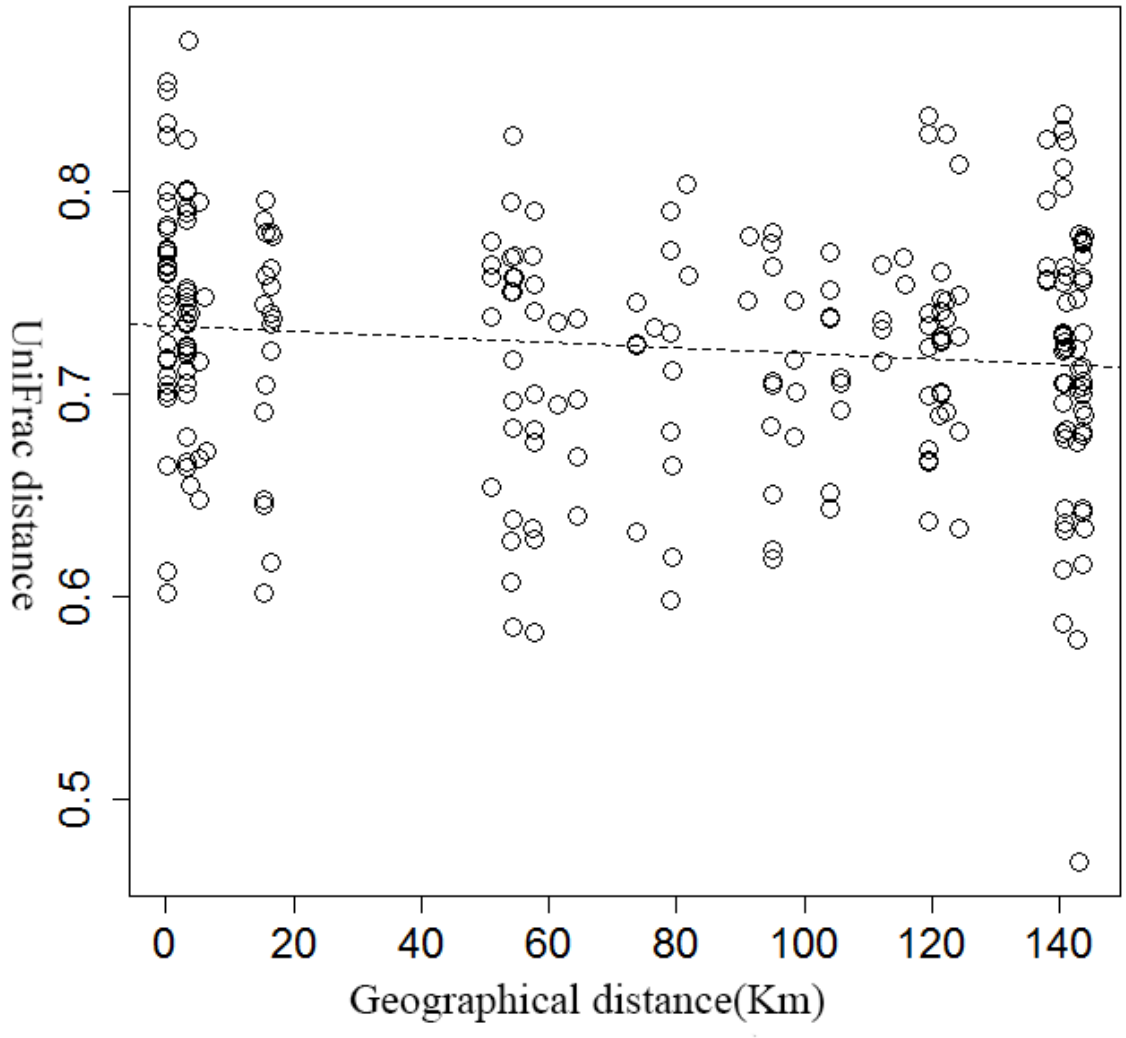

Figure 7. The relationship between unweighted UniFrac and geographical distance for nematode community in the Fynbos based on 18S rRNA gene dataset. The regression slope of the slopes is based on the Gausssian generalised model is shown with dashed line (statistically significant, Mantel test, 999 permutation, $p<0.05$)

Table2. Multiple regression on matrix (MRM) of phylogenetic matrices and the explanatory edaphic variables) with significance levels.

Community Matrix	Edaphic variable	P-Value
UniFrac	Geographical Distance	0.09
MNTD	NH_4^+	0.03*
Bray-Curtis	Calcium	0.05*
	Nitrogen	0.04*
	Potassium	0.02*

Discussion

This study is the first of its kind to investigate the nematode phylogenetic signal within a Mediterranean ecosystem. The use of phylogenetic signals has been widely documented in other microbial studies in order to infer ecological processes (L. C. Anderson et al. 2010; Cavender-Bares and Holbrook 2001; Pontarp et al. 2012; Stegen et al. 2012; Stegen et al. 2013; Wang et al. 2013b). There is overwhelming evidence to suggest that there is a clear relationship between ecological niches and the phylogenetic signal. In this study we found that over short phylogenetic distances nematodes were ecologically coherent. From this signal we can also infer that in the Fynbos the nematode community is phylogenetically conserved, and, phylogenetic niche conservatism seem to play a role within the nematode community. This phylogenetic clustering of closely related nematode in the Fynbos indicates that there niche overlap, in the absence of competitive exclusion of closely related taxa the results will be phylogenetic clustering due to habitat filtering and competition (Horner-Devine and Bohannan 2006).

The nematode community was significantly influenced by sulphate and potassium ions, it has been shown that in agricultural fields were K rich fertilisers were used there was an increase in root-associated nematode (Oteifa 1952; Pettigrew et al. 2005). Both Alluvial and Shale Fynbos vegetation types had the highest nutrient soils when compared to the other sites. It is then not surprisingly that they nematodes communities in these two site were influenced by Potassium. Conversely, SO_4^- fertiliser have been shown to be negatively associated to nematodes populations (Vincent 1979). Interestingly, the relative abundance of plant feeding nematodes was not influenced by neither SO_4^- nor K^+, but, by NH_4^+. Higher levels of NH_4^+ in soils are indicative of higher decomposition rates in the soil (Jenny et al. 1949; Pérez-Harguindeguy et al. 2000). Higher rates of decomposition would in part increase the relative abundance of bacteria feeding groups as there will a higher abundance of bacteria as they are

the primary agents of soil decomposition. However, bacterial feeding nematodes are in most systems always in higher abundance, and complexity of nematode community (presence of other higher trophic groups) has in in the past used as an indicator of soil health and productivity (H Ferris and Matute 2003; H. Ferris et al. 2012; Van Veen and Kuikman 1990). It possible to infer that in the Fynbos, the soils are matured and well developed as there a 'healthy' proportion of higher trophic (PF, FF and OP) across all soils.

The UniFrac matrix is sensitive towards the nodes of the phylogenetic tree and thus can be used to infer deeper evolutionary trends. The phylogenetic community structure based on the UniFrac matrix revealed that the community was not clustered by Fynbos vegetation type, indicating that all the lineages found in the Fynbos co-occurred. This is further corroborated by the MNTD matrix which also revealed a similar trend, MNTD unlike the UniFrac is more sensitive towards the tips of the phylogenetic trees. This clearly shows that in the Fynbos the different lineages have a long evolutionary history of co-occurrence. The ses.MNTD indicated that at the local scale (local scale= each sampling point) deterministic assembly processes (environmental selection) were important, whilst, ses.NTI also indicated that the nematode community in Fynbos was phylogenetically clustered. A mean NTI value greater than two indicates greater than expected turnover (turnover of clades between communities beyond the one expected if species turnover was independent of phylogeny (Olivier J Hardy et al. 2012b)).

To further discern which environmental variable were delimiting the phylogenetic community structure in the Fynbos, Mantel and MRM analysis were performed. Only geographical distance was able to explain the variation in the UniFrac matrix. A distance-decay relationship exists between these two matrices. The MRM showed that NH_4^+ greatly influenced the nematode phylogenetic community structure, whereas, Bray-Curtis matrix revealed that the community composition based on the taxonomic classification was

influences by Ca^{2+}, N, and K^{+} respectively. Overall, this is the first study to incorporate the assembly mechanisms of nematodes. The phylogenetic nematode community structure is clustered with closely related taxa occupying similar niches. It seems to be that in the Fynbos there has been very little phylogenetic divergence (ecological and genetic drift) of nematode lineages, this, further shown by both the UniFrac, MNTD, and Bray-Curtis matrices. All these indicating that there is no distinct nematode community that is adjusted to each of the five Fynbos vegetation types. Though, we know that deterministic processes govern nematode phylogenetic community assembly in the Fynbos, there was no environmental variable was correlated with the phylogenetic index. However, we propose that the geographical distance was important in the initial species sorting as it is the correlated with UniFfrac. This indicated initially neutral processes played a role in assembling communities, but do not seem to play a significant role now. This is in congruence with many community assembly studies that found similar patterns (Langenheder and Szekely 2011; Ren et al. 2015; Wang et al. 2012; Wang et al. 2013a)

3.1 Distinct Fungal Community in Fynbos Soils

Introduction

Edaphic variables and pedogenesis that control the distribution and abundance of plants across Mediterranean ecosystems have been studied for decades, however those that edaphic variable that control the distribution and abundance of micro-organism are still fairly poorly understood. In recent years studies using modern molecular and biomedical approaches have started exploring the soil microbial communities, and the edaphic variable that driving the diversity patterns observed (M. Kim et al. 2013; Martiny et al. 2006; Martiny et al. 2011; Ranjard et al. 2010; B. M. Tripathi et al. 2012; B. M. Tripathi et al. 2014; Binu M Tripathi et al. 2015).

Fungi are as ubiquitous in soil as other micro-organism in the soil matrix, and fungi play an essential role in maintain proper soil health and function. Together with other micro-organism they play an integral role in decomposition and nutrient cycling, but fungi also act as pathogens and their relationships with plants are well known (Rousk et al. 2010). A large portion of the fungal studies have come from tropics, agricultural pastures, and marine environment (Lauber et al. 2008; Rousk et al. 2010). There have been few studies that have attempted to categorise the fungal diversity and patterns in Mediterranean ecosystems.

In other Mediterranean systems De Marco *et al*., (2005) found that fire changes the soil biochemistry and this led to an increase in microbial biomass and soil metabolic quotient (qCO_2). Similarly, Goberna *et al*.,(2012) found that immediately after a fire, there were shifts in bacterial communities and these shifts were correlated to increased microbial biomass, activity, soil desiccation and increased macronutrients concentrations. However, Docherty *et al.*,(2012) indicated that these effects seem to be indiscernible 33 months post a fire.

The objectives for this study were 1) to determine the abundance, taxonomic diversity and composition of fungi in the Fynobs, 2) to determine the community structure of Ectomycorrhizal (EcM) fungi in the Fynbos, and 3) to test whether the fungal community in the Fynbos was affected by any edaphic variable, as suggested by (Fierer et al. 2009).

Sequence analysis and taxonomic analysis

The sequence data obtained was processed following the MiSeq SOP in Mothur (Schloss et al. 2009). All trimmed quality sequences were classified using UNITE database (Abarenkov et al. 2010), and EcM lineages were determined by recent phylogenetic and stable isotope data (Tedersoo et al. 2010).

Statistical analysis

All sampled were standardised by subsampling at randomly to 20,359 sequences per sample for cross-comparison between samples from 3, 036, 010 quality sequences.

The relationship between microbial community Bray Curtis distance and edaphic factors was assessed using the 'Vegan' package in R (R Development Core Team, 2007). Furthermore, a non-metric multidimensional scaling (NMDS) was used to visualise the microbial community structure, the Euclidean distance of edaphic variables and analysis of similarity using (PERMANOVA) with 999 permutations (PERMANOVA) was performed to assess the significance of the relationship (M. J. Anderson 2001).

Operational Taxonomic Units (OTUs; at 97% sequence similarity) and rarefaction values were calculated using the Mothur platform (Schloss et al. 2009). Regression analysis between diversity indices , abundance , and edaphic factors were visualised using SigmaPlot program (Systat Software, San Jose, CA, USA).

To determine which edaphic factors were more important in governing community composition, Mantel and partial Mantel tests were performed using the Bray Curtis matrices controlling for environment, geographical and elevation distance.. Furthermore, a multiple regression on matrix (MRM) was performed on the relative abundance of most dominant bacterial phyla regressing these against the individual edaphic variable matrices.

Deposited ITS1 region accession numbers

The ITS1 region sequence data has been submitted to MG-RUST (Meyer et al. 2008) under the accession numbers from 4641301.3-4641329.3

Results

A NMDS of the environmental variables indicated that the all the Fynbos vegetation types had distinct physical chemical properties from one another and they clustered significantly by Fynbos vegetation type (Figure 8, $p<0.05$) .All the edaphic variables were normalised prior to generating the NMDS, and the NMDS is generated using the Euclidean distance. PERMANOVA results indicated each Fynbos vegetation type was distinct.

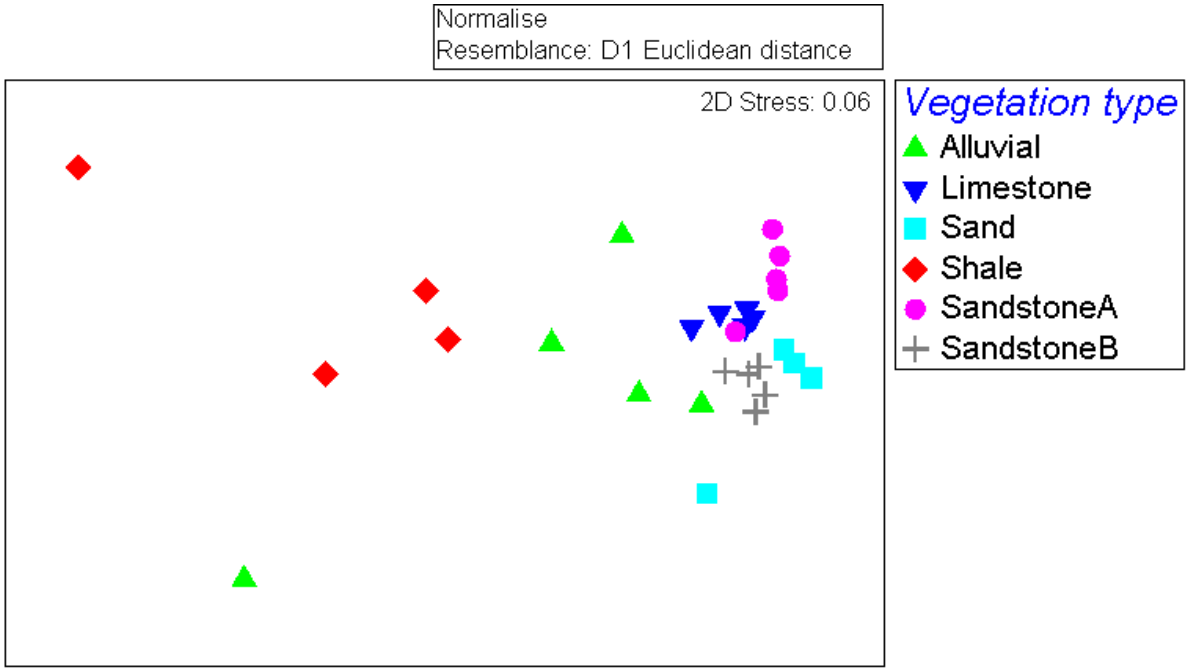

Figure 8 NMDS of normalised Euclidean distance of edaphic variables from the Fynbos, R^2= 0.57 and $p<0.01$. Pairwise comparison revealed that each Fynbos vegetation type was distinct ($p<0.05$).

