EUKARYOTIC DIVERSITY OF MIERS VALLEY HYPOLITHS

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ITS

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Microalgae

DECLARATION

I, Jarishma Keriuscia Gokul, declare that the thesis entitled "*Eukaryotic Diversity of Miers Valley Hypoliths"* is my own work. To the best of my knowledge, all sentences, passages or illustrations quoted in it from other bodies of work have been acknowledged by clear referencing to the author.

Jarishma Keriuscia Gokul

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ABSTRACT

The extreme conditions of Antarctic desert soils render this environment selective towards a diverse range of psychrotrophic microbial communities. Cracks and fissures in translucent quartz rocks permit an adequate amount of penetrating light, sufficient water and nutrients to support cryptic microbial development. Hypolithons colonizing the ventral surface of these quartz rocks have been classified into three types: cyanobacterial dominated (Type I), moss dominated (Type II) and lichenized (Type III) communities. Eukaryotic microbial communities were reported to represent only a minor fraction of Antarctic communities. In this study, culture independent techniques (DGGE, T-RFLP and clone library construction) were employed to determine the profile of the dominant eukaryotes, fungi and microalgae present in the three different hypolithic communities. The 18S rRNA gene (Euk for eukaryotes), internal transcribed spacer (ITS for fungi) and microalgal specific regions of the 18S rRNA gene, were the phylogenetic markers targeted for PCR amplification from hypolith metagenomic DNA. Results suggest that the three hypolith types are characterized by different eukaryotic, fungal and microalgal communities, as implied by nMDS analysis of the DGGE and T-RFLP profiles. Sequence analysis indicates close affiliation to members of Amoebozoa, Alveolata, Rhizaria (general eukaryote), Ascomycota (fungal) and Streptophyta (microalgal). Many of these clones may represent novel species. This study demonstrates that Dry Valley hypolithons harbour higher eukaryote diversity than previously recognised. Each hypolithon is colonized by specialized microbial communities with possible keystone species. The ecological role of the detected microorganisms in the hypolith environment is also theorized, and a trophic hierarchy postulated.

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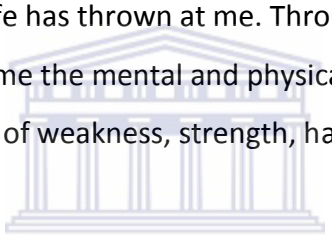

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LIST OF ABBREVIATIONS

°C	degrees Celsius
× *g*	times gravity
µ	micron
µg	microgram
µl	microlitre
µM	micromolar
APS	ammonium persulfate
ARDRA	amplified ribosomal DNA restriction analysis
ATP	adenosine triphosphate
bp	base pairs
BLAST	basic local alignment search tool
$CaCl_2$	calcium chloride
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ds	double stranded
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
FAM	phosphoamidite fluorochrome 5-carboxyfluorosceine
g	gram
HEX	4,7,2',4',5',7'-hexachlorofluorescein
IPTG	isopropyl β-D-thiogalactosidase
ITS	internal transcribed spacer
$km.h^{-1}$	kilometres per hour
KCl	potassium chloride

LB	Luria Bertani medium
LB_{amp}	Luria Bertani medium containing ampicillin
LSU	large subunit
M	molar
MCS	multiple cloning site
nMDS	non-metric Multi Dimensional Space
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
MVH	Miers Valley hypolith
ml	millilitre
mM	millimolar
min	minute
NaCl	sodium chloride
ng	nanogram
nm	nanometre
OD	optical density
OTU	operational taxonomic unit
PCR	polymerase chain reaction
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal oxyribonucleic acid
ROX	6-carboxyl-x-rhodamine
SDS	sodium dodecyl sulphate
SOC	super optimal broth with catabolite repression
SSU	small subunit
s	second
TAE	tris acetic acid EDTA
TE	tris EDTA
TEMED	tetramethylethylenediamine
T-RFLP	terminal restriction fragment length polymorphism

HCl	hydrochloric acid
UV	ultraviolet
v/v	volume per volume
V	volts
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

CHAPTER 1
LITERATURE REVIEW

1.1 The Antarctic Environment and the Dry Valleys

The ancient terrain and environmental extremes of Antarctica allows for it to be characterized as a nearly inhospitable continent (Onofri *et al*., 2004a). It is among a number of cold and hot hyper-arid environments around the globe which serve as model systems of early life and evolutionary processes on earth (Thomas, 2005). These sites include the Atacama Desert, Rio Tinto (Spain) and Australian plains and deserts. Each of these environments has unique geological, biological and climatic features, making them ideal terrestrial analogues for astrobiological investigations. (Ascaso and Wierzchos, 2002; Anderson *et al*., 1990). The continued interest in Antarctica is due to the presence of permafrost (permanent frozen sediment) that may be similar to icy extraterrestrial ecosystems (de Vera *et al*., 2010; West *et al*., 2010; Gilichinsky *et al*., 2007; Thomas, 2005).

A large proportion of the Antarctic landscape consists of ice plateaus (Fell *et al*., 2006; Hopkins *et al*., 2006). The Dry Valleys constitute approximately 0.35% of ground in Antarctica that is not overtaken by permanent ice and snow (Convey and Stevens, 2007; Hopkins *et al*., 2006). In this desert region, some of the most severe environmental conditions have been recorded. These include extremely cold temperatures with significant fluctuations, extremely low intrinsic soil moisture,

limited organic nutrients (Cary *et al*., 2010; Friedmann *et al*., 1993) and physical instability due to strong katabatic winds (Cowan, 2009; Fell *et al*., 2006). Extreme dessication, aridity, high soil salinity (Adhikari *et al*., 2009), frequent freeze-thaw (periglacial activity) and wet-dry cycles (Block *et al*., 2009) and unusually high solar radiation (UVB) (Cowan *et al*., 2010b) make it an unfavourable environment for microorganism development, as these factors vary both spatially and temporally (Block *et al*., 2009).

Mean temperatures in the Dry Valleys fluctuate significantly over the seasons. The air temperature fluctuates between -20°C and -50 °C in winter (Onofri *et al*., 2004b) and between -25°C and 3°C in summer (de la Torre *et al*., 2003). In winter, the soil surface maintains a temperature of about -20°C to -25°C; in summer months temperatures hover at approximately 0°C and reach an average high of approximately 15°C (Hopkins *et al*., 2006; Cowan and Ah Tow, 2004).

The soil is old and cold and has a moisture content of less than 2% (Cary *et al*., 2010; Friedmann *et al*., 1993). The dry katabatic winds that travel through the Transantarctic Mountains exceed 100 $km.h^{-1}$ (de la Torre *et al*., 2003) and contribute to the absence of snow in the Dry Valleys (Anderson *et al*., 1990). These extremely cold and dry conditions were thought to limit the growth of microbial communities and plant life (Boyd and Boyd, 1962). However, these are not the only factors that negatively impact growth in this environment. Low levels of carbon and organic

nitrogen, imbalanced salt concentrations and dessication create a disproportionate soil geochemical composition that directly influences vegetative and microbial growth (Moorhead *et al*. 1999; Boyd and Boyd, 1962). Additionally, the limited nutrient cycling, due to low metabolic activity as a result of the cold temperatures, severely impairs the potential for microbial growth (Moorhead *et al*., 1999).

Isolation from the ice plateau, and the low level of precipitation experienced in this valley, contributes to the Dry Valley possibly being the driest desert on earth (Hopkins *et al*., 2006; de la Torre *et al*., 2003). Intermittent snowfall during summer months is the only form of precipitation received by the region (Fountain *et al*., 2009; Hopkins *et al*., 2006; Claridge and Campbell, 1977).

Furthermore, water availability in the Dry Valley varies with time and space due to temperature fluctuations and the presence of the permafrost layer at the base of the mineral soil profile (Gilichinsky *et al*., 2007; Hopkins *et al*., 2006; Noy-Meir, 1973). The brief occurrence of water from permafrost during the summer months can sustain the growth of microorganisms. However, since the liquid water that can be obtained from it is insufficient, microbial activity is limited (Cowan and Ah Tow, 2004; Wynn-Williams, 1988). Moist and dry soils, however, have water films that are the habitat to protozoa, rotifers, tardigrades and nematodes (Bamforth, 2005). Recent studies show that these organisms are actually of ancient origin and their growth has persevered in isolation for millennia (Convey and Stevens, 2007).

Antarctic soil surface communities may receive elevated levels of UV irradiation periodically, particularly UVB (280-315 nm) and UVC rays (100-280 nm), due to the depletion of the ozone layer (Villar *et al*., 2003). Polar ozone layer depletion was thought to contribute to there being lower levels of diversity in Dry Valley soils, although, incident UV rays probably have little impact on sub-surface soil microbial communities (Cowan and Ah Tow, 2004).

1.2 Antarctic Environmental Niches

Despite the many adverse environmental constraints posed upon Antarctic microbial communities, this extreme ecosystem has been shown to support and promote colonization in niche environments (Pointing *et al*., 2009; Wood *et al*., 2008; Yergeau *et al*., 2007; O'Brien *et al*., 2005; Bérard *et al*., 2005; Anderson and Cairney, 2004; Cockell *et al*., 2003). A biologically diverse cohabitation of organisms, including bacteria, lichen, fungi, moss and algal components have been revealed by advanced molecular methods (Cary *et al*., 2010; Pointing *et al*., 2009).

Prokaryotes represent the largest biomass in Antarctic ecosystems. They colonize salt lakes, anaerobic sediments and thermally heated soils and rocks (Franzmann, 1996). However, diverse heterotrophic organisms have been found in ice-free Antarctic regions such as Dry Valley lakes, ice melts, ephemeral streams and moist soils at lake margins.

Ecological studies of the McMurdo Valley soils by Fell *et al.* (2006) have indicated that nearly all soils contain micro-eukaryotes. Two major groups, ascomycetes and basidiomycetes, were detected using both large subunit (LSU) and small subunit (SSU) ribosomal DNA analysis. Within these groups a number of known and unknown species were encountered (Fell *et al*., 2006).

Using the information gathered from several investigations, it is possible to assess the effects of environmental change, human, natural and unnatural interference, in the Antarctic environment (Cowan and Ah Tow, 2004). Such stochastic events could bring about drastic changes in the primary production in the environment. This could affect the chemical content of the soils, thus impacting the surrounding trophic structures (Cowan and Ah Tow, 2004; Doran *et al*, 2002a).

1.2.1 Lake and Wet Sediment Diversity

Coastal and sub-Antarctic environments have nutrient rich and water rich ornithogenic and fellfield soils (Cowan *et al*., 2002). They support higher and more diverse microbial life, such as bacteria, microalgae, flagellates, ciliates, fungi, and other micro-eukaryotes (Fenchel, 1992) than numerous other Antarctic habitats (Stoek and Epstein, 2003). These marine-influenced environments are, however, biologically and environmentally very different from terrestrial Dry Valley ecosystems, due to the climate, water availability and plant growth achieved. Glacial stream, saline lake and moist soil habitats support a wide range of bacterial

phylotypes, including Acidobacteria, Proteobacteria and Cyanobacteria (Smith *et al*., 2006). The organic matter content of these marine environments are also higher due to the impact of mummified seal carcasses, penguin guano and sea-spray (Cary *et al*., 2010; Convey *et al*., 2009) and as such can support distinct microbial communities.

Sub-glacial sub-oxic and anoxic sediments and waters of the Taylor Glacier also display microbial growth in the absence of sunlight and under permanently cold and elevated nutrient stresses (Mikucki and Priscu, 2007). These communities may survive due to chemoautotrophic or chemoorganotrophic abilities to gain nutrients from ancient soil minerals or even by heterotrophic respiration of iron or sulphur compounds (Mikucki and Priscu, 2007).

1.2.2 Open Soil Diversity

Primary colonization of exposed soils is by pioneering organisms such as cyanobacteria, green algae, lichens, mosses, fungi and heterotrophic bacteria (Cary *et al*., 2010; Smith *et al*. 2006; Breen and Lévesque, 2006), which are capable of utilizing the nutrients that are present in the soil. These species contribute to a significant fraction of the terrestrial microbial population and play a major role in biogeochemical cycling in Antarctic soils (Cary *et al*., 2010).

Previous culture dependant studies describe Dry Valley soils as containing low richness of microbial cells (Friedmann, 1993; McKay, 1993; Vishniac, 1993; Wynn-Williams, 1990; Vincent, 1988; Vishniac and Mainzer, 1972; Cameron *et al*., 1970). Metagenomic analyses in the last decade have, however, reported higher levels of microbial diversity than previously encountered (Cowan and Ah Tow, 2004; Cowan *et al*., 2002). ATP analysis has shown that the Dry Valley environment has a biomass value 3-4 times greater than initially observed (Cowan and Ah Tow, 2004; Cowan *et al*., 2002). Additionally, in the pioneer study performed by de la Torre *et al*. (2003), Dry Valley soils were shown to harbour a diverse range of microbial communities. These soils have yielded isolates of cosmopolitan and indigenous fungal, yeast and protozoan species (Cowan and Ah Tow, 2004).

Moorhead *et al*. (1999) suggested that current soil organic matter was a legacy of previous primary producers (Moorhead *et al*., 1999). Another theory is that modern lacustrine cyanobacteria contribute to the soil organic carbon and nitrogen necessary for Dry Valley systems (Hopkins *et al*., 2010). However, it is possible that a combination of these two factors contribute to the biogeochemical cycling in Dry Valley soils (Cary *et al*., 2010). As such, the homogeneity of microbial ecology across all Dry Valley soils is no surprise (Cary *et al*., 2010). Recent studies show, however, that soil has a lower level of species diversity, with the exception of the areas beneath quartz rocks that are in direct contact with it (Pointing *et al*., 2009). These microenvironments have greater and more distinct diversity when compared to exposed soils (Khan *et al*., 2011; Cowan *et al*., 2010b; Khan, 2008).

1.2.3 Lithic Habitat Diversity

Extreme environmental conditions in the Antarctic Dry Valley's force the colonization of refuge microenvironments, such as the sub-surfaces of sandstones and the ventral surfaces and crevices of marble and quartzite (Cowan *et al*., 2010b; Barrett *et al*., 2006; Cockell *et al*., 2003). A diverse range of microorganisms are distributed throughout these niches (Babalola *et al*., 2009; Cowan *et al*., 2009; Aislabie *et al*., 2006).

Microbial communities (lithobionts) that inhabit the ecological niche within hard mineral substrates (Golubic *et al*., 1981) are associated with rocks that are capable of storing or retaining liquid water internally for several days. They grow on the external surface (epiliths), on the interior of (endoliths) or beneath (hypoliths) (Cowan, 2009; Golubic *et al*., 1981) rocks that contain sufficient moisture and are able to filter surface light to tolerable intensities (Cowan *et al*., 2010b), as depicted by Figure 1.1. This withdrawal of microorganisms to the protected environment can therefore be viewed as a stress avoidance technique (Wong *et al*., 2010).

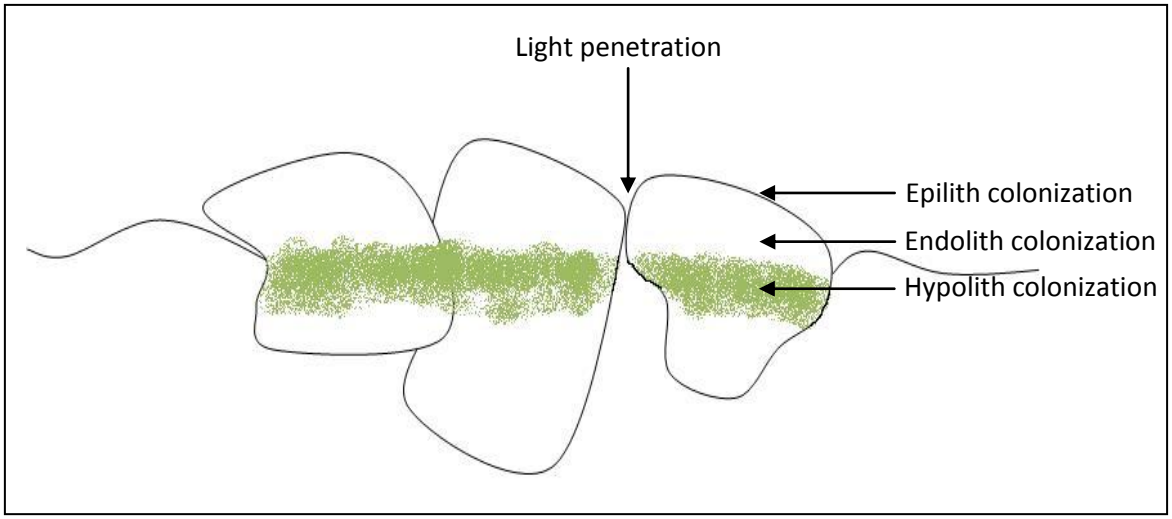

Figure 1.1 Area on quartzite and dolomite rocks in the Dry Valley colonized by epilithic, endolithic and hypolithic communities (adapted from Cockell and Stokes, 2006).

1.2.3.1 Chasmolithic Microbial Communities in the Dry Valleys

The sheared surface, cracks and fissures of weathering rocks provide the perfect niche for the growth of chasmolithic communities. While epilithic growth is observed in the less extreme coastal regions, Dry Valley rock surfaces are more ideally suited to endolithic and hypolithic colonization (Friedmann and Ocampo-Friedmann, 1984; cited in Cowan *et al*., 2010a). The endoliths, which colonize the pore spaces within rocks, and chasmoendoliths, that colonize cracks and fissures in rocks, display the greatest diversity (Wong *et al*., 2010; Pointing *et al*., 2009).

1.2.3.2 Dry Valley Hypolithons

The hypolithic environment provides refuge for photosynthetic microbes from the intense UV irradiation (Khan, 2008). The overlying translucent rock allows sufficient light penetration to support photosynthesis (Pointing *et al*., 2009). Hypolithic

communities, or 'hypolithons', have been documented in a number of other hyper-arid deserts, e.g. the Atacama (Chile) (Warren-Rhodes *et al*., 2006), Mojave (USA) (Schlesinger *et al*., 2003), Negev (Israel) (Berner and Evenari, 1978) and Namib (Africa) (Budel and Wessels; 1991) Deserts.

Hypolithic communities in the Dry Valley region colonize the ventral surface of quartz rock at the rock-soil interface. Each hypolithon exhibits a clear zonation in growth pattern. This divides them into microbial morphological classes or types that separate the cyanobacterial (Type I), moss (Type II) and lichenized (Type III) communities (Cowan *et al*., 2010c; Cowan, 2009; Khan, 2008).

Cyanobacteria occupy the rock surface and seem to form a biofilm-like layer; moss exist on the ground itself and can only be observed once the quartz rocks are dislodged from the ground; the lichenized communities exist as masses of fungal filaments attached to the lower surface of the rock (Cowan, 2009; Khan, 2008) (Figure 1.2). Table 1.1 describes the microbial communities and the location of the 3 types of hypolithons.

Table 1.1 The 3 categories of hypolithic communities observed in the soils of the Miers Valley, Antarctica (Cowan *et al*., 2010c).

	Predominant Species	**Location**
Type I	Cyanobacterial dominated communities	Found adhering to the underside of quartz rock as a greenish biofilm
Type II	Moss dominated communities	Occur on soil directly beneath the rock, but are not adhered to the rock itself
Type III	Lichenized communities	Appear as dry, fragile filaments on the ventral surface of the quartz rock

Hypolith development is influenced by low light intensities, filtered UVA and B, thermal and humidity buffering, higher water availability and physical stability (Cowan *et al.,* 2010c). Recent research shows that hypolithons may develop in succession, where Type I communities give rise to Type II and then Type III communities (Cary *et al*., 2010; Cowan *et al*., 2010c).

Figure 1.2 A quartz hypolith in the Miers Valley. These show (A) quartz rock, colonized by hypolithic cyanobacterial (Type I) and moss (Type II) communities, and (B) a hypolithic lichen (Type III) community (Photo's courtesy of Prof. Don Cowan).

It is known that altitude has an impact on the growth and survival of lichens in moist soils (Smith *et al*., 2006). A similar conclusion was developed in the course of investigation of Miers Valley hypoliths along the valley floor and slopes (Cowan *et al*., 2010). Here, Type I cyanobacterial dominated hypoliths were found to survive under low light conditions at both high and low altitudes, but the occurrence of the eukaryotic Type II and III communities diminished with an increase in altitude. On the Dry Valley floor itself, an abundance of all three hypolith communities was detected. This suggests that water from melting permafrost at lower altitudes, light and temperature may be the principal drivers of metabolic activity and thus hypolith community development in the Miers Valley (Cowan *et al*., 2010c; Pointing *et al*., 2009).

1.3 Prokaryotic and Eukaryotic Hypolith Diversity

Cryptic communities are often described as one of the main sources of microbial diversity in Dry Valley soils (de la Torre *et al*., 2003). Microenvironments promote growth of both prokaryotes and lower eukaryotes, which exist at high biomass levels in suitable niches in hot and cold environments (Cowan *et al*., 2010c; Cowan and Ah Tow, 2004).

Prior to 1988, research and knowledge of prokaryotic diversity was limited to bacteria (Franzmann, 1996). With the advances of molecular techniques, archaea and cyanobacteria were encountered (Hogg *et al*., 2006). Psychrophilic and

psychrotolerant cyanobacterial species (sourced from the surrounding lakes in the Dry Valley), heterotrophic bacteria and archaea, therefore represent the dominant prokaryotes in Dry Valley hypolithic communities (Hoover and Pikuta, 2010; Khan, 2008). Cyanobacteria in particular are ideally suited to survive in the extreme hypolithic environment. These dessication and radiation tolerant photoautotrophs may constitute the primary producers in hypolithic mineral soils (Cowan *et al*., 2010b; Warren-Rhodes *et al*., 2006; Cockell and Stokes, 2004).

Bacteria were initially thought to dominate Dry Valley soil microbial diversity. However, these soils have been reported to support low levels of eukaryotic microbial communities (Rao *et al*., 2011; Cowan, 2009; Pointing *et al*., 2009; Lawley *et al*., 2004). The application of culture independent molecular approaches to Dry Valley hypolithons has increased the knowledge about the composition, dynamics and role of these micro-eukaryote communities (Khan *et al*., 2011; Cowan *et al*., 2010c).

Cold desert hypolithons have shown evidence of photosynthetic unicellular algae and cyanobacteria (Thomas, 2005; Cockell and Stokes, 2004). These provide essential nutrients for the support of an entire niche ecosystem in the extreme Antarctic environment (Thomas, 2005). Based on microscopic and phylogenetic evidence, we now know that the Antarctic hypolithon supports algal forms, moss, lichen

communities and micro-arthropods (Cowan *et al*., 2010c; Block *et al*., 2009; Cockell and Stokes, 2004; Broady, 1980).

1.4 Metagenomic Methods Employed in Diversity Studies

Early ecological studies carried out on Dry Valley soils were based on traditional culture methods and morphological examination of the microorganisms *in situ* (Aislabie *et al*., 2006; Fell *et al*., 2006). Culture-based studies are now known to under-represent microbial abundance and diversity (Valášková and Baldrian, 2009; Hartmann *et al*., 2005). In addition, the organisms observed may not necessarily be dominant or physiologically important in the community (Hogg *et al*., 2006; Diez *et al*., 2001).

Culture independent methods and molecular techniques have contributed significantly to our knowledge of microbial community structure in environmental samples (Wong *et al*., 2010; Diez *et al*., 2001; Muyzer, 1998; Franzmann, 1996; Muyzer *et al.*, 1993). PCR and genetic fingerprinting have allowed for a fast and sensitive alternative to cultivation methods through DNA extraction directly from environmental samples (Muyzer and Smalla, 1998; Liu *et al*., 1997).

Microbial taxonomy and phylogenetic characterization has been revolutionized with molecular techniques such as 16S rRNA gene sequencing, PCR amplification and

cloning (Gast *et al*., 2004; Diez *et al*., 2001; Franzmann, 1996). When used in a polyphasic approach, culture independent techniques are able to provide a higher resolution of microbial diversity than microscopy and morphological methods (Cary *et al*., 2010; Pointing *et al*., 2009; Smalla *et al*., 2007).

Metagenomic techniques are based on extraction of total DNA, followed by cloning and sequence analysis of the small subunit (16S or 18S) ribosomal RNA gene (Fell *et al*., 2006). Fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single strand conformation polymorphism (SSCP) and terminal restriction fragment length polymorphism (T-RFLP) analysis are now routinely used in microbial ecology (Campbell *et al*., 2009; Malosso *et al*., 2006; Muyzer *et al*., 2003; Muyzer and Smalla, 1998; Muyzer *et al*., 1993). These highly reproducible methods allow rapid, simultaneous identification and comparison of microbial community profiles (Valášková and Baldrian, 2009; Diez *et al*., 2001). Insight into the structure and spatial distribution of microbial populations develops an understanding of ecological processes, biogeography and biogeochemical dynamics (Khan *et al*., 2011; Cary *et al*., 2010; Zeglin *et al*., 2009).

1.4.1 Metagenomic DNA Extraction

Metagenomic methods yield a greater amount of information from environmental samples than traditional culture dependant methods (Handelsman, 2004). Total

metagenomic DNA can be screened for phylogenetic markers or anchors (Handelsman, 2004; Muyzer and Smalla, 1998) such as the 16S rRNA gene region, 18S rRNA gene region and/or other conserved gene sequences. Phylogenetic information obtained from these regions can provide valuable insights into the structure and evolution of microbial communities (Cary *et al*., 2010; Moreira *et al*., 2006; Dorigo *et al*., 2002).

General methods of DNA isolation from different organisms vary, depending on the complexity of the cell wall of the organism. Cell extraction and direct lysis techniques are mainly employed to optimally extract cellular DNA (Keyster, 2007; Robe *et al*., 2003; Moré *et al*., 1994). This prevents the failure of cell lysis or over-lysis, and thus fragmentation of metagenomic DNA.

Cell extraction requires the initial isolation and culturing of whole cells for DNA extraction (Miller *et al*., 1999). This process can be more time consuming and laborious than direct lysis. Additionally, only 25-50% of the total endogenous microbial community is accounted for with this method. Direct extraction has proved more effective, with reported total DNA yields of 86-100% (Miller *et al*., 1999; Moré *et al*., 1994). Cell lysis can be achieved by vigorous agitation using glass beads, phenol-chloroform extraction, enzymatic lysis and rapid freezing and thawing (Zhou *et al*., 1996). A combination of two of these methods provides high levels of total metagenomic DNA from environmental samples. Subsequent purification steps are,

however, required in order to obtain DNA free of phenolic/humic acid content and of sufficiently high quality and yield for amplification and cloning.

1.4.2 Phylogenetic Marker Genes

rRNA genes are reliable markers for ascertaining diversity since they are present in all organisms. The 16S and 18S rRNA genes are universal prokaryotic and eukaryotic rRNA gene regions, respectively (Gardes and Bruns, 1993; White *et al*., 1990). They contain both conserved and variable regions that enable design of general or specific primers (using the conserved regions) and allow differentiation between individual taxonomic groups (using the variable regions) (Valášková and Baldrian, 2009; Muyzer, 1998). Typically, the sequence analysis of universal rRNA gene regions provides identification to the genus or family level (Vilgalys, 2010; Valášková and Baldrian, 2009; White *et al*., 1990). PCR amplification of combinations of variable regions allows a more effective evaluation of microbial diversity in a community or environment.

In eukaryotes, the 18S rRNA gene sequence does not yield a sufficient level of sequence variation across the major eukaryotic phyla (Thies, 2007). The use of specific primers permits the characterization of phyla of interest. The fungal internal transcribed spacer (ITS) regions are species specific for most fungi (Martin and Rygiewicz, 2005). They are possibly the most commonly sequenced eukaryotic rRNA gene regions since they provide a more reliable level of similarity to the conserved

sequences in fungal community samples (Thies, 2007; Martin and Rygiewicz, 2005; Kirk *et al*., 2004). This is due to the higher degree of variation in non-coding ITS regions as a result of their rapid evolution (White *et al*., 1990). To selectively amplify regions in fungal DNA, the ITS1 and ITS4 primers were modified by Gardes and Bruns (1993) to amplify basidiomycete ITS sequences, creating the ITS1F and the ITS4 sequences depicted in Figure 1.3 (Gardes and Bruns, 1993).

Fungi in lichenized habitats commonly exist in symbiotic relationships with photosynthetic cyanobacteria and algae (Friedmann and Sun, 2004; Galun *et al*., 1971). PCR analysis of microalgal DNA using a new set of universal primers, P45 and P47 (Dorigo *et al*., 2002) shown in Figure 1.3, amplify a ± 400 bp region in the 18S rRNA gene. These primers were created to detect the most common algal classes, Bacillariophyceae, Chlorophyceae, Cryptophyceae and Chrysophyceae (Dorigo *et al*., 2002). Species within these classes have been observed, initially through microscopic studies (Broady, 1981) and later through ribosomal RNA sequencing (Vincent, 2000) in the sublithic environment of the McMurdo Dry Valleys, the Ross Sea and Antarctic sea ice and lakes.

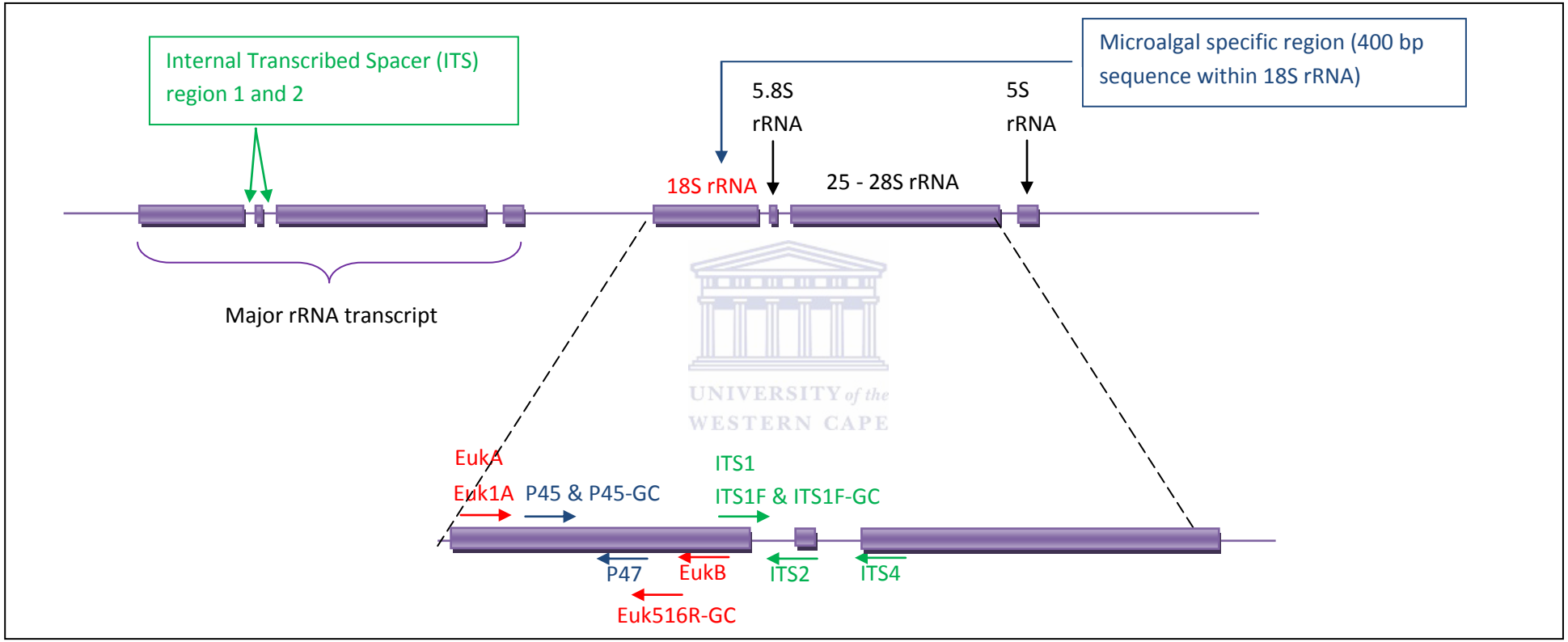

Figure 1.3 Schematic representation of the regions on the rRNA gene that are amplified from total metagenomic environmental DNA using the general eukaryotic (Euk), fungal specific (ITS) and microalgal specific (P45 & P47) primers.