A regression analysis showed that OTU richness was significantly influenced by Potassium (K^+) concentration in Fynbos soil (Figure 9 $p<0.05$).

The NMDS of pairwise Bray Curtis dissimilarity revealed that fungal communities were clustered significantly by Fynbos vegetation type (PERMANOVA: $R^2=0.63$, $p<0.01$; Figure 9). Also, Figure 9 illustrates all the environmental variable that were significantly delimiting the fungal community in the Fynbos. A Mantel and partial Mantel tests performed on the Bray-Curtis matrix showed that the fungal community in the fynbos was significantly influenced and delimited by both environment and elevation (Table 3).

To further capture the community composition, the relative abundance of the most dominant phyla was calculated (Figure 10). The most dominant phyla were *Ascomycota, Basidiomycota, and Zygomycota*, with only *Ascomycote* and *Zygomycota* showing significant difference across the sites (Figure 3).

The EcM community in the Fynbos also to an extend clustered by Fynbos vegetation type, however pairwise comparison showed that some Fynbos vegetation were indistinguishable overall there was a significant difference (Figure 13).

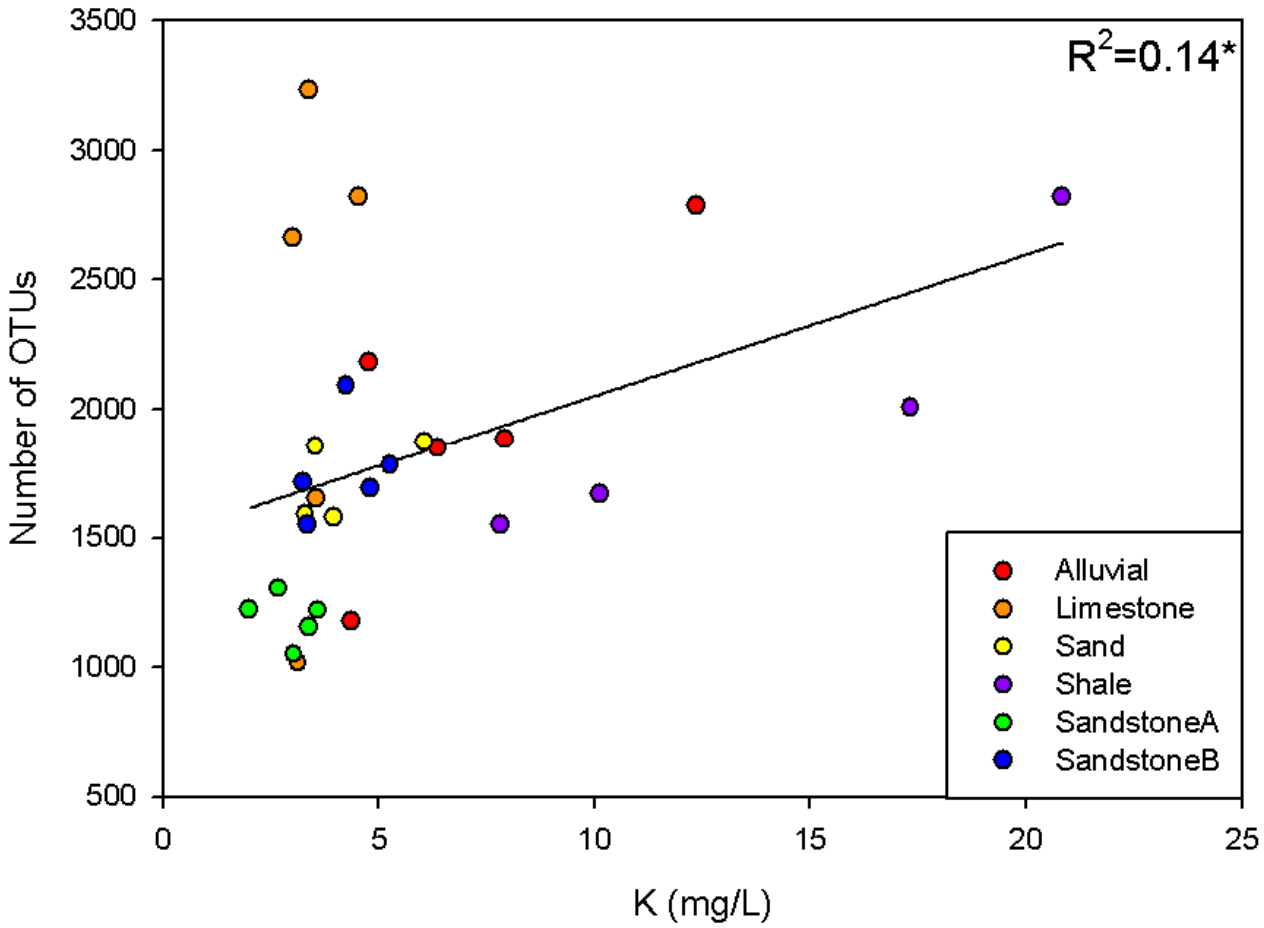

Figure 9. Regression analysis of the OTU richness against Potassium (K) in the Fynbos.

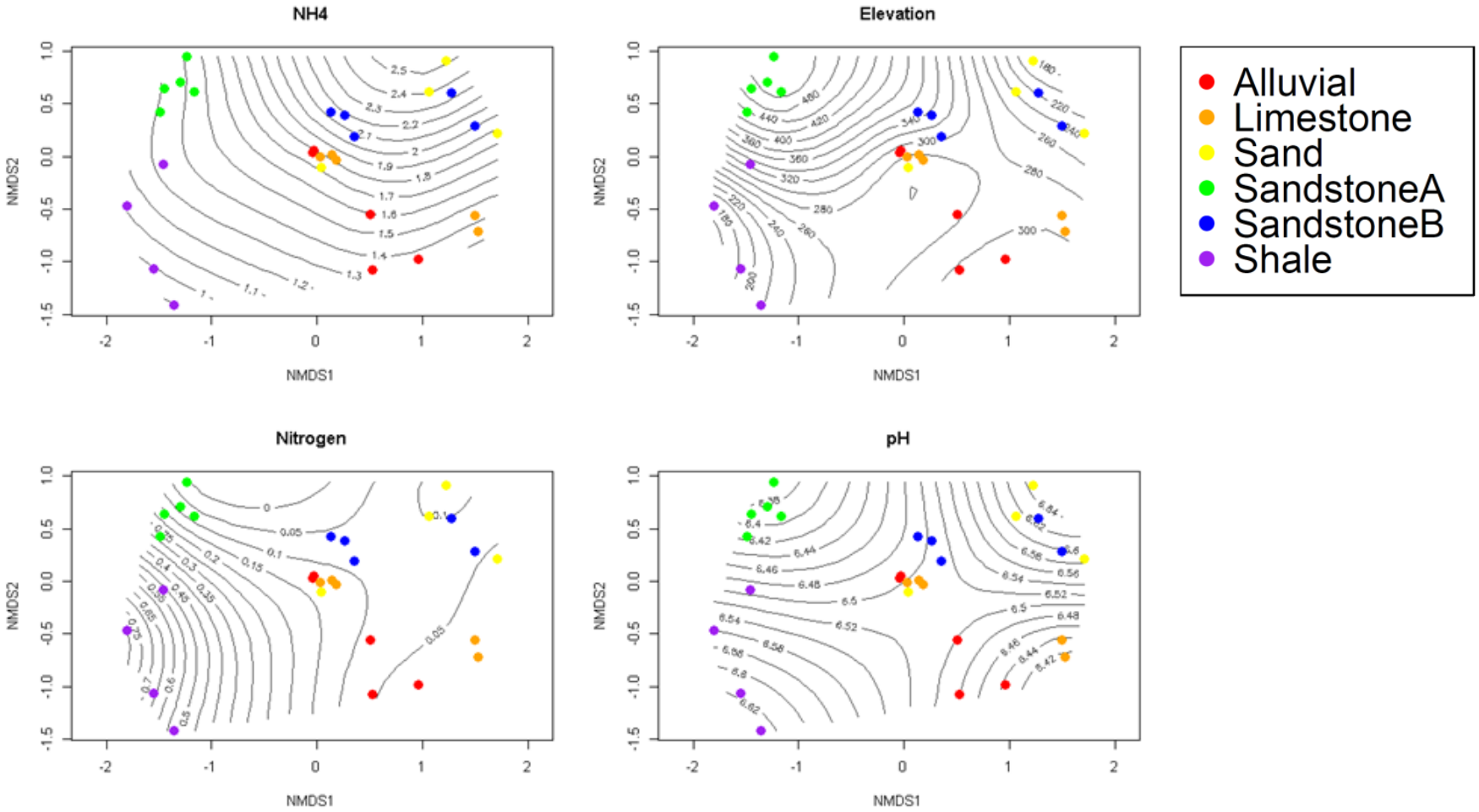

Figure 10 NMDS of fungal community structure in the Fynbos with edaphic variables that delimit community composition ($p<0.05$).

Table 3. Mantel and partial Mantel tests for the correlation between Bray Curtis and the explanatory distances (geographic, environment and elevation) using Spearman's rho in the Fynbos.

Effect of	Controlling for	Fynbos
Geographic		0.1340*
Environment		0.3598***
Elevation		0.2322**
Geographic	Environment and Elevation	0.1134
Environment	Geographic and Elevation	0.3500***
Elevation	Geographic and Environment	0.2422**

Significant level:***<0.001; **<0.01;*,0.05.

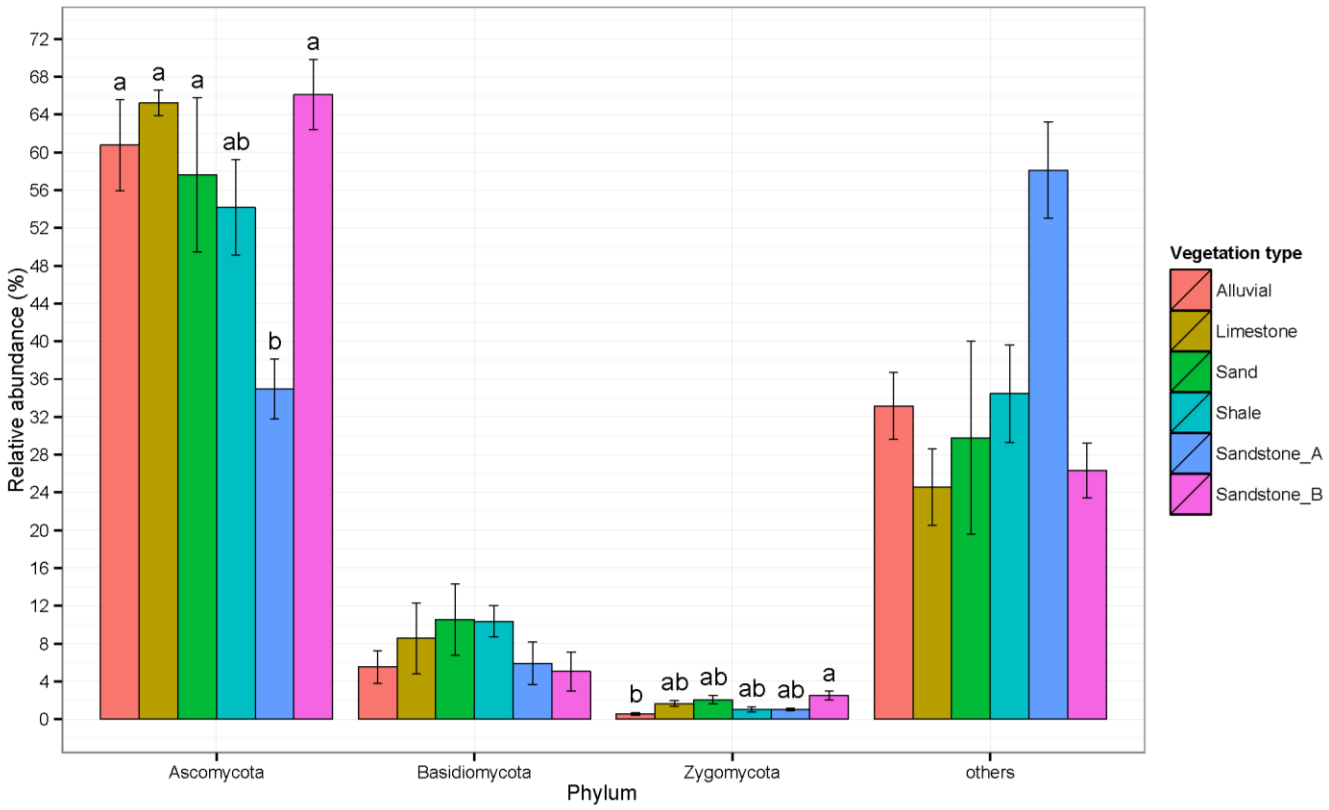

Figure 11. Percentage abundance of the dominant fungal phyla across the different Fynbos vegetation types.

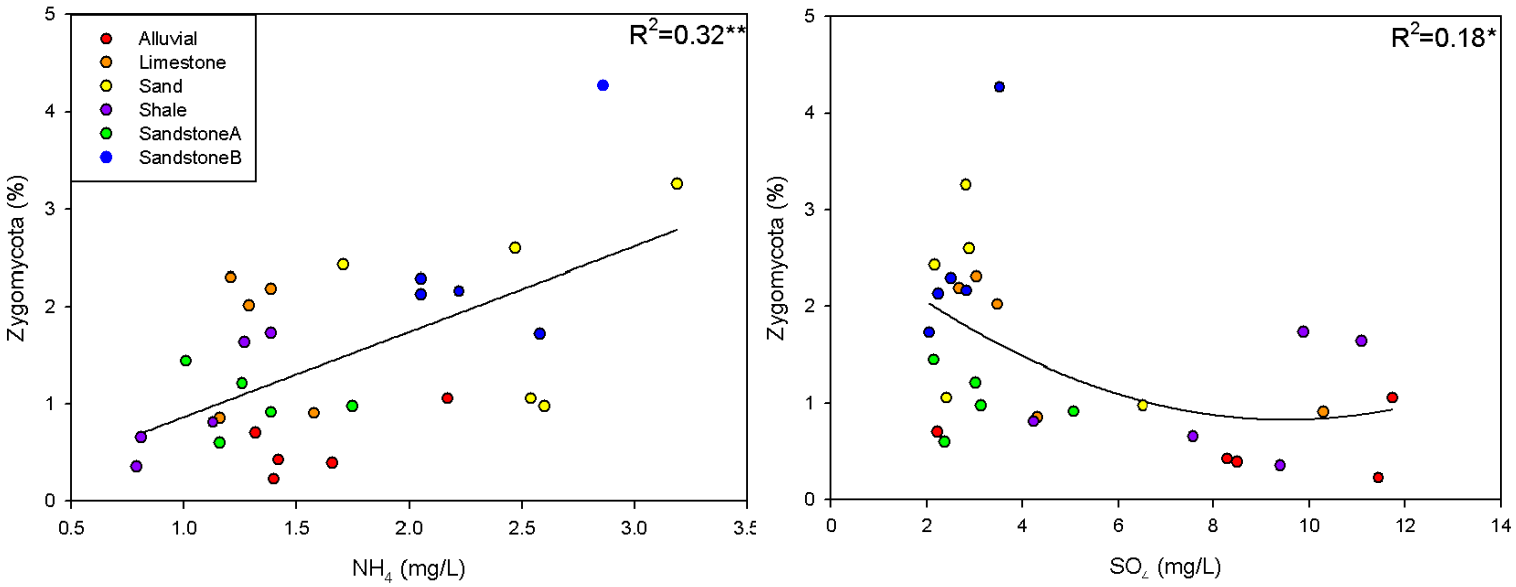

Figure 12 Regression analysis of *Zygomycota* abundance and environmental variables that significantly influence its' abundance.

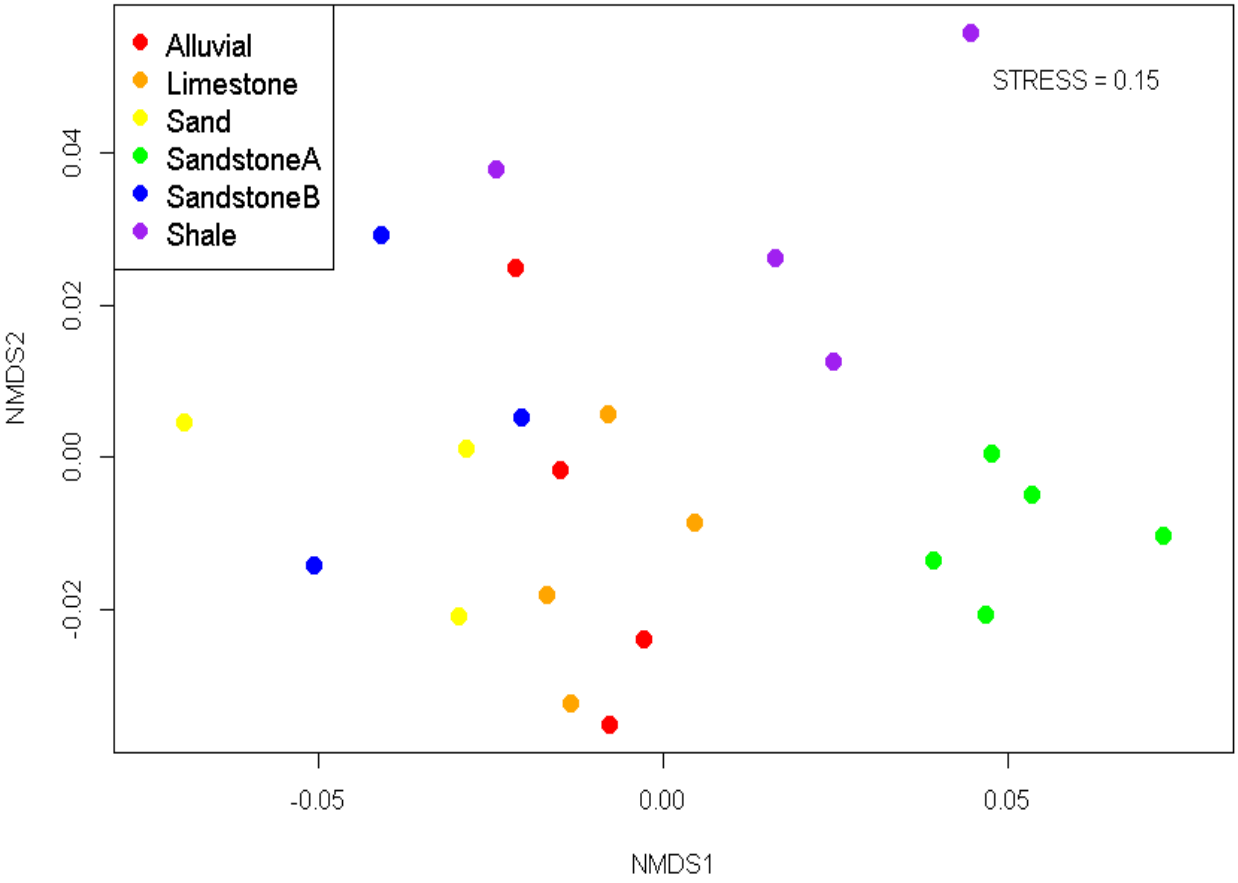

Figure 13 NMDS of the EcM community composition in the Fynbos, PERMANOVA: R^2=0.55, p<0.01.

Discussion

The results showed that in the Fynbos the fungal community is overall adjusted to each Fynbos vegetation type, and that these communities are delimited in their structure by certain edaphic variables

In this study we found that overall OTU richness varied across the different Fynbos vegetation types, and Potassium was positively correlated with richness as seen in figure 9. The role that edaphic variable play on fungal abundance and richness is widely documented (Fierer et al. 2009; Lauber et al. 2008; Rousk et al. 2010). Previously, Jones and Jennings (1965)showed that cations directly affected fungal growth and development, and that K+ stimulates faster growth and increased biomass accumulation. Although, fungal biomass was not measured in this study, it is possible to infer that sites that have higher K^+ concentration will according to (Jones and Jennings 1965) have higher biomass.

The overall community composition of the fungal community was determined using the Bray Curtis distance matrix, and it showed that the each Fynbos vegetation type had its own distinct community structure (Figure 10). In figure 10 it is possible to notice which edaphic variable directly influenced community composition. Another cation that not influences the abundance but also the community structure is NH_4^+, and NH_4^+ has been linked to increased levels of soil decomposition (Jenny et al. 1949; Pérez-Harguindeguy et al. 2000; Porazinska et al. 2003; Van Veen and Kuikman 1990). Similarly, total Nitrogen was found to influence the community structure within the Fynbos. Expectedly, pH significantly structured the community. The role of pH is widely documented (Dolan 2006; Dong et al. 2015; Fierer and Jackson 2006; Fierer et al. 2009; Jagersten 1956; Kennedy and Papendick 1995; Lauber et al. 2008; Martiny et al. 2006; Rousk et al. 2010; B. M. Tripathi et al. 2012; B. M. Tripathi et al. 2014; Binu M Tripathi et al. 2015; Vreulink et al. 2007; Zavarzin 1994). Previous studies have used environment as a proxy for environment showing that even within the same

landscape, elevation seemed to delimit the total community structure (D. Singh et al. 2013; D. Singh et al. 2014; Wang et al. 2012). It is therefore not surprising that in this study we find that elevation strongly influenced fungal community, however in this study we have distinct multiple landscapes (Fynbos vegetation types) and the two (edaphic variable and elevation) are entangled . Further corroborated by the Mantel and partial Mantel test which showed that that environmental distance (calculated as the Euclidean distance of all edaphic variables) and elevation were significantly influencing the community structure (Table 3).

There was a distinct difference in the abundance of the dominant phyla (Figure 11), with only *Ascomycota* and *Zygomycota* varying significantly across all Fynbos vegetation types respectively. Only the variation in abundance of *Zygomycota* was significantly influenced by edaphic variables (SO_4^- and NH_4^+) as seen in figure 12.

The total EcM community in the Fynbos also to an extend clustered by Fynbos vegetation type, however pairwise comparison showed that some Fynbos vegetation were indistinguishable overall there was a significant difference (Figure 13).

Overall, our study showed that in the Fynbos the environment is important in delimiting community structure and abundance. This corroborates previous studies on fungi and our study further sheds more light on importance of fungi in ecological process that occur in the soil.

CHAPTER 3. Bacterial Community Structure and Assembly Processes in Fynbos Soils.

3 Influence of Edaphic Variable on Bacterial Community Structure and Diversity in Fynbos soils

Introduction

The complexity of the soil matrix is often credited with structuring and delimiting microbial composition and diversity (Fierer and Jackson 2006; M. Kim et al. 2013; Lauber et al. 2008; B. M. Tripathi et al. 2012).Understanding horizontal and vertical distribution of bacteria and how it is influenced by soils has been extensively studied (Chu 2011; Cycon et al. 2013; Fierer et al. 2005; Fierer and Jackson 2006; Hanson et al. 2012; Hossain and Sugiyama 2011; Landa et al. 2014; Lauber et al. 2008; Martiny et al. 2006; Rousk et al. 2010; D. Singh et al. 2014). However, within the Mediterranean climate zones most studies have often investigated how different agricultural management practices influence microbial community composition and structure (Garcia-Orenes et al. 2013; Sofo et al. 2010).

The Mediterranean region of South Africa is characterised by a mild Pleistocene climate with a patchy landscape that is a mixture of nutrient poor and nutrient rich soils (Allsopp 2014; Wintle et al. 2011). The Cape Floral Region (CFR) has very high plant diversity, endemism, and the drivers for this high diversity is widely reported (Allsopp 2014; Cowling et al. 2003; Mucina and Rutherford 2006). However, only a few studies have investigated bacterial processes and patterns (Slabbert et al. 2010b; Slabbert et al. 2010a; Slabbert et al. 2014; Stafford et al. 2005). These studies as invaluable as they are, only investigate soils from certain Fynbos vegetation types (Alluvial and Sand Fynbos), this study will investigate bacterial communities from multiple soil samples from difference Fynbos vegetation types. Many of the plants species of the CFR are obligate seeders and rely on fire for regeneration, and it is reported that climate change will increase the frequency of fires in the Fynbos

(Allsopp 2014; Bond and Keeley 2005; Dell et al. 1986; Keith et al. 2008; K. E. Marais et al. 2014; Wintle et al. 2011).

In other Mediterranean systems De Marco *et al*., (2005) found that fire changes the soil biochemistry and this led to an increase in microbial biomass and soil metabolic quotient (qCO_2). Similarly, Goberna *et al*.,(2012) found that immediately after a fire, there were shifts in bacterial communities and these shifts were correlated to increased microbial biomass, activity, soil desiccation and increased macronutrients concentrations. However, Docherty *et al*.,(2012) indicated that these effects seem to be indiscernible 33 months post a fire.

Here we investigate the roles of elevation, geographical distance, and edaphic variables in delimiting the bacterial community structure and composition. Using 16S rRNA datasets from soils from four Fynbos vegetation types, we examined total bacterial community of Fynbos soils. This study aims to not only shed more light on bacterial ecology in Mediterranean climate zones, but, also discern which edaphic variables are important in structuring bacterial communities. Our objective was to look at Fynbos soils and to examine how the different edaphic variable delimit bacterial community structure and diversity. Also, to discern the relative importance of elevation, environment and geographic distance on influencing bacteria community structure and diversity.

Material and Methods

Quantitative PCR analysis

The relative abundance of the 16S rRNA gene copy numbers of bacteria were measured by quantitative PCR (qPCR) using primers and PCR conditions as described in (Fierer et al. 2005; Lauber et al. 2008). Standard curves to estimate the 16S rRNA gene abundance were generated using a 10-fold dilution of plasmid containing a full length copy of *E.coli* 16S rRNA gene. The 10µl qPCR mixture contained 5µl of ABgene SYBR Master Mix (ABgene, Rochester, NY, USA), 0.25µl (10pmol μl^{-1}) of both forward and reverse primers, 3.5µl sterile DNA-free water, and 1µl of template DNA (1ng). The reaction was carried out using Eco Real-time PCR system (Illumina, San Diego, CA, USA).

Statistical analysis

The sequence data obtained was processed following the MiSeq SOP in Mothur (Schloss et al. 2009). All trimmed quality sequences were classified using EzTaxon-e reference bacterial taxonomy (Chun et al. 2007).

All sampled were standardised by subsampling at randomly to 3317 reads per sample for cross-comparison between samples. The relationship between microbial community Bray Curtis distance and edaphic factors was assessed using the 'Vegan' package in R (R Development Core Team, 2007). Furthermore, a non-metric multidimensional scaling (NMDS) was used to visualise the microbial community structure, the Euclidean distance of edaphic variables and analysis of similarity using (PERMANOVA) with 999 permutations

(PERMANOVA) was performed to assess the significance of the relationship (M. J. Anderson 2001).

Operational Taxonomic Units (OTUs; at 97% sequence similarity) and rarefaction values were calculated using the Mothur platform (Schloss et al. 2009). Regression analysis between OTU richness, 16S rRNA gene copy numbers, and edaphic factors were visualised using SigmaPlot program (Systat Software, San Jose, CA, USA).

To determine which edaphic factors were more important in governing community composition, Mantel and partial Mantel tests were performed using the Bray Curtis matrices controlling for environment, geographical and elevation distance.. Furthermore, a multiple regression on matrix (MRM) was performed on the relative abundance of most dominant bacterial phyla regressing these against the individual edaphic variable matrices.

Deposited 16S rRNA gene dataset accession numbers

The 16S rRNA gene sequence data has been submitted under the NCBI SRA accession number SRP049314.

Results

From the 20 samples, at the 97% sequence similarity we obtained 15,627 OTUs from 262,084 high quality sequences. Rarefaction curve of OTU distribution indicates that both Shale and Alluvial soils had the highest abundance respectively (Figure 14).

The soils from the four Fynbos vegetation types were distinctly different from each other in both geology and composition (Mucina and Rutherford 2006). There was very little intra-variability edaphic variable from each vegetation type.