1.4.3 Molecular Fingerprinting Methods

Genetic fingerprinting of an environmental sample facilitates the phylogenetic identification of the dominant members of a population (Valášková and Baldrian, 2009; Diez *et al*., 2001). Depending on the methods employed, a quantitative and/or qualitative representation of the fingerprint data is provided. While techniques like DGGE are more suitable for the analysis of low or moderately complex soil communities, or for providing an efficient snap-shot of the dominant microorganisms, T-RFLP techniques may be a superior tool for monitoring the overall complexity and spatial distribution of more diverse communities (Schwartzenbach *et al*., 2007; Lukow *et al*., 2000)

1.4.3.1 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is a fingerprinting technique that is based on the electrophoretic mobility of a partially melted DNA molecule in a polyacrylamide gel. A urea and formamide based chemical gradient separates DNA of the same length but with different GC content due to sequence variation (Malosso *et al*., 2006; Muyzer *et al*., 1993). DGGE provides the initial insight that is required to further investigate diversity using quantitative and qualitative molecular techniques.

1.4.3.2 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

T-RFLP analysis is a semi automated molecular fingerprinting technique. It permits phylogenetic identification of microorganisms (Hartmann *et al*., 2005) by detecting differences in the localization of restriction sites in DNA sequences (Kitts, 2001). It is an extremely reproducible method that provides a high resolution profile of diverse microbial communities detected in soil when compared to other PCR-based fingerprinting methods (Thies, 2007; Hartmann *et al*., 2005).

1.4.4 Clone Libraries

The cloning of selectively amplified small subunit (SSU) rRNA genes from environmental samples has been used extensively to assess the community structure of marine and soil environments, including hot and cold desert hypoliths (Lacap *et al*., 2011; Wong *et al*., 2010; Pointing *et al*., 2009; Diez *et al*., 2001; Guillou *et al*., 1999). It involves the incorporation of a PCR amplicon into a suitable vector by ligation and transformation of the ligated product into a host organism (Sambrook and Russel, 2001). This generates a large number of clones for further analysis.

Amplified ribosomal DNA restriction analysis (ARDRA) with one or more restriction enzymes is used to dereplicate and analyse PCR amplified products of mixed populations. When performed on clone sequences, it eliminates the need to sequence multiple common inserts and instead allows for dereplication and

phylotyping based on restriction fragment length polymorphism profiles (Gich *et al*., 2000).

1.4.5 Molecular Systematics

"Systematics is the scientific study of the kinds of organisms and of any and all relationships among them" (Simpson, 1961). Molecular systematics would therefore be defined as the use of molecular structures to investigate relationships between organisms, both within and between species. These relationships are depicted on a phylogenetic tree created from the level of similarity between organisms (Hillis *et al*., 1996).

DNA and RNA sequences can be used to reconstruct phylogenies, as well as in the identification and classification of organisms. Direct sequencing of metagenomic DNA isolated from soil samples provides the data needed for community phylogenetic studies. Through the comparison of universal and phylotype-specific rRNA genes, the total diversity in an environmental sample can be observed in an unbiased manner (de Souza *et al*., 2004).

Primary sequence identity assessment, known as BLAST (Basic Local Alignment Search Tool) analysis, available at the NCBI website (http://blast.ncbi.nlm.nih.gov/), is performed. It allows the comparison of nucleotides to those stored in web-based

gene databases (Soltis *et al*., 1998). Alignment of suitable sequence data is done using software tools such as ClustalW in BioEdit or MEGA (Tamura *et al*., 2011; Harrison and Langdale, 2006).

Phylogeny reconstruction using the resultant alignments is performed to generate a phylogenetic tree. This tree can be either rooted with the sequence of a common ancestor, to show evolutionary distance, or unrooted to investigate the relationship between organisms that are analysed (Tamura *et al*., 2011). Phylogenetic trees can be generated using computer algorithms that determine maximum parsimony, maximum likelihood and neighbour-joining (Harrison and Langdale, 2006).

Maximum parsimony was originally designed for use on morphological characteristics but is also used for nucleotide and amino acid analysis. This technique, however, is time consuming when aligning a large number of sequences (Nei and Kumar, 2000). Maximum likelihood models create a number of plausible cladograms or trees, and thereafter choose the tree that best represents the data-set. It is a robust method to analyse the evolutionary relatedness of sequences as it utilizes user-specified parameters (P-distance, Jukes-Cantor, Kimura 2P) to test the distance between the sequences (Tamura *et al*., 2011). While these methods reliably generate phylogenetic trees, the neighbour-joining method is preferred in many diversity studies as a result of the rapid performance (Hall, 2001). Neighbour-joining results in the creation of a single tree that is unrooted, but can be rooted using an

outgroup taxon (Tamura *et al*., 2011). A statistical analysis of the confidence level for each branch in the resulting tree can be provided using the bootstrap and jack-knife support values (Harrison and Langdale, 2006; Soltis *et al*., 1998). Bootstrap values are commonly used in diversity studies. The reliability of any branch in a phylogenetic tree is assessed such that a 95% confidence level implies correct tree topology (branching) (Tamura *et al*., 2011).

1.5 Aim

The main aims of this study were (I) to determine the eukaryotic microbial constituents of hypolith communities in Miers Valley, Antarctica and (II) to compare this composition between the cyanobacterial (Type I), moss (Type II) and lichen (Type III) dominated hypolithons. An investigation of the eukaryotic communities in hypolithons may be able to provide insight into understanding the community structure and ecosystem function in the Dry Valleys and other cold deserts.

1.6 Objectives

To successfully elucidate the dominant microorganisms present in the Miers Valley hypolithon, as well as accomplish phylogenetic analysis of the eukaryotic diversity, community fingerprinting and sequencing methods were used. The following molecular techniques were applied based on previous studies (including unpublished work at the Institute for Microbial Biotechnology and Metagenomics (IMBM)):

- PCR amplification of isolated metagenomic DNA from Type I, II and III hypolith communities, targeting the 18S rRNA gene (universal and microalgal specific), and the fungal internal transcribed spacer (ITS) region,
- Denaturing gradient gel electrophoresis (DGGE) to obtain a snap-shot of the total dominant eukaryotic members of the communities,
- Terminal restriction fragment length polymorphism (T-RFLP) analysis for microbial community structure characterization and to provide a quantitative analysis of the microorganisms present,
- Clone library construction of the amplified genes, followed by characterization of different phylotypes and sequencing of selected clones, enabling phylogenetic relatedness and microbial diversity assessment.

CHAPTER 2
MATERIALS AND METHODOLOGY

2.1 Samples and Collection

Hypolith samples were obtained from Miers Valley, Antarctica, courtesy of Prof. Don Cowan, during November - December 2009. Samples from the various sites were collected aseptically and stored in WhirlPak® bags at 4°C in the field and during transport. Long term storage was at -80°C in the laboratory, prior to further analysis. A total of 7 hypolith samples (Table 2.1) were chosen for metagenomic DNA extraction, based on the presence of green/brown biomass (moss) (Cowan *et al.*, 2010).

Table 2.1 Nov-Dec 2009 Expedition: Miers Valley hypolith samples used for metagenomic DNA isolation.

Community Type	Hypolith Sample	Collection Site
Cyanobacterial dominated Type I	MVH 09 23	Stream region of W camp. South slope and at top of S slope transect
	MVH 09 65	Traverse to lower Miers north side i-button transect
	MVH 09 75	Alti Piano
Moss dominated Type II	MVH 09 113	Lower altitude upper Miers sites
	MVH 09 134	Lower altitude upper Miers sites
Lichenized Type III	MVH 09 50	Camera location
	MVH 09 79	Alti Piano

2.2 Metagenomic DNA Extractions

Total soil DNA was extracted using the 50-50-50 buffer-chloroform-phenol method (Von Sigler, 2004). One millilitre of extraction buffer (50 mM NaCl; 50mM Tris-HCl at pH 7.6; 50 mM EDTA; 5% SDS) was added to 1 g of each soil sample in 2 ml vials containing 0.4-0.5 ml mesh sea-sand. Thereafter, 1 µl of 1 M dithiothreitol (DTT) was added to the lid of each vial and mixed by vortexing briefly. All samples were shaken for 15 minutes at maximum speed on the Vortex Genie 2 (Scientific Industries Inc., USA) followed by 3 minutes of centrifugation at 14 000× *g* in an Eppendorf 4524 Centrifuge, to separate the soil and sea-sand from the supernatant. The supernatant was carefully decanted into sterile 2 ml microcentrifuge tubes. Once the final volume of supernatant was determined, 0.5× volumes of both phenol and chloroform/isoamyl alcohol was added to the tubes, which were vortexed until the solution appeared milky. This was followed by centrifugation at 14 000× *g* for 3 minutes, to separate the aqueous phase (containing the DNA) from the other phases. The aqueous phase was transferred to another 2 ml sterile tube, and an equal volume of chloroform was added, vortexed and centrifuged as before, to separate the phases. The aqueous phase was transferred to a sterile 1.5 ml tube for nucleic acid precipitation and volume determination. Thereafter, 0.1 volumes of 3 M sodium acetate and 0.7 volumes of isopropanol were added to the solution and mixed gently by inversion. DNA was precipitated by centrifugation at 14 000× *g* for 30 minutes at 10°C. The isopropanol-sodium acetate solution was aspirated, being careful to not dislodge the pellet. The pellet was then washed by the addition of 0.5 ml of 70% ethanol and gentle inversion of the tube. This was centrifuged for 5

minutes at 14 000× *g*, the ethanol aspirated and the pellet allowed to air dry before resuspension in 25 µl of sterile distilled water.

The presence of DNA was confirmed by gel electrophoresis on a 1% agarose gel made up with 1× TAE Buffer (40 mM Tris-HCl; 10 mM glacial acetic acid; 1 mM EDTA at pH 8.0), viewed using the AlphaImager 3400 (AlphaInnotech) imaging system and quantified using the NanoDrop® ND-1000 UV/Vis Spectrophotometer (NanoDrop Technologies, USA) in triplicate.

2.3 PCR Amplification of rRNA Genes

The PCR primers and parameters described in Table 2.2 were used for the amplification of the 18S (EukA/EukB/Euk516R-GC/Euk1A/EukA-FAM), fungal (ITS1F/ ITS4/ITS1F-GC/ITS2/ITS1F-FAM) and microalgal 18S (P45/P47/P45-GC/P45-FAM) rRNA gene regions from purified metagenomic DNA. The basic components of a 25 µl reaction consisted of 12-20 ng metagenomic DNA, 1× DreamTaq™ reaction buffer (Fermentas, USA), 0.2 mM of each dNTP, 0.5 µM of each primer and 0.2 U DreamTaq™ DNA polymerase (Fermentas, USA). Control reactions were included in all rounds of PCR: Positive controls for all reactions contained DNA sourced from Ascomycota fungus, while negative controls contained all the reagents with no template DNA. All PCR reactions were optimized for annealing, in addition to various forms of troubleshooting steps being performed, to reduce the generation of non-specific amplifications.

Table 2.2 PCR primers used to amplify universal 18S, 5.8S, Internal Transcribed Spacer and microalgal 18S regions of ribosomal RNA genes from eukaryotic microorganisms, and their respective PCR cycling parameters.

Primer Set	Sequence (5' - 3')	Region of Amplification	PCR Parameters	Reference
EukA **EukB**[a]	AACCTGGTTGATCCTGCCAGT TGATCCTTCTGCAGGTTCACCTAC	18S rRNA gene	94°C for 3 min **30 cycles**: 94°C for 45 s, 50°C for 1 min, 72°C for 3 min 72°C for 20 min	Diez *et al*., 2001
EukA-FAM **EukB**[b]	AACCTGGTTGATCCTGCCAGT TGATCCTTCTGCAGGTTCACCTAC	18S rRNA gene	94°C for 3 min **30 cycles**: 94°C for 45 s, 50°C for 1 min, 72°C for 3 min 72°C for 20 min	Diez *et al*., 2001
Euk1A **Euk516R-GC**[c]	CTGGTTGATCCTGCCAG CGCCCGGGGCGCGCCCCGGGCGGG GCGGGGGCACGGGGGGACCAGACT TGCCCTCC	18S rRNA gene	94°C for 3 min **8 cycles**: 94°C for 45 s, 60°C touchdown to 56°C for 45 s, 72°C for 2 min **27 cycles**: 94°C for 45 s, 56°C for 45 s, 72°C for 2 min 72°C for 15 min	Diez *et al*., 2001
ITS1F **ITS4**[a]	CTTGGTCATTTAGAGGAAGTAA TCCTCCGCTTATTGATATGC	5.8S, ITS1 and ITS2	94°C for 5 min **35 cycles**: 94°C for 1 min, 50°C for 1 min, 72°C for 1 min 72°C for 20 min	Gardes and Bruns, 1993 White *et al*., 1990
ITS1F-FAM **ITS4**[b]	CTTGGTCATTTAGAGGAAGTAA TCCTCCGCTTATTGATATGC	5.8S, ITS1 and ITS2	94°C for 5 min **35 cycles**: 94°C for 1 min, 50°C for 1 min, 72°C for 1 min 72°C for 20 min	Gardes and Bruns, 1993 White *et al*., 1990
ITS1F-GC **ITS2**[c]	CGCCCGCCGCGCCCCGCGCCCGGCC CGCCGCCCCCGCCCCTCCTCCGCTTA TTGATATGC GCTGCGTTCTTCATCGATGC	ITS1	94°C for 5 min **5 cycles**: 94°C for 1 min, 64°C for 1:20 min, 72°C for 1 min **5 cycles**: 94°C for 1 min, 64°C for 1:20 min, 72°C for 1 min **25 cycles**: 94°C for 1 min, 65°C for 1:20 min, 72°C for 1 min 72°C for 25 min	Gardes and Bruns, 1993 White *et al*., 1990

[a] primers employed in first round standard PCR amplification of metagenomic DNA
[b] FAM-labelled primers used in T-RFLP
[c] PCR primers containing GC clamps used in nested PCR amplification for DGGE analysis
[d] primers targeting the M13 region on the pGEM-T Easy vector

Table 2.2 Continued.

Primer Set	Sequence (5' - 3')	Region of Amplification	PCR Parameters	Reference
P45 **P47**[a]	ACCTGGTTGATCCTGCCAGT TCTCAGGCTCCCTCTCCGGA	400 bp fragment within 18S rRNA gene	94°C for 1 min **37 cycles**: 92°C for 50 s, 57°C for 50 s, 72°C for 50 s 72°C for 10 min	Dorigo *et al*., 2002
P45-FAM **P47**[b]	ACCTGGTTGATCCTGCCAGT TCTCAGGCTCCCTCTCCGGA	400 bp fragment within 18S rRNA gene	94°C for 1 min **37 cycles**: 92°C for 50 s, 57°C for 50 s, 72°C for 50 s 72°C for 10 min	Dorigo *et al*., 2002
P45-GC **P47**[c]	CGCCCGCCGCGCCCCGCGCCCGGCC CGCCGCCCCCGCCCCACCTGGTTGAT CCTGCCAGT TCTCAGGCTCCCTCTCCGGA	400 bp fragment within 18S rRNA gene	94°C for 3 min **8 cycles**: 94°C for 45 s, 60°C touchdown to 56°C for 45 s, 72°C for 20 s **27 cycles**: 94°C for 45 s, 56°C for 45 s, 72°C for 2 min 72°C for 15 s	Dorigo *et al*., 2002
M13fw **M13rev**[d]	AGCGGATAACAATTTCACACAGG CCCAGTCACGACGTTGTAAAACG	M13 Cloning Vector	94°C for 5 min **10 cycles**: 94°C for 30 s, 65°C touchdown to 55°C for 30 s, 72°C for 1:30 min **25 cycles**: 94°C for 30 s, 55°C for 30 s, 72°C for 1:30 min 72°C for 5 min	Promega, USA

[a] primers employed in first round standard PCR amplification of metagenomic DNA
[b] FAM-labelled primers used in T-RFLP
[c] PCR primers containing GC clamps used in nested PCR amplification for DGGE analysis
[d] primers targeting the M13 region on the pGEM-T Easy vector

Gel electrophoresis was performed using 5 µl of the PCR products on 1% agarose to confirm amplification of the correct sized inserts. PCR amplicons from the first round of amplification, i.e. EukA & EukB, ITS1F & ITS4 and P45 & P47 amplicons, were purified with the GFX™ PCR DNA and Band Purification Kit (Illustra™) and quantified using the NanoDrop® ND-1000 (NanoDrop Technologies, USA).

2.4 DGGE

DGGE was carried out using the Bio-Rad DCode™ Universal Mutation Detection System (Bio-Rad, USA), according to Muyzer *et al*. (1993), with slight modifications to the protocol. DGGE PCR primers described in Table 2.2 were used in the nested PCR amplification of the initial round of PCR. Products containing 5' GC-rich clamps were separated on 9% polyacrylamide gel containing a high and low chemical gradient of denaturing gel solutions consisting of 0% and 100% denaturant stock solutions. The 0% solution consisted of 40% acrylamide (37.5:1 acrylamide:*N,N*-methylene bisacrylamide and 1× TAE buffer). The 100% solution consisted of 7 M urea and 40% deionised formamide in addition to the 40% acrylamide and 1× TAE buffer. Both solutions were filtered through a 0.45 µ filter and stored light protected at 4°C. TEMED and ammonium persulfate (APS) were added to a final concentration of 0.09% (v/v).

Broad range polyacrylamide denaturing gradients (20-60%, 30-70% and 20-50%) were used to separate 18S, fungal ITS and microalgal rRNA gene amplicons,

respectively. Duplicate wells of the gel were loaded with 45 µl of nested PCR, with 10× Orange G (BDH Laboratory Supplies, England) functioning as a loading dye. Electrophoresis was performed at a voltage of 100 for 16 hours in 1× TAE Buffer at 60°C. Once removed from the buffer chamber and gel sandwich, the gel was stained using 0.5 µg/ml EtBr in 1× TAE buffer for 15 minutes and thereafter de-stained in 1× TAE buffer for 15 minutes. Gels were viewed and captured under UV with the AlphaImager 3400 (AlphaInnotech) imaging system. Prominent bands observed on the gels were excised, allowed to diffuse into 50 µl of sterile distilled water for 4-5 hours, re-amplified via PCR with the same DGGE primer set, and electrophoresed on a polyacrylamide gel to the same parameters as for the initial DGGE.

The band excision, diffusion and PCR processes were repeated to retrieve the most prominent bands, before purification of the products using the Illustra™ GFX™ PCR DNA and Band Purification Kit. Purified products were then sent for sequencing with Euk1A (general eukaryotic rRNA gene), ITS2 (fungal ITS rRNA gene) and P47 (microalgal 18S rRNA gene) primers (Table 2.2), at the University of Stellenbosch Sequencing Facility. Analysis of the DGGE profiles was performed using image analysis software GelCompar® II, Version 5.0 (Applied Maths), and was based upon presence or absence of bands. Individual bands represented a single OTU. Multidimensional scaling (MDS) and dendrograms were generated using the Pearson correlation coefficient with the Unweighted Pair Group Method using Arithmetic averages (UPGMA) algorithm.

2.5 T-RFLP

PCR was carried out on the extracted metagenomic DNA using 5' labelled FAM (phosphoamidite fluorochrome 5-carboxyfluorosceine) fluorescent labelled primers. These were specific to the 18S rRNA gene region, the fungal ITS rRNA gene region and the microalgal 18S rRNA gene region. The FAM labelled forward primer and unlabelled reverse primers for each set were used to amplify the target genes, using the same cycling parameters as the standard PCR (Table 2.2). Agarose gel electrophoresis was performed to confirm amplification and the products were purified using the NucleoSpin® Extract II PCR Clean Up Kit (Macherey-Nagel), prior to quantifying using the NanoDrop® ND-1000.

Restriction of the purified products using *Alu*I, *Ava*I, *Hae*III, *Hin*fI, and *Msp*I was carried out according to the manufacturer's instructions. A digest was performed on each product, where 10 µl of PCR product was cleaved in 2 µl of restriction buffer, 1 µl of restriction enzyme and the volume adjusted to 30 µl with sterile double distilled H_2O. The combinations of enzymes utilized for the sample digests were:

- 18S Type I, II and III - *Hae*III and *Alu*I,
- ITS Type I and II - *Hin*fI and *Msp*I,
- ITS Type III - *Ava*I and *Msp*I,
- Microalgal 18S Type I, II and III - *Ava*I and *Msp*I.

Digested products were purified with the NucleoSpin® Extract II PCR Clean Up Kit and sequenced at the University of Stellenbosch Sequencing Facility. Sequences were analysed by peak identification and fragment sizing using Peak Scanner™ Software, Version 1.0 (Applied Biosystems) with a GeneScan™-500LIZ® size standard.

T-RF's were filtered for noise, aligned and converted into a binary matrix using T-REX (Culman *et al*., 2009). PRIMER 6 (Primer-E, UK) was used for statistical analysis of the T-RFLP profiles. Species richness (d) and distribution across the community as well as univariate diversity indices were calculated by using PRIMER 6 on the T-RF generated matrix data. The Shannon Index (H'), the Simpson Index (1-λ') and Pielou's evenness (J') were calculated for the statistical determination of alpha and beta diversity.

Phylogenetic assignment of the T-RF's was achieved through the online Phylogenetic Assignment Tool, PAT (Kent *et al*., 2003). Databases for comparison of these T-RF's were created using the virtual digest function on the Microbial Community Analysis, MiCA, website (Shyu *et al*., 2007) using all known eukaryotes from the SILVA online eukaryote database (Pruesse *et al*., 2007).

2.6 Clone Library Construction and Screening

Purified PCR products of 18S, ITS and microalgal 18S rRNA genes from the first round of amplification of the 3 hypolith types (section 2.3) were ligated into the pGEM®-T

Easy vector system (Promega, USA), as per the manufacturer's instructions. Ligation reactions of 10 µl, consisting of 5 µl of 2× Rapid Ligation Buffer, 1 µl of pGEM®-T Easy vector, 1 µl of T4 DNA ligase and 3 µl of the purified PCR product, were prepared and incubated overnight at 4°C, in order to produce the maximum number of transformants. The ligations were transformed into chemically competent *E. coli* GeneHogs™ (Invitrogen), with positive and negative controls for both ligation and transformation.

2.6.1 Preparation of Chemically Competent Bacterial Cells

One millilitre of overnight *E. coli* GeneHogs™ grown at 37°C was diluted to 30 ml in SOC medium (20 g bactopeptone; 5 g yeast extract; 7.2 g glucose; 1.9 g $MgCl_2$; 4.93 g $MgSO_4$; 0.36 g KCl per litre). This culture was incubated for 1-2 hours at 37°C on a shaking incubator until the optical density (OD) reached 0.375 at 590 nm. The cultures were then placed on ice for the remainder of the procedure, to stop cell growth and maintain cell viability.

Cells were then pelleted at 7 000× *g* in an Eppendorf Centrifuge 5417R for 10 minutes. The supernatant was decanted and the cells resuspended in 15 ml ice cold 100 mM $CaCl_2$. This centrifugation and resuspension procedure was repeated. After the second resuspension, the sample was incubated for 20 minutes on ice. A final centrifugation was performed, the supernatant was discarded and the cells were resuspended in a 2 ml $CaCl_2$/glycerol solution (100 mM $CaCl_2$; 10% glycerol)

(Sambrook and Russel, 2001). Cells were dispensed into 100 µl aliquots and stored overnight at 4°C. The cells were then stored at -80°C for later use.

2.6.2 Transformation

Transformation was carried out using the heat shock protocol, as per Sambrook and Russel (2001). Two microlitres of each ligation reaction was added to 50 µl of competent cells and gently mixed and placed on ice for 20 minutes. The cells were heat shocked for 45 seconds in a 42°C water bath and incubated on ice for 2 minutes. Nine hundred and fifty microlitres of SOC medium was added to the cells which were then incubated for 1.5 hours at 37°C in a shaking incubator. Aliquots of 100 µl of the transformants were transferred and spread onto duplicate LB plates (1% peptone; 0.5% yeast extract; 1% NaCl; 1.5% agar) containing ampicillin (100 µg/µl), X-Gal (80 µg/ml) and IPTG (0.5 mM). These were incubated overnight at 37°C and later transferred to 4°C to allow further development of blue colonies.

Positive recombinant clones were selected by blue-white colony screening. These recombinants were picked using sterile toothpicks and placed into separate 96 well plates containing TE buffer (10 mM Tris-HCl; 1 mM EDTA at pH 8) and LB_{amp} broth(1% peptone; 0.5% yeast extract; 1% NaCl). TE samples were stored at -20°C for use in downstream PCR applications, while LB_{amp} samples were incubated overnight at 37°C to create 50% glycerol stocks that were stored at -80°C.

2.6.3 PCR-based Clone Library Screen

The presence of the correct sized insert was verified with a PCR-based screen using the M13fw and M13rev vector primers (Fermentas, USA), using the cycling parameters described in Table 2.2. Single clone colonies from the TE stocks (section 2.6.2) were used as the PCR templates. Using the positively amplified products obtained from this screen, a nested PCR with 18S, ITS and microalgal 18S rRNA gene fragment primers was performed to further verify the presence of inserts.

2.6.4 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Clones were screened by ARDRA, where amplicons from the nested PCR (section 2.6.3) were digested with restriction enzymes (section 2.5). The digest was performed in 15 or 30 µl volumes and incubated overnight at 37°C. Digested products were electrophoresed on 2.5% agarose gels. After capturing the image, phylotypes were manually assessed and clones of each phylotype were purified and sequenced.

2.6.5 Plasmid Purification

Selected clones of each phylotype were inoculated from the 50% glycerol stock (section 2.6.2) into separate McCartney bottles containing 5 ml of LB_{amp} broth and were incubated for 16 hours at 37°C in a shaking incubator. The cells were then harvested by centrifugation for 3 minutes at 17 900× *g* in an Eppendorf 5424

Centrifuge and the supernatants discarded. All proceeding centrifugations were performed at 17 900× *g*. Plasmid DNA purification was performed using the QIAprep® Spin Miniprep Kit protocol (QIAGEN, GmbH, USA), according to the manufacturer's instructions. Each plasmid preparation was quantified using the NanoDrop® ND-1000. Aliquots of 20-30 µl of ± 100 ng/µl of each preparation were sequenced by the Molecular and Cellular Biology Department at the University of Cape Town.

2.6.6 Phylogenetic Analysis

Estimates of the phylotype richness and sampling efficiency of clone libraries were performed using the online tools of the Association for the Societies of Limnology and Oceanography (http://www.aslo.org/lomethods/free/2004/0114a.html) (Kemp and Aller, 2004). Mothur software (Schloss *et al*., 2009) was used to perform chimera analysis, description and comparison of communities as well as species richness calculations. Multiple sequence alignments of the cloned sequences and their top NCBI BLASTn hits was performed in MEGA version 5 software (Tamura *et al*., 2011), using the ClustalW alignment tool. Thereafter, phylogenetic and molecular evolutionary analysis was performed to generate an evolutionary tree using the neighbour-joining method. *Escherichia coli* 16S rRNA region was used as an outgroup for rooting. The p-distance evolutionary model was used with 1000 bootstrap replications. Bootstrap values that are depicted on phylogenetic trees correlate to the reliability of the phylogenetic tree. Low bootstrap values between organisms on the nodes of an evolutionary tree imply low confidence. Taxa depicted on the

phylogenetic trees were the sequences that displayed the highest % identity to the cloned sequences using BLASTn comparisons. Hits that displayed e-values below 0.0 were included in the phylogenetic trees. All other sequences were excluded from further analysis.

CHAPTER 3
RESULTS AND DISCUSSION

3.1 Introduction

Hypoliths are the major contributors to microbial diversity and primary production in the Dry Valleys (Khan *et al*., 2011; Smith, 2006; Broady, 1981). Antarctic hypolithons were once recognised as being primarily cyanobacteria dominated (Wong *et al*., 2010; Wood *et al*., 2008; Cockell and Stokes, 2004). However, recent studies have described cyanobacterial, moss and lichen dominated communities that survive beneath the quartz rocks on the desert pavement. These communities are categorised by gross morphology and are the Type I, Type II and Type III hypolithons, respectively (Khan *et al*., 2011). Each hypolithon has been shown to harbour distinct microbial life and this is supported by 16S rRNA gene studies (Khan *et al*., 2011; Cowan *et al*., 2010b; Pointing *et al*., 2009). The eukaryotic composition of hypolith systems in the Dry Valley, however, is yet to be described.

Eukaryotic diversity in Dry Valley Deserts is often underestimated (Fell *et al*., 2006), however, in lower valley regions, algal, moss, lichen and arthropod life is evident (Cowan *et al*., 2010c; Block *et al.,* 2009; Cockell and Stokes., 2004; Broady, 1980). Due to the contribution of hypoliths to primary production in Antarctica (Hopkins *et al*., 2006) and the evidence of eukaryotic diversity by the hypolith niche, lower eukaryotes are likely to contribute significantly to primary production. As such,

understanding the phylogenetic composition of the eukaryotic, fungal and microalgal diversity in each of the three hypolithons is important. The result of this phylogenetic characterization will demonstrate whether they are in fact the major contributors in these terrestrial niches.

3.2 Metagenomic DNA Isolation

Total metagenomic DNA was isolated from each hypolith, as described in section 2.2. Hypolith samples yielded high quality DNA (Figure 3.1), where concentrations ranged from 9.85-345.5 ng/µl (Table 3.1). Each DNA isolation was used for downstream PCR applications.

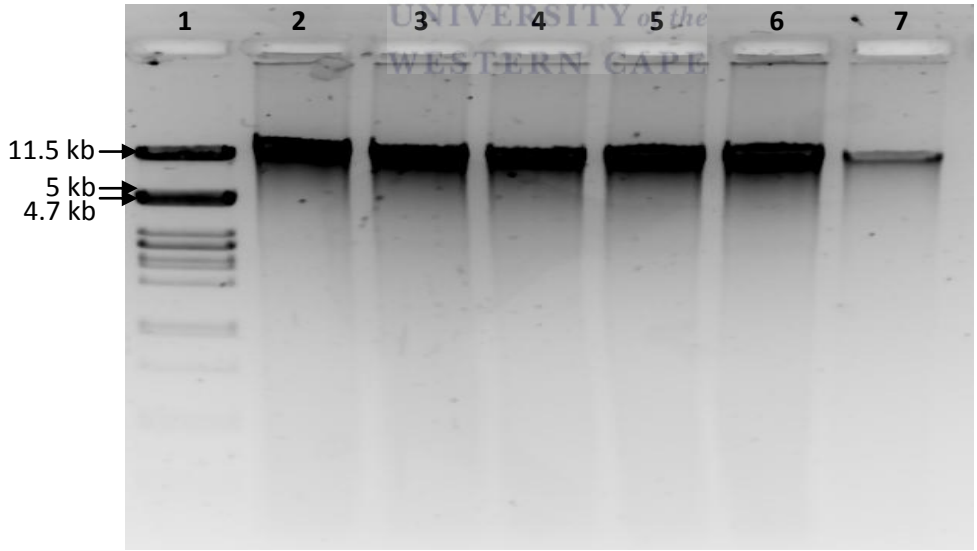

Figure 3.1 Metagenomic DNA isolation from Type I, II and III hypoliths, performed on 6 hypolith communities. Lane 1: Molecular weight marker, Lane 2 and 3: Type I hypolith, Lane 4 and 5: Type II hypolith, Lane 6 and 7: Type III hypolith.