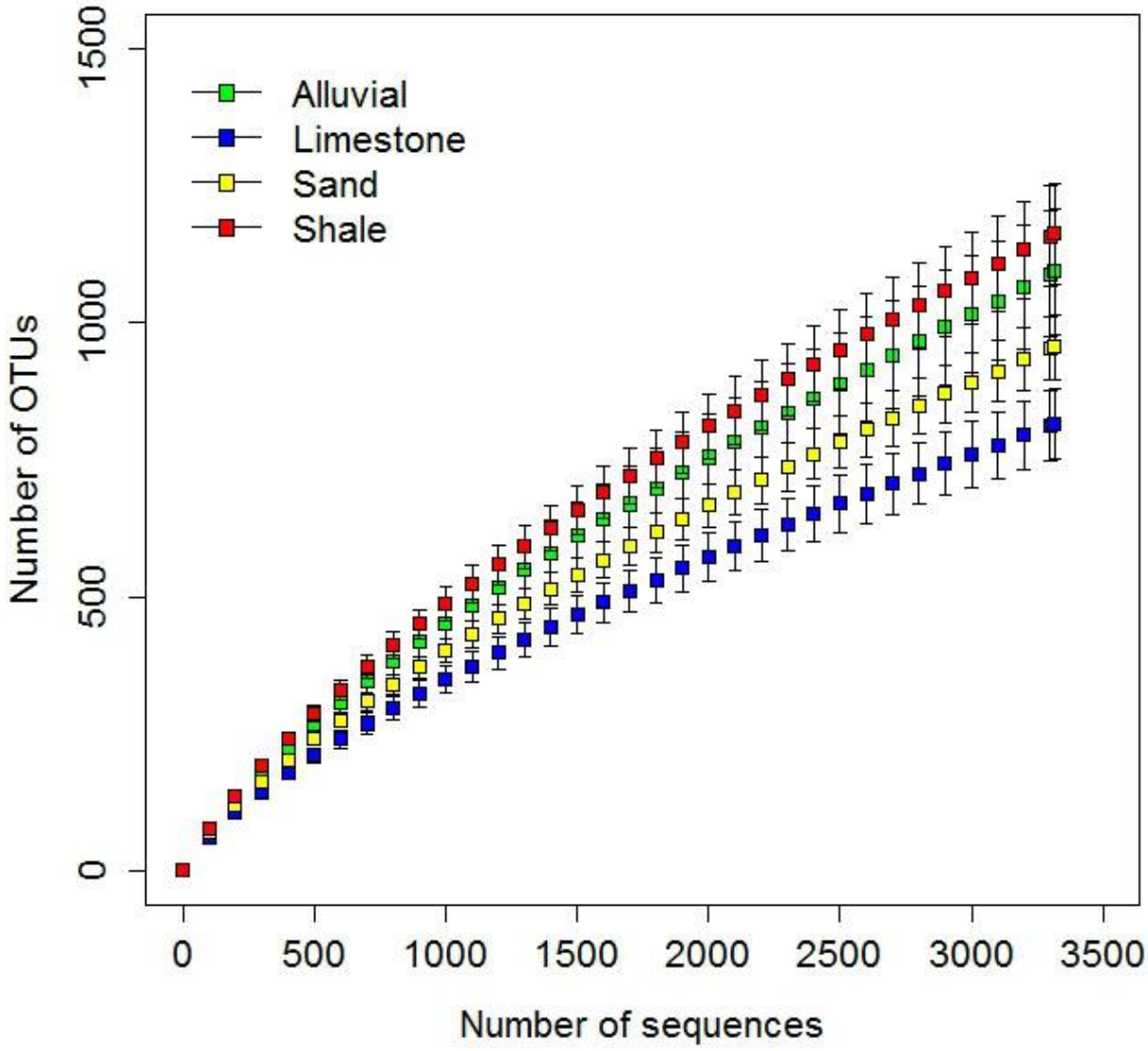

Figure 14. Rarefaction Curve of OTU richness in the Fynbos. At the 97% identity level, the final OTU table consisted of 262 087 sequences distributed into 15 627 OTUs

The NMDS drawn from the Euclidean distance of the edaphic variables showed that there was a significant clustering of individual samples by vegetation type (Figure 2 and 8, $p \leq 0.05$). Environment and geographical distance at each Fynbos vegetation type significantly correlated with bacterial community structure using a Mantel and partial Mantel tests (Table 4). Despite differences in edaphic properties, the bacterial community did not cluster by vegetation type, however, Calcium (Ca^{2+}) was the only edaphic variable that significantly influenced the bacterial community structure (Figure 15 , $p \leq 0.05$). Furthermore, Ca^{2+} was strongly correlated with OTU richness (R^2=0.44, $P \leq 0.001$; Figure 16), and, 16S rRNA gene copy number (R^2=0.65, $P \leq 0.001$; Figure 16). Soil texture (silt and clay %) was also strongly correlated with OTU richness (R^2=0.41, $P \leq 0.001$; Figure 16), and, 16S rRNA gene copy number (R^2=0.54, $P \leq 0.001$; Figure 16).

There was an overall significant difference in the abundance of most dominant phyla across the different Fynbos soil, *Acidobacteria*, *Chloroflexi*, *Bacteroidetes*, *Gemmatimonadetes* and *Verrucomicrobia* ($P \leq 0.05$; Figure 17). Post-hoc pairwise comparison test (Tukey) showed that there were differences within the different soil types (Figure 17)

However, *Actinobacteria* and *Proteobacteria* which were the most dominant were not significantly different across soil types (Figure 17). To determine which edaphic variable was important in delimiting the relative abundance, we used MRM. Of all the edaphic variables, total organic carbon (TOC) explained the relative abundance of *Bacteroidetes* ($P \leq 0.05$; Table 5), Ca^{2+} only marginally for *Gemmatimonadetes* (P=0.061; Table 5) and for *Proteobacteria* ($P \leq 0.01$; Table 1S), soil texture for *Chloroflexi* ($P \leq 0.04$; Table 5) and *Gemmatimonadetes* ($P \leq 0.001$; Table 5) and sulphate (SO_4^{-2}) for Proteobacteria ($P \leq 0.01$Table 5). Interestingly, at the class level more edaphic variables seem to be influencing the relative abundance of certain taxa (Table S1).

Table 4. Measured edaphic variables for each sample and site, with (*) indicating variables that significantly varied across all sites (C/N= carbon: nitrogen, MAP=mean annual precipitation, and MAPE=mean annual potential evaporation)

Sample	Soil type	Classification	Clay*	Silt*	Sand*	pH	EC *	P *	Na *	K*	Ca*	Mg*	Cl*	SO_4	NH_4*	NO_3*	P Brayll*	N*	C*	C/N*	Silt & Clay*	MAP*	MAPE*	Elevation*
ID	ID	ID	(%)	(%)	(%)		(mS/m)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/kg)	(%)	(%)	(%)	(%)	(mm)	(mm)	(m)
A1	alluvial	LmSa	6.4	8	85.6	6.5	4	0.22	3.44	4.78	3.19	0.99	30.84	2.22	1.32	0.93	19.47	0.02	0.86	43	14.4	400	2130	323
A2	alluvial	Lm	20.4	28	51.6	5.9	8	0.64	9.36	6.37	6.2	3.23	17.62	8.5	1.66	0.69	15.39	0.03	1.79	59.66	48.4	400	2130	323
A3	alluvial	SaLm	16.4	16	67.6	6.6	11	0.49	8.75	7.91	15.28	2.81	22.03	11.45	1.4	0.88	14.48	0.03	2.91	97	32.4	400	2130	323
A4	alluvial	Lm	22.4	20	57.6	7	5	0.14	5.27	4.37	3.57	1.2	13.22	8.29	1.42	0.47	8.84	0.06	1.31	21.83	42.4	400	2130	323
A5	alluvial	Lm	20.4	22	57.6	6.5	21	0.29	11.68	12.35	27.67	6.44	26.44	11.74	2.17	19.44	26.3	0.13	2.09	16.07	42.4	400	2130	323
L1	Limestone	Sa	4.4	6	89.6	6.5	6	0.39	6.69	4.54	2.06	1.33	8.81	10.31	1.58	0.39	1.65	0.09	1.41	15.66	10.4	484	1761	236
L2	Limestone	LmSa	6.4	8	85.6	6.3	5	0.34	7.28	3.55	1.59	1	13.22	4.31	1.16	0.97	0.68	0.01	1.48	148	14.4	484	1761	236
L3	Limestone	Sa	4.4	6	89.6	6.5	3	0.52	5.28	3.01	0.67	0.46	13.22	2.68	1.39	0.58	0.23	0.2	1.77	8.85	10.4	484	1761	236
L4	Limestone	Sa	4.4	4	91.6	6.4	4	0.59	6.88	3.37	0.97	0.69	8.81	3.04	1.21	0.57	0.27	0.1	1.66	16.6	8.4	484	1761	236
L5	Limestone	Sa	4.4	4	91.6	6.4	3	0.5	5.9	3.12	0.89	0.66	8.81	3.47	1.29	0.54	1.65	0.05	1.33	26.6	8.4	484	1761	236
SF1	Sand	Sa	4.4	0	95.6	6.4	5	0.6	3.78	3.29	1.31	0.79	13.22	2.89	2.47	0.48	4.02	0.27	0.27	1	4.4	565	2034	63
SF2	Sand	Sa	4.4	0	95.6	6.7	5	0.76	4.19	3.53	0.52	0.44	8.81	2.41	2.54	2.09	5.23	0.28	0.25	0.89	4.4	565	2034	63
SF3	Sand	Sa	4.4	0	95.6	6.8	5	0.89	4.82	3.95	1.42	0.84	13.22	2.16	1.71	2.92	7.68	0.01	0.44	44	4.4	565	2034	63
SF4	Sand	Sa	4.4	2	93.6	6.6	8	0.79	10.12	6.05	1.51	0.81	22.03	6.52	2.6	0.62	51.43	0.02	0.86	43	6.4	565	2034	63
SF5	Sand	Sa	4.4	2	93.6	6.6	7	0.66	5.42	4.16	1.81	1.13	8.81	2.82	3.19	5.02	11.94	0.01	0.43	43	6.4	565	2034	63
SL1	Shale	Lm	23	28	49	6.7	12	0.23	15.96	7.81	7.61	3.11	26.44	9.4	0.79	3.51	10.27	0.51	4.11	8.05	51	717	1941	205
SL2	Shale	Lm	24	27	49	6.8	16	0.5	15.44	17.32	11.81	5.35	17.62	9.88	1.39	10.76	11.31	0.57	2.68	4.7	51	717	1941	205
SL3	Shale	Lm	24	27	49	6.8	11	0.3	14.72	10.12	5.95	2.85	30.84	7.58	0.81	4.14	10.05	0.61	1.81	2.97	51	717	1941	205
SL4	Shale	Lm	21	22	57	6.6	21	0.6	20.59	20.84	16.32	8.08	35.25	11.1	1.27	1.26	66.09	0.95	5.44	5.7	43	717	1941	205
SL5	Shale	SaLm	17	18	65	6.5	13	0.25	13.7	8.55	7.53	4.2	13.22	4.24	1.13	3.36	11.65	0.44	4.09	9.29	35	717	1941	205

Table 5. Mantel and partial Mantel tests for the correlation between Bray-Curtis and the explanatory distances (geographic, environment and elevation) using Spearman's rho in the Fynbos.

Bray-Curtis	Controlling for	Fynbos
Geographic		0.027
Environment		-0.100
Elevation		-0.040
Geographic	Environment and Elevation	4.765***
Environment	Geographic and Elevation	0.029*
Elevation	Geographic and Environment	0.114

Significant level:*** ≤0.001; **≤0.01;* ≤0.05.

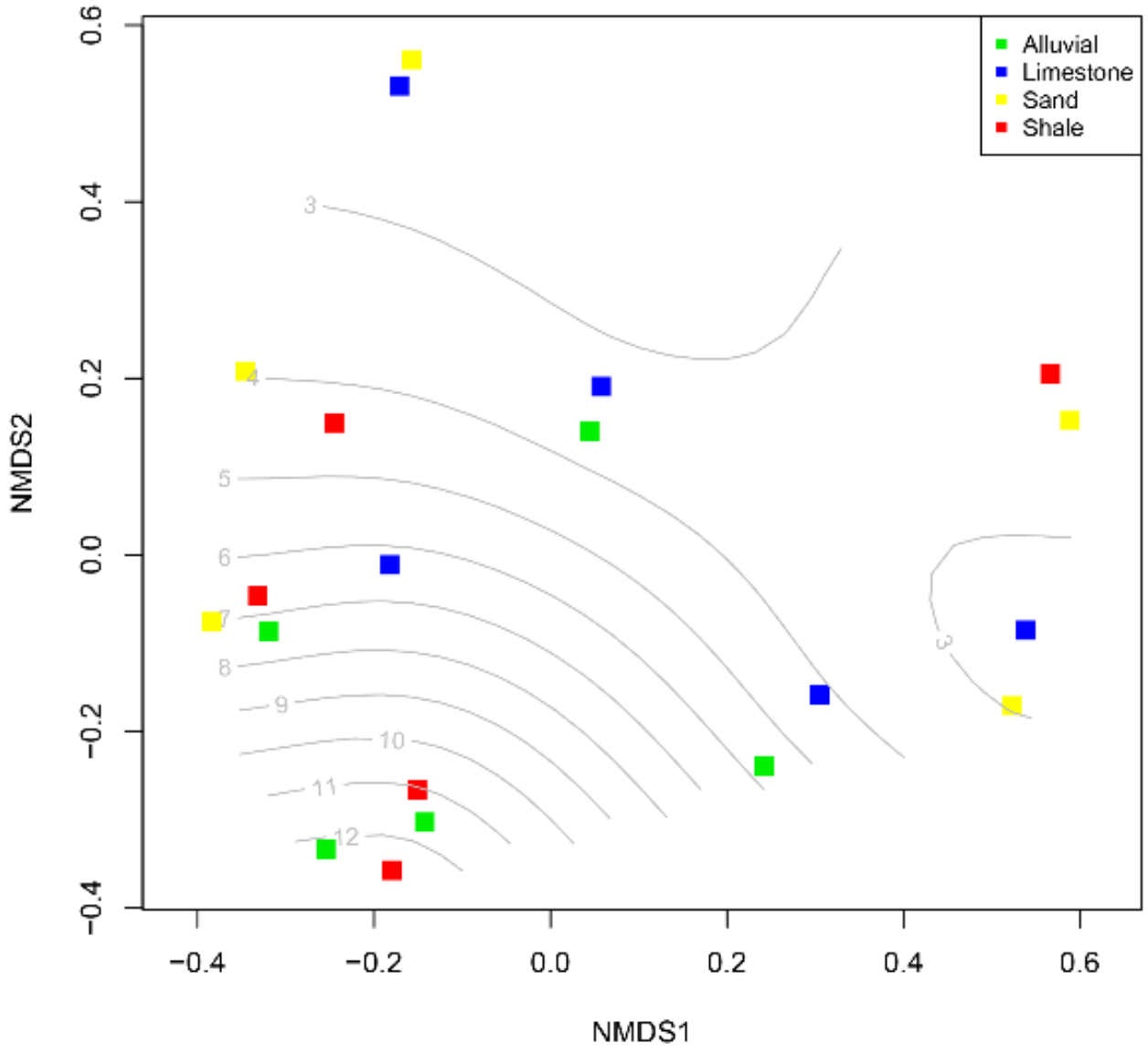

Figure 15. NMDS of bacterial community composition (Bray-Curtis dissimilarity) between different Fynbos soils, the overlaid contour lines shows the Ca^{2+} concentration gradient and how the community composition is affected by Ca.

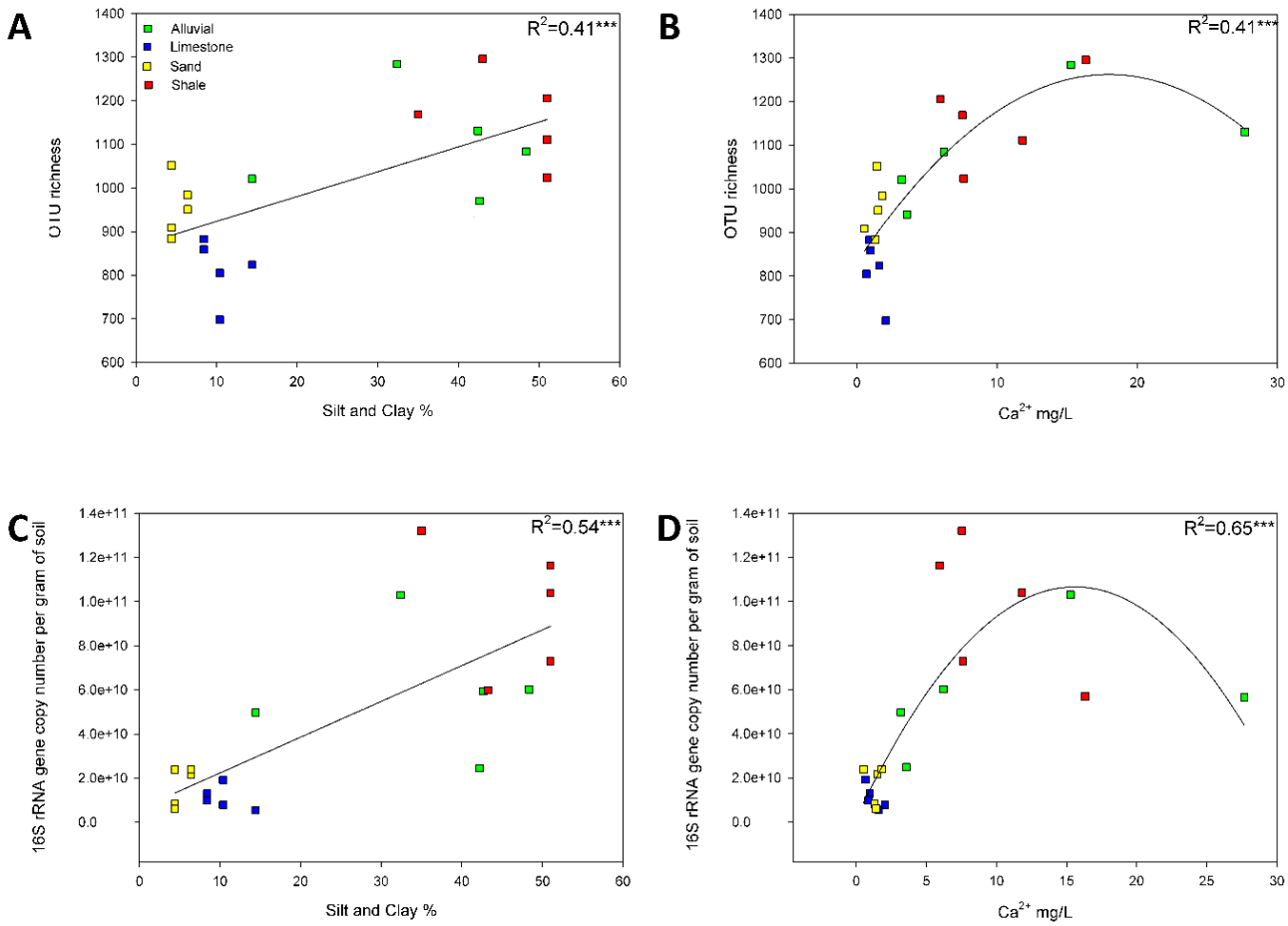

Figure 16 Relationship between OTU richness and soil texture (A) and between Ca concentrations (B), and relationship between 16S rRNA gene copy number and soil texture (C) and Ca concentrations (D). Adjusted R^2 values are shown and the lines represent the best-fit model to the data. Significant level :***P $\leq$0.001; **P $\leq$0.01 ;*P $\leq$0.05.

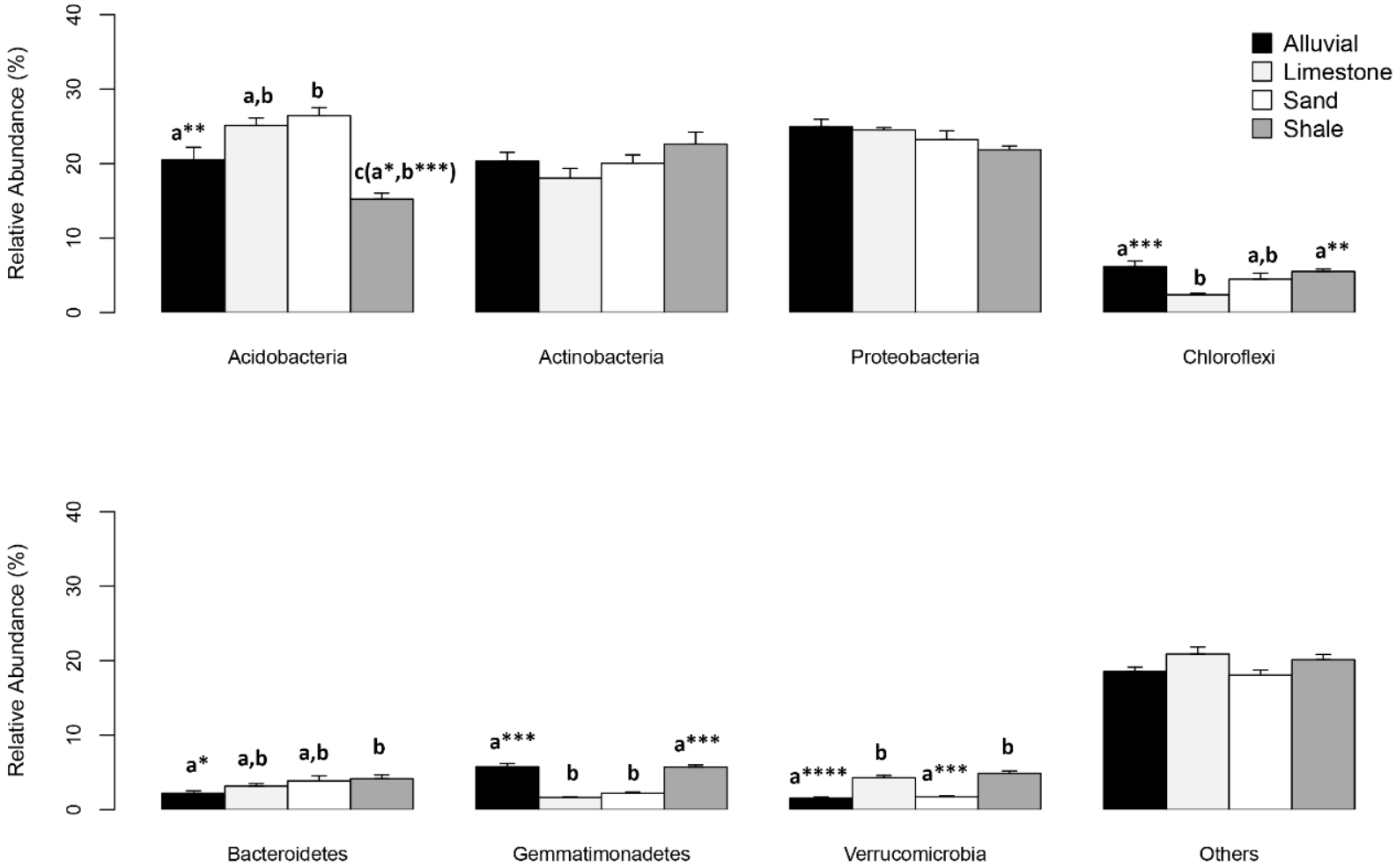

Figure 17 Relative abundance of (mean ± SD) the most abundant phyla in different vegetation types. Post-hoc Tukey pairwise comparisons are shown, different letters denote significant difference between the Fynbos soils. Significant level :***P ≤0.001; **P ≤0.01 ;*P ≤0.05.

Table 6. Multiple regression on matrix (MRM) of dominant phyla and the explanatory edaphic variables) with significance levels.

Dominant Phyla	Edaphic variable	P-Value
Bacteriodetes	Total Carbon (TOC)	0.04*
Chloroflexi	Soil Texture (Silt and Clay)	0.04 *
Gemmatimonadetes	Soil Texture (Silt and Clay)	4.595e-05 ***
	Calcium (Ca)	0.06141
Proteobacteria	Calcium (Ca)	0.006563 **
	Sulphate (SO_4^{-2})	0.015608 **

Significant level:***P ≤0.001; **P≤0.01;* P ≤0.05.

Discussion

Our survey of bacterial community in the Fynbos, revealed that certain edaphic variables were more important in influencing the community structure and relative abundance of taxa. We attribute the documented diversity pattern and richness to the unique edaphic properties at each site. Edaphic properties have been shown in certain systems to be consistent across sites with little variation, even within similar land-use types (Girvan et al. 2003; Jesus et al. 2009; Johnson et al. 2003; Lauber et al. 2008; Murty et al. 2002; D. Singh et al. 2013).However, our investigation revealed that the edaphic properties of soils from each Fynbos vegetation type were significantly different (Fig. 1). Vreulink *et al.*,(2007) also found similar trends when they investigated sandy Fynbos soils. Hopper (2009) described the landscape of the CFR as old, climatically buffered, infertile landscapes (OCBILs) and the region has a long history of leaching. However, the pedogenesis of Fynbos soils includes complex geomorphic processes, and unique vegetation that have resulted in a complex heterogeneous landscape that has a mixture of young fertile and old infertile soils (Allsopp 2014; Cowling et al. 2009; Mucina and Rutherford 2006). Our findings further reiterate the nature and extend of heterogeneous landscapes that exists within the Fynbos. Allsopp (2014) succinctly summarised and surmised the pedogenesis of Fynbos soils, moreover, the influence of plant community structure and litter in influencing edaphic variable is well documented (R. D. Bardgett and Shine 1999; Richard D. Bardgett 2005; Ehrenfeld 2003; Jhonson and ebrary Inc. 2009; J. S. Singh et al. 1989). We propose that the combination of the documented different vegetation types (Mucina and Rutherford 2006) and unique geomorphic processes in the each of the Fynbos vegetation types are key ultimate factors for this observed difference. Furthermore, we suggest that this observed variation in our soils samples is a natural feature of the Fynbos. Interestingly, this difference in edaphic variables

did not produce a bacterial communities unique to soils from each of the four Fynbos vegetation types. Previous studies have found that environment and geographical distance are important in shaping microbial community, albeit at local scale (Martiny et al. 2011; Ramette and Tiedje 2007; B. M. Tripathi et al. 2014). The Mantel tests gives support that both environment and geographical distance are significantly correlated with community structure (Table 2).Though, we found no bacterial community unique to each of the four Fynbos soils, Ca was found to be a factor that influenced community structure. The role that Ca plays in maintaining prokaryote cell structure, motility and sporulation is widely documented (Dominguez 2004; Norris et al. 1996). Previous studies have also highlighted the role of Ca in soils and how it shapes bacterial community (Allison et al. 2007; Jesus et al. 2009; Vreulink et al. 2007; Zornoza et al. 2008).

A highly heterogeneous soil environment has been shown at local scales to affect the distribution of bacteria, and, this fragmentation is known to cause the heterogeneous distribution of bacteria (Bent et al. 2003; Bundt et al. 2001; Grundmann and Debouzie 2000; Nunan et al. 2002; Nunan et al. 2003). Our results showed that in heterogeneous Fynbos soils, there was a significantly high correlation between OTU richness, 16S rRNA gene copy numbers, and silt and clay (Fig.3). Using modern DNA extractions methods, our results corroborate those of Chau *et al.*,(2011) found that relative sand %, and silt and clay content did not influence the amount of genomic DNA extracted, furthermore, they also showed that bacterial richness was positively correlated with silt and clay .

We suggest that the variation that was observed from soils across the different Fynbos vegetation types, is the key proximate factors that cause this strong correlation with bacteria richness and 16S rRNA gene copy abundance. Soils that consistently had the highest silt and

clay content (Alluvial and Shale) also had the highest OTU richness and 16S rRNA gene copy numbers

Further investigation of the taxonomic breakdown of the three most dominant phyla, *Protebacteria* and *Actinobacteria,* did not differ significantly across all Fynbos soil samples, whereas *Acidobacteria* varied significantly in all Fynbos soils (Fig.4). Barnard *et al.*,(2013) and Felske *et al.*, (2000)found that *Acidobacteria* relative abundance was insistently high in both dry and wet soils and it was the one of most dominant phyla in soils. This is not surprising as sampling was conducted during the wet season. Although, in this study elevation was not an explanatory variable it has been shown that *Acidobacteria* abundance was higher at lower elevations and mesic conditions (Van Horn et al. 2013; Zhang et al. 2014). We found that *Acidobacteria* abundance was highest in the Sand Fynbos soils, which incidentally has the lowest elevation of all the other sites (Fig.4; Table 2S). Interestingly, the abundance of *Proteobacteria* was highly influenced by Ca concentration (Table S2). Previous culture based experiments have shown that bacteria (*Proteobacteria*) have an affinity to attach more easily to Calcium than Silica substrates (Rodriguez-Navarro et al. 2012). We suggest this is the reason that the soils of both Alluvia and Shale Fynbos vegetation types have such a high OTU richness and consequently high 16S rRNA gene copy numbers (Fig.3; Fig. S1).Soils that have been Ca enriched have been shown to favour certain bacterial phyla such as *Bacteroidetes* and *Proteobacteria* (Sridevi et al. 2012), in this study we found that only the abundance of *Proteobacteria* was influenced by Ca concentrations (Table S2). Of all the three most dominant phyla, *Proteobacteria* had the most abundance across all Fynbos soils. We propose that *Proteobacteria* could be driving the lack of a distinct bacterial community in Fynbos soils, as it is the most abundant taxa and shows no variation in abundance across all sites.

5. Conclusion

As our soil analysis confirm, soils from across the Fynbos are chemically highly heterogeneous, variations that seem in part to be caused by both the unique pedogenesis processes and plant community in each soil type. Despite the strong differences in edaphic properties by Fynbos type the bacterial community structure was widespread and generalised across the Fynbos types. However, it was highly influenced by calcium, which was not related to soil type but instead varied between individual samples both within and between the Fynbos types. In addition, calcium and silt and clay content also influenced the abundance and richness of the bacterial taxa. Along with the unique geomorphic processes, we found that edaphic variables play a role in structuring the soil bacterial community. Further investigation is needed to discern the importance of these edaphic variables in delimiting bacterial community assemble processes in the Fynbos.

4.3 Deterministic Process Governing Bacterial Community Structure in Fynbos, South Africa

Introduction

The use of the 16S rRNA gene has become a very useful tool for microbial ecologists to study and survey soil microbial diversity (Dunbar et al. 2002; Torsvik et al. 1990; Torsvik and Ovreas 2002). More recent studies have focused not only on microbial diversity but also biogeography (Bryant et al. 2008; Fierer and Jackson 2006; Hanson et al. 2012; Martiny et al. 2006) and this has shed more light on the factors influencing the structure of soil microbial communities.

The soil matrix is immensely complex and its heterogeneous nature has often been considered to be one of the factors that could explain microbial diversity and biogeography (Chau et al. 2011; Ettema and Wardle 2002; Mcarthur et al. 1988; Ramette and Tiedje 2007). Spatial and temporal dynamics are also understood to influence microbial diversity and biogeography; their roles in significantly shaping microbial communities is widely reported (Cottenie 2005; Hanson et al. 2012; Martiny et al. 2011; Yergeau et al. 2010).

The South African Cape region is recognised as one of the biological hotspots; with its high levels of plant species diversity and endemism, the region is characterised by highly heterogeneous soils and mild Pleistocene climate (Allsopp 2014; Wintle et al. 2011). The Fynbos (Mediterranean heathland) is the major vegetation type of the region. Many of the plant species of the Fynbos are obligate seeders and rely on fire for regeneration, climate change is likely to increase the frequency of fires in the Fynbos (Keith et al. 2008; Wintle et al. 2011). The South African Fynbos has been widely studied, and the causes of its very high plant diversity have been widely discussed (Allsopp 2014; Cowling 1992; Cowling et al. 2003; Mucina and Rutherford 2006). However, little is known about Fynbos microbial

community patterns and processes. Some efforts have been made to try and fill this knowledge gap (Slabbert et al. 2010b; Slabbert et al. 2014; Stafford et al. 2005). The work that has been done invaluable though it is mostly based on describing overall similarities and differences in community composition.