Table 3.1 Concentration of metagenomic DNA yielded from each hypolithon sample.

Hypolith	Community Type	Average Concentration (ng/µl)
MVH 09 23	Type I	345.5 ± 12.8
MVH 09 65		9.85 ± 0.05
MVH 09 75		142.9 ± 1
MVH 09 113	Type II	61.05 ± 0.05
MVH 09 134		185.35 ± 7.05
MVH 09 50	Type III	154.7 ± 0.1
MVH 09 79		98.45 ± 0.95

3.3 Eukaryote Community Profiling of the 18S rRNA Gene Region

The general eukaryotic region of the rRNA gene was targeted using the universal 18S rRNA gene region PCR primers, EukA and EukB (Table 2.2). At least one of each hypolith community provided sufficient amplification for DGGE and T-RFLP analyses, and the expected fragment sizes were obtained (Figure 3.2). Despite the non-specific amplification in lane 4, the nested PCR was performed, as the mere presence of an amplicon in this initial PCR permits further analysis. The multiple bands could imply multiple or truncated copies of the 18S rRNA gene, as the number of copies of small subunit rRNA genes present in uncultured organisms is as yet unknown (Farrelly *et al*., 1995). They could additionally be a result of the formation of heteroduplex molecules during PCR (Aguilera *et al*., 2006).

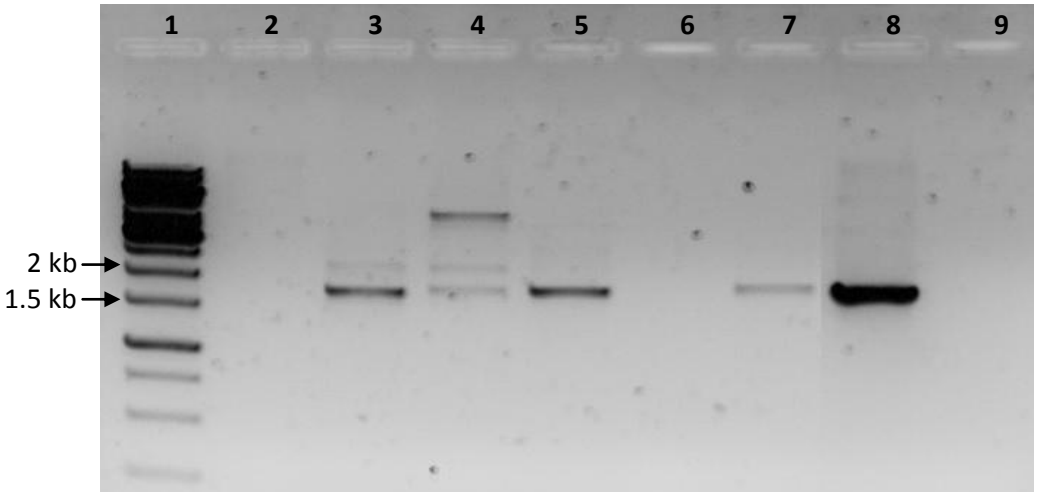

Figure 3.2 PCR amplification using universal 18S rRNA gene PCR primers on metagenomic template DNA. Lane 1: Molecular weight marker, Lane 2 and 3: Type I hypolith, Lane 4 and 5: Type II hypolith, Lane 6 and 7: Type III hypolith, Lane 8: Ascomycota positive control, Lane 9: negative control. Fragments of interest are located between 1.5 kb and 2 kb.

3.3.1 Denaturing Gradient Gel Electrophoresis Fingerprinting of the 18S rRNA Gene Region

A nested PCR was performed with primers Euk1A and Euk514R-GC, with the EukA-EukB PCR amplicons (section 3.3) acting as template DNA (Figure 3.3). Non-specific amplifications were excluded in the downstream fingerprinting analysis, as the nested PCR step was used to generate amplicons with increased specificity. Non-specificity was unable to be completely eliminated, especially in nested PCR reactions. As such, the data analysis of the proceeding DGGE's was performed by sequencing the PCR generated amplicons extracted from excised DGGE bands, in addition to band matching to interpret the result.

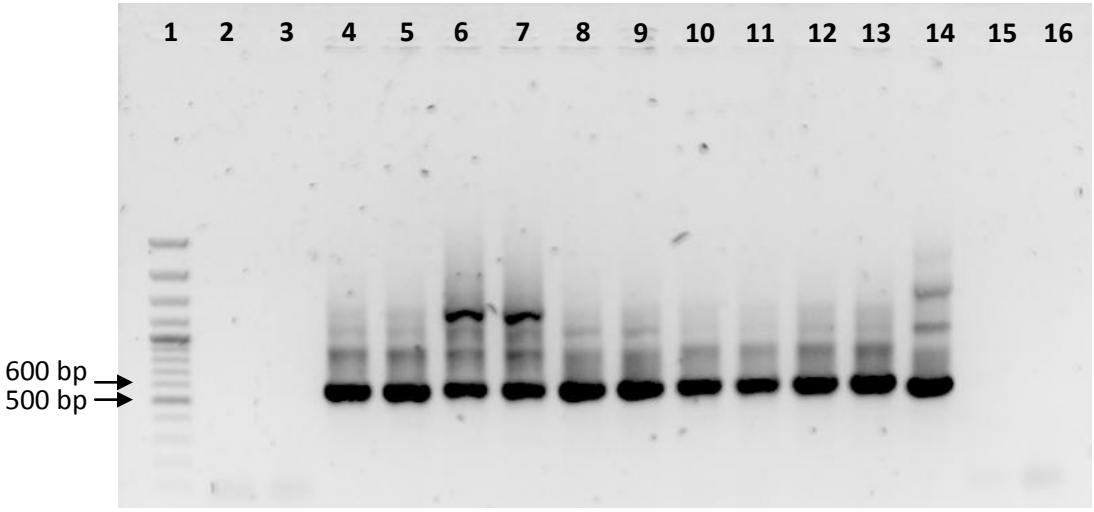

Figure 3.3 Nested PCR amplification of the 18S rRNA gene. Lane 1: Molecular weight marker, Lane 2-4: Type I hypoliths in duplicate, Lane 5-8: Type II hypoliths in duplicate, Lane 9-12: Type III hypoliths in duplicate, Lane 13: Ascomycota positive control, Lane 14: negative control. Bands of interest are located between the 500 bp and 600 bp position.

PCR products separated on 9% polyacrylamide gels with a 20-60% denaturing gradient showed a number of similar and dissimilar OTU's (Figure 3.4). The unique bands that were excised from each hypolithon fingerprint corresponded to *Tetracladium marchalianum* and *P. truncata* in Type I hypoliths*, P. truncata* and *Trebouxiophyceae* sp. in Type II hypoliths and *P. truncata* in Type III hypoliths.

The number of OTU's observed for Type I, II and III communities were 7, 6 and 5 respectively. The dendrograms generated by band-matching are depicted in Figure 3.4. This indicates that Type I and III hypolithons cluster together with a 93.10% similarity but are separate from the Type II hypolithon.

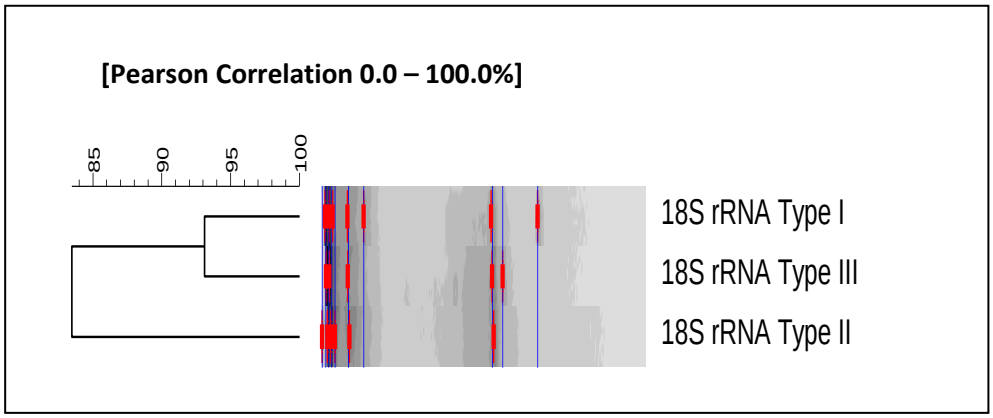

Figure 3.4 Dendrogram depicting the cluster analysis of the Type I, II and III hypolith communities from the assessment of diversity of the total eukaryotes that dominate these hypolithons. Type I and III communities appear to have more similarity to each other than to the Type II community.

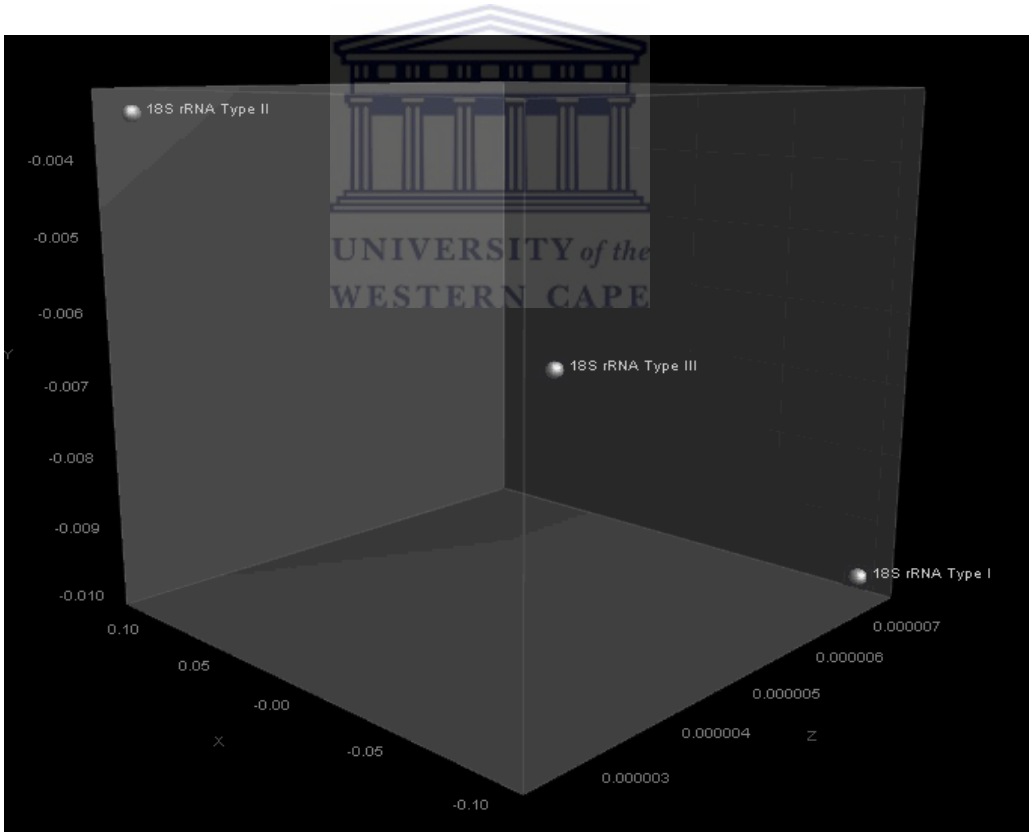

Figure 3.5 Non-metric MDS ordination of the Type I, II and III hypolith communities using the 18S rRNA gene region. This shows how distinct the composition of each hypolithon is by the large spatial distance between the samples (white circles).

nMDS ordinations in Figure 3.5 show the overall similarity of the community fingerprints. The spatial distribution in these plots indicate that the three hypolithon communities cluster separately, but that Type I and III are more similar to each other while Type II is isolated.

3.3.2 Diversity Assessment and Phylogenetic Assignment of the 18S rRNA Gene Terminal Restriction Fragments

Peak profiles with Peak Scanner™ (Applied Biosystems) displayed a number of peaks for enzyme digests of amplified 18S rRNA gene regions from Type I, II and III community DNA. Each different enzyme pair provided a different profile, as shown in Figure 3.6.

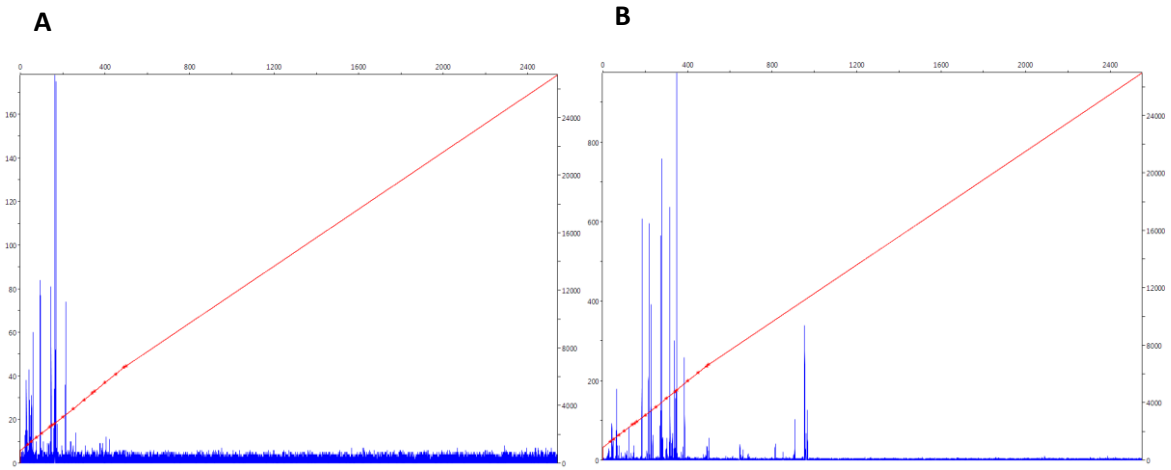

Figure 3.6 Peak profiles of 18S rRNA gene T-RF's observed in Peak Scanner™. Blue lines represent the different sized peaks within each hypolith amplicon digest with (A). *Alu*I and (B) *Hae*III. These peaks represent the abundance and the organisms, several of which may share the same T-RF. The red indicates the GS500LIZ size standard marker used in the capillary system.

The height and area of the peaks represented by the electropherograms indicate the number and relative abundance of the OTU's within each community. The OTU's for each enzyme are displayed in Table 3.2.

Table 3.2 OTU's observed for each 18s rRNA gene T-RF enzyme digest. Each OTU is defined as a single peak in the peak profile.

Community Type	*Alu*I Digest OTU's	*Hae*III Digest OTU's
Type I A	8	23
Type I B	7	20
Type II A	13	15
Type II B	12	17
Type III A	6	14
Type III B	7	16

The cluster and 2 dimensional MDS ordination diagrams generated via PRIMER 6 are displayed in Figure 3.7 and Figure 3.8, using Bray Curtis Similarity at the fourth root. It is evident that Type II and III communities appear to be more closely related than Type I communities. Duplicate samples in the cluster analysis (Figure 3.7) show a high degree of similarity, which is additionally supported in the MDS-cluster overlay (Figure 3.8). Close to 100% similarity exists in these duplicate hypolith samples.

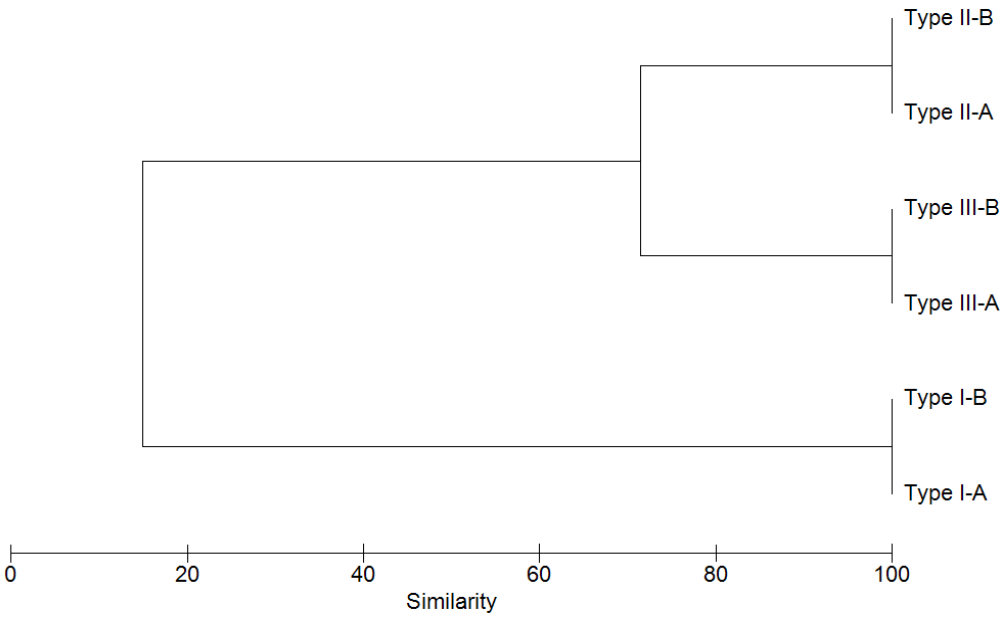

Figure 3.7 Cluster analysis of the diversity between Type I, II and III communites using the 18S rRNA gene produced by PRIMER 6, using Bray Curtis similarity to the 4th root. A 70% similarity is observed between Type II and III hypolithons, with Type I hypoliths having less than 20% similarity to these two communities.

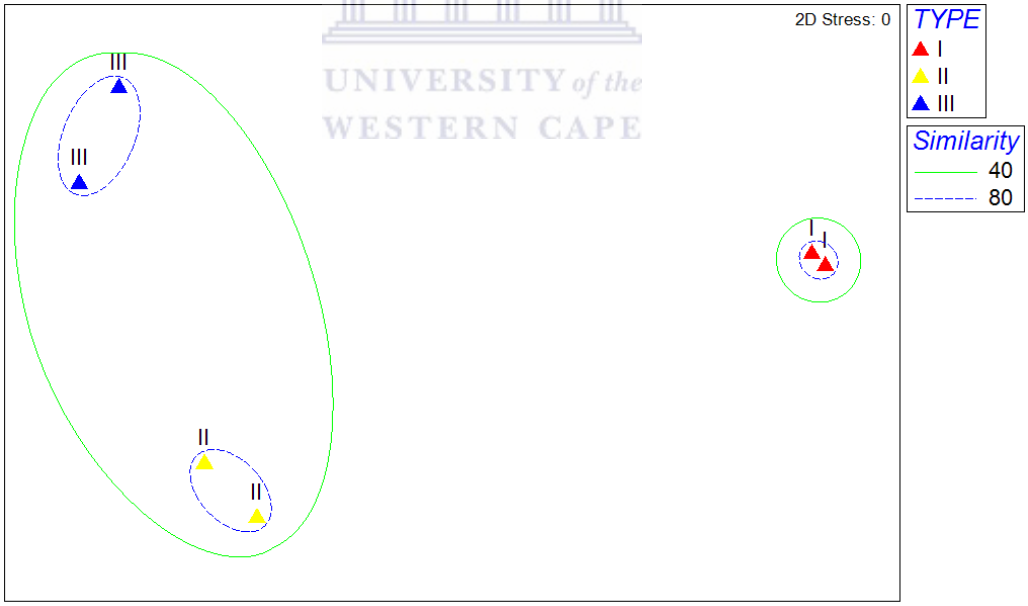

Figure 3.8 2-D multi-dimensional scale of the universal 18S rRNA gene T-RF's with an overlay of the cluster analysis depicting the percentage similarity with the green and blue lines.

Univariate diversity indices were estimated using PRIMER 6 (Table 3.3). The Shannon Index, H'(logE), shows that Type I and III have a lower species richness than Type II

communities. The diversity, estimated using the Simpson Index (1-λ'), approaches 1 in all hypolith communities. This indicates high diversity of the OTU's in the 18S rRNA gene T-RF's. However, the high J' (Pielou's Evenness) value implies low variation within these OTU's.

Table 3.3 Diversity indices obtained from universal 18S rRNA gene T-RF's.

Sample	S	d	J'	H'(logE)	1-λ'
18S rRNA Type I A	8	1.52	0.9499	1.975	0.8569
18S rRNA Type I B	8	1.52	0.9499	1.975	0.8569
18S rRNA Type II A	13	2.606	0.8657	2.221	0.8591
18S rRNA Type II B	13	2.606	0.8657	2.221	0.8591
18S rRNA Type III A	7	1.303	0.9588	1.866	0.8408
18S rRNA Type III B	7	1.303	0.9588	1.866	0.8408

*S indicates the total species per sample, d shows the species richness, J' shows Pielou's Evenness, H'(logE) shows the Shannon Index, 1-λ is the Simpson Index.

By combining the data of the two restriction enzyme digests a putative phylogenetic assignment of the composition of each hypolithon (Figure 3.9) was generated. Overall, high diversity is observed, with Type II displaying the highest and Type III the lowest. A significant fraction of each community remains unassigned, 34.69%, 33.07% and 34.38% for the Type I, II and III hypolith T-RF's, respectively.

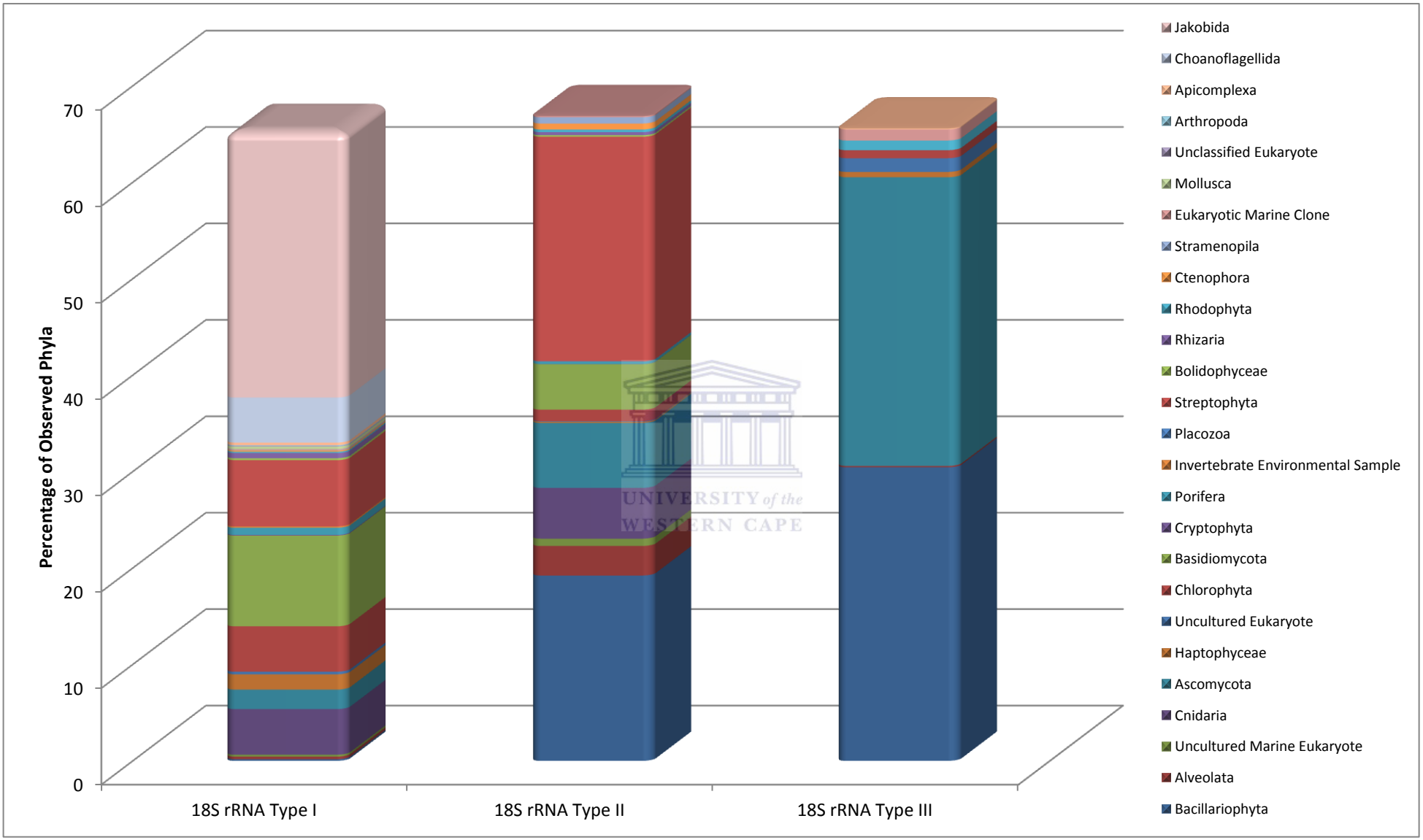

Figure 3.9 Putative phylogenetic assignment of 18S rRNA gene T-RF's based upon database comparison and similarity matrix analysis. This shows a large level of diversity within Type I, II and III hypolithons. Greatest diversity is displayed in the Type II (moss dominated) community (See Appendix 1 for taxonomy).

3.3.3 Phylogenetic Assessment of 18S rRNA Gene Clone Libraries

Clone libraries (section 2.6.2) of the PCR-amplified 18S rRNA gene were constructed. Positive recombinants were initially assessed by blue-white screening and PCR screened in a two-step process,

- Colony PCR with M13fw and M13rev primers (Figure 3.10),
- Nested PCR with EukA and EukB primers (Figure 3.11).

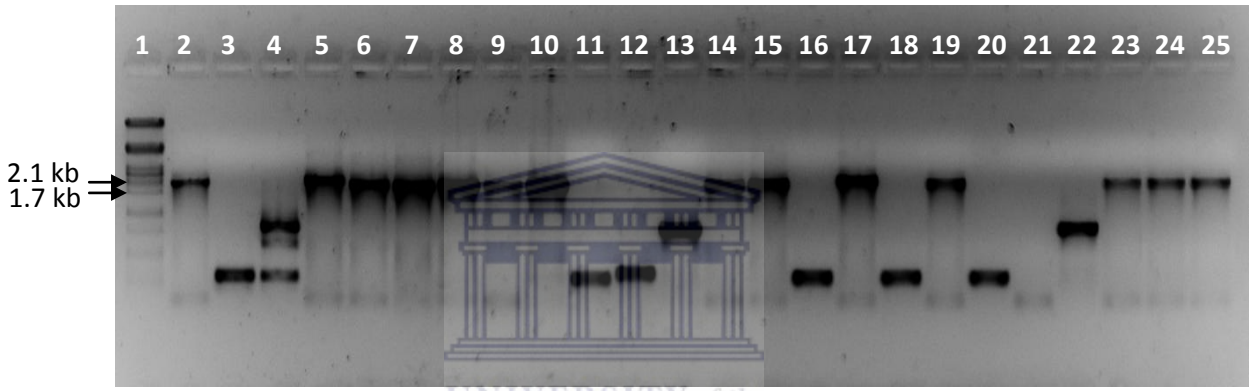

Figure 3.10 M13 colony PCR screen of a selection of clones from a Type I library to confirm the 18S rRNA gene insert. Lane 1: Molecular weight marker, lane 2-25: PCR products from Type I clones (C1-12 and D1-12). The presence of a band approximately 2.1 kb is an indication of a positive recombinant. Only positive recombinants were used in the following screening method.

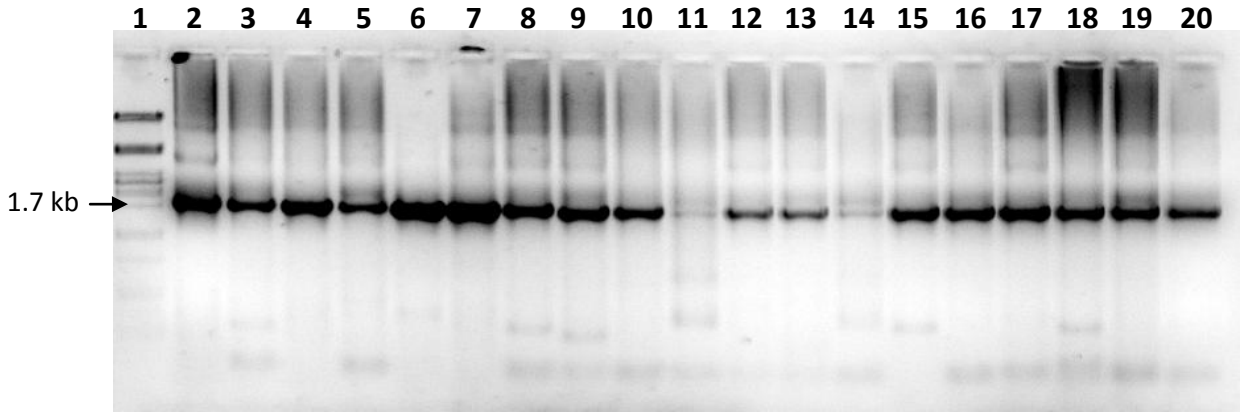

Figure 3.11 Nested PCR screen of the M13 colony PCR of the 18S rRNA gene of a Type I library. Lane 1: Molecular weight marker, lane 2-25: PCR products from Type I clones. The band located at position 1.7 kb is the positive EukA-EukB amplicon used in ARDRA. Blank lanes indicated clones negative for the insert, and were not used for the downstream screen.

A total of 96 recombinant clones per hypolith community type were PCR-screened (Table 3.4). Restriction patterns produced via ARDRA with *Alu*I and *Hae*III digests (Figure 3.12) were manually assigned phylotypes for the Type I, II and III clone libraries.

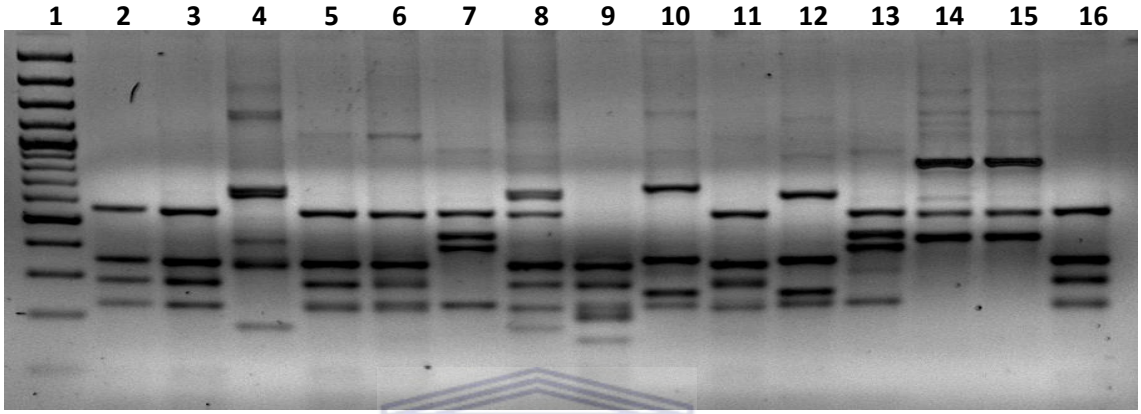

Figure 3.12 Example of the ARDRA digestion patterns observed for Type I 18S rRNA gene clones. Lane 1: Molecular weight marker, Lane 2-16: 18S PCR ARDRA digest with restriction enzyme *Hae*III, electrophoresed on 2.5% agarose.

The collectors curves (Figure 3.13) show the predicted sampling efficiency. Typically, a slope tending towards zero (an asymptotic curve) implies that sampling is comprehensive. It is clear that asymptotes have been reached for the Type II (yellow) 18S rRNA gene clone library, but not for the Type I (red) or Type III (blue) eukaryote libraries. Thus, further screening is required in these two hypolithon clone libraries.