Community ecology not only focuses on patterns of diversity but also the processes governing these patterns. (Vellend 2010) succinctly revised and restated that there were four major processes that governed community assembly; speciation, dispersal, selection and ecological drift. In recent years there has been a growing body of work showing the relative importance of these processes in varying systems; microcosm experiments also found that species-sorting (community assembly regulated by local environment/niche processes) and stochastic process interacted during bacterial assembly and their relative importance was dependent on species abundance (Langenheder and Szekely 2011). In stream biofilms and subsurface environments, a few studies have found that the role of probabilistic dispersal ergo neutral processes tend to be more important in structuring community (Besemer et al. 2012; Stegen et al. 2012), however, these studies do not forego the contribution of non-neutral assembly (deterministic) processes. Dumbrell et al. (2010) also found that arbuscular mychorrhizial fungi community was structured by both niche and neutral processes. Similarly, in a global study of extreme deserts, Caruso et al. (2011) found that in multi trophic system the use of single assembly rules (niche or neutral) was unrealistic.

Initially proposed by Webb et al. (2002), the field of phylogenetic community ecology has grown substantially in recent years (Morlon et al. 2011; Stegen et al. 2012; Stegen et al. 2013; Wang et al. 2012; Wang et al. 2013a; Wang et al. 2013b; Zaneveld et al. 2011), and was first applied to plant and animal communities, including the Fynbos.(Morlon et al. 2011) Highlighted the importance of spatial patterns in phylogenetic diversity, and proposed the initial theoretical predictions of increased phylogenetic diversity with area and loss of

phylogenetic similarity with distance in the Fynbos. They also showed that in the Fynbos phylogenetic diversity depends less strongly on environment and more on geographical distance under a random assembly model, which is steeped in neutral theory (Chave 2004; Hubbell 2001; Nemergut et al. 2013). Furthermore,(Etienne et al. 2006; Latimer et al. 2005) when applying Hubble's neutral models to plants in the Fynbos, found that the neutral model is appropriate for testing hypothesises about speciation and dispersal limitation. To discern the relative influence of stochastic and deterministic processes, ecologist use null models. In particular null models that couple phylogenetic community composition and randomisations has become common practice (Graham and Fine 2008; Stegen et al. 2012; Webb 2000; Webb et al. 2002).There are only few studies that have looked at the processes governing phylogenetic community structure assembly in Mediterranean climate system.

Here we investigate the roles of both deterministic (niche-based) and neutral process play in delimiting the microbial phylogenetic community structure of Fynbos soils. Using a 16S rRNA gene dataset that was compiled from sample sets in four different Fynbos vegetation types, we examined the phylogeny of the microbial communities in these four habitats. The present study aims not only to gain a better understanding of microbial ecology in the Fynbos, but also to discern what underlying ecological processes are important in phylogenetic community structure assembly.

We hypothesise that:

4. Each Fynbos vegetation type will harbour distinct microbial communities and have evolved distinct microbial lineages, and , the phylogenetic signal (likelihood of related species to resemble one another more than they are chosen at random from a phylogenetic tree (Blomberg and Garland 2002)) from the Fynbos will indicate that closely related bacterial Operational Taxonomic Unit (OTUs) occupy similar niches, due to conservatism in their ability to adapt and exploit the environment.

5. Phylogenetic community structure assembly in the Fynbos will be greatly influenced by deterministic processes rather than neutral processes, parallel to most microbial community assembly studies (Ofiteru et al. 2010; Stegen et al. 2013; Wang et al. 2013b)

I used an array of phylogenetic diversity indices to infer differences in community composition between Fynbos vegetation types, and, analysed patterns in phylogenetic signalling in relation to apparent ecological niches of OTUs. Furthermore, we used phylogenetic turnover ('turnover of clades between communities beyond the one expected if species turnover was independent of phylogeny', (O. J. Hardy et al. 2012a)) to infer the relatives influences of community assembly processes on community structure. Finally, we compared the relative roles of environment, elevation, and geographical distance in delimiting phylogenetic community structure assembly.

Material and Methods

Sequencing processing and analysis

The sequence data obtained was processed following the MiSeq SOP in Mothur (Schloss et al. 2009). All trimmed quality sequences were classified using EzTaxon-e reference bacterial taxonomy (Chun et al. 2007).

Statistical analysis

The number of sequences was standardised to 3317 per sample for cross-comparison between samples. All statistical analyses were performed using the Vegan (Oksanen et al. 2013), Picante v1.4 (Kembel et al. 2010),for the R program (R Development Core Team, 2007). A non-metric multidimensional scaling (NMDS) was created by calculating the Euclidian distance of environmental variables to visualise the difference in soil physical and chemical properties of the each Fynbos type.

Phylogenetic community composition analysis

A maximum likelihood tree was inferred from all aligned sequences using Fast Tree 2 under the Jukes and Cantor model (Price et al. 2010). Using the resultant tree, Mantel correlograms were used to evaluate the phylogenetic signal in the 16S rRNA gene across a range of phylogenetic depths and significance was drawn from 999 permutations (Diniz et al. 2010). Phylogenetic distances were portioned into classes (0.02 units) and the correlation coefficient was relating OTU phylogenetic distance to environmental-optimum distance (Diniz et al. 2010). An environmental-optimum for each OTU was calculated for each environmental variable as in Stegen et al. (2012) and Wang et al. (2013b). Between OTU optimum

difference was calculated as Euclidean distances using optima for all environmental variables. Phylogenetic diversity Faith's PD (Faith 1992) was also calculated using the phylogenetic tree, this index calculates the branch length of the community in each sample using the phylogeny. The resultant phylogenetic signal (see results) elicited the calculation of both un-weighted UniFrac matrix (Lozupone and Knight 2005) which detects phylogenetic distance closer to branches, and, ses. βMNTD matrix which detects phylogenetic distance closer to leaves of the phylogenetic tree. To visualise the bacterial community composition, a non-metric multidimensional scaling (NMDS) and analysis of similarity using (PERMANOVA) with 999 permutations (M. J. Anderson 2001) were performed using R 'Vegan' package. The community was visualised using the un-weighted UniFrac matrix and the ses. βMNTD matrices. Furthermore, to infer the phylogenetic community structure, we used the nearest taxon index (NTI) to characterise the community composition within each sample point (Webb et al. 2002). For each community NTI that is greater than two indicates phylogenetic clustering; whilst NTI less than two indicates phylogenetic over-dispersion. A mean NTI of all samples that is greater than 0 indicates clustering and NTI with a mean less that 0 indicates overall over-dispersion.

Phylogenetic community turnover and assembly processes

Phylogenetic turnover was quantified using the UniFrac and the mean nearest taxon distance (βMNTD) (Fine and Kembel 2011; Stegen et al. 2012). βMNTD is the mean phylogenetic distance to closest relative in a paired community of taxa (Fine and Kembel 2011) and is sensitive to the changes of lineages close to the phylogenetic tips. Ses. βMNTD was computed as the number of standard deviations that observed βMNTD depart from the mean null distribution (999 null iterations) based on random shuffling of OTU labels across the tips

of the phylogeny (Fine and Kembel 2011; Stegen et al. 2012; Stegen et al. 2013; Wang et al. 2013b). This randomisation hold constant observed species richness, occupancy, and turnover. Thus, it provides an expected level of βMNTD given observed species richness, occupancy, and turnover.

To understand and discern which assembly processes were important in delimiting bacterial community, Mantel and partial Mantel tests were performed using the phylogenetic matrices controlling for environment, geographical and elevation distance to determine their relative roles in explaining the phylogenetic community composition in the Fynbos (Wang et al. 2013a; Wang et al. 2013b). A multiple regression on matrices (MRM) (UniFrac and ses. β MNTD) was performed on the (environmental, geographical and elevation) distance matrices (Legendre et al. 1994). Furthermore, a multiple regression on matrix using individual environmental variables made it possible to assess the relative importance of each variable, reverse selection (in which all the individual environmental matrices were regressed against the phylogenetic matrices, and those environmental matrices that were non-significant were the removed.) was performed.

Finally, using the ses. βMNTD matrix, whose absolute magnitude reflects the influence of deterministic processes; the larger it is the greater the influence of niche-based processes. Using this it was possible to discern which assembly processes were important in delimiting bacterial phylogenetic community in the Fynbos.

Results

At the 97% identity level, the final OTU table consisted of 262,087 sequences distributed into 15,627 OTUs, of which 5,113 were represented by more than one sequence. Rarefaction curves indicated that both alluvial and shale Fynbos had the highest number of OTUs when compared to limestone and sand Fynbos types.

A NMDS of the environmental variables indicated that the all the Fynbos vegetation types had distinct physical chemical properties from one another and they clustered significantly by Fynbos vegetation type.

Phylogenetic community composition analysis

The phylogenetic signal using the Mantel correlogram showed significant correlations over short phylogenetic distances ($p<0.05$, Figure 18).The signal showed significantly both negative and positive correlations across short phylogenetic distances. Indicating that there are instances of ecological coherence (closely related OTUs occupying the same/similar niches) and ecological incoherence (distantly related OTUs occupied the same/similar niches).

The phylogenetic diversity (Faith's PD) index showed that Alluvial and Shale vegetation types had the highest phylogenetic diversity, whilst, Sand and Limestone vegetation types had intermediate and lowest diversity respectively (Figure 19).

The non-metric multidimensional scaling from the UniFrac revealed that samples clustered according to Fynbos vegetation type, PERMANOVA results showed that clustering by Fynbos vegetation type was overall significant whilst ses βMNTD NMDS did not show any clustering by Fynbos vegetation types($p<0.05$, Figure 20).

A T-test revealed that the overall NTI index mean significantly deviated from zero, indicating that the phylogenetic community composition was clustered (t_{20} = -58.034, $P \ll 0{,}0001$). Although, not all communities exhibited the overwhelming clustering phenomenon, only 15% of the samples did not significantly deviate from zero.

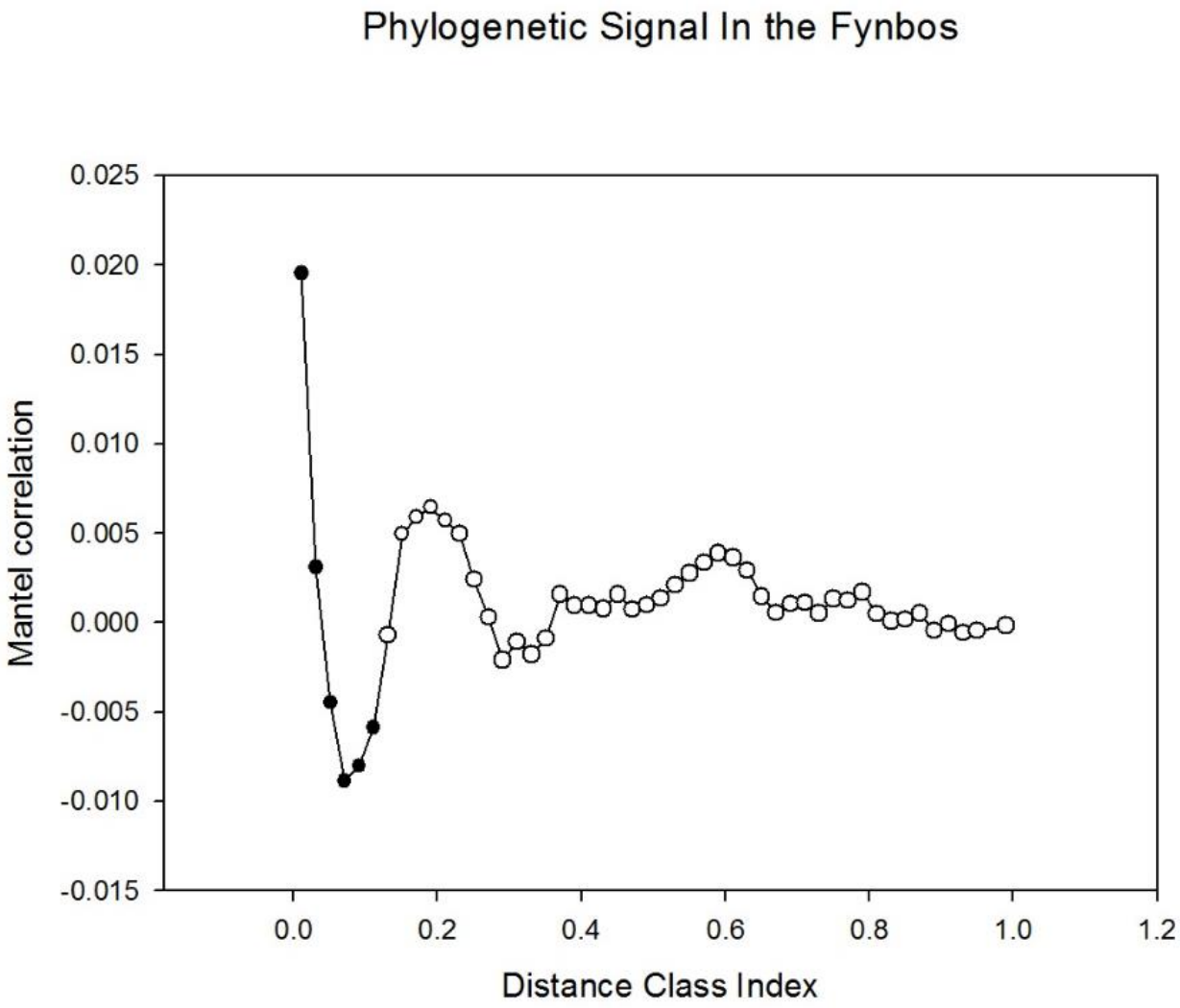

Figure 18 Mantel correlogram between the pairwise distance of OUT niche distance and phylogenetic distance. Significant correlation ($p<0.05$, solid circles) phylogenetic signal in species ecological niches

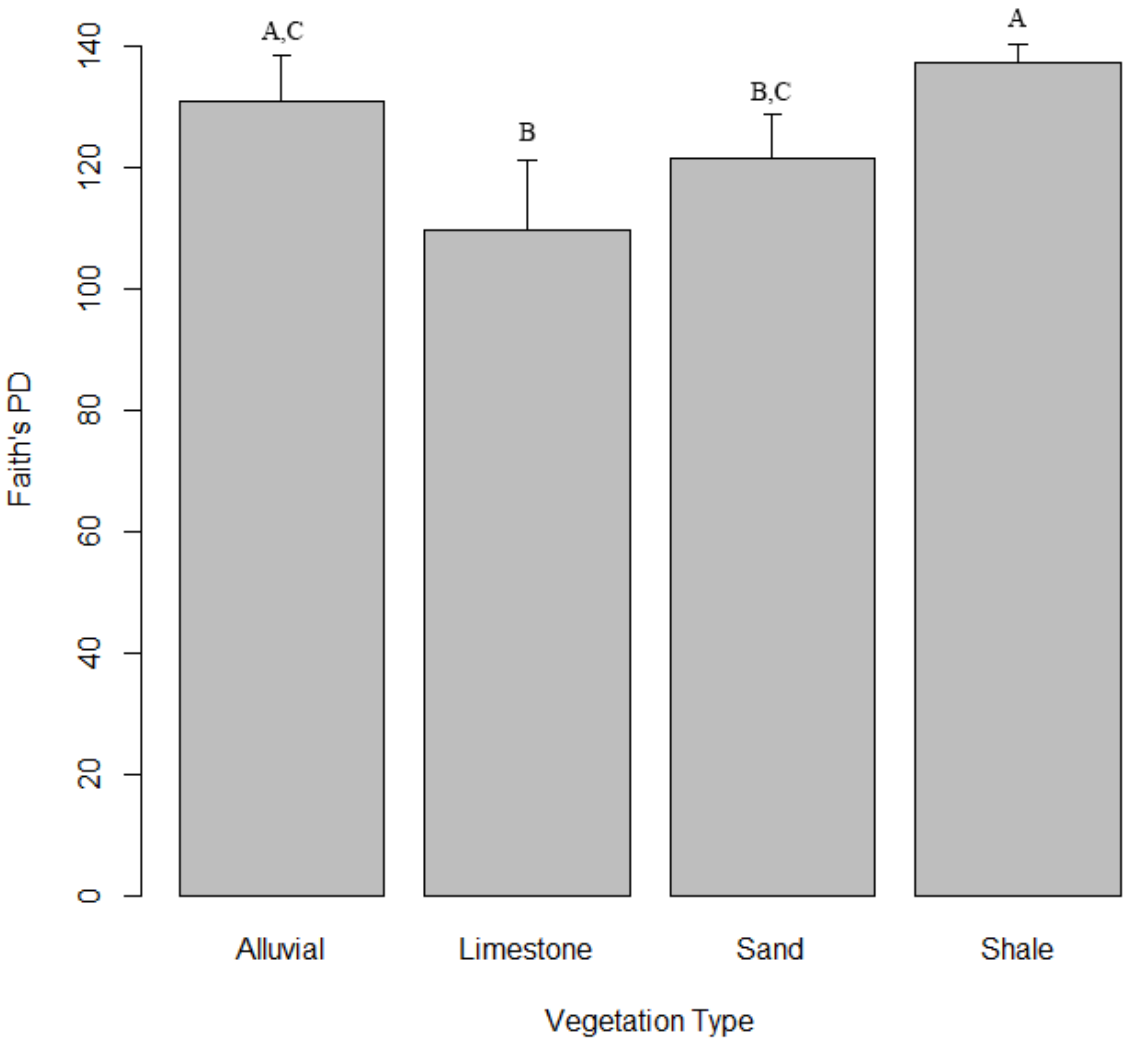

Figure 19 Faith's PD showed that overall the four fynbos types were significantly different ($P<0.05$); further post-hoc Tukey's test analysis revealed that both alluvial and shale sites were not significantly different from each other as was the case with sand and limestone also alluvial and sand .

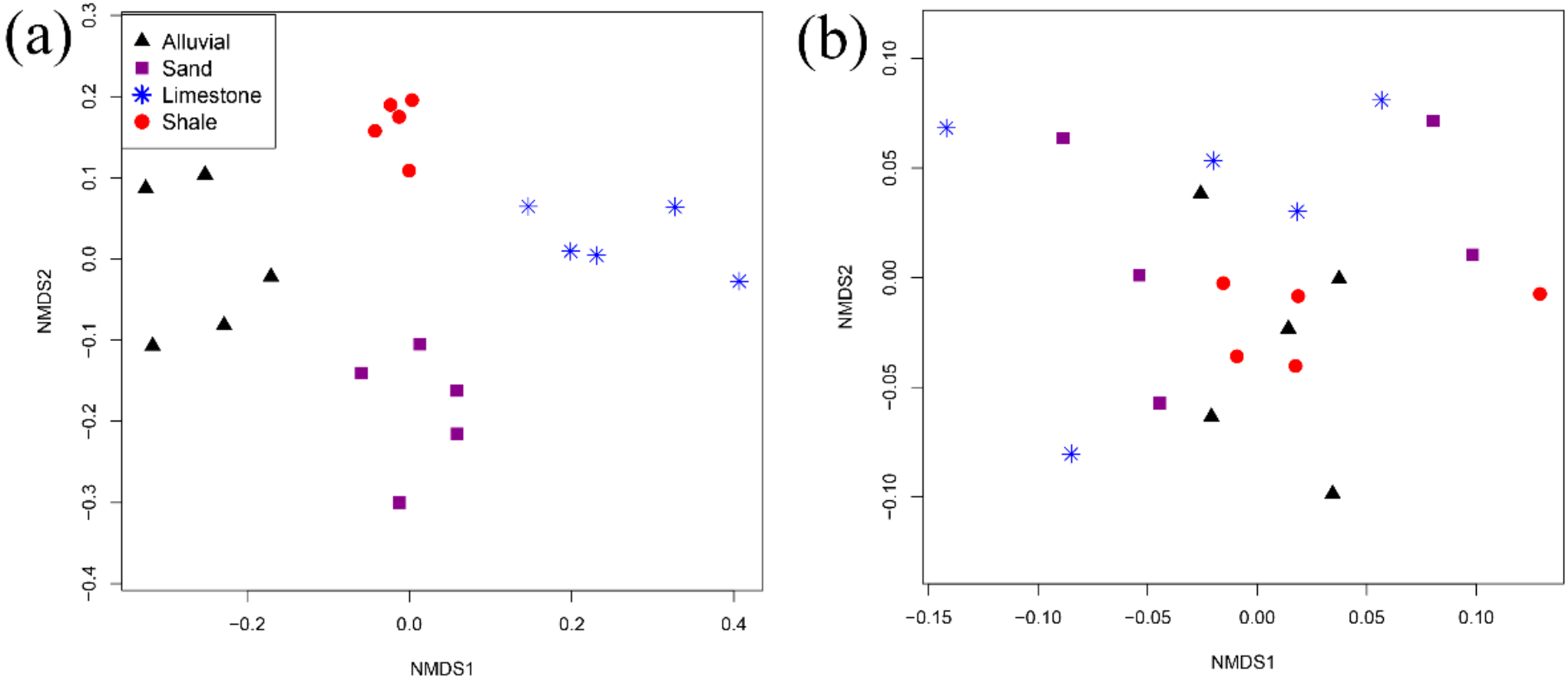

Figure 20 Non-Metric Dimensional Scaling from a) UnFrac matrix which was significantly clustered ($p<0.05$) and b) ses .βMNTD.

Phylogenetic community turnover and assembly processes

To infer the underlying processes that govern community turnover and assembly, we found that phylogenetic turnover rates in the Fynbos to be higher than expected (Figure 21). And, ses.βMNTD mean was significantly different from the expected value of zero (T-test, $t_{190} = -958.2552$, $P \ll 0{,}0001$, Figure 21), indicating that deterministic processes govern phylogenetic community assembly in the Fynbos.

The Mantel tests showed that both βMNTD and UniFrac were significantly correlated with geographical distance, with UniFrac also being correlated with elevation and ses. βMNTD with elevation (Table 6). When controlling for the effects of both geographical and environmental distance, environmental distance was not correlated with any of the indices (UniFrac, βMNTD and ses. βMNTD).

MRM analysis performed on the ses.βMNTD matrix with individual environmental matrices indicated that soil texture was the most important identifiable environmental variable that influenced community assembly in the Fynbos ($p<0.01$). A similar analysis using the UniFrac matrix revealed that soil texture, Potassium (K) and Nitrate (NO_3) were important ($P<0.001$).

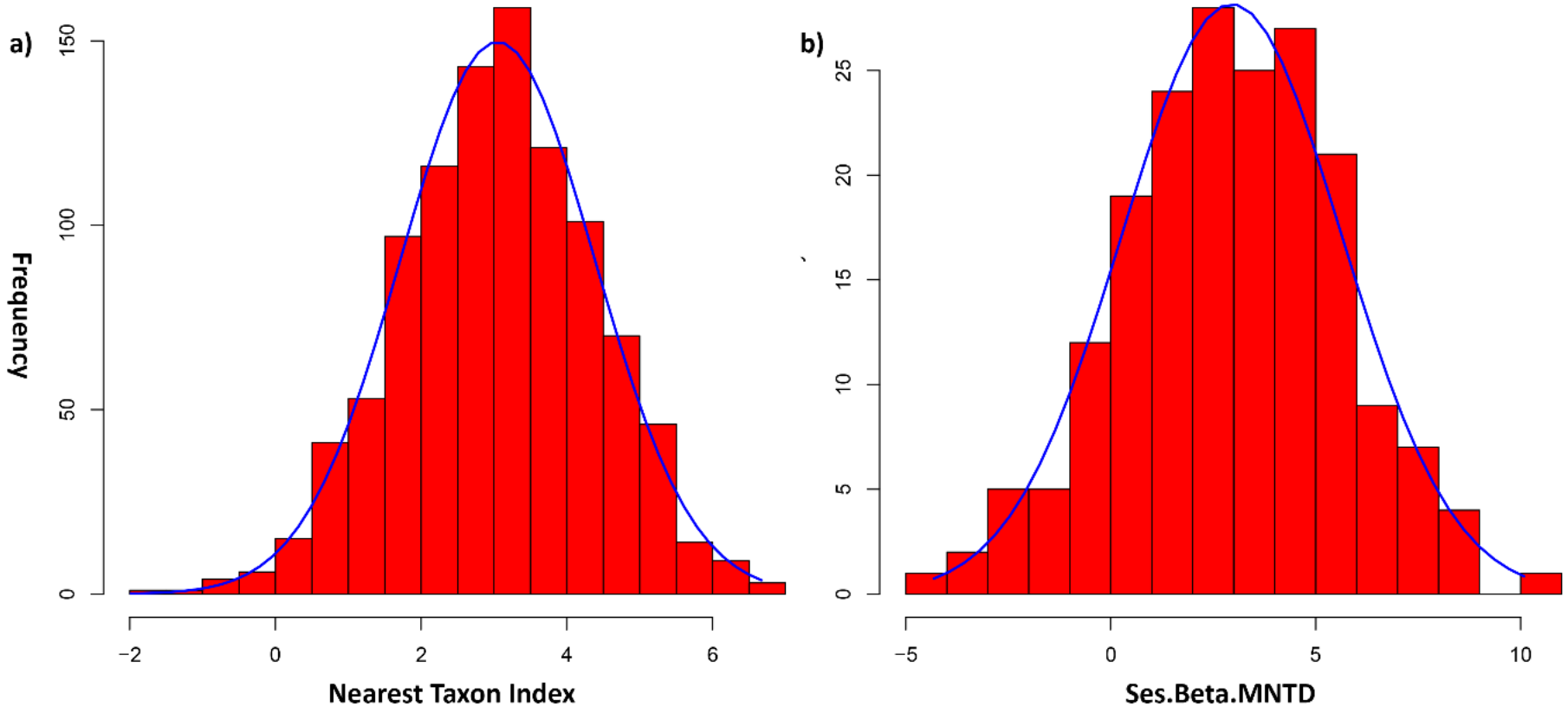

Figure 21.Frequency estimates for distribution of Nearest Taxon Index (NTI, mean=3.01) and ses.Beta.MNTD (mean=2.95).Each observation is the number of null model standard deviations the observed value is from the mean of its associated null distribution. Ses.Beta.MNTD values <-2 indicate less than expected turnonver; values >+2 indicate greater than expected turnover. Both mean of NTI and Ses.Beta.MNTD were significantly different from expected value of zero for random data (P<0.01, t-test)

Table7. Mantel and partial Mantel tests for the correlation between Unifrac, βMNTD and ses.βMNTD and the explanatory distances (geographic, environment and elevation) using Spearman's rho in the fynbos.

Effect of	Controlling for	Fynbos
Unifrac		
Geographic		0.4043***
Environment		0.1828
Elevation		0.3855***
Geographic	Environment and Elevation	0.4024**
Environment	Geographic and Elevation	0.1619
Elevation	Geographic and Environment	0.3444**
βMNTD		
Geographic		0.2149*
Environment		-0.0395
Elevation		0.0810
Geographic	Environment and Elevation	0.2045*
Environment	Geographic and Elevation	-0.0434
Elevation	Geographic and Environment	0.0640
ses.βMNTD		
Geographic		0.0571
Environment		0.1299
Elevation		0.1702*
Geographic	Environment and Elevation	0.0440
Environment	Geographic and Elevation	0.1042
Elevation	Geographic and Environment	0.1438

Significant level:***<0.001; **<0.01;*,0.05.

Discussion

In this study we analysed the Mediterranean climate Fynbos ecosystem to understand soil bacterial phylogenetic community composition and assembly. Overall, we discovered from the NTI and ses.βMNTD indices that the bacterial community is phylogenetically clustered, and that environmental filtering delimits phylogenetic community structure assembly. Nevertheless, stochastic factors seem to play a role, albeit secondary.

Phylogenetic community composition analysis

To our knowledge this study is the first to investigate the microbial phylogenetic signal within a Mediterranean ecosystem. However, various other studies have used phylogenetic signals to infer ecological processes in other ecosystems and taxonomic groups (L. C. Anderson et al. 2010; Cavender-Bares and Holbrook 2001; Pontarp et al. 2012; Stegen et al. 2012; Stegen et al. 2013; Wang et al. 2013b)These studies have shown that there is a clear relationship between phylogenetic signal and ecological niches. We observed that over short phylogenetic distances, there was a close relationship with ecological niche. This is in accordance with Brownian niche evolution (Ackerly et al. 2006; Diniz et al. 2010).

We predicted that a clear relationship between phylogenetic signal and ecological niche would occur, with closely related OTUs being ecologically coherent. However, our results (Figure 18) showed that in the CCR system, the opposite was the case: with majority of distantly related OTUs in the Fynbos being ecologically coherent and co-occurred over short phylogenetic distance, conversely a small proportion of closely related OTUs did co-occur. Overall, these results indicate that at close phylogenetic distance the bacterial community is phylogenetically over-dispersed. At close phylogenetic distances related taxa were not ecologically coherent, and, these taxa were not phylogenetically conserved in their niches.

This over-dispersion of closely related OTUs in the Fynbos may be predicted to result from avoidance of niche overlap, Morlon et al. (2011)proposed that when competitive exclusion of close relatives operates at a local scale, the result is over-dispersion in relation to environmental factors. Competitive exclusion and habitat filtering both act to produce the observed community. However, the roles of competition and habitat filtering have been shown to be important in structuring bacterial communities (Horner-Devine and Bohannan 2006). We propose that the heterogeneous nature of the Fynbos soils (Allsopp 2014), will result in a narrow range of bacterial ecotypes that will likely produces the observed ecological incoherence in the phylogenetic signal .The phylogenetic diversity was consistently lower in soil samples from Limestone and Sand Fynbos vegetation types, and, highest in both Alluvial and Shale Fynbos soil samples (Figure 19). The influence of pH and edaphic properties on bacterial phylogenetic diversity is widely reported (Lauber and Fierer 2009; Ren et al. 2015; D. Singh et al. 2014; B. M. Tripathi et al. 2012; Wang et al. 2012). However, in this present study we found distinction in edaphic variable across all Fynbos types. We suggest that the unique pedogenesis process in each Fynbos vegetation type could be producing this observed difference in phylogenetic diversity. These results prompted further investigation into the phylogenetic community composition within the fynbos.