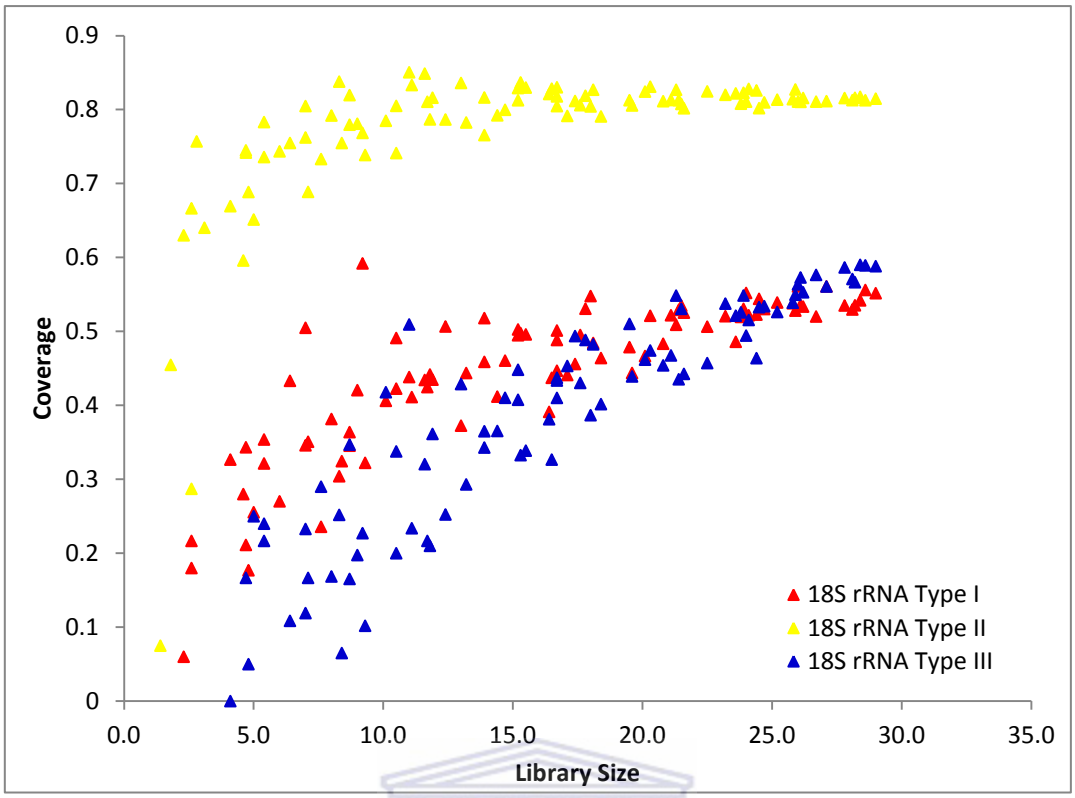

Figure 3.13 Collectors curves of the universal 18S rRNA gene clone library for Type I, Type II and Type III hypoliths. The appearance of an asymptote for the Type II library suggests that sampling was sufficient, however, further sampling is required for Type I and III libraries.

In addition, the S_{Chao1} prediction value (indicating whether a clone library is sufficiently large to provide a reliable estimate of phylotype richness) was determined (Table 3.4). The results show that while the clone library was sufficiently screened, a larger library needs to be generated to establish a better representation of the overall eukaryotic diversity of the hypolithon.

Table 3.4 ARDRA results of Type I, II and III 18S rRNA gene clone libraries showing the richness of sampling (S_{Chao1}) of the libraries generated.

Hypolith Community	Number of Clones	Number of Phylotypes	Observed Phylotypes/ Predicted S_{Chao1}
Type I	29	17	46.74%
Type II	54	13	23.31%
Type III	17	11	65.88%

Forty one different phylotypes from the 18S rRNA gene clone libraries were sequenced, using primers targeting the M13 region of the vector. Partial sequences were compared to the closest matching sequences on the NCBI database using BLASTn. These matches and the percentage identity to the closest match are shown in Appendix B1.

Similarity values of all the sequences ranged from 68-100%, and BLASTn data showed a variety of bryophytes and previously uncultured environmental sample clones, as well as Amoebozoans, Chlorophtyes, Cercozoans and Apicomplexans. Approximately 39% of the sequences displayed low identity, when using a cut-off of 97%. The composition of the individual libraries suggests that Type I and III hypolith communities are dominated by Bryophyta, while Type II is dominated by uncultured eukaryotes (Figure 3.14).

A neighbour-joining tree was constructed with MEGA 5 using the p-distance model with 1000 replications. Four distinct clusters are displayed in the rooted tree (Figure

3.15). Cluster 1 is made up of most of the Type I hypolith clones. A fraction of Type II and III clones cluster together, forming group 2. Group 3 displays Type I and II hypoliths with resemblance to Amoebozoa, Alveolata and Viridiplantae sequences. The remaining Type II and III clones that cluster together (cluster 4) are similar to bryophyte species and previously uncultured clones. Additionally, it appears that the clone sequences in clusters 1 and 2 are more similar to each other than to NCBI BLASTn matches. This is an indication of the distinct community structure of these hypolithons. The low bootstrap values observed in this tree indicate the level of divergence of the sequences, in addition to illustrating very low confidence in the clustering pattern within the tree. This clustering is, therefore, not very reliable to determine the relationships of known sequences to those in the clone libraries. To competently analyse the evolutionary relationships of eukaryotes observed in the hypolithon, generation of a tree containing additional sequences of cultured isolates would be essential.

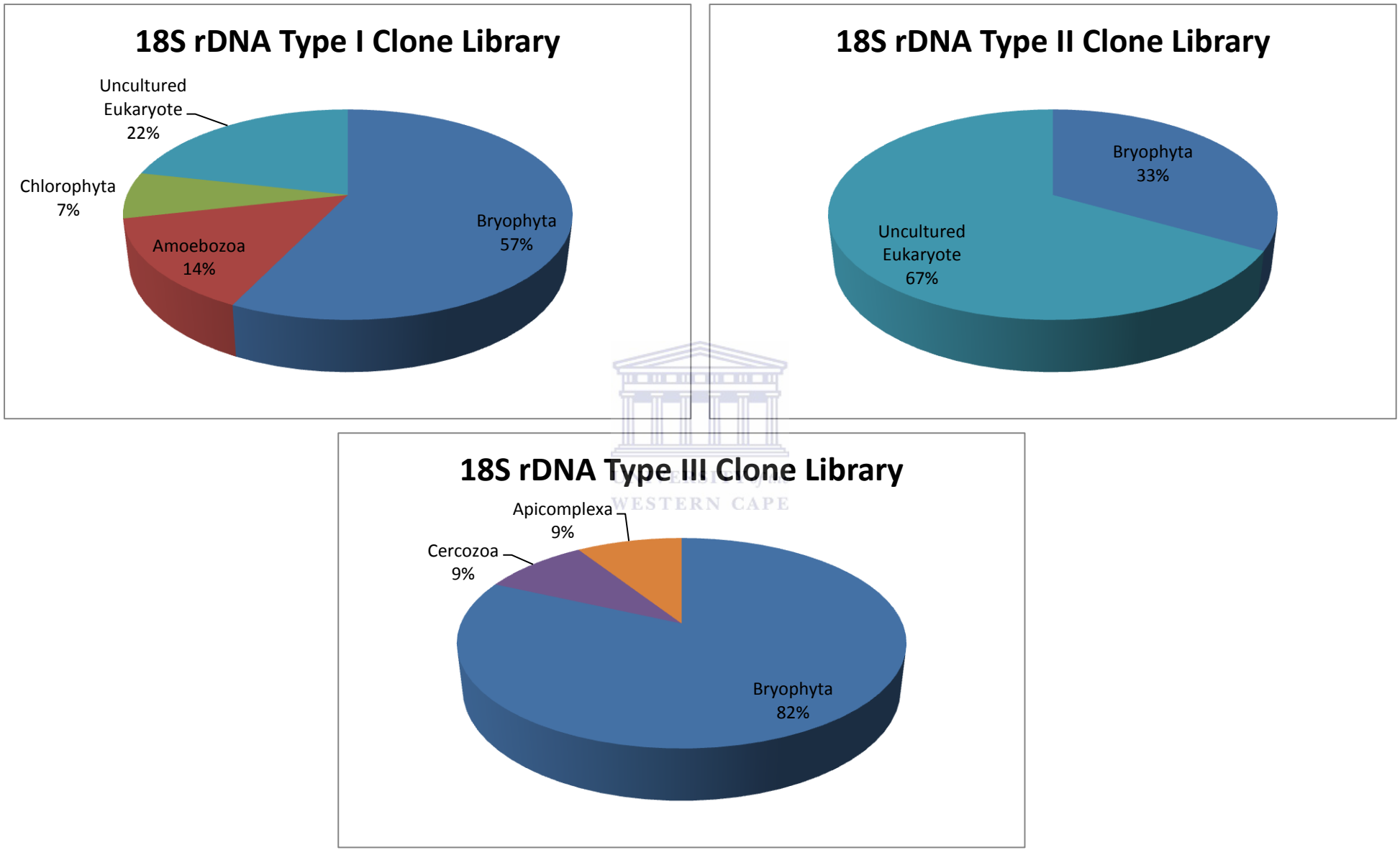

Figure 3.14 The relative percentage distribution of the universal 18S rRNA gene clone library phylotypes identified from Type I, II and III hypolith communities.

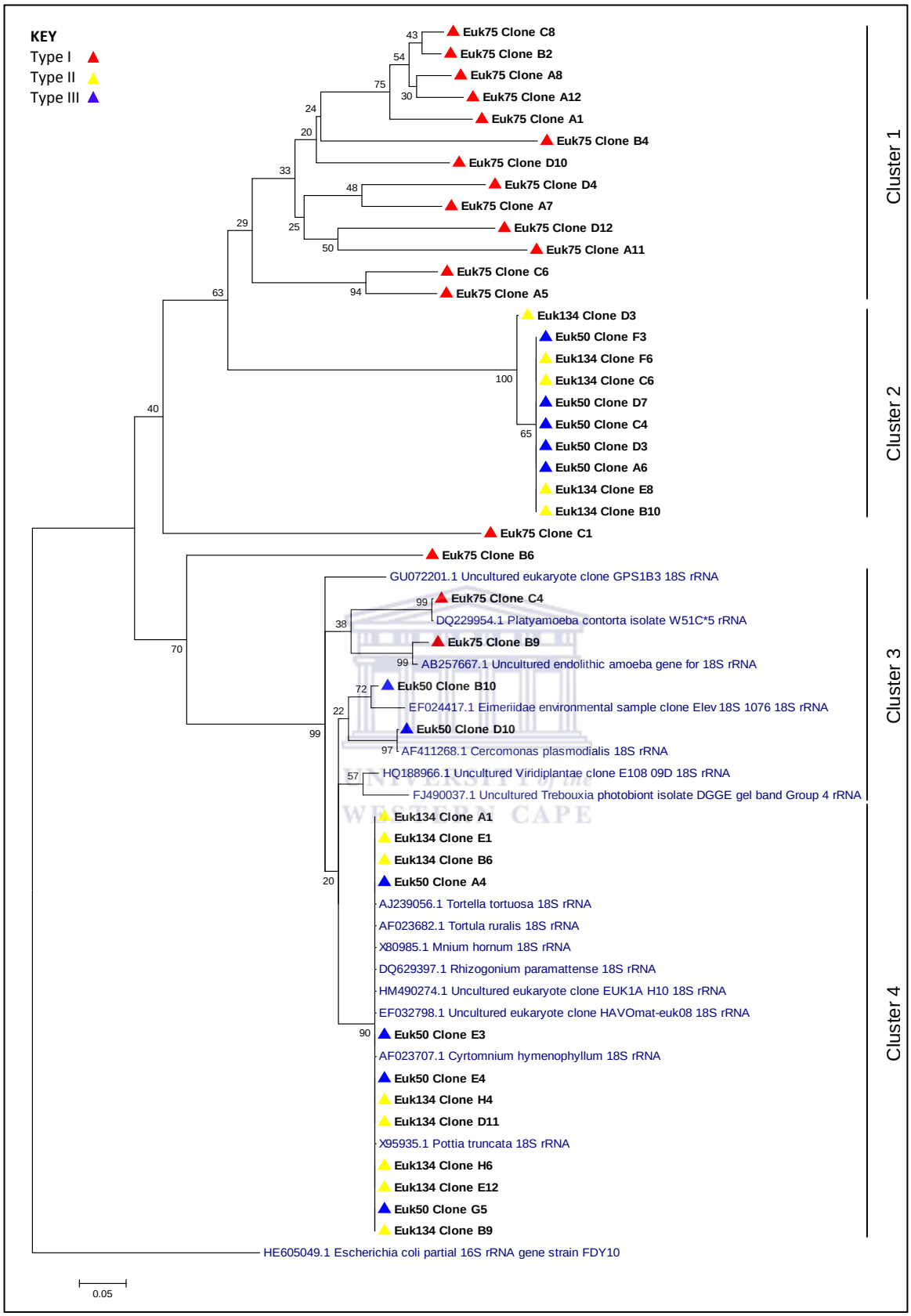

Figure 3.15 Neighbour-joining tree of 41 18S rRNA gene sequences for Type I, Type II and Type III hypoliths. Bootstrap values were inferred from 1000 replicates and evolutionary distances were calculated using the p-distance method in MEGA 5.

The phylogenetic tree was used to perform parsimony analysis of the clone libraries. This revealed a pars score of 4 (P<0.001). Pairwise comparisons of the three community types yielded the following pars scores, showing that each hypolithon has a distinct and unique community structure:

- Type I : Type II - 2 (P<0.001),
- Type I : Type III - 2 (P<0.001),
- Type II : Type III - 3 (P<0.001).

The overall Shannon Index was 2.197 supporting the high diversity indicated by DGGE and T-RFLP investigations. The observed species richness between the communities (Figure 3.16) and the 1 OTU overlap between Type II and III communities is depicted in the Venn diagram below.

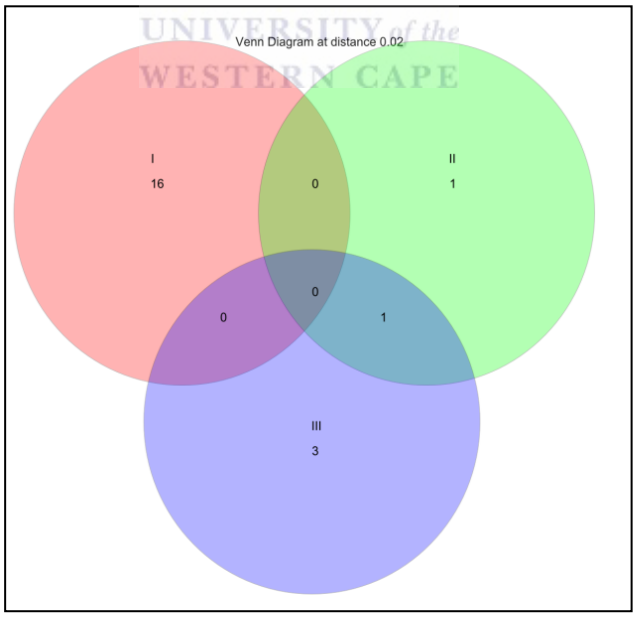

Figure 3.16 Venn diagram illustrating the OTU richness observed between the 18S rRNA gene clone sequences of Type I, Type II and Type III hypolith communities. There is an OTU richness of 16, 2 and 4 for Type I, II and III hypolithon sequences respectively, with an overlap of 1 OTU between Type II and III communities.

3.3.4 Fingerprinting and Clone Library Analysis of Hypolithic Eukaryote 18S rRNA Diversity

The universal 18S rRNA gene region provides ample information to generate the diversity profiles required for taxonomic identification (Marande *et al*., 2009). This rRNA region is an ideal phylogenetic marker as it contains well defined regions that are conserved over the entire eukaryal kingdom (Khan, 2008; de la Torre *et al*., 2003). When applied to PCR-DGGE, a molecular fingerprint of the dominant community members is observed. From this a description of the relatedness of these communities can be provided.

DGGE microbial fingerprint data shows us that all three communities appear to be very distinct. However, the Type I and Type III communities have a closer level of similarity compared to Type II communities, as is depicted by the cluster and non-metric MDS spatial distribution analysis. The T-RFLP cluster dendrograms further describe the unique composition of each community. Here, the similarities between the Type II and Type III communities are greater than the Type I community.

The differing results observed in these two techniques could be explained by the primers used in DGGE and T-RFLP. DGGE primers used in this study amplify 500-600 bp fragments of PCR-produced DNA (Valášková and Baldrian, 2009). T-RFLP primers, however, were developed from the universal primers used in clone library

generation, yielding amplicons of 1.5-2kb. They therefore cover a larger region of the 18S rRNA gene and could contribute to the greater diversity observed in the T-RF's.

The richness estimates support the high diversity and low variation of the hypolithons. Parsimony analysis, Shannon Index and OTU richness showed that each community is very distinct in composition, with 0-1 overlapping species. However, clone libraries were unable to recover the entire eukaryote diversity in the three hypolithons, since the collector's curve (Figure 3.13) and Chao1 predictions (Table 3.4) show that the total diversity of each of the hypolithons was not fully represented. It can therefore be inferred that there is much more diversity to be sampled, analysed and uncovered.

In clone library sequences, Type II and Type III dominated communities are more similar to each other, as with the T-RFLP results. Bryophytes (moss species), Amoeboid protozoan's, Cercozoans and Apicomplexans are probably the major contributors to hypolithon diversity. The phylogenetic associations observed in the neighbour-joining tree (Figure 3.15) show that some clone sequences have more similarity to each other than to their closest BLASTn match. This is supported by the low percentage (less than 97%) of identity between clone sequences and previously cultured species. This observation implies that most of the Type I, II and III 18S rRNA clones may belong to novel species of Amoebozoa, Alveolata and Chlorophyta.

Sequences that displayed highest identity to previously uncultured eukaryotic clones illustrate that vast quantities of Dry Valley soil eukaryotes are as yet un-described. These microorganisms may have crucial functions in hypolithic communities. However, the limitations of current culturing techniques have prevented their isolation and characterization (Adams *et al*., 2006).

In environments closer to the wetland sections of Antarctica, bryophyte dominance has been observed (Cary *et al*., 2010; Thomas, 2005). However, their widespread dispersal and presence in moist Dry Valley soils implies no particular preference to either maritime or terrestrial environments (Convey *et al*., 2008). Mosses primarily act as stabilizers, favouring the presence and sustenance of other microorganisms (Ugolini and Bockheim, 2008). Secondarily, they contribute to organic carbon cycling (Bamforth, 2005). Their abundance in hypoliths can be explained by the positive thermal buffering condition, water availability and light concentration that are conducive for moss survival (Broady, 1981).

Apicomplexans are an interesting group to encounter, as these are pathogens to humans or vertebrates (Leander *et al*., 2003). With the interference from penguins, seals and humans in Dry Valley's, however, their presence may not be unexpected (Vincent, 2000). The activity of Apicomplexans provides other microbial species with a suitable habitat and essential nutrients. Protozoa and Cercozoa are commonly referred to as bacterial grazers in soil communities and may therefore be involved in

bio-control in the hypolith (Manzano *et al*., 2007). Still, it is unknown if the observed species of Amoeba, Cercozoa and Apicomplexa in these communities are active or encysted and dormant (Bamforth *et al*., 2005). Future analysis with RNA-based molecular methods would provide more insight into the metabolically active eukaryotic communities in hypolithons.

3.4 Fungal Community Profiling of the ITS rRNA Gene Region

The fungal hyper-variable ITS rRNA gene regions were PCR amplified with group-specific fungal primers (section 2.3) and analysed by DGGE, T-RFLP and clone library sequencing.

3.4.1 Denaturing Gradient Gel Electrophoresis Fingerprinting of the ITS rRNA Gene Region

Nested PCR amplicons generated were between 200 bp and 300 bp (Figure 3.17). Additional bands were also observed at approximately 500 bp for all three hypolithons. This could be an indication of multiple rRNA repeats which are frequently observed in most species (Bridge and Spooner, 2001; Kennedy and Clipson, 2003; Vilgalys *et al*., 2004), the presence of eukaryotes with differing lengths of the ITS rRNA gene or non-specific amplifications.

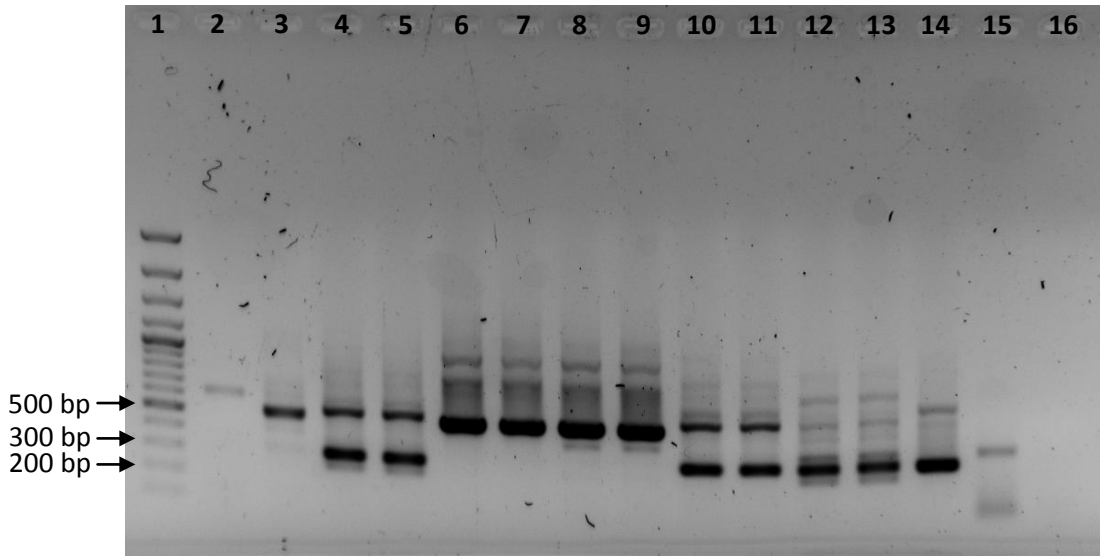

Figure 3.17 Visualization of the fungal specific internal transcribed spacer (ITS) region PCR using nested primers, ITS1F-GC and ITS2. Lane 1: Molecular weight marker, Lane 2-5: Type I hypolith, Lane 6-9: Type II hypolith, Lane 10-13: Type III hypolith, Lane 14: Ascomycota positive control, Lane 15: negative control (standard PCR), Lane 16: negative control (nested PCR).

The fungal ITS DGGE community analysis was performed on a 30-70% polyacrylamide gradient. Sequencing of the excised bands showed predominantly uncultured fungal clones (Type I and III hypolithons), *Phaeophyscia* sp. (Type I hypolithon), *Thelidium papulare* (Type II hypolithon) and *Lecanora dispersa* (Type III hypolithon).

The number of OTU's obtained for Type I, II and III hypolithons were 8, 7 and 13, respectively. The cluster analysis (Figure 3.18) showed that Type I and II communities have more resemblance to each other than to Type III communities.

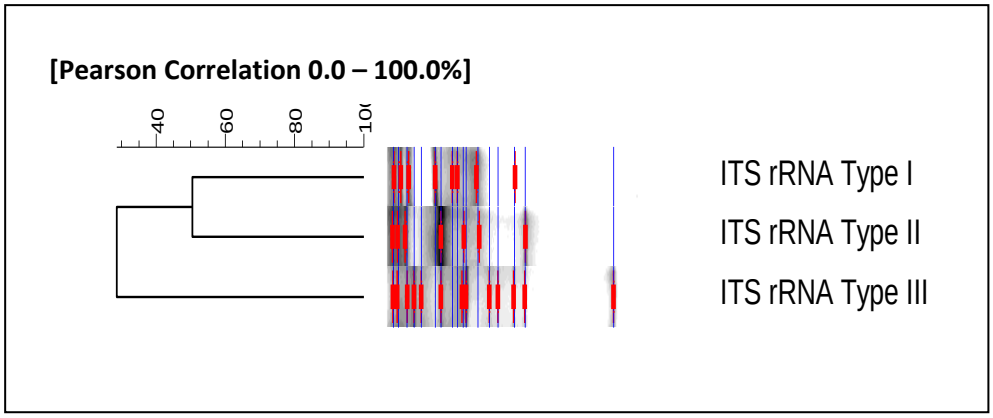

Figure 3.18 Cluster diagram of the ITS rRNA gene region of the Type I, II and III hypoliths. These show that the Type I and II communities are more similar to each other than to the Type III community.

The similarity values of Type I and II versus the Type III hypolithon communities are 50.47% and 28.57% respectively. The nMDS plots generated from the cluster analysis (Figure 3.19) shows that these three communities are distinctly different from each other.

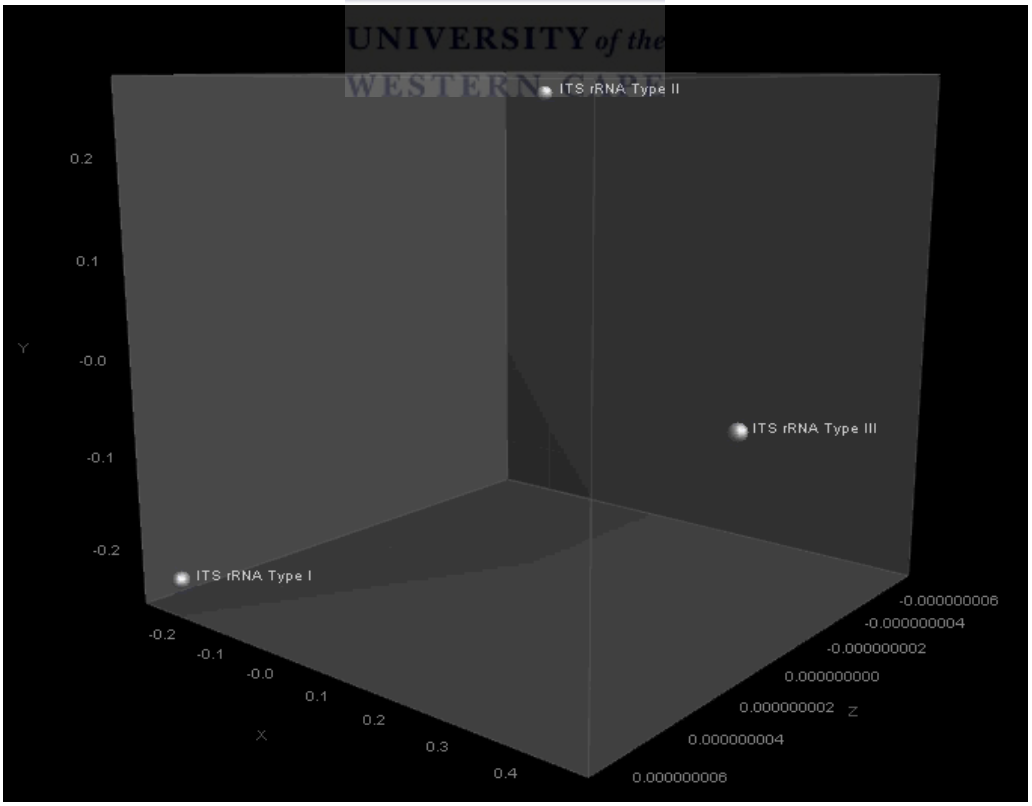

Figure 3.19 nMDS ordination of the fungal ITS componant of Type I, II and III hypolith communities from the ITS rRNA gene region. Despite Type I and II hypolithons showing a level of similarity, each hypolithon is isolated from the next.

3.4.2 Diversity Assessment and Phylogenetic Assignment of ITS rRNA Gene Terminal Restriction Fragments

Peak profiles of the restriction enzyme digests revealed the OTU numbers displayed in Table 3.5.

Table 3.5 OTU's observed for each ITS rRNA gene region enzyme digest. Two compatible enzymes were used for each community type.

Community Type	*Ava*I Digest OTU's	*Hae*III Digest OTU's	*Msp*I Digest OTU's
Type I A	-	17	16
Type I B	-	15	16
Type II A	-	10	7
Type II B	-	12	2
Type III A	4	-	11
Type III B	6	-	11

* Dashes (-) indicate enzymatic digests that were not performed for any particular sample.

Cluster analysis (Figure 3.20) of the *Ava*I, *Hae*III and *Msp*I T-RF's shows that the duplicate Type I ITS rRNA T-RF's group together and display similarity to Type III ITS rRNA T-RF's. Type II T-RF's appear to be independent of the other two hypolithons. However, an extremely low level of similarity is generally observed within the fungal ITS component between the hypolithons, as shown in Figures 3.20 and 3.21. Interestingly, the two Type III (lichenized) hypolithons also display clear separation, as opposed to the Type I and II T-RF's that show at least 20% similarity. The duplicates of the fungal ITS region are, however, highly disparate. This could be an indication of each individual hypolith having unique dominant fungal species.

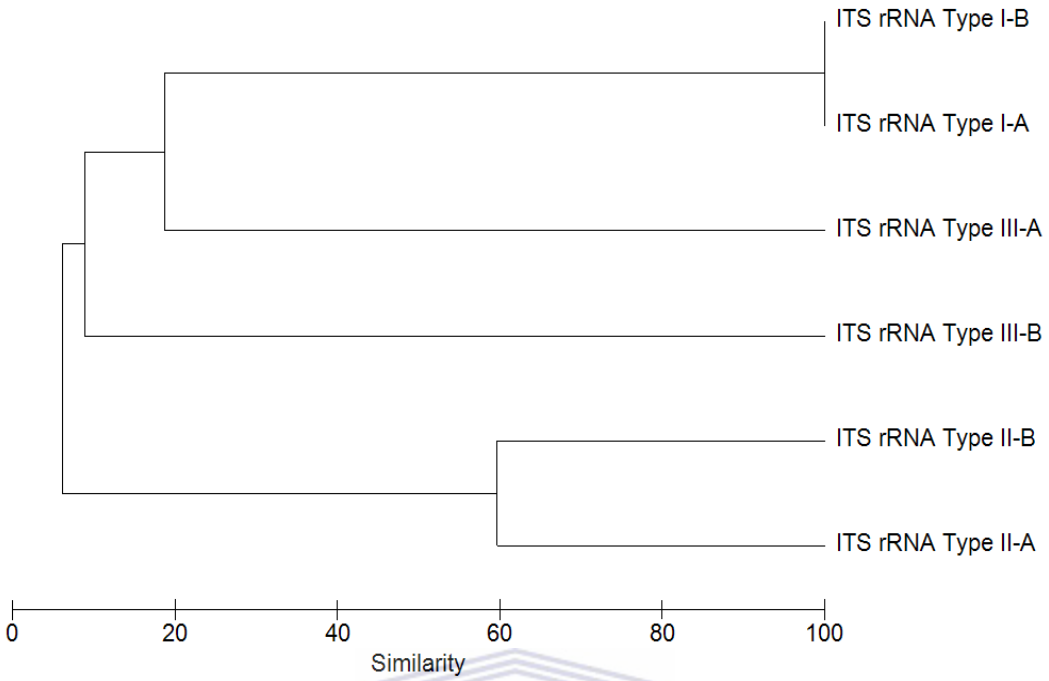

Figure 3.20 PRIMER 6 based diversity assessment of the fungal ITS rRNA gene T-RF's in Type I, II and III hypolith communities, presented in duplicate. Here we see the cluster analysis in tree form showing the relation between Type I, II and III hypolith communities.

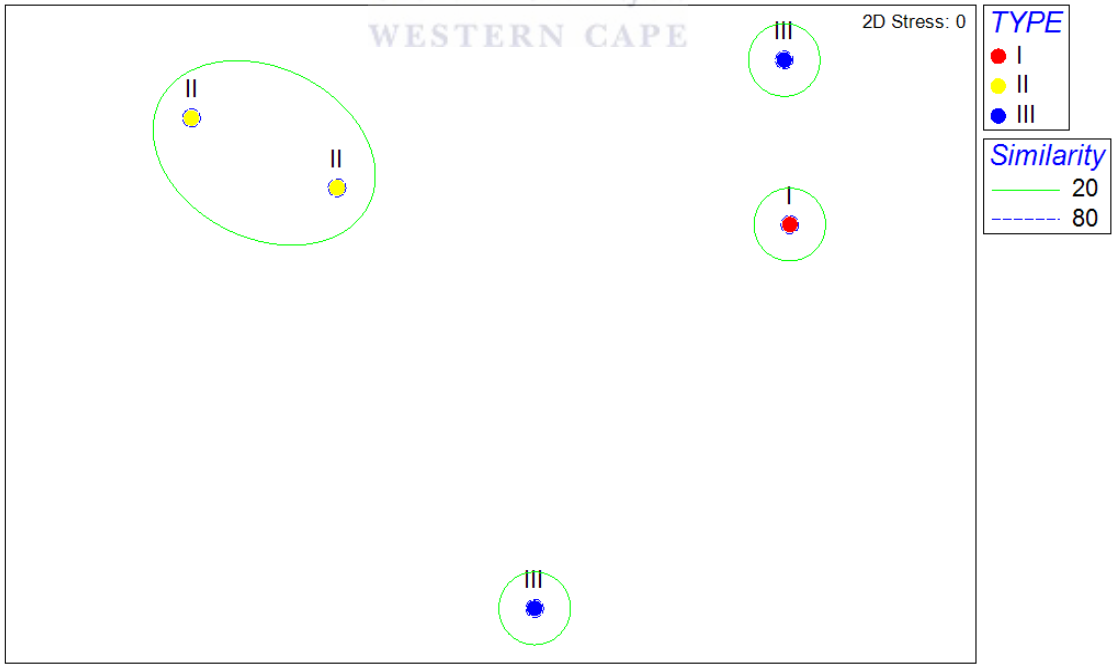

Figure 3.21 A 2-dimensional MDS representation of the fungal ITS rRNA gene region data combined with the cluster analysis.

The diversity indices generated from the fungal ITS rRNA gene T-RF's (Table 3.6) indicate low species richness overall, with Type II displaying the lowest species richness (d). Distinct variation is observed in the OTU's of the Type I, II and III hypolithons (J'). Higher species diversity is observed in the Type I and III samples according to the Simpson index.

Table 3.6 Univariate diversity indices for the fungal ITS rRNA gene generated from T-RF data.

Sample	S	d	J'	H'(logE)	1-λ'
ITS Type I A & B	16	3.257	0.6624	1.836	0.7301
ITS Type II A	7	1.303	0.5768	1.122	0.5403
ITS Type II B	2	0.2171	0.781	0.5413	0.3596
ITS Type III A	11	2.171	0.6319	1.515	0.7187
ITS Type III B	10	1.954	0.7849	1.807	0.789

*S indicates the total species per sample, d shows the species richness, J' shows Pielou's Evenness, H'(logE) shows the Shannon Index, 1-λ is the Simpson Index.

A comparison of the T-RF peak alignments permitted phylogenetic allocation of the fungal ITS T-RF's to Ascomycota, Basidiomycota and Glomeromycota species. It can be deduced from Figure 3.22 that the overall known diversity observed within the fungal component of these three hypolithons, is extremely low for the Type I and III communities. The levels of unknown diversity are 97.43% and 96.04% for Type I and III respectively. The Type II hypolith, however, displays only 23.17% of unknown diversity. This hypolith is dominated by Glomeromycota (43.9%), with Basidiomycota and Ascomycota contributing 10.98% and 21.95% of the phylotypic assignments, respectively.

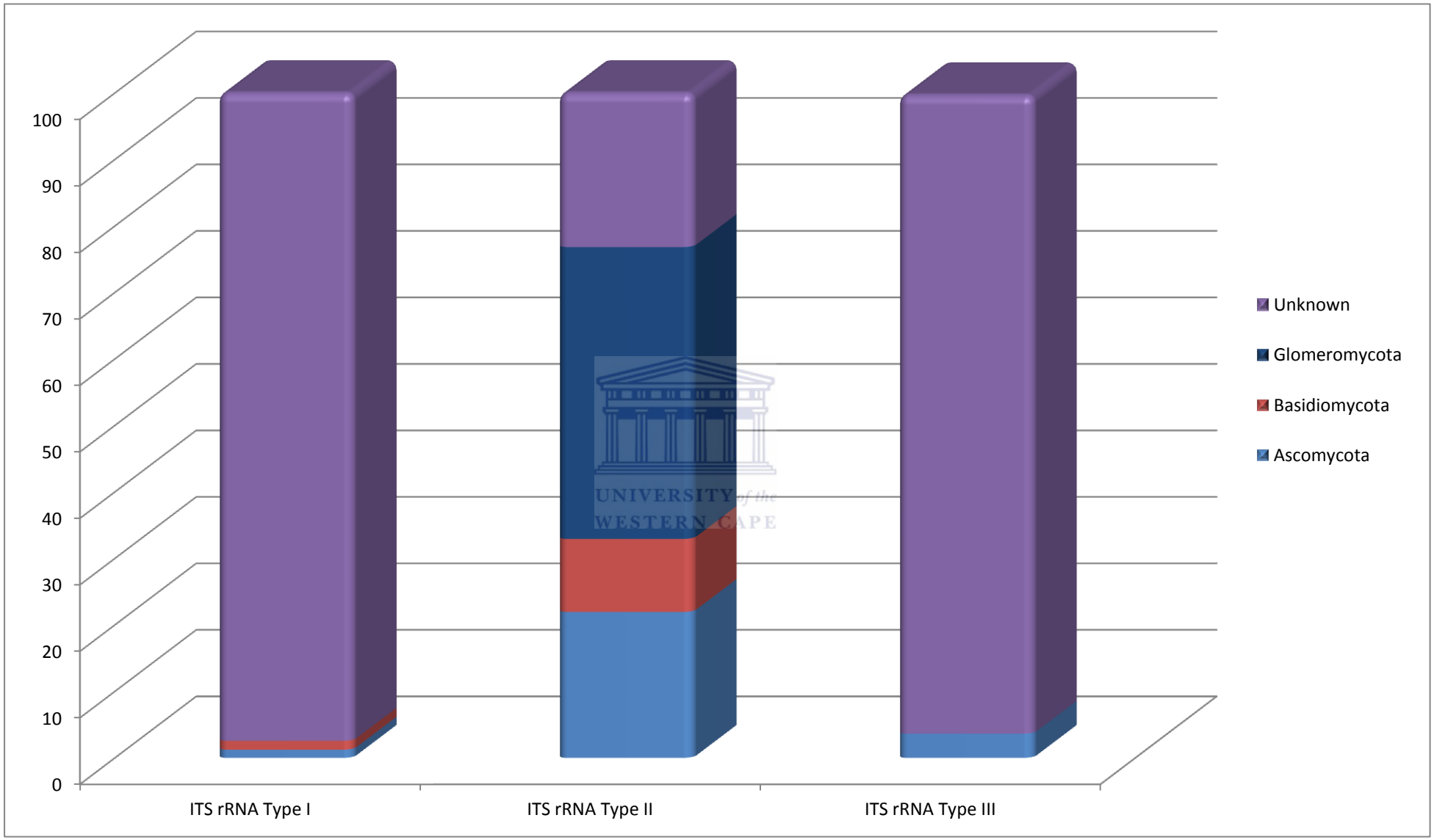

Figure 3.22 Putative phylogenetic assignment of the fungal ITS rRNA gene, using T-RF's created with enzyme *Msp*I. Type II hypoliths, display the most fungal diversity, however, a very low level of diversity is observed overall since majority of the microbial component is unknown (See Appendix A for taxonomy).

3.4.3 Phylogenetic Assessment of Fungal ITS rRNA Clone Libraries

Clone libraries were screened using M13 PCR primers in a colony PCR screen (Figure 3.23) for positive recombinants, followed by a nested PCR performed with the fungal ITS specific primers, ITS1F and ITS4 (Figure 3.24).

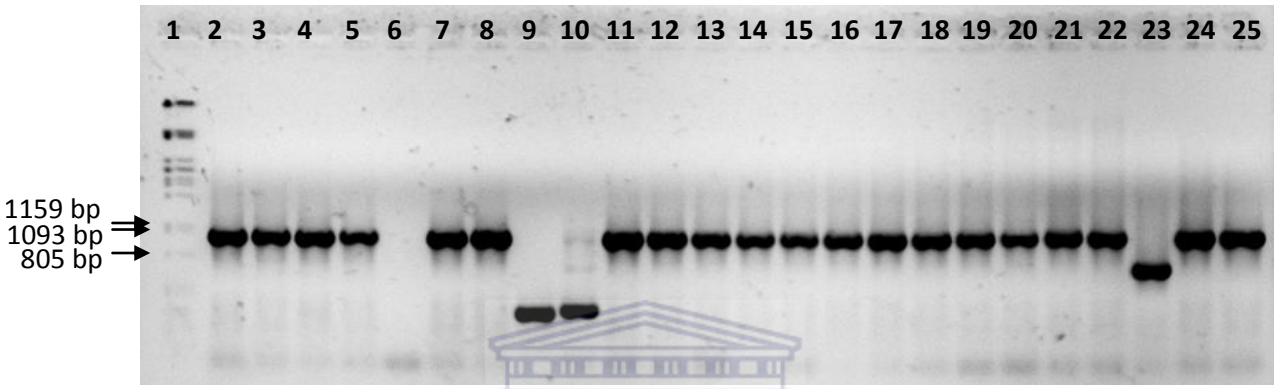

Figure 3.23 M13 colony PCR of the fungal ITS rRNA gene in Type II hypoliths. Lane 1: Molecular weight marker, Lane 2-24: Amplicons from clones C1-12, D1-12. The presence of a band approximately 1 kb is an indication of a positive recombinant.

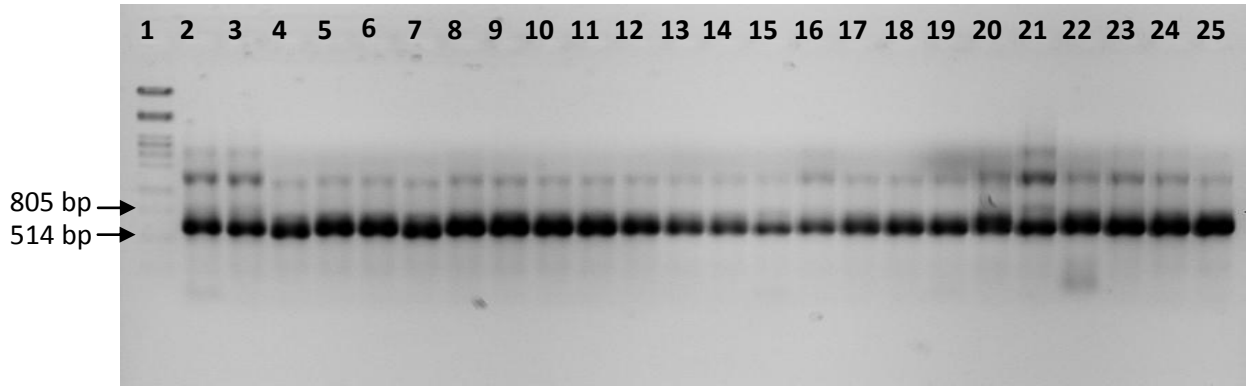

Figure 3.24 Nested PCR screen of the fungal ITS rRNA gene in a Type II library. Lane 1: molecular weight marker, lane 2-25: PCR products from the Type II M13 colony PCR.

All positive amplicons of ± 600-700 bp generated in the nested PCR were ARDRA screened, using enzymes *Ava*I, *Msp*I and *Hinf*I and electrophoresed on 2.5% agarose gels (Figure 3.25).

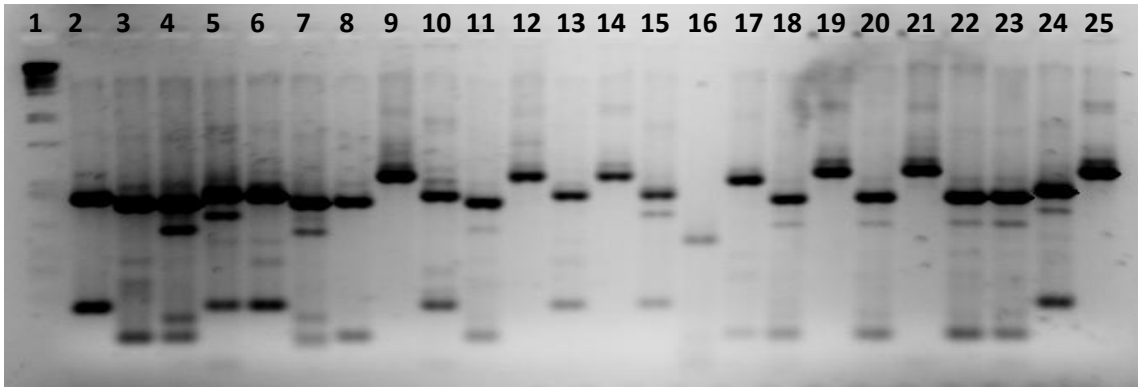

Figure 3.25 Example of the ARDRA digest performed on fungal ITS rRNA gene PCR in Type II clone amplicons. Lane 1: Molecular weight marker, Lane 2-16: fungal ITS PCR ARDRA digest with restriction enzyme *Msp*I, electrophoresed on 2.5% agarose.

Fungal rRNA gene clone libraries showed the following phylotypes after assessment of the ARDRA profiles:

- Type I fungal ITS rRNA gene clone library - 31 phylotypes,
- Type II fungal ITS rRNA gene clone library - 32 phylotypes,
- Type III fungal ITS rRNA gene clone library - 32 phylotypes.

An assessment of the richness of sampling (section 2.6.6) was performed using these phylotypic results. The collectors curves (Figure 3.26) show the possible approach of an asymptote, suggesting that the screening in the generated clone libraries was sufficient.

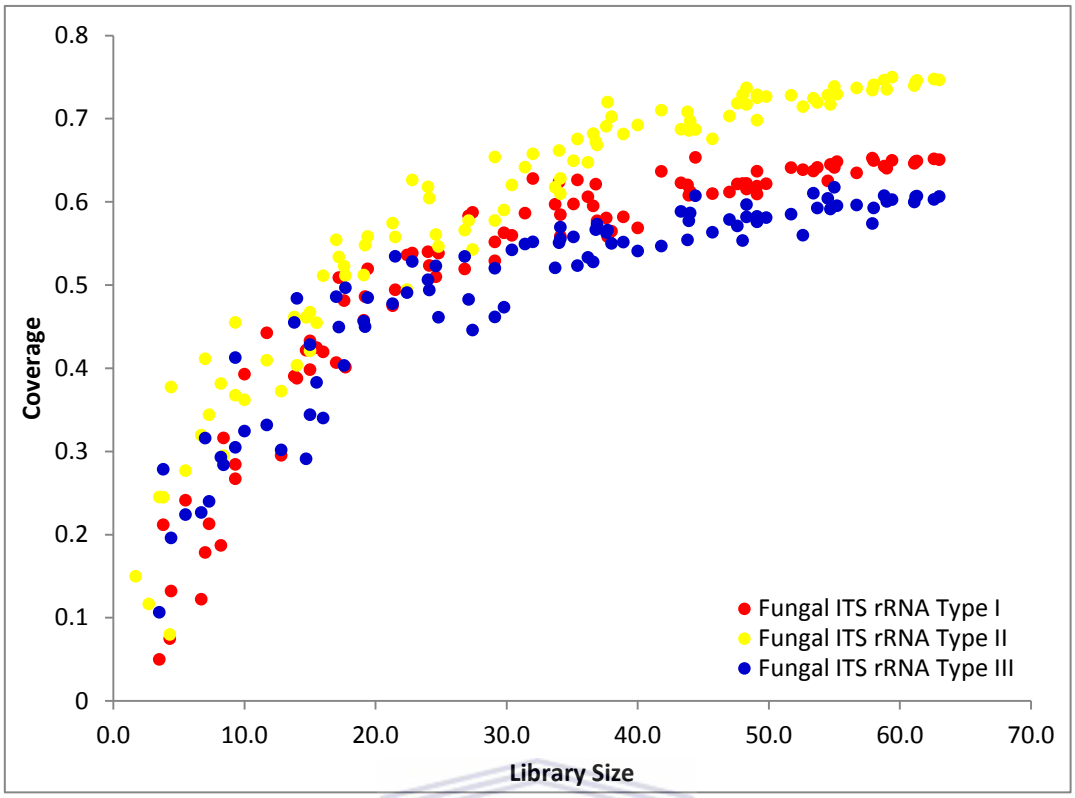

Figure 3.26 Collectors curve of fungal ITS rRNA gene clone library ARDRA phylotypes for Type I, Type II and Type III hypoliths. All curves reach asymptote implying sufficient sampling of the libraries.

Table 3.7 provides an estimate of the percentage of species richness based on the phylotypes. The Chao1 value indicates that the predicted diversity is higher than the observed diversity. A larger library therefore needs to be screened for an improved representation of the diversity within these 3 hypolith communities.

Table 3.7 ARDRA results of Type I, II and III ITS rRNA gene clone libraries showing the richness of sampling (S_{Chao1}) of the libraries generated.

Hypolith Community	Number of Clones	Number of Phylotypes	Observed Phylotypes/ Predicted S_{Chao1}
Type I	63	31	33.34%
Type II	75	32	52.64%
Type III	61	32	27.57%

A total of ninety five clones were sequenced from the fungal ITS rRNA gene clone libraries. The NCBI BLASTn matches to the partial DNA sequences are listed in Appendix B2.

Sequence identity ranged from 71-100%. Identified sequences were all Ascomycota species. The most abundant identity was to *Acremonium rutilum* and *Verrucaria anziana* ITS rRNA gene sequences. Approximately 39% of the sequences displayed low identity values. Analysis of the sequence data provided by the libraries shows that the dominant fungal phylotypes in Type I, II and III hypolith communities belong to Ascomycota. However, the Type I community shows a 5% occurrence of Streptophyta species.

A phylogenetic neighbour-joining tree of the ITS rRNA gene sequences (Figure 3.27) was generated with the p-distance model (bootstrap = 1000 replications). The tree displays seven main clusters. Type II hypolith clones group together in the first and second clusters. These sequences display resemblance to the Ascomycota subphylum Pezizomycotina.

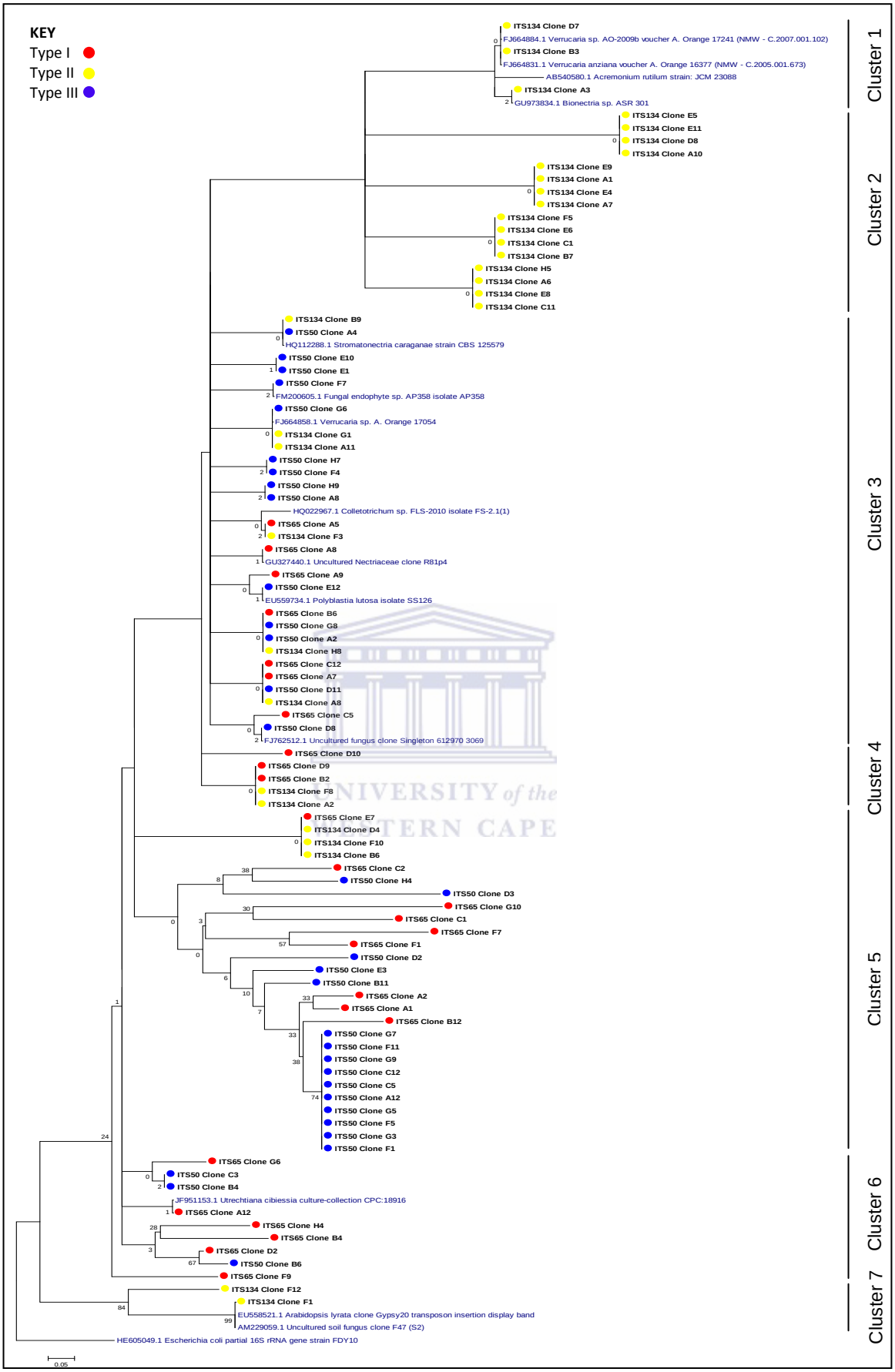

Figure 3.27 Neighbour-joining tree of the fungal ITS clone libraries for Type I, II and III hypolith communities, generated in MEGA 5. A total of 89 nucleotide sequences were analysed via the p-distance evolutionary model and a bootstrap replication value of 1000 was employed.

The third cluster is made up of Type I, II and III clone sequences that are similar to a variety of Pezizomycotina. This cluster additionally has a number of distinct internal nodes that clearly separate some clone sequences. Clusters 4 and 5 are primarily comprised of sequences that are more similar to each other than to their closest homologous BLASTn sequence. These are composed of Type I and III hypolithon sequences in cluster 4 and Type I and II sequences in cluster 5. The Type I and II clone sequences form the major component of the sixth cluster. Again, these sequences display more similarity to each other than to the closest known identified sequence. Cluster 7 is composed of two Type II sequences that resemble an *Arabidopsis lyrata* clone sequence and a previously uncultured soil fungus.

Very low bootstrap values were generated in the ITS rRNA sequence tree, with many of the internal nodes displaying values of 0. This is due to some ITS rRNA gene sequences being too divergent for alignment, and is supported by the low percentage of identity described in Appendix B2. While these clusters have been determined upon the general appearance of the phylogenetic tree, it is of extreme importance to recognise that the inferences made from this are minimal, as a result of the low bootstrap values, thus low reliability of the clustering in the tree. More reliable conclusions can be inferred from the sequence data and % identity of the clone library sequences (Appendix B).

According to the parsimony analysis the pars score was 14 (P<0.001) for Types I, II and III hypoliths. Pairwise pars scores and significance was also measured and showed the following:

- Type I : Type II - 5 (P<0.001),
- Type I : Type III - 9 (P<0.001),
- Type II : Type III - 2 (P<0.001).

These results imply that each hypolith community differs significantly from each other. The observed overall Shannon Index was 3.36, implying that all hypoliths were very diverse. A Venn diagram depicting the shared OTU richness and the observed species richness appears in Figure 3.28, and the lack of overlap supports the statistical observation that each hypolith type supports a unique community structure.

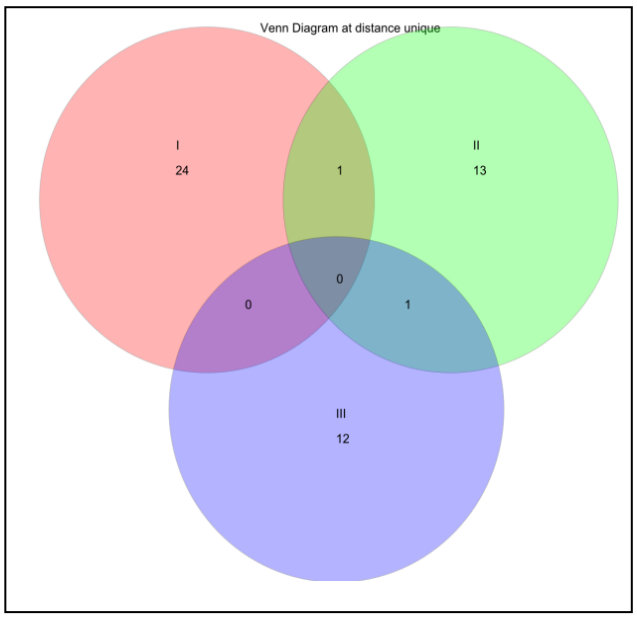

Figure 3.28 Venn diagram for the OTU richness in ITS rRNA gene clone library sequences. This indicates a total OTU richness of 51. The number of overlaps indicates that 1 OTU was shared between Type I and Type II communities as well as between Type II and Type III communities.

3.4.4 Fingerprint and Clone Library Analysis of Hypolithic Fungal ITS rRNA Diversity

Fungal community profiling reveals high fungal diversity in the Type I, Type II and Type III dominated communities. However, due to the reduced ability to assign fungal species, the known diversity appears to be very low. Still, a relationship between these communities can be developed using the generated data. The dominant fungal OTU's in Type I and Type II communities have a similar fingerprint in DGGE gels. This is supported by the clustering of Type I and II hypolithons in the nMDS plots with ± 50% similarity (Figures 3.18 and 3.19). Putative T-RF assignment shows that Type I and Type III communities have similar diversity (Ascomycota and Basidiomycota).

The differences in the clustering patterns of Type I, II and III communities in DGGE, T-RFLP and clone library sequences is an interesting observation. Here, each technique generates a different dendrogram of the relationship between Type I, II and III hypolith communities. It is possible, however, that due to the high complexity of fungal diversity, the phylogenetic data generated by each technique differs, resulting in contrasting clustering. Each technique targets a different aspect of community profiling analysis, i.e. a snap-shot of the dominant community members is observed via DGGE, the relative abundance and presence or absence of T-RF's, and thus differences in community structure are shown via T-RFLP and the most likely identity of the organisms is specified via sequencing and phylogenetics. As such, it is

important to use these techniques polyphasically, as each provides specific information towards answering the research question.

The statistical measurements of the fungal biodiversity shows that while the total fungal diversity may be high (Figure 3.22), the ability to assign the contributing members is difficult. The overall known species richness and evenness of Type I and Type III communities is therefore extremely small, despite the high OTU variation of clone library estimates (Table 3.6). This implies that further studies of hypolithon fungal diversity is required to fully appreciate the complete diversity supported by these niche communities.

The similarity of the Type I and Type III communities shown by T-RFLP analysis is supported by sequence data. The ITS rRNA phylogenetic tree shows a close correlation of the clone library sequences to each other. This displays the novelty of the species that were discovered and is supported by the low identity (less than 97%) to previously sequenced ITS rRNA gene regions. The few sequences that do display high identity correspond to yeast type fungi. They are, however, randomly distributed between the three hypolithons, implying no specific pattern to fungal species development.

Saprophytic, filamentous and yeast species of Ascomycota and Basidiomycota are among the known symbionts of cyanobacterial communities (Glenn *et al*., 1996). The

saprophytic *A. rutilum* for example, may feed on the detritus of organic matter from Type I and III hypolithons. Endolithic fungi that also exist in cyanobacterial communities produce succinic and oxalic acids, which are required for nutrient mobilization (Wynn-Williams, 1996).

The Type II T-RF data shows that the community is dominated by a single Glomeromycota species, *Glomus geosporum*. These spore formers are the largest genus in Glomeromycota (Redecker and Raab, 2006). They form an essential plant-microbe association in vascular plants and algae. Photobiontic Glomeromycota depend on these associations for mineral nutrient uptake (Redecker and Raab, 2006). They have additionally been shown to confer resistance to plant pathogens (Kauserud *et al*., 2008). With hypoliths showing a distinct lack of vascular phototrophs, *Glomus* species may associate with moss thalli and green algae. Filamentous fungi have also been observed in bryophyte dominated habitats in Terra Nova and Wood Bays, Antarctica (Adams *et al*., 2006). The significant presence of fungal species in these regions supports the high percentage of known diversity in Type II communities.

Typically a lichenized community is a symbiotic relationship between fungal mycelia and algae (Nash, 2004). Morphological assessment of Type III hypolithons revealed that fungal communities were closely associated to green pigmented cell clusters (Cowan *et al*., 2010c). There is a large likelihood of these cells being green algae

(Chlorophytes). The symbiotic relationship of hypolithon fungi and green algae are supported by the occurrence of *Lecanora* and *Trebouxia* species (phycobionts) in the DGGE (Galun *et al*., 1971).

Previous studies have shown that the yeasts and fungi detected in Antarctic soil systems are mostly cosmopolitan, globally distributed species (Wynn-Williams, 1996). Due to classical taxonomy rather than nucleic acid analysis characterization, there is a vast quantity of unknown fungal species yet to be identified (Adams *et al*., 2006). Additionally, online databases specific to fungal taxonomy are not very well represented, limiting the extent to which phylogenetic association of fungi can be made.

3.5 Microalgal Community Profiling of the Microalgal 18S rRNA Gene Region

The standard PCR amplification of the microalgal specific rRNA gene region (section 2.3) yielded a ± 400 bp band for each hypolith community. This was analysed via DGGE after nested PCR amplification.

3.5.1 Denaturing Gradient Gel Electrophoresis Fingerprinting of the Microalgal 18S rRNA Gene

Amplicons of approximately 400 bp were obtained using microalgal specific DGGE primers (Figure 3.29).

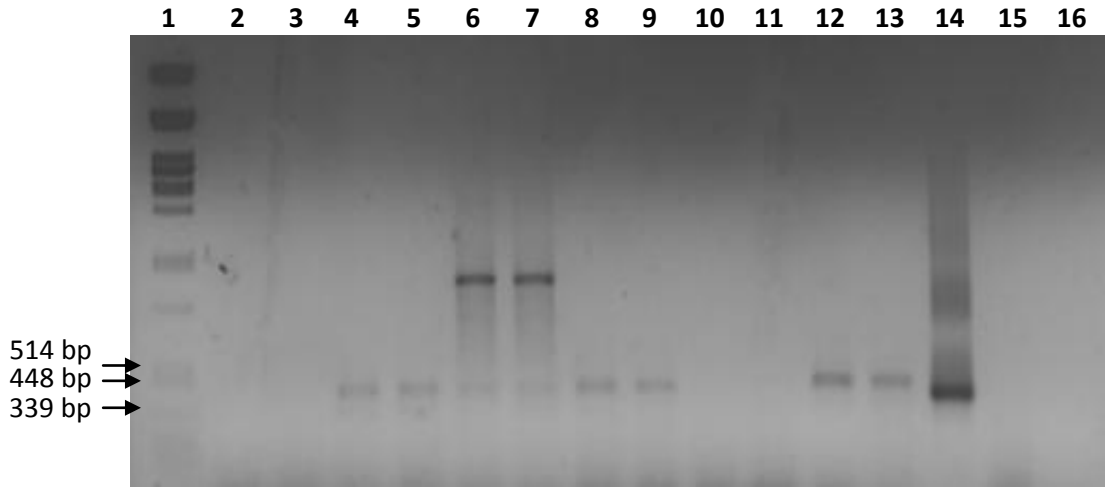

Figure 3.29 Nested PCR of a ± 400 bp microalgal specific 18S rRNA gene region from Type I, II and III hypolith communities. Lane 1: Molecular weight marker, Lane 2-5: Type I, Lane 6-9: Type II, Lane 10-13: Type III, Lane 14: positive control, Lane 15: negative control of the standard PCR, Lane 16: negative control of the DGGE PCR.