Previous studies that investigated bacterial communities in the Fynbos; Fynbos rhizospheres (Stafford et al. 2005), sand Fynbos (Slabbert et al. 2010b; Slabbert et al. 2010a) and riparian alluvial Fynbos (Slabbert et al. 2014) have indicated that microbial communities within the Fynbos are to some extent distinct between Fynbos vegetation types. Our prediction that each Fynbos vegetation type would have a distinct bacterial community was supported by the UniFrac result (Figure 20), clearly illustrating that the phylogenetic community is highly clustered by vegetation type. This clustering is much stronger than has been reported in other studies that investigated the phylogenetic community structure in Fynbos soils (Slabbert et al.

2014). The UniFrac is sensitive closer to the nodes, indicating that the distinctions in the soil microbial communities are ‘ancestral’ (i.e. relatively ancient) in nature. Ses.βMNTD results indicate that at species level there was no clustering by vegetation type (Figure 20), ses. βMNTD, which is more sensitive towards the tips of the tree, suggesting that there had been rapid speciation events within the microbial community of each vegetation type. However, unlike previous studies that had investigated bacterial communities using these matrices, there was a discordance between the two matrices. A comprehensive study by Wang et al. (2013b) using UniFrac and ses. βMNTD revealed that the community was significantly clustered by habitat type. To further infer phylogenetic local community composition (local community= each sampling point), we used the NTI index for each sampling point.. The mean NTI values for all the local communities was significantly positive ($p<0.05$, Figure 21), indicating that overall the local communities are phylogenetically clustered as result of deterministic processes (Kembel 2009; Stegen et al. 2012; Wang et al. 2013b; Webb 2000) .Wang et al. (2012) argued that environmental heterogeneity is more important than spatial scale when studying local ecological processes. However, we found no correlation between NTI and elevation. Furthermore, the complexity of the soil matrix makes it challenging to discern with absolute confidence which environmental variable is driving this local ecological process in the Fynbos

It is worth noting that 10% of samples were not significantly clustered (NTI < 2), and most of these were from Limestone Fynbos. Phylogenetic clustering is not only the result of environmental filtering but also of an adaptive radiation event (i.e. speciation) from adaptation to different environment (Graham and Fine 2008; Horner-Devine and Bohannan 2006). There has no study that has investigated bacterial community structure in the Limestone Fynbos soils. We propose that the nature of Limestone Fynbos soils would create a bacterial community that has adapted to the elevated calcium carbonate concentrations. And,

very few bacterial lineages can adapt to such levels. This would decrease the phylogenetic diversity, as indicated by the phylogenetic signal (Figure 18) there were instances of closely related taxa co-occuring.

The striking difference in both abiotic and biotic properties of each of the Fynbos sites is widely documented (Mucina and Rutherford 2006; Witkowski and Mitchell 1987). Allsopp (2014) succinctly summarised and surmised the pedogenesis of Fynbos soils, moreover, the influence of plant community structure and litter in influencing edaphic variable is well documented (R. D. Bardgett and Shine 1999; Richard D. Bardgett 2005; Ehrenfeld 2003; Jhonson and ebrary Inc. 2009; J. S. Singh et al. 1989). We propose that the combination of the documented different vegetation types (Cowling 1992; Cowling et al. 2009; Mucina and Rutherford 2006) and unique geomorphic processes in the each of the Fynbos vegetation types are key ultimate factors for this observed difference.

Phylogenetic community turnover and assembly processes

The NTI index revealed that the local community was phylogenetically clustered and that overall mean was greater than two. This indicates that though deterministic processes play a crucial role in delimiting community assembly at the local scale, some stochastic process still act in delimiting community assembly. Previous studies that used elevation as a proxy for environment (Singh *et al.*,2014). Wang *et al.*(2013) argued that environmental heterogeneity is more important than spatial scale when studying local ecological processes. However, we found no correlation between NTI and elevation.

Furthermore, the complexity of the soil matrix makes it challenging to discern with absolute confidence which environmental variable is driving this local ecological process in the Fynbos. Though, deterministic processes were governing community assembly, role of neutral processes are also crucial in determining the overall community composition (Weiher et al. 2011).

Phylogenetic turnover and community dynamics

To determine which ecological processes were delimiting composition within communities and turnover; we used the NTI and $ses.\beta$MNTD indices (Figure 21). The results also indicated that $ses.\beta$ MNTD significantly deviated from zero. Interestingly, it was significantly different from zero to 65% of pairwise comparisons, proving that though within and overall deterministic processes govern phylogenetic turnover in the Fynbos, stochasticity also has an influence. Furthermore, the mean of the ses.βMNTD was greater than two, indicating that that though the community was over-dispersed, this over-dispersion is explained by the differences in environmental conditions across the sites (Wang et al. 2013a; Wang et al. 2013b). And, these results are in congruence with other similar studies (Fine and Kembel 2011; Stegen et al. 2012; Stegen et al. 2013).

To understand which environmental variable was most important in delimiting phylogenetic turnover, regression on matrices revealed that soil texture (clay and silt %) was positively correlated to phylogenetic turnover (Figure 22), though weak in nature. A study by Vreulink et al. (2007), found that soil moisture was an important variable that delimited growth of heterotrophic microbes in Fynbos soils.A Mantel test performed on the $ses.\beta$MNTD reveals that elevation may play a role in delimiting phylogenetic turnover (Table 5). Wang et al. (2012) found that elevation seems to play a crucial role in delimiting phylogenetic community composition, and they suggest that the difference in temperature could be key to this difference. We conducted the sampling during the winter rainy season; we propose that the combined environmental variables of each Fynbos type are more important. Similar to the other Mediterranean ecosystems, the Fynbos has wet winters and dry summers. Thus, the soils had higher moisture content (field capacity).

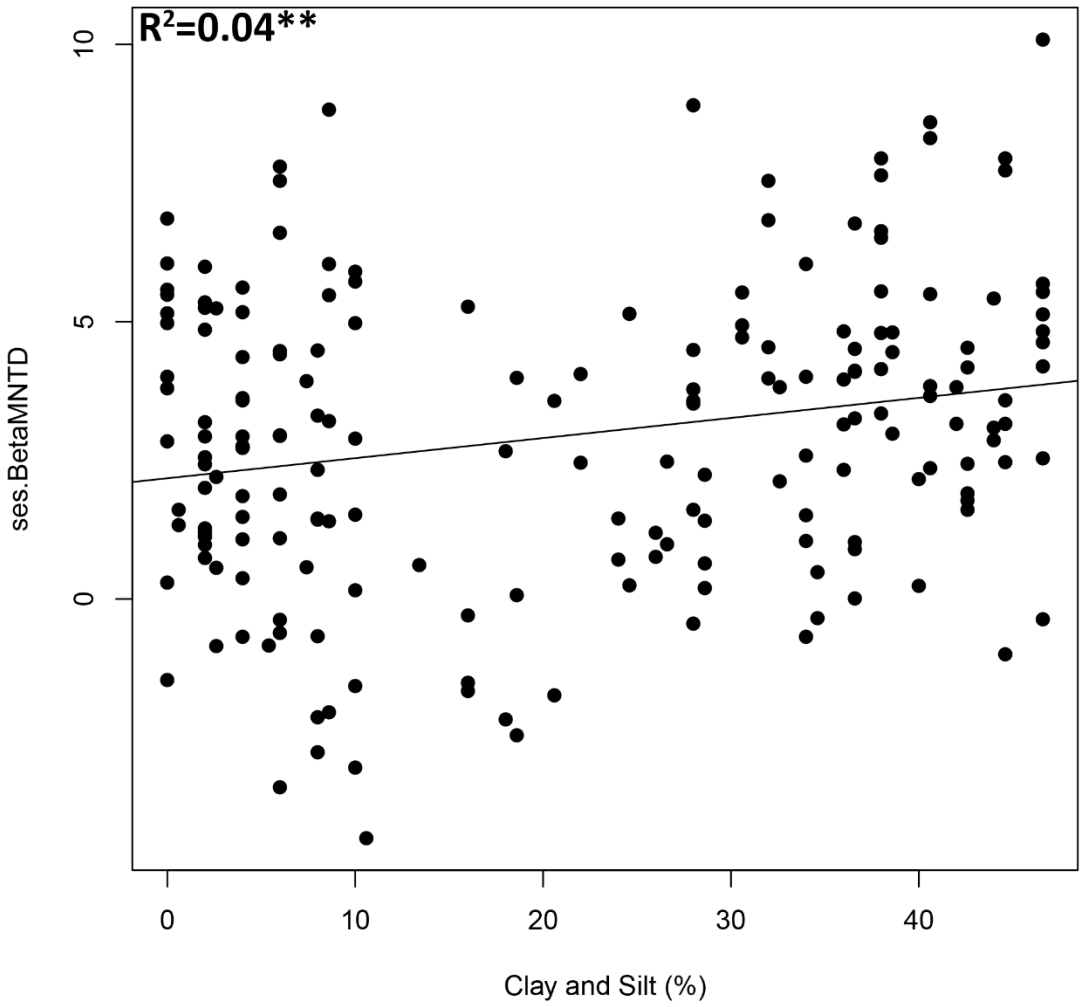

Figure 22. Regression analysis show that phylogenetic turnover (ses. βMNTD,) was positively correlated to soil texture (clay and silt). Significant level:***<0.001; **<0.01;*<0.05.

De Marco et al. (2005) found that after a fire had gone through a Mediterranean *Marquis*, there was a drastic change in the soil biochemistry and this led to increase change in microbial biomass and soil metabolic quotient (qCO_2). Because of varying fire regimes in the different Fynbos vegetation types, we propose that this could be the reason for this difference in physical and chemical properties within the Fynbos, (Neary et al. 1999) found the fire intensity and regime affects belowground diversity. Our study sites were conducted in undisturbed nature parks, and the management plan for these areas includes prescribed burning. However, there had not been any fires in recent years, we suggest that this could be the reason for the decreased lack of a strong environmental correlation despite the environment playing such a crucial role in community assembly. We propose that there are unmeasured environmental variables that are delimiting community assembly within the Fynbos.

Previous studies have shown that species sorting and neutral process play crucial role during the initial assembly of bacterial communities (Langenheder and Szekely 2011). The rapid speciation events that characterise the Fynbos (as characterised by the high turnover rate, $ses.\beta$MNTD) are constantly changing the phylogenetic tips of the tree, and this could cause this influence of the neutral process as these process have been shown to be important in the beginning.

A number of caveats to be considered with this study are that. Firstly we did not record the detailed plant community structure, although each sample was located within a known mapped area of a particular fynbos community type, and was determined during fieldwork to be broadly representative of that type in terms of taxonomic composition and vegetation structure (Allsopp 2014; Cowling et al. 2003; Mucina and Rutherford 2006). Secondly, although we measured a large array of different edaphic variables, it is always possible that

some other unmeasured variable that we did not measure is actually important in determining soil community structure. Indeed, the fact that we detected determinism in bacterial community structure without being able to explain most of it through our measured variables, suggests that there must be some other variable or set of variables that is important.

General Conclusions

The results of this study reveal several interesting conclusions that may provide insight into the ecology of soil micro-organism in Mediterranean ecosystems. In the Fynbos, the pedogenesis processes that are unique to each vegetation type have led to an incredibly distinct edaphic properties. This distinction in soil properties it seems to be affecting the different soil micro-organisms differently. The mobile nematode community does not seem to show any habitat pretences and showing great species niche overlap of closely related taxa. And, this community seems in part to be influenced by the edaphic properties to a lesser extent than the fungi. The fungal community is highly specialised to each vegetation site, and this distribution is largely influenced by the unique edaphic conditions at each site. The prokaryote community seems to resemble the nematode community- in that there is no habitat preference and the community seems to be only delimited by divalent cations. Interestingly, the prokaryote community seems to show that only distantly related taxa tend to co-occur.

Overall, this study sheds lights on the processes that govern microbial phylogenetic community in the Fynbos. There is clearly an important role of deterministic processes in microbial assembly within the Fynbos. However, as in many other natural systems, neutral processes also appear play some role. We found that a relatively large proportion of the taxa that tend to co-occur were distantly related and only a relatively small proportion of closely related taxa co-occurred. Further studies are needed to discern whether similar community assembly processes occur in other Mediterranean climate zones. It is proposed here that initially neutral processes played a role in assembling communities, but do not seem to play a significant role now. This is in congruence with many community assembly studies that found similar patterns.

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Appendix

Table S1. Regression analysis (relative abundance) of dominant class and explainatory edaphic variables

Dominant Class	Edaphic variable	Adj Rsqr	P-value
Soilbacteres	Clay	0.1625	0.0441
Acidiobacteria	Clay	0.3248	0.0051
Gemmatimonadetes	Clay	0.2716	0.045
Acidiobacteria	silt	0.2946	0.02
Gemmatimonadetes	silt	0.2426	0.0159
Acidiobacteria	sand	0.3085	0.0169
Gemmatimonadetes	sand	0.2658	0.0477
Alphaproteobacteria	pH	0.4847	0.0033
Soilbacteres	pH	0.379	0.0136
Gemmatimonadetes	pH	0.188	0.0321
Deltaproteobacteria	pH	0.4477	0.0056
Thermoleophilia	pH	0.3397	0.0217
Betaproteobacteria	pH	0.4244	0.0077
Sphingobacteria	pH	0.2819	0.0405
TM7	pH	0.3337	0.0232
Acidiobacteria	EC	0.2266	0.0196
Gemmatimonadetes	Total P	0.3711	0.015
Gemmatimonadetes	Na	0.1899	0.0313
Acidiobacteria	Ca	0.2074	0.0251
Acidiobacteria	Mg	0.2305	0.0419
Soilbacteres	Cl	0.1938	0.0298
Actinobacteria	SO_4	0.2304	0.042
Actinobacteria	NH_4	0.1605	0.0452
Gemmatimonadetes	NH_4	0.4967	0.0011
Deltaproteobacteria	NH_4	0.3019	0.0183
Verrucomicrobiae	NH_4	0.3469	0.02
Thermoleophilia	NH_4	0.3803	0.0067
Betaproteobacteria	NH_4	0.3023	0.0182
Planctomycetacia	NH_4	0.2805	0.0237
Sphingobacteria	NH_4	0.1648	0.0428
Soilbacteres	Available P	0.1582	0.0465
Verrucomicrobiae	N	0.2466	0.035
Gemmatimonadetes	C	0.1924	0.0303
Verrucomicrobiae	C/N	0.5776	0.0007
Sphingobacteria	C/N	0.2274	0.0433
Acidiobacteria	Silt and Clay	0.2956	0.0078
Gemmatimonadetes	Silt and Clay	0.2658	0.0477
Gemmatimonadetes	MAP	0.1669	0.0418
Sphingobacteria	MAP	0.2574	0.031

Alphaproteobacteria	MAPE	0.649	<0.0001
Actinobacteria	MAPE	0.304	0.0069
Soilbacteres	MAPE	0.7094	<0.0001
Acidiobacteria	MAPE	0.2946	0.0079
Deltaproteobacteria	MAPE	0.4479	0.0007
Verrucomicrobiae	MAPE	0.7538	<0.0001
Thermoleophilia	MAPE	0.6275	0.0003
Betaproteobacteria	MAPE	0.2662	0.0475
Planctomycetacia	MAPE	0.691	<0.0001
Sphingobacteria	MAPE	0.6963	<0.0001
TM7	MAPE	0.5985	0.0005