A 20-50% optimized polyacrylamide gradient on 9% polyacrylamide was required for efficient band separation of the PCR amplicons depicted in Figure 3.29. Distinct banding patterns were visible for each community type suggesting unique microalgal composition. Prominent bands excised from the gel corresponded to *Plectidae* sp. (Type I), *Stichococcus bacillaris* (Type II) and an uncultured eukaryote clone (Type I, II and III). Type I, II and III communities had 7, 8 and 7 OTU's, respectively. Band-matching of these OTU's shows that Type II and III communities are closely related, while the Type I community appears on a separate node (Figure 3.30).

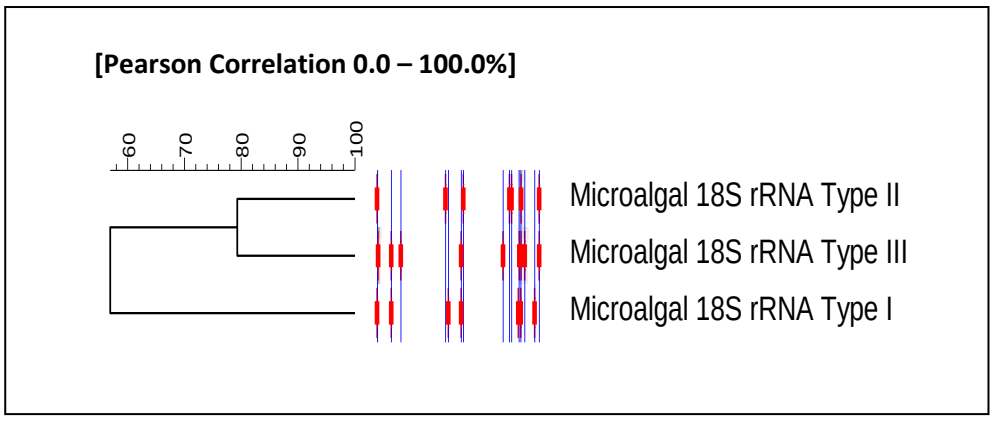

Figure 3.30 Microalgal 18S rRNA gene region cluster diagram generated using the Pearson coefficient and UPGMA algorithm. Type II and III communities show greater similarity to each other than to the Type I community.

The percentage of similarity between Type II and III is 79.31%. This cluster shows 56.93% similarity to the Type III hypolithon. The nMDS plot of this result (Figure 3.31) shows the 3 communities are separate and distinct in their composition.

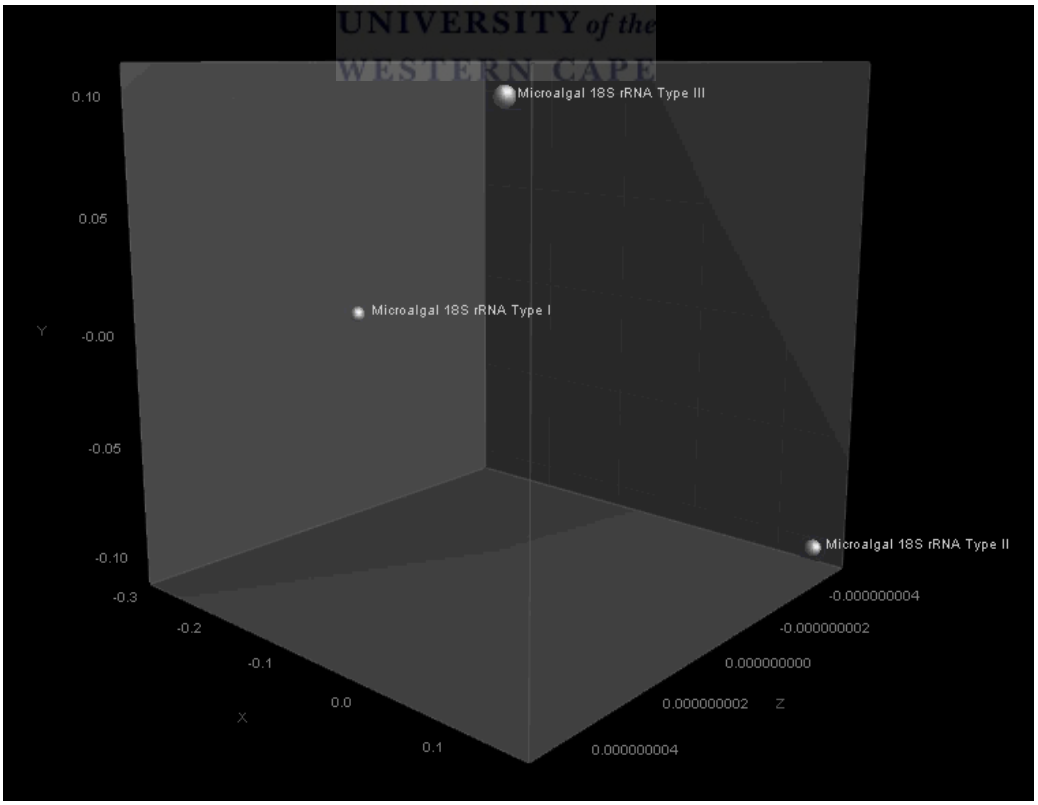

Figure 3.31 nMDS ordination of Type I, II and III hypolith communities using the the microalgal 18S rRNA gene region. Each hypolithon displays unique microbial diversity composition.

3.5.2 Diversity Assessment and Phylogenetic Assignment of Microalgal 18S rRNA Gene Terminal Restriction Fragments

Each peak produced in the T-RF fingerprint represents a single OTU. Table 3.8 shows the total number of OTU's observed for each digest in the microalgal 18S rRNA T-RF's.

Table 3.8 OTU's observed for each microalgal 18S rRNA gene enzyme digest.

Community Type	*Alu*I Digest OTU's	*Msp*I Digest OTU's
Type I A	45	47
Type I B	38	40
Type II A	22	43
Type II B	20	45
Type III A	17	30
Type III B	10	25

Cluster (Figure 3.32) and 2 dimensional MDS plots (Figure 3.33) of the T-RF's from *Ava*I and *Msp*I digests show a close relationship between Type I and II hypoliths (± 47% similarity), while the Type III hypolith community forms a separate branch. Additionally, the duplicate hypolith T-RF's are clearly similar, almost identical, to each other (Figure 3.32).

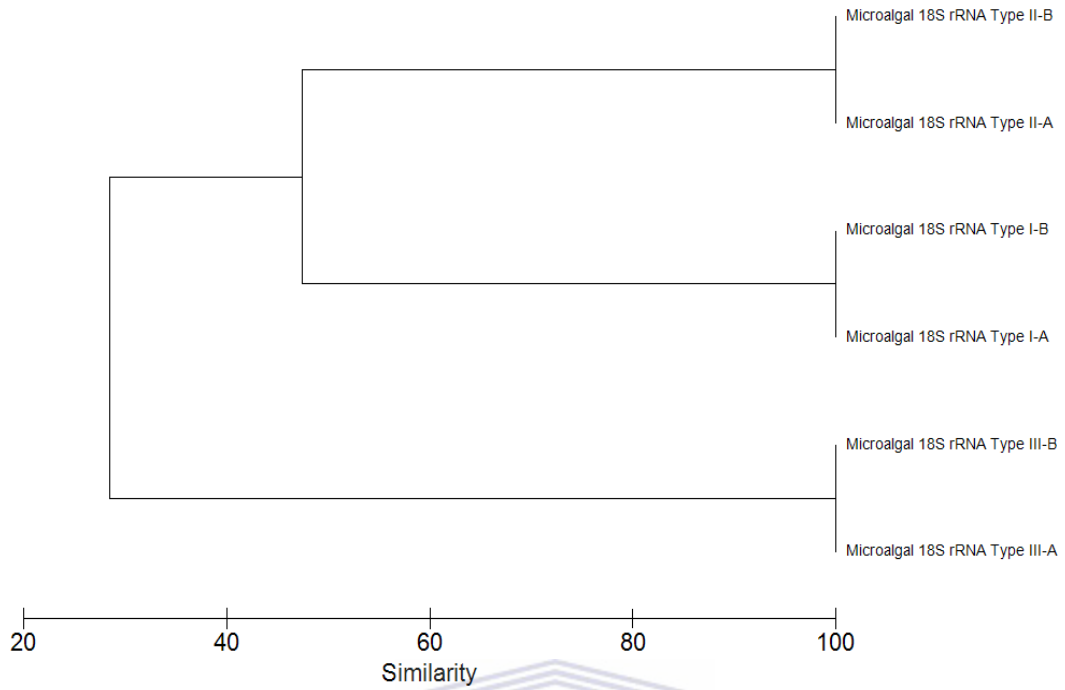

Figure3.32 Cluster analysis of the microalgal 18S rRNA gene componant of Type I, II and III hypolith communities, presented in duplicate. Close relation of Type I and II hypolith communities is observed while Type III communities exist on a separate node.

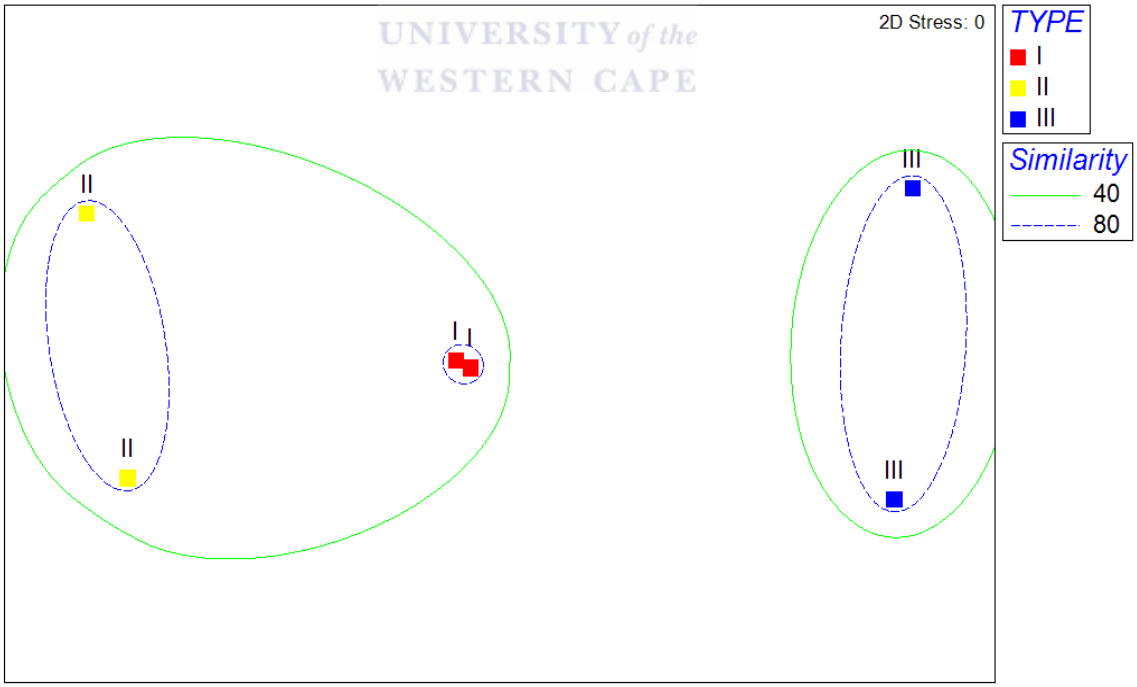

Figure3.33 A 2-dimensional MDS plot of the microalgal 18S rRNA gene T-RF's combined with the cluster analysis showing Type I and II communities having over 80% similarity to each other. Type III microalgal communities appear to be very distinct from those in the Type I and II hypolithons.

Diversity indices for the T-RF's of the microalgal 18S rRNA gene are shown in Table 3.9. The OTU variation (J') is consistent in each duplicate samples of the three hypolithons. Type I and II communities show higher species diversity (1-λ'), richness and evenness (H'(logE)), while that of the Type II community is lower.

Table 3.9 Univariate diversity indices for microalgal 18S rRNA gene T-RF data.

Sample	S	d	J'	H'(logE)	1- λ'
Microalgal 18S rRNA Type I A	46	9.772	0.6597	2.526	0.8422
Microalgal 18S rRNA Type I B	46	9.772	0.6597	2.526	0.8422
Microalgal 18S rRNA Type II A	42	8.903	0.6434	2.405	0.8618
Microalgal 18S rRNA Type II B	42	8.903	0.6434	2.405	0.8618
Microalgal 18S rRNA Type III A	30	6.297	0.5706	1.941	0.7256
Microalgal 18S rRNA Type III B	30	6.297	0.5706	1.941	0.7256

*S indicates the total species per sample, d shows the species richness, J' shows Pielou's Evenness, H'(logE) shows the Shannon Index, 1-λ is the Simpson Index.

Figure 3.34 shows the putative phylogenetic assignment of the T-RF's obtained for the microalgal 18S rRNA gene signatures. The percentage of known diversity in this data set is exceptionally high - more than 90% for all three hypolithons (Table 3.10).

Highest diversity is apparent in the Type II hypoliths, with phylum Euglenozoa contributing 16.51% of the diversity. The most abundant Type I phylotype was uncultured marine eukaryotes (10.81%), while the Type III dominant T-RF correlated to Arthropoda (83.37%).

Table 3.10 Known and unknown diversity of the microalgal 18S rRNA gene T-RF's.

Hypolith Community	% Known Diversity	% Unknown Diversity
Type I	93.0283	6.9717
Type II	96.7388	3.2612
Type III	94.7103	5.2897

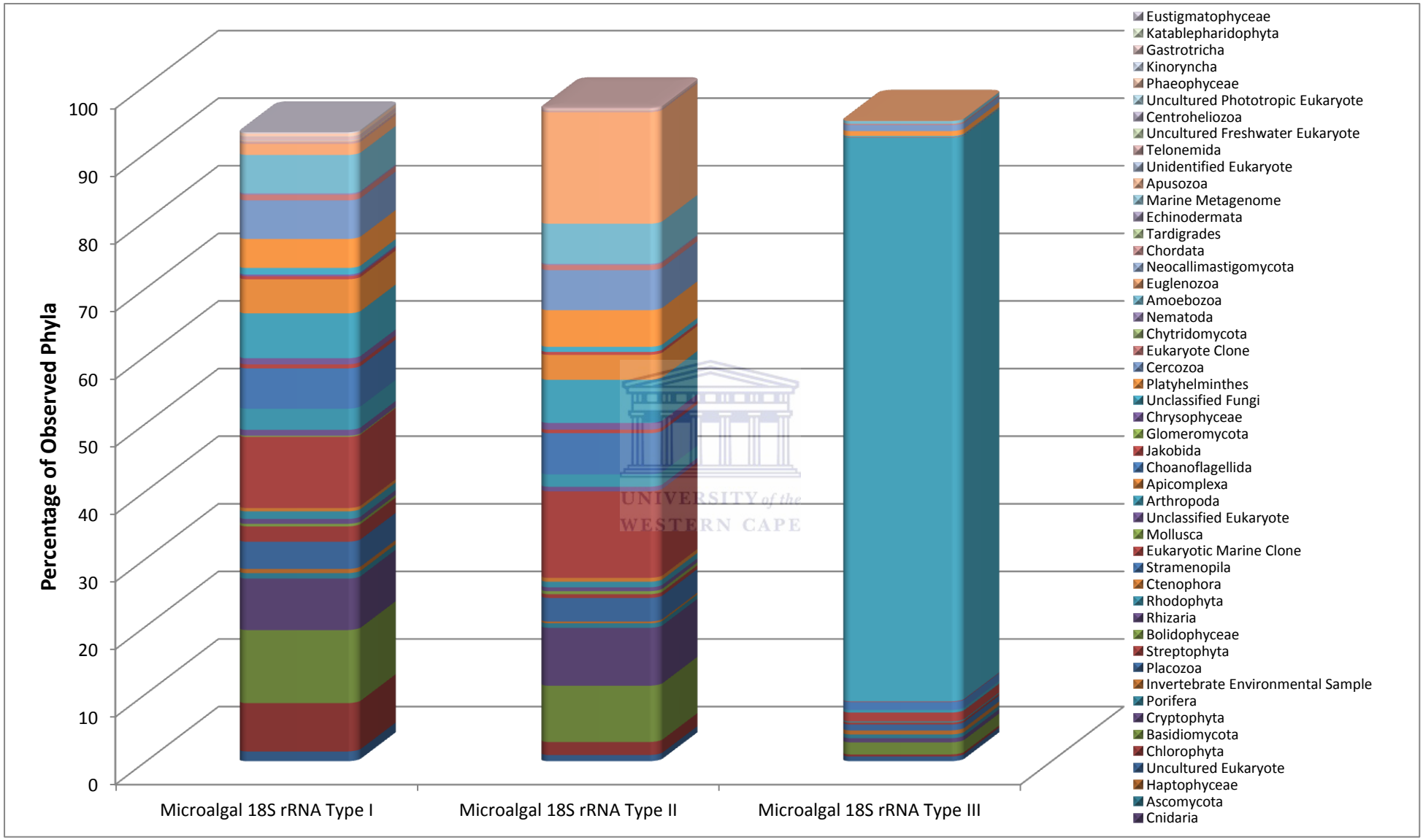

Figure 3.34 Putative phylogenetic assignment of microalgal 18S rRNA gene T-RF's based upon database and similarity matrix analysis. Type II hypoliths display the highest level of diversity, however, overall, there is an extremely high level of microalgal diversity within the dataset (see Appendix A for taxonomy).

3.5.3 Phylogenetic Assessment of Microalgal 18S rRNA Gene Clone Libraries

Positive recombinants were selected by blue-white screening and further dereplicated by PCR and ARDRA (sections 2.6.3 and 2.6.4). The presence of a ± 700 bp insert in the M13 colony PCR indicated positive recombinants (Figure 3.35).

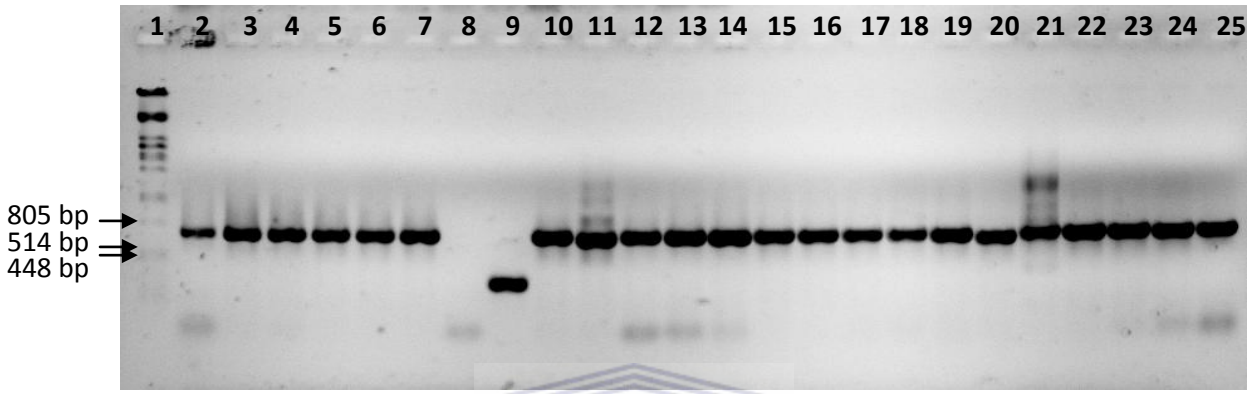

Figure 3.35 M13 PCR colony screen of a portion of the Type III microalgal 18S rRNA gene clone libraries from C1-12 and D1-12. Lane 1: Molecular weight marker, Lanes 2-24: M13 colony PCR of the microalgal 18S clones. Lanes 8 and 9: negative amplifications from within the clone library.

The microalgal specific nested PCR, yielded a ± 400 bp fragment (Figure 3.36). A large number of positive recombinants were obtained from the clone libraries.

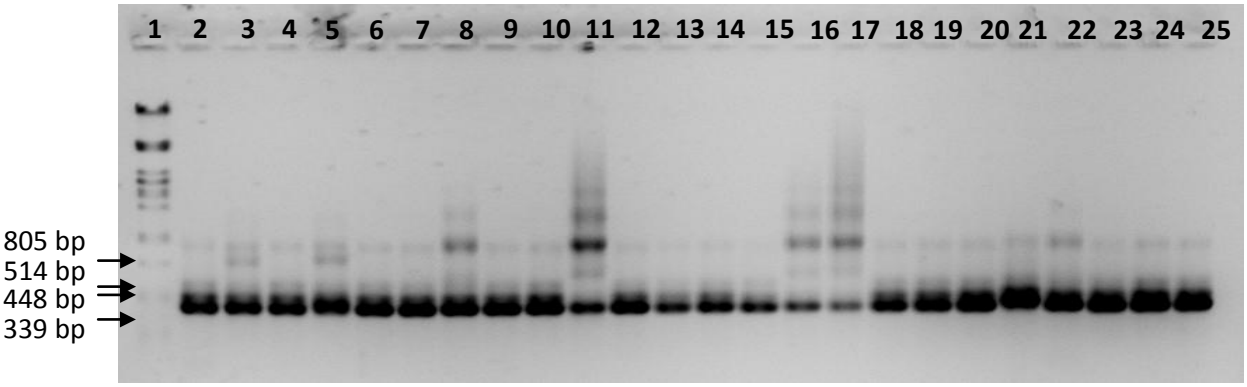

Figure 3.36 Nested PCR of a portion of the positive recombinants from the microalgal 18S rRNA gene clone screen using microalgal specific primers, P45 and P47. Lane 1: Molecular weight marker, Lane 2-25: Nested PCR of M13 colony PCR using microalgal specific primers.

The total number of positive clones for each hypolith was as follows:

- Type I microalgal 18S rRNA gene clone library - 59,
- Type II microalgal 18S rRNA gene clone library - 89,
- Type III microalgal 18S rRNA gene clone library - 73.

The ARDRA screen with *Ava*I and *Msp*I, displayed various banding patterns (Figure 3.37) which was indicative of different phylotypes within the microalgal 18S rRNA gene clone libraries.

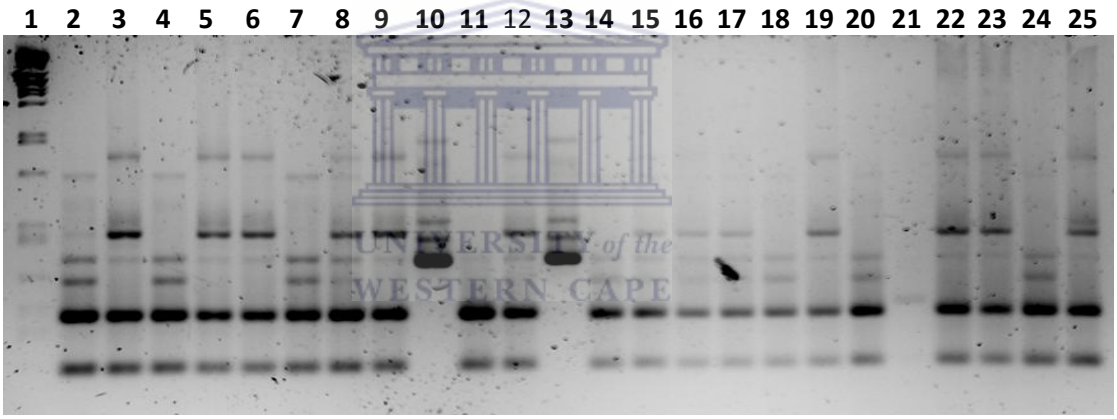

Figure 3.37 Example of the ARDRA screen of the microalgal 18S rRNA gene clone libraries created using a dual endonuclease digestion with enzymes *Ava*I and *Msp*I.

The number of phylotypes observed in the microalgal 18S rRNA gene clone library is displayed in Table 3.11. It additionally provides estimations on the richness of sampling for Type I, II and III hypolith communities.

Table 3.11 ARDRA results of Type I, II and III microalgal 18S rRNA gene clone libraries showing the richness of sampling (S_{Chao1}) of the libraries generated.

Hypolith Community	Number of Clones	Number of Phylotypes	Observed Phylotypes/ Predicted S_{Chao1}
Type I	59	21	30%
Type II	83	21	55.42%
Type III	70	23	24.17%

The collectors curve (Figure 3.38) shows a plateau in Type I, II and III communities. This implies that sampling within the libraries was sufficient. The phylotype/S_{Chao1} percentage (Table 3.11) infers that the library generated for each hypolithon was not representative of all possible OTU's. More screening is therefore required for an improved estimation of the diversity.

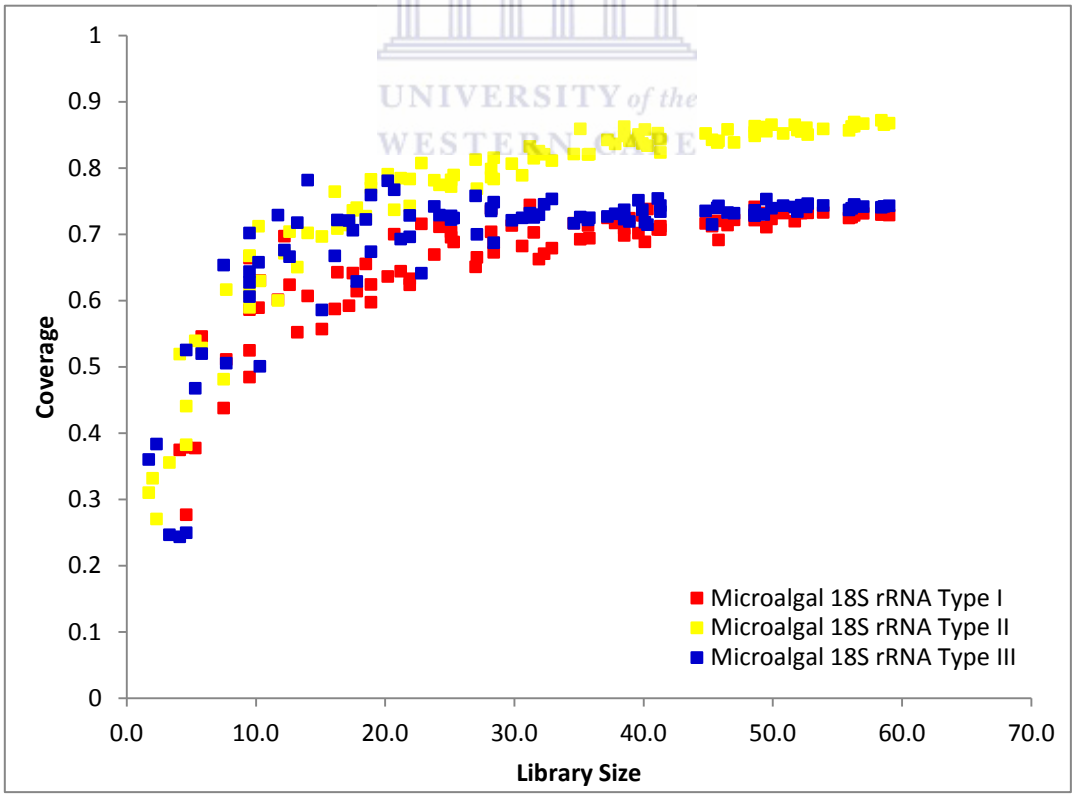

Figure 3.38 Collectors curve of the microalgal 18S rRNA gene clone libraries after ARDRA phylotype assignment. These show that the libraries were sufficiently sampled.

Overall, sixty five microalgal specific 18S rRNA gene clones were sequenced. Of these, ten Type I clones were unable to be sequenced. The remaining partial DNA BLASTn matches are listed in Appendix B3. The identity of these sequences ranged from 79-100%. Type I and II communities are dominated by uncultured eukaryote clones (Figure 3.39). Additionally, Type I clone sequences show 36% similarity to bryophytes. In the Type II hypolith clone library, 89% of sequences were similar to a single uncultured eukaryote clone from a previous hypolith study (HM490274.1). The Type III community is dominated by Bryophyta, with uncultured eukaryotes constituting the remaining 13% of OTU richness.

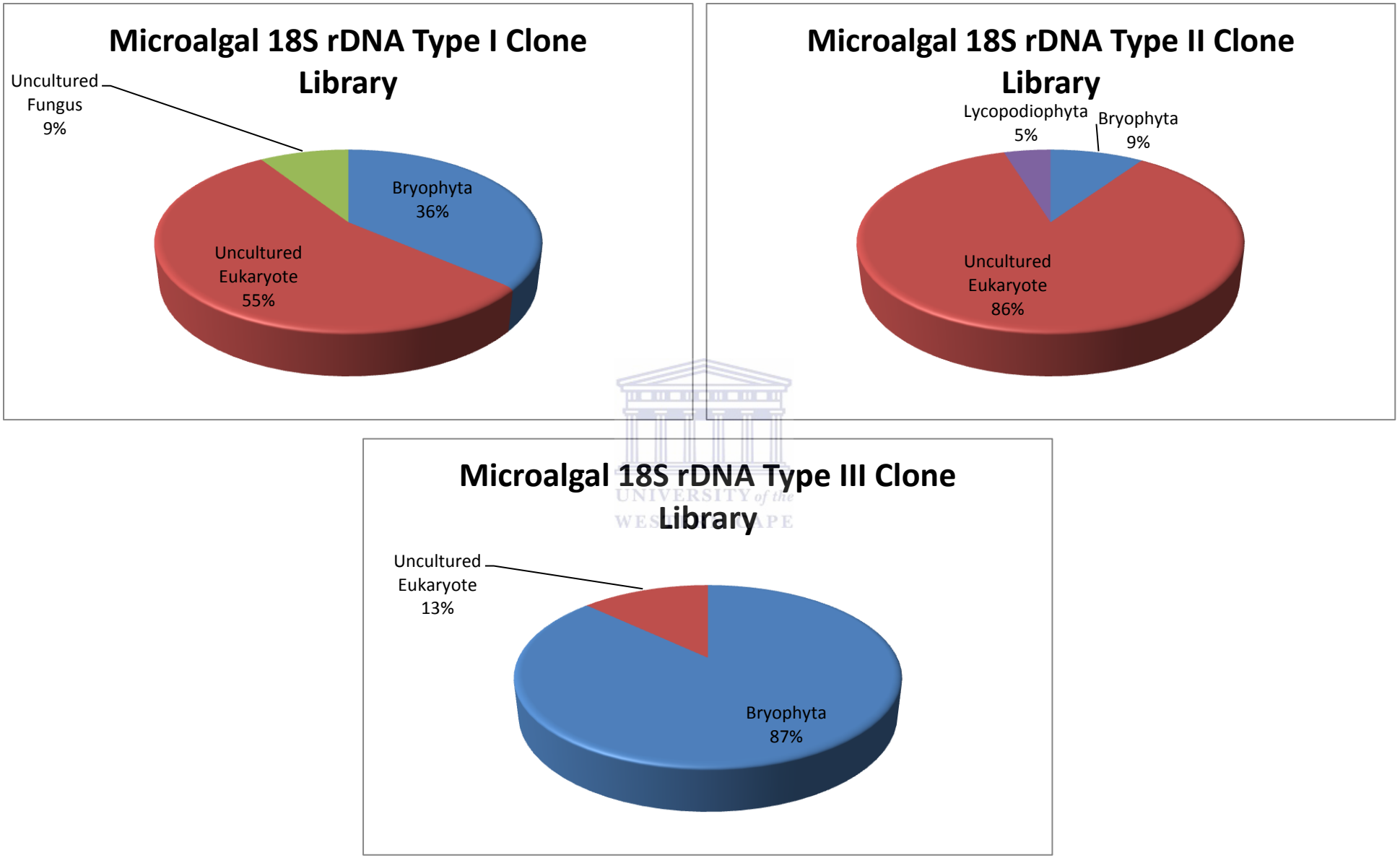

Figure 3.39 The relative percentage distribution of the microalgal 18S rRNA gene clone library phylotypes identified from Type I, II and III hypolith communities.

The neighbour-joining tree generated with the p-distance model (1000 replications) displayed five distinct clusters (Figure 3.40). Cluster 1 shows that one Type III clone has high identity to *T. ruralis*. The Type I clone in cluster 1 has sequence similarity to two different uncultured clones as well as to five different moss species. Sequences in the second cluster, however, do not resemble any of the BLASTn sequences obtained for the Type I clones. This implies that they are more similar to each other than to known sequences of microalgal specific RNA gene regions. A similar pattern is observed in clusters 3 and 5. Both clusters are made up of Type II and III clone sequences. Cluster 4 constitutes one Type II sequence and two Type III sequences that display similar sequence identity to a previously uncultured eukaryotic clone, in addition to a separate node of Type II and Type III clone sequences.

MEGA 5 once again was unable to align some sequences as they were too divergent. This could explain the extremely low bootstrap values observed at internal nodes of the Type II and III communities in cluster 5. The result of this low confidence implies that clusters 4 and 5 have little to no support. The topology is therefore not reliable enough to determine evolutionary relationships for most of the Type II and Type III organisms that contain microalgal 18S rRNA regions in the hypolithon. However, clusters 1-3 display sufficient bootstrap support and can be used to determine an evolutionary relationship between these hypolith sequences.

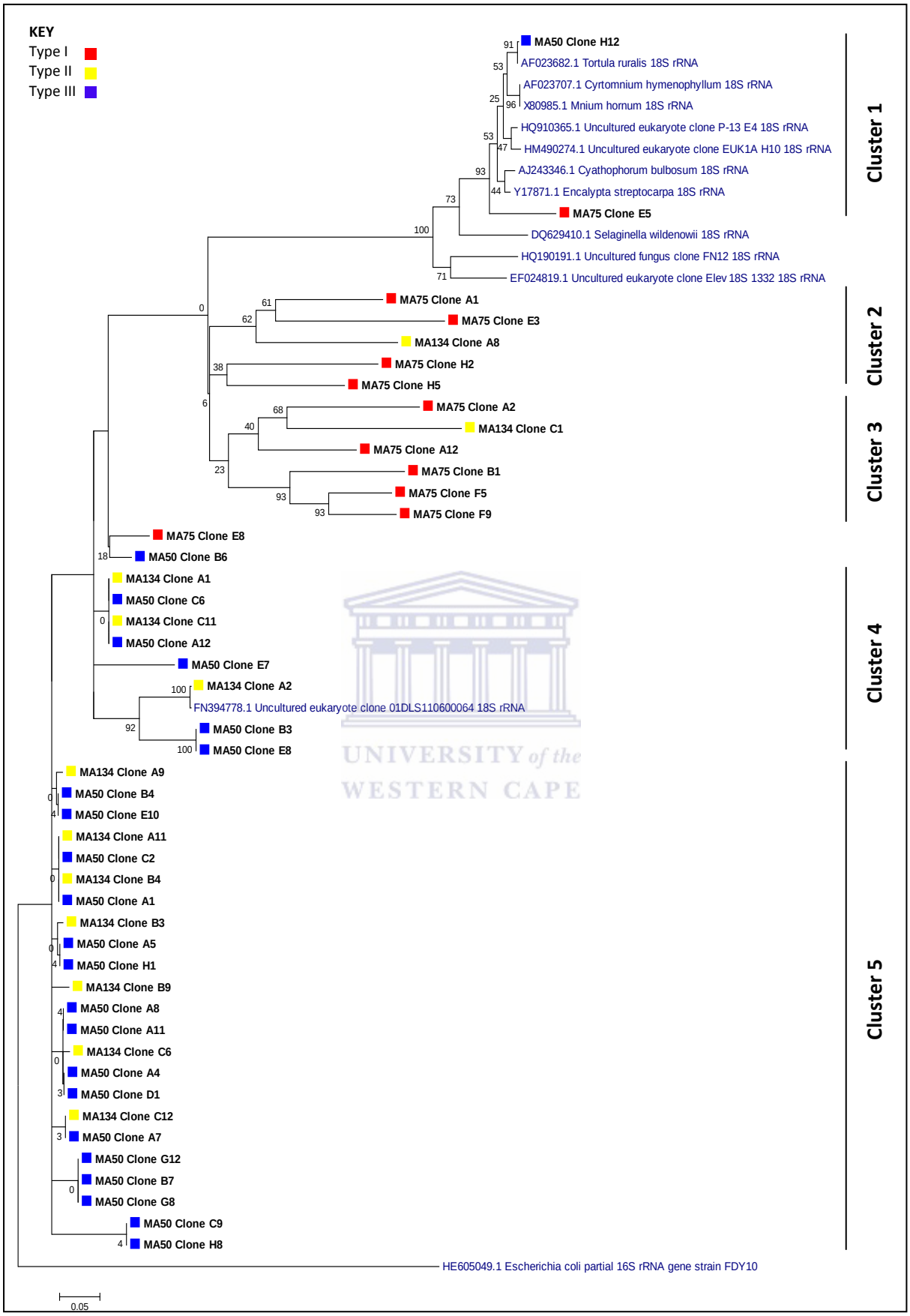

Figure 3.40 Neighbour-joining tree of the microalgal specific 18S rRNA gene clone library, performed with MEGA 5. A cumulative 46 clones were analysed from the Type I, Type II and Type III libraries, using 1000 bootstrap replications with the p-distance evolutionary model.

Parsimony analysis of the microalgal 18S rRNA tree provided a pars score of 13 with a significance of P<0.002. Each hypolithon displayed the following pairwise parsimony statistics,

- Type I : Type II - 3 (P<0.001),
- Type I : Type III - 2 (P<0.001),
- Type II : Type III - 10 (P<0.588).

The scores imply that these hypolithons are distinct. However, there is a noticeably lower pars score between Type II and III communities inferring a level of similarity between them. Shannon Index for the microalgal rRNA gene sequences was 2.163, implying high biodiversity of the hypolithons. The Venn diagram (Figure 3.41) displays the observed species richness.

Figure 3.41 Venn diagram of the OTU richness in the microalgal 18S rRNA gene sequences. These show an overall OTU richness of 19. An overlap of 1 OTU exists between Type II and III hypolithons.

3.5.4 Fingerprint and Clone Library Analysis of Hypolithic Microalgal 18S rRNA Diversity

Research into the metagenomic microalgal constituents, a previously unstudied microbial group in Dry Valley hypoliths, has shown that the biodiversity of this division is remarkable. DGGE cluster analysis (section 3.5.1) shows closer relation of Type II and III communities while T-RF analysis (section 3.5.2) shows more resemblance between Type I and II communities. This can be explained by PCR primer bias towards four specific microalgal classes. Additionally, the differences in the basic principles of the two techniques could limit the level of OTU assignment.

Dominant species in the putative T-RF assignment showed correlation to unclassified Stramenopila (also known as Heterokonta), Euglenozoa and Arthropods. Interestingly, some microalgae (Chrysophyceae, Bacillariophyceae and Phaeophyceae) fall under the kingdom Stramenopila (Anderson, 2004). It can therefore be postulated that a number of microalgal communities remain unknown in the hypolithon due to the limited level of taxonomic characterization of such groups.

Microalgal 18S rRNA gene sequences display high species diversity, richness and evenness according to alpha and beta diversity indices (Table 3.9). Chao1 and collectors curves, however, imply that a large amount of the contributing species is still to be determined.

The phylogenetic tree based upon the sequence data shows correlation to DGGE dendrogram clustering. The unique composition of each of these three communities can be observed from the phylogenetic associations of the clone library sequences. Like the universal eukaryotic and fungal ITS rRNA gene sequences, the microalgal 18S rRNA gene sequences are more similar to each other than to their highest homologous NCBI BLASTn hits. Those that do exhibit high percentage of identity, generally correspond to bryophyte species. This data also confirms that the Type II and III communities have a level of similarity that is not shared with the Type I community.

It is essential to acknowledge that the four classes of microalgae (Chlorophyceae, Bacillariophyceae, Cryptophyceae and Chrysophyceae) that were used to design the primers employed in this study are the most commonly observed microalgal classes in natural systems (Dorigo *et al*., 2003). However, records of more than 300 algal taxa have been found in Antarctic Dry Valleys. These belong to the divisions Chlorophyta, Bacillariophyceae, Xanthophyceae, Chrysophyceae, Dinophyta, Cryptophyta and Euglenophyta. Most of these species are, however, observed in freshwater communities, aquatic environments and cyanobacterial mats (Adams *et al*., 2006). While this study shows similarity to some of these microalgal classes (Chrysophyceae, Chlorophyta and Cryptophyta), the hypolithon may support additional microalgal classes that could actively contribute to hypolithon development and sustainability. However, these currently remain uncharacterized, contributing to the level of unknown species postulated by the statistical predictions.

The detection of a number of non-microalgal sequences, despite the use of microalgal specific primers could also be explained by the primers being located within the 18S rRNA gene region (Dorigo *et al*., 2003). The development of more specific primers would alleviate this problem and possibly detect more microalgal classes.

Many moss species have optimized their functioning or developed adaptations for survival in harsh environments. *Stichococcus bacillaris,* the dominant microalgal chlorophyte observed in DGGE sequences, is responsible for nitrogen assimilation in autotrophic and heterotrophic conditions (Ahmed and Hellebust, 1986). The high nutrient and water content may permit the survival and dominance of various moss communities such as *Cyathophorum* species (Carrigan and Gibson, 2003). When viewed in addition to the adaptations of *T. ruralis* and *E. streptocarpa* to this environment (dessication tolerance and recurved leaf margins) (Crandall-Stotler and Bartholomew-Began, 2007), it appears that this hypolithon may sufficiently support diverse microbial species.

The Euglenids are phototrophic protozoans that are thought to have derived this function from symbiotic green algae (Keeling, 2004). They are mostly observed in fresh water ecosystems (Finlay and Esteban, 1998). Even though they are a recognized phylum of the algal division, these species may function as grazers on

bacteria. Previous research of their function shows that they also act as a food source for higher trophic levels (Finlay and Esteban, 1998).

Arthropods, the largest endemic terrestrial invertebrates, are known to inhabit regions of high moisture content in Antarctic Dry Valleys (Hogg *et al*., 2006). Many arthropods feed on microalgae and mosses (Adams *et al*., 2006). Moist environments such as Dry Valley hypoliths, favour the colonization of mosses (Cowan *et al*., 2010b). However, it has been stated that the co-occurrence of these species may be a coincidence. Their symbiosis, however, may contribute to the composition of nutrients in the hypolith via arthropod grazing (Teuben and Roelofsma, 1990).

CHAPTER 4
FINAL DISCUSSION AND CONCLUSION

4.1 Introduction

Early studies on Antarctic soil showed indications of low levels of viable microbial diversity (Cameron *et al*., 1970; cited in Smith *et al*., 2006). However, small invertebrates, lower plants and bacterial communities were abundantly observed when investigated by microscopy, gross morphology and culturable methods (Convey and Stevens, 2007). These communities are dominated by nematodes, tardigrades, rotifers, mosses, lichens, cyanobacteria, actinobacteria and proteobacteria. Through the development of molecular methods, the ability to distinguish and describe microbial diversity in the harsh environment of the Dry Valley soils has been enhanced (Cowan *et al*., 2004; Blackwood *et al*., 2003; Dorigo *et al*., 2002).

Microorganisms that can survive the environmental constraints of Dry Valley hyper-arid ecosystems colonize refuge environments (Cowan *et al*., 2010c). These refuges are mostly translucent rocks on the desert pavement (Cockell and Stokes, 2004). Microbial growth on the ventral surface of translucent rocks forms crypto-endolithic and hypolithic communities (Cowan *et al*., 2010c). Here they are able to survive and proliferate despite the fluctuating external temperature, intense UV, dessication and limited nutrient availability (Cowan and Ah Tow, 2004).

Hypolithons have been described on gross morphology and are categorized as Type I, II and III hypoliths (Khan *et al*., 2011). They display dominance visually by cyanobacteria, moss and lichen communities, respectively. In this study the eukaryotic diversity associated with these hypolithons was analysed by the use of three molecular techniques (DGGE, T-RFLP and clone libraries) targeting three different regions (18S, ITS and microalgal 18S rRNA genes).

4.2 Culture Independent Techniques and Diversity Studies

Recent investigations of microbiota surviving the Antarctic habitat indicate a richer diversity than initially recognised (Rao *et al*., 2011; Cowan *et al*., 2010c; Pointing *et al*., 2009; de la Torre *et al*, 2003). This is due to the application of molecular techniques that provide a larger amount of information on community content and structure than culture-based approaches (Anderson and Cairney, 2004). An analysis of soil or hypolith metagenomic DNA gives insight into the previously unrecognized/unknown microbial diversity in a community. Used in a polyphasic manner, culture independent approaches can provide understanding of the dynamics and ecological role of communities (Garbeva *et al*., 2004; Kitts *et al*., 2001; Muyzer and Smalla, 1998). This study of the eukaryotic diversity of Dry Valley hypolithons has displayed tremendous species diversity and richness using the three different molecular techniques.

According to DGGE principles, each band separated on the polyacrylamide gel represents a single operational taxonomic unit (OTU). Sequencing of these bands has proven that they may not represent a single OTU (Sekiguchi *et al*., 2001). While a large number of bands can be matched using the DGGE band-matching technique, this does not imply that each matched band has closer affiliation to one microorganism over another. Additionally, DGGE can only provide an assessment of the most dominant OTU's in a community and detects significantly fewer OTU's when compared to T-RFLP. This technique, however, was efficient in the detection of dominant community members in each hypolithon. It indicated high diversity within and between the Type I, II and III hypolithons in the universal 18S rRNA gene region, as well as the fungal ITS and microalgal specific 18S rRNA gene regions. This provided the initial evidence and support to warrant further in-depth investigation of the microbial community profiles of the three hypolithons.

For quantitative and qualitative assessment of communities, T-RFLP is employed. Together with DGGE, these techniques provide a temporal and spatial representation of microbial profiles, allowing an understanding of the distribution and structure of the communities. By understanding this structure, theories on the ecological and biogeochemical cycling roles of these microorganisms can be developed.

Ecological studies (human and environmental) have previously expressed inconsistencies when comparing these two fingerprinting techniques (Matovelle *et al*., 2007). Analysis of a DGGE gel is only as good as the image of the band separation that is captured. T-RFLP MDS analysis, though, is based upon the relative peak size and height obtained from electrophoresis. It is therefore considered a more reliable tool than a DGGE image for phylogenetic inference. All three hypolithons display an unexpected high level of species richness and diversity, especially in the fungal and microalgal centred investigations. Although PCR bias plays a particularly important role in the over-estimation of OTU's in standard PCR based T-RFLP studies, in this instance it proves that the high diversity indicated by DGGE fingerprints and nMDS plots is plausible and therefore required further investigation.

T-RFLP analysis has demonstrated immense value in describing complex microbial communities at high resolution (Schwartzenbach *et al*., 2007; Lukow *et al*., 2000; Liu *et al*., 1997). In this study T-RF results show the distinct community structure of these three hypolithons. The database peak-matching process, however, is putative. Additionally, molecular techniques, including T-RFLP, are based upon all recoverable DNA signatures and not necessarily only the metabolically active organisms, as is represented in the fungal study by Rao *et al*. (2011). In this study the combined data implies a richer eukaryotic diversity in hypolithons than previously encountered. It also provides an indication of the most abundant fungal and microalgal divisions in hypolithon systems in Antarctic Dry Valley hypolithons. Quantitative RT-PCR analyses

would be able to distinguish between preserved diversity and the metabolising community.

Clone library screens are deemed an important facet of any phylogenetic study. The sequence data it provides allows for an estimation of their evolutionary relationship when compared to seemingly homologous organisms. This enables an estimation of the interaction between microbes and their roles in the environment (Torsvik and Øvreås, 2002). The clustering pattern of the hypolithons displayed irregularity when the diversity using the three different phylogenetic target marker rRNA gene regions were compared. However, when one considers the symbiotic relationship that each hypolithon (Type I, II or III) offers the different moss, fungal, amoeboid and microalgal species, the differing dendrograms may be a direct interpretation thereof. Many sequences from the clone libraries display very low percentage identity (less than 97%) to NCBI database sequences. This, as well as the inability to align extremely divergent sequences implies that the majority of the identified sequences belong to novel species of moss, fungi and previously uncultured eukaryotes (Appendix B1, B2 and B3). The limitation to the clone library data in this study was the inability to recover the entire eukaryote, fungal and microalgal diversity of Type I, II and III hypolithons, despite the appearance of an efficiently sampled library in the collector's curves (Figures 3.13, 3.26 and 3.38). As such, more sampling and analysis, as well as improved characterization of these organisms, is necessary to uncover the true diversity of this environmental niche.

Laboratories across the world are currently attempting to re-define classical culture techniques and media (Vartoukian *et al*., 2010). With the revolution in characterizing the diversity of previously unculturable microorganisms, taxonomy will be greatly expanded (Vartoukian *et al*., 2010; Sharma *et al*., 2005). Due to metagenomics and high throughput sequencing, many environmental studies result in the generation of vast amounts of sequence data representing uncultured organisms. However, the analysis of the functional role of these organisms is limited (Edgecomb *et al*., 2011; Schnetzer *et al*., 2010). This study has been an example in which the majority of the sequences generated contain previously uncultured moss, fungal and microalgal clones that appear to be very novel (display less than 97% similarity to currently known species). Additionally, research of the metabolically active communities in the three hypolithons is necessary for a more complete analysis of the community structure and the role that these organisms play in these environments. This would reduce the amount of unusable data that can be generated by community profiling investigations. It proves particularly important for hypolithon communities such as those in the Miers Valley where the presence of dormant or inactive DNA is potentially high as a result of the ideal conditions for preservation (Cowan, 2009).

Current next-generation sequencing platforms have demonstrated the remarkable improvement in detection, sensitivity and costs associated with 16S rRNA based studies (Tamaki *et al*., 2011). In combination with cDNA libraries and metagenomic sequencing projects, high throughput sequencing has the potential to access the undetected microbial diversity worldwide, and specifically of Antarctic Dry Valley

hypolithons. Again, these techniques generate copious amounts of data, much of which is unusable for species extrapolation (Edgecomb *et al*., 2011; Schnetzer *et al*., 2010), as comprehensive methods of analysis of this data have yet to be developed (Edgecomb *et al*., 2011; Tamaki *et al*., 2011; Schloss *et al*., 2009). The culturing and determination of the physiological and biochemical characteristics of the observed species, therefore, becomes critical to future analysis and interpretation, to support microbial community profiling in the environment.

4.3 Eukaryotic Diversity of Miers Valley Hypolithons

Culture independent techniques have consistently been applied in Antarctic microbial diversity studies. However, many studies focus on the characterization of microbiota in sea ice, lake sediments, cyanobacterial mats and open soils (Wood *et al*., 2008; Shravage *et al*., 2007; Franzmann, 1996). Recently the hypolithon has been investigated due to the favourable conditions this unique microenvironment offers microbial life (Cowan, 2009; Pointing *et al*., 2009). Phylogenetic studies of hypolith communities using metagenomic DNA have previously yielded a vast quantity of information on organism abundance and diversity (Khan *et al*., 2011; Cowan *et al*., 2009; Pointing *et al*., 2009).

The data obtained in this study displays very high species richness and diversity. This is supported by the MDS plots and cladograms of the universal eukaryotic (section

3.3), fungal ITS (section 3.4) and microalgal 18S (section 3.5) rRNA gene regions, generated through two different molecular fingerprinting techniques.

The trees generated in this study to date are merely a representation of relatedness, as they are neighbour-joining trees. This limits their use in the estimation of evolutionary pathways that exist in the hypolithon. To obtain an improved indication of the evolutionary distance between the sequences observed in the phylogenetic trees (Figures 3.15, 3.27, 3.40), a maximum likelihood model and analysis parameters need to be applied. When attempting to perform such an alignment and phylogeny on cluster 1 of the 18S rRNA gene region, the branch bootstrap values showed improvement, but were still low (Figure 4.1.) This pattern of confidence level is similar to those illustrated in Figures 3.15, 3.27 and 3.40. It supports the claim that the sequences are likely to be closely related to each other. It additionally illustrates that there is currently insufficient data available to precisely determine phylogenies and evolutionary relationships within this dataset.

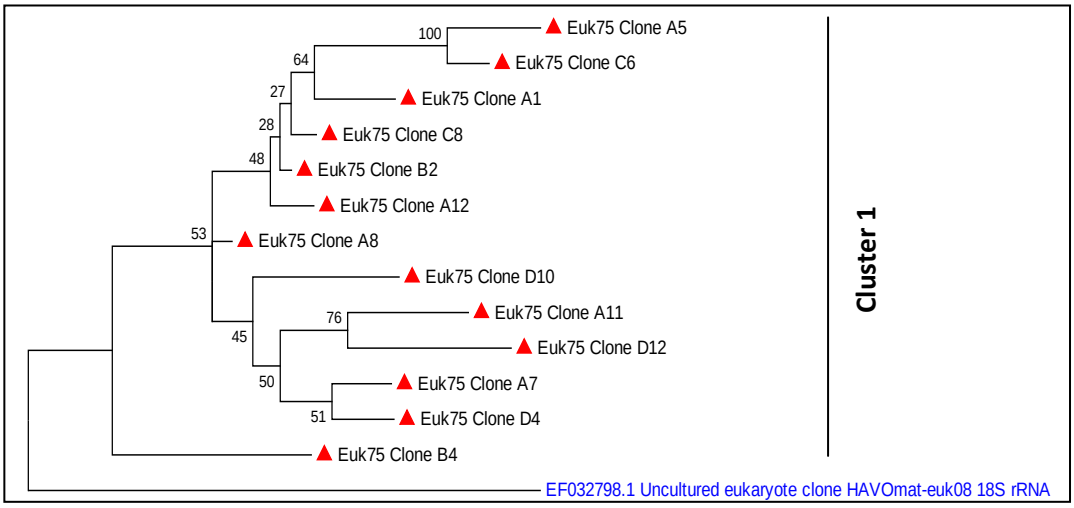

Figure 4.1 Phylogenetic tree of cluster 1 of the 18S rRNA gene region, generated using the maximum likelihood model using the Kimura-2 parameter. Improved bootstrap values that are observed are still insufficient to reliably deduce the evolutionary pathway in hypolithons.

However, sufficient sequence data was obtained which has contributed significantly to a better understanding of the hypolithon community structure. What is observed overall is the stable representation of photosynthesizers in hypolith eukaryotes. These include numerous characterized and previously encountered bryophyte species (*C. bulbosum*, *T. ruralis*, *E. streptocarpa*, *P. truncata*, *M. hornum* and *T. tortuosa*), in addition to a high percentage of physiologically and biochemically uncharacterized moss species (Figures 3.15, 3.27 and 3.40).

The ecological roles of microorganisms in hypolithons are often unknown or underestimated (Fell *et al*., 2006; Moon-van der Staay *et al*., 2006). Cyanobacterial, chlorophyte and fungal dominated communities are presumed to be the primary photobionts in Dry Valley cryptic communities (de le Torre *et al*., 2003). In this study these species were well represented in the universal eukaryotic and the fungal study. The most prominent eukaryote was Ascomycota in Type I and Type III communities, with the spore forming Glomeromycota forming the major colonizer of Type II communities. Despite the low species diversity observed in some instances, sequence data depicts a large amount of novelty in the fungal hypolith component. These species may provide the main form of carbon, nitrogen and minerals required for higher trophic level sustenance (Rao *et al*., 2011; Convey and Stevens, 2007). As such, further investigations of hypolithic eukaryote diversity and the dominant Antarctic soil kingdoms (fungi and microalgae), is necessary to ascertain the extent of their involvement in nutrient cycling in the hypolithon.

A variety of different moss, algae and fungi were present in the three hypolithons. The eukaryotic communities are structured differently in each of the hypolith types, as has been demonstrated for the prokaryotes (Khan *et al*., 2011; Pointing *et al*., 2009). This provides sufficient evidence from which a possible mutualistic relationship between fungi, cyanobacteria, moss and algae can be derived.

Type I hypoliths are cyanobacteria dominated, and *Oscillatoriales* can comprise up to 95% of the cyanobacterial biomass (Khan *et al*., 2011; Cowan *et al*., 2010c; Pointing *et al*., 2009). As such, they are imperative in maintaining the carbon and nitrogen balances and cycles in sparsely vegetated environments (Cowan *et al*., 2011; Cockell and Stokes, 2004). Cyanobacteria are known to colonize bryophytes in a mutualistic manner in drier micro habitats (Rai *et al*., 2003). These bryophytes are detected at high abundance, and are acknowledged as the primary colonizers (Breen and Levesque, 2006) and vegetative structures in barren landscapes such as the Dry Valleys.

Cyanobacteria are known to have a symbiotic (endo-symbiotic or mutualistic) relationship with fungi (Redecker and Raab, 2006). The occurrence of relatively high levels of fungal activity is likely to be the main source of decomposition in the Type I hypolithon, enhancing the nutrients available to other colonizing organisms. When analysing some of the colonizing species, they appear to have unique and useful abilities. *A. rutilum* and *S. caraganae* are fungi that create a stable support matrix

and additionally provide protection to cells in the hypolithon against dehydration and radiation (Glenn *et al*., 1996). Amoebozoa and Nematoda that are present at low levels in the Type I community, form a tremendously important component as grazers of diatoms, green algae and unicellular and filamentous cyanobacteria (Finlay and Esteban, 1998). This exerts population control in the ecosystem and maintains the structure of the community (Finlay and Esteban, 1998).

The Type II hypolithon displayed a remarkable abundance of the Glomeromycota in addition to the Ascomycota and Basidiomycota (Figure 3.22). Fungi are known to naturally associate with moss species (Stevens *et al*., 2007), as evidenced by the high percentage of fungal signals observed. Fungi and algae (Chlorophyta) also exist as symbionts of bryophyte species (Hogg *et al*., 2006). It is possible, however, that the moss species detected in the microalgal 18S rRNA gene region study are the dominant photosynthesizers in the Type II hypolithon. They may provide additional physical stability to the hypolithon, preventing soil erosion as a result of the katabatic winds. However, both mosses and microalgae could potentially provide the food resources necessary for heterotrophic colonization as well as a nutrient-rich habitat for future algal colonization.

Lichen dominated hypoliths (Type III communities) are known to contain algal and fungal communities which are frequently described as mutualistic ecosystems (Friedmann and Sun, 2005). Yeast and filamentous fungi types are therefore

expected in this hypolithon. Bryophytes show high abundance in Type III communities. The observed species may have interacted with the microalgal community of the hypolithons, and could have surpassed their growth due to their mutually beneficial relationships with lichens. Population control in the Type III hypolithon could be achieved by the tertiary level Cercozoa (*Cercomonas* species). They feed upon bacteria on the surface of sediments using pseudopodia (Finlay and Esteban, 1996). The Apicomplexans observed in this study are parasitic protists and occur at a low abundance. However, the low percentage (91%) of sequence similarity to current *Eimeriidae* could imply that this species is an ancestor to present day parasitic Apicomplexans (Stoek *et al*., 2007).

The data therefore shows that each hypolithon is represented by a very unique composition of organisms. These contribute to the survival and efficient functioning of hypolithons in the cold, arid conditions of the Miers Valley. While some phyla are common to all hypolithons (Ascomycota, Bryophyta, Chlorophyta), others are hypolithon specific (Amoebozoa and Nematoda in Type I communities and Apicomplexans and Cercozoa in Type III communities).

Antarctic Dry Valley ecosystems are typically simplistic as a result of the extreme environmental and physiological factors that persist (Cary *et al*., 2010). With the refuge environments, however, some of these external factors do not have a great effect on the survival of communities. In this study we confirm the presence of three

trophic levels in the hypolithon environment. A brief explanation of the role of the organisms distinguished in this study, as well as a hypothesis on the interaction and relationship between trophic levels can be formulated for the first time.

4.4 Potential Role of Microorganisms in Hypolith Communities

Microbial populations have an extremely unique composition in hyper-arid deserts. The roles that organisms play in the environment are of great importance, since microbiota are pivotal in biogeochemical cycling and ecological dynamics (Barrett *et al*., 2006). As such, it is important to distinguish the microorganisms that survive the harsh conditions of extreme environments. Additionally, these organisms may have useful information and implications towards the study of adaptive mechanisms, climate change, anthropogenic effects and even astrobiology (Barrett *et al*., 2006; Hogg *et al*., 2006).

Hyper-arid desert regions such as the Dry Valleys, display a trophic dependency of less than three trophic levels (Cary *et al*., 2010). These levels constitute the most important life-forms in this refuge environment. Soil food webs (Figure 4.2) are predominantly inhabited by cyanobacteria, algae, fungi, moss, yeasts, bacteria, protozoa and metazoa (Bamforth *et al*., 2005; Freckman and Virginia, 1997; Friedmann *et al*., 1993; Schwarz *et al*., 1993). Most soils, however, are capable of providing a rich source of predators to maintain the balance between pioneer species, vertebrates and invertebrates despite the low species diversity.

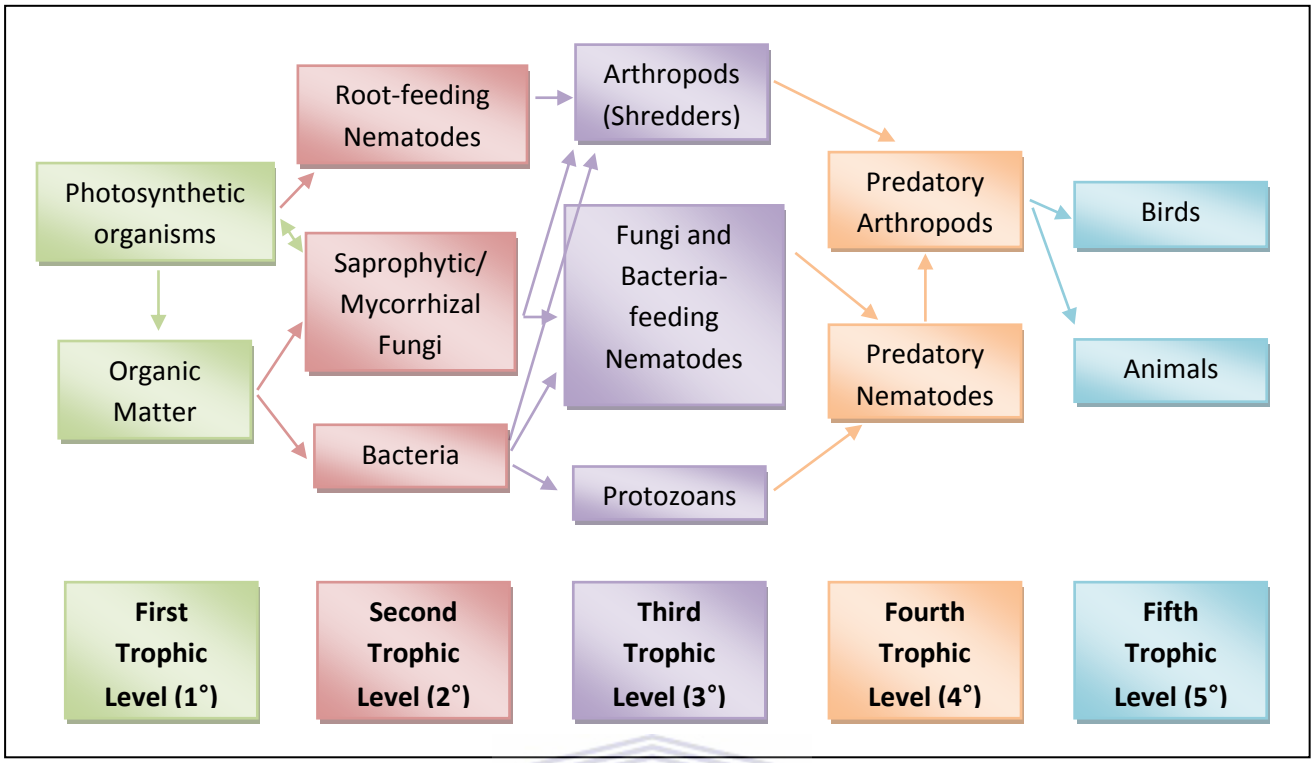

Figure 4.2 Representation of the general soil food web (adapted from Cary *et al.*, 2010). Antarctic systems have been shown to predominantly support the first three trophic levels, implying a rather simplistic food web in hyper-arid ecosystems, compared to other soils.

Miers Valley hypolithons display high bacterial diversity and richness (Khan *et al.*, 2011). These prokaryotes may perform essential functions in nutrient cycling and make provisions for the eukaryotic trophic levels, as well as benefit from the interactions with them (Lacap *et al.*, 2011; Cary *et al.*, 2010) (Table 4.1). It appears that a large primary trophic level consisting of photosynthetic eukaryotes forms the base of the hypolithon nutrient structure (Figure 4.3). These photobionts are comprised of bryophytes, such as the dessication tolerant *Tortula ruralis*, lycopodiophytes (the oldest extant vascular plant) and streptophytes.

Table 4.1 Role of eukaryotic microorganisms in soil communities.

Phyla/Division/Class	Function in Soil Communities	Trophic level
Amoeboid protozoa	Grazers that feed on bacteria, protozoa, organic matter, fungi	3°
Apicomplexa	Intracellular vertebrate parasites	3°
Arthropoda	Shredders, predators, herbivore or fungal-feeders	3° or 4°
Ascomycota	Decomposers that support detritivores	2°
Bryophytes	Water and nutrient cycling, insulating permafrost	1°
Cercozoa	Grazers	3°
Chlorophyta	Enhances soil fertility and organic matter therefore organic carbon, prevent soil erosion, oxygen producers (pioneer organisms)	1°
Lycopodiophyta	Vascular plant	1°
Nematoda	Plant feeders, grazers, predators	3° or 4°
Streptophyta	Vascular plant	1°

In Antarctic regions, and hypolithons in particular, microalgae are one of the main photosynthetic entities (Broady, 1981). They require the low light levels generated by the overlying translucent rock for their growth (Broady, 1981). Additionally, microalgae are the main oxygen producers in sublithic and chasmolithic niches (Broady, 1981). The diverse range of microalgal genera detected in this study indicates that they may perform the same function in this refuge habitat. In addition to the chlorophyll producing cyanobacteria, microalgae support nutrient and water cycling in this isolated environment. They therefore provide the necessary organic matter, carbon and nitrogen (through fixation by cyano-lichen species and mineralization by protozoans) in the hypolithon (Rai *et al*., 2003).

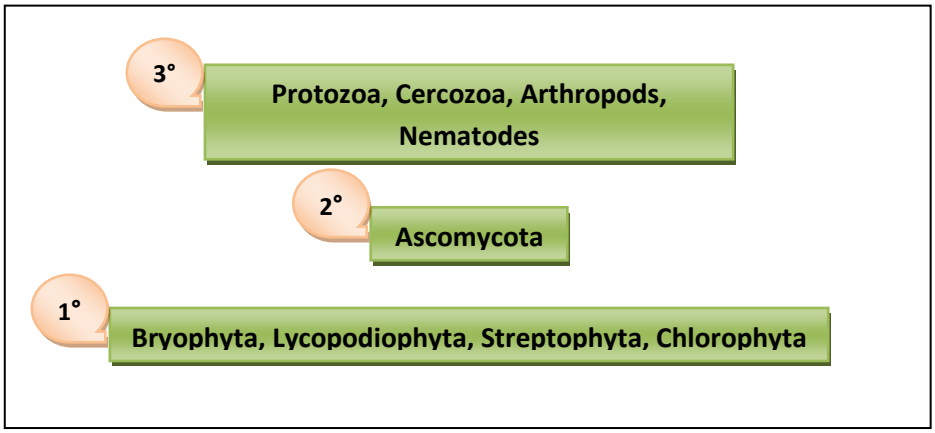

Figure 4.3 Diagrammatic representation of the potential eukaryotic food web observed in Miers Valley hypolithons.

The small fungal component at the secondary trophic level consists mainly of heterotrophic Ascomycota. These fungi may be involved in the decomposition of organic matter, as suggested by Baublis *et al*. (1991). Some yeast Ascomycota also produce exopolysaccharide capsules, promoting dessication tolerance and soil stabilization of the hypolith (Onofri *et al*., 2004b). Fungal growth requires organic compounds, perhaps sourced from cellulose and photosynthesis for energy (Adams *et al*., 2006). This would explain their close association with mosses, algae and cyanobacteria (Friedmann and Sun, 2004).

The lower and higher invertebrates form the third trophic level, which is supported by modestly diverse fungal communities. Typically, nematodes function as grazers, like the Protozoa and Cercozoa (Wynn-Williams, 1996). These organisms may be responsible for bio-control and as such, add to the maintenance of the geochemical balance of hypolithons (Leander *et al*., 2003).

Open soils and hypoliths display clear distinction in their microbial ecology (Khan *et al*., 2011). However, there is evidence of temporal and spatial changes in hypolith communities due to atmospheric, edaphic and wind dispersal conditions (Cowan *et al*., 2010b; Pointing *et al*., 2009; Noy Meir, 1973). Light, moisture, carbon, nitrogen and salinity have the most important ecological role in hypolithons. Physico-chemical analysis performed by Khan (2008), however, showed that the constantly fluctuating relative humidity and temperatures are not the main limiting factor for hypolith microbial colonization (Khan, 2008). Altitude has a distinct role in hypolith development. Valleys display richer microbial growth of Type II and III hypolithons when compared to samples observed on slopes as a result of the decrease in moisture with altitude (Cowan *et al*., 2010b).

These conditions may all affect open soil and hypolith communities by influencing and promoting physiological adaptations for cold desert and hypolith survival (Hogg *et al*., 2006). Antarctic ecosystems and Dry Valley hypolithon development and diversity are therefore strongly influenced by abiotic factors (Cowan *et al*., 2010b; Hogg *et al*., 2006).

4.5 Conclusion

The diversity of hot and cold desert environments is investigated to determine the microorganisms that can adapt and survive under radical and harsh environmental constraints (Hopkins *et al*., 2010; Potts, 1994). It is evident from the use of molecular

methods that Dry Valley mineral soils display low abundance yet high diversity and heterogeneity of a number of species (Cowan *et al*., 2010c). The significant eukaryote and microalgal diversity and species richness in the three hypolithons infer that these communities are undoubtedly unique. The low level of assigned diversity in the fungal constituent of the hypolithons, however, displays the lack of knowledge of species richness and ecosystem functioning in this phylum (Adams *et al*., 2006).

Initiatives to characterize the unknown species have been undertaken (Adams *et al*., 2006; Cowan and Ah Tow, 2004). However, due to unculturability and the vast amount of data generated by molecular techniques, a large percentage of the microorganisms may remain unassigned. This limits complete characterization of the microbial diversity of under-represented environments such as that of Antarctic hypolithons.

Based on what is currently known of the hypolith ecosystem, a trophic structure can be theorized by the analysis of organisms detected in cyanobacterial, moss and fungal dominated hypolith communities. Each hypolithon could support soil biota by accumulation and generation of water, residue and metabolites from the decomposition of photosynthesizers (Barrett *et al*., 2006; Davey and Rothery, 1993; Schwarz *et al*., 1993). Organic matter (carbon in particular) has been proposed to accumulate in Antarctic soils from two mechanisms, aeolian transport and intrinsic

soil carbon. The former theory is as a result of lacustrine cyanobacteria that generate the carbon and nitrogen necessary to support higher microbial life (Hopkins *et al*., 2010). The latter refers to the ancient organic carbon that has survived in the cold-preserved Antarctic soils (Moorhead *et al*., 1999). When one considers these two theories of organic matter deposition in Antarctic hypolith systems, organic nutrient availability may result from a combination of these processes.

Those species that are capable of surviving may represent keystone pioneer species essential for biogeochemical cycling in refuge environments. Their roles in stability, maintenance of moisture and the release, immobilization and storage of nutrients, are essential for growth in this hyper-arid desert niche (Breen and Levesque, 2006).

This study illustrates that each hypolithon has substantial differences in community structure. The cyanobacterial, moss and lichen dominated hypolithons are characterized by a diverse range of cosmopolitan and novel eukaryotic, fungal and microalgal communities. However, moss seems to comprise the largest proportion of eukaryote diversity in all hypolith communities. This implies that the morphological description and characterization of these hypoliths may not necessarily represent the dominant extant community members. Additionally, specific interactions between hypolithon microbial species exist. Mutualistic symbioses by colonizing microbiota in combination with different abiotic factors, drives Dry Valley hypolith microbial life. A concerted effort combining biochemical, physiological, molecular

and traditional techniques will be able to further describe microbial functioning in hypolith ecosystems.

The data generated in this study can provide a foundation for further research of these established eukaryote refuge communities. Inferences from the biological-environmental interactions of Antarctic Dry Valley communities can be used to understand the impact of climate change, global warming, as well as anthropogenic effects. These factors will be of utmost importance in the retention of the pristine condition of this polar region. It could additionally serve as a useful reference for the interpretation and comparison of results in other cold systems. As a widely recognized Mars analogue site, insight into Antarctic cryptic microbial life could supplement current knowledge of survival and growth of life in Martian conditions (West *et al*., 2010; Brown *et al*., 2005; Onofri, 2004). The ancient frozen Antarctic environment may therefore be able to provide an indication of early earth and extra-terrestrial life where extremophiles may have dominated the lithic environment.

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APPENDIX A

Appendix A Taxonomy of a representative number of eukaryotic microorganisms observed in the T-RF phylogenetic assignment for the 18S, ITS and microalgal 18S rRNA gene regions.

Phyla/Division	Taxonomy
Alveolata	*Cryptoperidiniopsis brodyi* - Eukaryota; **Alveolata**; Dinophyceae; Peridiniales; Pfiesteriaceae; Cryptoperidiniopsis
	Lacrymaria marina - Eukaryota; **Alveolata**; Ciliophora; Intramacronucleata; Litostomatea; Haptoria; Haptorida; Lacrymariidae; Lacrymaria
	Mesodinium pulex - Eukaryota; **Alveolata**; Ciliophora; Intramacronucleata; Litostomatea; Haptoria; Cyclotrichida; Mesodiniidae; Mesodinium
	Phialina salinarum - Eukaryota; **Alveolata**; Ciliophora; Intramacronucleata; Litostomatea; Haptoria; Haptorida; Lacrymariidae; Phialina
Amoebozoa	*Acanthamoeba* sp. KA/E2 - Eukaryota; **Amoebozoa**; Centramoebida; Acanthamoebidae; Acanthamoeba; unclassified Acanthamoeba
	Protophysarum phloiogenum - Eukaryota; **Amoebozoa**; Mycetozoa; Myxogastria; Myxogastromycetidae; Physariida; Protophysarum
Apicomplexa	*Cryptosporidium baileyi* - Eukaryota; Alveolata; **Apicomplexa**; Coccidia; Eucoccidiorida; Eimeriorina; Cryptosporidiidae; Cryptosporidium
	Cryptosporidium canis - Eukaryota; Alveolata; **Apicomplexa**; Coccidia; Eucoccidiorida; Eimeriorina; Cryptosporidiidae; Cryptosporidium
	Eimeria nieschulzi - Eukaryota; Alveolata; **Apicomplexa**; Coccidia; Eucoccidiorida; Eimeriorina; Eimeriidae; Eimeria
	Sarcocystis muris - Eukaryota; Alveolata; **Apicomplexa**; Coccidia; Eucoccidiorida; Eimeriorina; Sarcocystidae; Sarcocystis
	Syncystis mirabilis - Eukaryota; Alveolata; **Apicomplexa**; Gregarinia; Neogregarinorida; Syncystidae; Syncystis
	Theileria sp. China 1 - Eukaryota; Alveolata; **Apicomplexa**; Aconoidasida; Piroplasmida; Theileriidae
	Uncultured Colpodellidae - Eukaryota; Alveolata; **Apicomplexa**; Colpodellidae; environmental samples
Apusozoa	*Amastigomonas mutabilis* - Eukaryota; **Apusozoa**; Apusomonadidae; Amastigomonas
	Apusomonas proboscidea - Eukaryota; **Apusozoa**; Apusomonadidae; Apusomonas
	Uncultured Apusozoa - Eukaryota; **Apusozoa**; environmental samples
Arthropoda	*Betamorpha africana* - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Coelomata; Protostomia; Panarthropoda; **Arthropoda**; Mandibulata; Pancrustacea; Crustacea; Malacostraca; Eumalacostraca; Peracarida; Isopoda; Asellota; Janiroidea; Munnopsidae; Betamorpha
	Munnopsis typica - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Coelomata; Protostomia; Panarthropoda; **Arthropoda**; Mandibulata; Pancrustacea; Crustacea; Malacostraca; Eumalacostraca; Peracarida; Isopoda; Asellota; Janiroidea; Munnopsidae; Munnopsis
	Paramunnopsis sp. 3 D17 - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Coelomata; Protostomia; Panarthropoda; **Arthropoda**; Mandibulata; Pancrustacea; Crustacea; Malacostraca; Eumalacostraca; Peracarida; Isopoda; Asellota; Janiroidea; Munnopsidae; Paramunnopsis
	Zonophryxus quinquedens - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Coelomata; Protostomia; Panarthropoda; **Arthropoda**; Mandibulata; Pancrustacea; Crustacea; Malacostraca; Eumalacostraca; Peracarida; Isopoda; Cymothoida; Dajidae; Zonophryxus

Appendix A Continued.

Phyla/Division	Taxonomy
Ascomycota	*Aureobasidium pullulans* - Eukaryota; Opisthokonta; Fungi; Dikarya; **Ascomycota**; saccharomyceta; Pezizomycotina; leotiomyceta; dothideomyceta; Dothideomycetes; Dothideomycetidae; Dothideales; Dothioraceae; mitosporic Dothioraceae; Aureobasidium
	Candida parapsilosis - Eukaryota; Opisthokonta; Fungi; Dikarya; **Ascomycota**; saccharomyceta; Saccharomycotina; Saccharomycetes; Saccharomycetales; mitosporic Saccharomycetales; Candida
	Eurotium amstelodami - Eukaryota; Opisthokonta; Fungi; Dikarya; **Ascomycota**; saccharomyceta; Pezizomycotina; leotiomyceta; Eurotiomycetes; Eurotiomycetidae; Eurotiales; Trichocomaceae; Eurotium
	Penicillium purpurogenum - Eukaryota; Opisthokonta; Fungi; Dikarya; **Ascomycota**; saccharomyceta; Pezizomycotina; leotiomyceta; Eurotiomycetes; Eurotiomycetidae; Eurotiales; Trichocomaceae; mitosporic Trichocomaceae; Penicillium
	Phoma sp. RMF1 - Eukaryota; Opisthokonta; Fungi; Dikarya; **Ascomycota**; saccharomyceta; Pezizomycotina; leotiomyceta; dothideomyceta; Dothideomycetes; Pleosporomycetidae; Pleosporales; Pleosporineae; Didymellaceae; mitosporic Didymellaceae; Phoma
	Pleosporales sp. RMF2 - Eukaryota; Opisthokonta; Fungi; Dikarya; **Ascomycota**; saccharomyceta; Pezizomycotina; leotiomyceta; dothideomyceta; Dothideomycetes; Pleosporomycetidae; Pleosporales; unclassified Pleosporales
	Trichoderma viride (*Hypocrea rufa*) - Eukaryota; Opisthokonta; Fungi; Dikarya; **Ascomycota**; saccharomyceta; Pezizomycotina; leotiomyceta; sordariomyceta; Sordariomycetes; Hypocreomycetidae; Hypocreales; Hypocreaceae; Hypocrea
Bacillariophyta	*Asterionellopsis glacialis* - Eukaryota; stramenopiles; **Bacillariophyta**; Fragilariophyceae; Fragilariophycidae; Fragilariales; Fragilariaceae; Asterionellopsis
	Asteroplanus karianus - Eukaryota; stramenopiles; **Bacillariophyta**; Fragilariophyceae; Fragilariophycidae; Fragilariales; Fragilariaceae; Asteroplanus
	Guinardia delicatula - Eukaryota; stramenopiles; **Bacillariophyta**; Coscinodiscophyceae; Rhizosoleniophycidae; Rhizosoleniales; Rhizosoleniaceae; Guinardia
	Rhaphoneis belgicae - Eukaryota; stramenopiles; **Bacillariophyta**; Fragilariophyceae; Fragilariophycidae; Rhaponeidales; Rhaphoneidaceae; Rhaphoneis
Basidiomycota	*Leucoagaricus gongylophorus* - Eukaryota; Opisthokonta; Fungi; Dikarya; **Basidiomycota**; Agaricomycotina; Agaricomycetes; Agaricomycetidae; Agaricales; Agaricaceae; Leucoagaricus
Bolidophyceae	*Bolidomonas pacifica* - Eukaryota; stramenopiles; **Bolidophyceae**; Bolidomonas
Centroheliozoa	Uncultured Centroheliozoan - Eukaryota; **Centroheliozoa**; environmental samples
Chlorophyta	*'Chlorella' luteoviridis* - Eukaryota; Viridiplantae; **Chlorophyta**; Trebouxiophyceae; Trebouxiophyceae incertae sedis; Chlorella
	Chlorella trebouxioides - Eukaryota; Viridiplantae; **Chlorophyta**; Trebouxiophyceae; Chlorellales; Chlorellaceae; Chlorella
	Prototheca zopfii - Eukaryota; Viridiplantae; **Chlorophyta**; Trebouxiophyceae; Chlorellales; Chlorellaceae; Prototheca
Choanoflagellida	*Acanthocorbis unguiculata* - Eukaryota; Opisthokonta; **Choanoflagellida**; Acanthoecidae; Acanthocorbis
	Diaphanoeca grandis - Eukaryota; Opisthokonta; **Choanoflagellida**; Acanthoecidae; Diaphanoeca
	Salpingoeca napiformis - Eukaryota; Opisthokonta; **Choanoflagellida**; Salpingoecidae

Appendix A Continued.

Phyla/Division	Taxonomy
Chordata	*Ciona savignyi* - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Coelomata; Deuterostomia; **Chordata**; Tunicata; Ascidiacea; Enterogona; Phlebobranchia; Cionidae; Ciona
	Kryptopterus bicirrhis - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Coelomata; Deuterostomia; **Chordata**; Craniata; Vertebrata; Gnathostomata; Teleostomi; Euteleostomi; Actinopterygii; Actinopteri; Neopterygii; Teleostei; Elopocephala; Clupeocephala; Otocephala; Ostariophysi; Otophysi; Siluriphysi; Siluriformes; Siluridae; Kryptopterus
	Latimeria chalumnae - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Coelomata; Deuterostomia; **Chordata**; Craniata; Vertebrata; Gnathostomata; Teleostomi; Euteleostomi; Sarcopterygii; Coelacanthimorpha; Coelacanthiformes; Coelacanthidae; Latimeria
Chrysophyceae	Chrysophyta sp. JZH-2007-002 - Eukaryota; stramenopiles; **Chrysophyceae**; unclassified Chrysophyceae
	Spumella sp. GOT220 - Eukaryota; stramenopiles; **Chrysophyceae**; Chromulinales; Chromulinaceae; Spumella; unclassified Spumella
Chytridiomycota	*Hyaloraphidium curvatum* - Eukaryota; Opisthokonta; Fungi; **Chytridiomycota**; Monoblepharidomycetes; Monoblepharidales; Monoblepharidales incertae sedis; Hyaloraphidium
	Rhizidium endosporangiatum - Eukaryota; Opisthokonta; Fungi; **Chytridiomycota**; Chytridiomycetes; Chytridiales; Chytridiaceae; Rhizidium
Cnidaria	*Craspedacusta sowerbyi* - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; **Cnidaria**; Hydrozoa; Hydroida; Limnomedusae; Olindiidae; Craspedacusta
	Haliscera conica - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; **Cnidaria**; Hydrozoa; Trachylina; Trachymedusae; Halicreatidae; Haliscera
	Hydra circumcincta - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; **Cnidaria**; Hydrozoa; Hydroida; Anthomedusae; Hydridae; Hydra
	Microhydrula limopsicola - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; **Cnidaria**; Hydrozoa; Hydroida; Limnomedusae; Microhydrulidae; Microhydrula
Cryptophyta	*Pyrenomonas salina* - Eukaryota; **Cryptophyta**; Pyrenomonadales; Pyrenomonadaceae; Pyrenomonas
	Uncultured freshwater cryptophyte - Eukaryota; **Cryptophyta**; environmental samples
Ctenophora	*Charistephane fugiens* - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; **Ctenophora**; Typhlocoela; Cydippida; Mertensiidae; Charistephane
	Hormiphora sp. - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; **Ctenophora**; Typhlocoela; Cydippida; Pleurobrachiidae; Hormiphora
Echinodermata	*Amphipholis squamata* - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Coelomata; Deuterostomia; **Echinodermata**; Eleutherozoa; Asterozoa; Ophiuroidea; Ophiuridea; Ophiurida; Ophiurina; Gnathophiurina; Gnathophiuridea; Amphiuridae; Amphipholis
	Paracentrotus lividis - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Coelomata; Deuterostomia; **Echinodermata**; Eleutherozoa; Echinozoa; Echinoidea; Euechinoidea; Echinacea; Echinoida; Echinidae; Paracentrotus
Euglenozoa	*Bodo rostratus* - Eukaryota; **Euglenozoa**; Kinetoplastida; Bodonidae; Bodo
	Perkinsiella-like sp. AFSM3 - Eukaryota; **Euglenozoa**; Kinetoplastida; Bodonidae; unclassified Bodonidae
Eustigmatophyceae	*Eustigmatos magnus* - Eukaryota; stramenopiles; **Eustigmatophyceae**; Eustigmatales; Eustigmataceae; Eustigmatos
	Vischeria helvetica - Eukaryota; stramenopiles; **Eustigmatophyceae**; Eustigmatales; Eustigmataceae; Vischeria
Gastrotricha	*Chaetonotus neptuni* - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Pseudocoelomata; **Gastrotricha**; Chaetonotida; Paucitubulatina; Chaetonotidae; Chaetonotus

Appendix A Continued.

Phyla/Division	Taxonomy
Glomeromycota	*Glomus geosporum* - Eukaryota; Opisthokonta; Fungi; **Glomeromycota**; Glomeromycetes; Glomerales; Glomeraceae; Glomus
Haptophyceae	*Chrysochromulina* cf. herdlensis - Eukaryota; **Haptophyceae**; Prymnesiales; Prymnesiaceae; Chrysochromulina
Jakobida	*Reclinomonas americana* - Eukaryota; **Jakobida**; Histionidae; Reclinomonas
Katablepharidophyta	*Roombia truncata* - Eukaryota; **Katablepharidophyta**; Roombia
Kinorhyncha	*Pycnophyes kielensis* - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Pseudocoelomata; **Kinorhyncha**; Homalorhagida; Pycnophyidae; Pycnophyes
Mollusca	*Ctenoides annulata* - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Coelomata; Protostomia; **Mollusca**; Bivalvia; Pteriomorphia; Limoida; Limidae; Ctenoides
Nematoda	*Laxus oneistus* - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Pseudocoelomata; **Nematoda**; Chromadorea; Desmodorida; Desmodoridae; Stilbonematinae; Laxus
	Strongyloides stercoralis - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Pseudocoelomata; **Nematoda**; Chromadorea; Rhabditida; Panagrolaimoidea; Strongyloididae; Strongyloides
Neocallimastigomycota	*Orpinomyces* sp. OUS1 - Eukaryota; Opisthokonta; Fungi; **Neocallimastigomycota**; Neocallimastigomycetes; Neocallimastigales; Neocallimastigaceae; Orpinomyces
Placozoa	*Trichoplax adhaerens* - Eukaryota; Opisthokonta; Metazoa; **Placozoa**; Trichoplax
Platyhelminthes	*Bathycestus brayi* - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Acoelomata; **Platyhelminthes**; Cestoda; Eucestoda; Pseudophyllidea; Triaenophoridae; Bathycestus
	Marsipometra hastata - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Acoelomata; **Platyhelminthes**; Cestoda; Eucestoda; Pseudophyllidea; Triaenophoridae; Marsipometra
	Parabothriocephalus segmentatus - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Acoelomata; **Platyhelminthes**; Cestoda; Eucestoda; Pseudophyllidea; Parabothriocephalidae; Parabothriocephalus
	Philobythoides stunkardi - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Acoelomata; **Platyhelminthes**; Cestoda; Eucestoda; Pseudophyllidea; Triaenophoridae; Philobythoides
	Ptychobothrium belones - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Acoelomata; **Platyhelminthes**; Cestoda; Eucestoda; Pseudophyllidea; unclassified Pseudophyllidea; Ptychobothrium
Porifera	*Aplysina aerophoba* - Eukaryota; Opisthokonta; Metazoa; **Porifera**; Demospongiae; Ceractinomorpha; Verongida; Aplysinidae; Aplysina
	Smenospongia aurea - Eukaryota; Opisthokonta; Metazoa; **Porifera**; Demospongiae; unclassified Demospongiae; Smenospongia
Rhizaria	*Dorataspis* sp. 813 - Eukaryota; **Rhizaria**; Acantharea; Arthracanthida; Sphaenacanthida; Dorataspididae; Dorataspis
	Nuclearia-like filose amoeba N-Por - Eukaryota; **Rhizaria**; Cercozoa; unclassified Cercozoa
	Platyreta germanica - Eukaryota; **Rhizaria**; Cercozoa; Vampyrellidae; Platyreta
	Uncultured taxopodid-like - Eukaryota; **Rhizaria**; Sticholonchida; environmental samples

Appendix A Continued.

Phyla/Division	Taxonomy
Rhodophyta	*Dasya villosa* - Eukaryota; **Rhodophyta**; Florideophyceae; Ceramiales; Dasyaceae; Dasya
	Hypoglossum hypoglossoides - Eukaryota; **Rhodophyta**; Florideophyceae; Ceramiales; Delesseriaceae; Hypoglossum
Stramenopiles	*Cafeteria* sp. GOT180 - Eukaryota; **stramenopiles**; Bicosoecida; Cafeteriaceae; Cafeteria
	Rictus lutensis - Eukaryota; **stramenopiles**; Bicosoecida; unclassified Bicosoecida; Rictus
	Uncultured bicosoecid - Eukaryota; **stramenopiles**; Bicosoecida; environmental samples
	Uncultured ochromonad - Eukaryota; **stramenopiles**; Synurophyceae; Ochromonadales; environmental samples
Streptophyta	*Coleochaete scutata* - Eukaryota; Viridiplantae; **Streptophyta**; Streptophytina; Coleochaetophyceae; Coleochaetales; Coleochaetaceae; Coleochaete
	Cyathophorum bulbosum - Eukaryota; Viridiplantae; **Streptophyta**; Streptophytina; Embryophyta; Bryophyta; Bryophytina; Bryopsida; Bryidae; Hypnanae; Hookeriales; Hypopterygiaceae; Cyathophorum
	Leptobryum pyriforme - Eukaryota; Viridiplantae; **Streptophyta**; Streptophytina; Embryophyta; Bryophyta; Bryophytina; Bryopsida; Bryidae; Bryanae; Splachnales; Meesiaceae; Leptobryum
	Pisum sativum - Eukaryota; Viridiplantae; **Streptophyta**; Streptophytina; Embryophyta; Tracheophyta; Euphyllophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; rosids; fabids; Fabales; Fabaceae; Papilionoideae; Fabeae; Pisum
Tardigrada	*Macrobiotus hufelandi* - Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Coelomata; Protostomia; Panarthropoda; **Tardigrada**; Eutardigrada; Parachela; Macrobiotidae; Macrobiotus; Macrobiotus hufelandi group
Telonemida	*Telonema subtile* - Eukaryota; unclassified eukaryotes; **Telonemida**; Telonema
Unclassified Eukaryote	*Macropharyngomonas halophila* - Eukaryota; Heterolobosea; **unclassified Heterolobosea**; Macropharyngomonas

APPENDIX B

Appendix B1 Universal 18S rRNA gene sequences from the clone libraries. The percentage of similarity to the closest homologues in GenBank and the taxonomic affiliations are shown.

Clone	Community Type	Accession Number	Closest BLASTn Match	% Identity	Phyla/Division
EUK75-A1		EF032798.1	Uncultured eukaryote clone HAVOmat-euk08 18S rRNA	81	Uncultured eukaryote
EUK75-A5		HQ188966.1	Uncultured Viridiplantae clone E108_09D 18S rRNA	82	Uncultured eukaryote
EUK75-A7		AF023682.1	*Tortula ruralis* 18S ribosomal RNA	73	Bryophyta
EUK75-A8		AF023682.1	*Tortula ruralis* 18S ribosomal RNA	82	Bryophyta
EUK75-A12		AF023682.1	*Tortula ruralis* 18S ribosomal RNA	86	Bryophyta
EUK75-B2		AF023682.1	*Tortula ruralis* 18S ribosomal RNA	89	Bryophyta
EUK75-B4		DQ629397.1	*Rhizogonium paramattense* 18S rRNA	68	Bryophyta
EUK75-B9	I	AB257 667.1	Uncultured endolithic amoeba gene for 18S rRNA	98	Amoebozoa
EUK75-C4		DQ229954.1	*Platyamoeba contorta* isolate W51C#5 rRNA	98	Amoebozoa
EUK75-C6		FJ490037.1	Uncultured *Trebouxia* photobiont isolate DGGE gel band Group 4 rRNA	87	Chlorophyta
EUK75-C8		AF023682.1	*Tortula ruralis* 18S ribosomal RNA	93	Bryophyta
EUK75-D4		AF023707.1	*Cyrtomnium hymenophyllum* 18S rRNA	73	Bryophyta
EUK75-D10		DQ629380.1	*Eurhynchium hians* 18S rRNA	71	Bryophyta
EUK75-D12		GU072201.1	Uncultured eukaryote clone GPS1B3 18S rRNA	79	Uncultured eukaryote
EUK134-A1		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	100	Uncultured eukaryote
EUK134-B6		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
EUK134-B9		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
EUK134-B10	II	X95935.1	*P. truncata* 18S rRNA	99	Bryophyta
EUK134-C6		X95935.1	*P. truncata* 18S rRNA	99	Bryophyta
EUK134-D3		X95935.1	*P. truncata* 18S rRNA	99	Bryophyta
EUK134-D11		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	100	Uncultured eukaryote

Appendix B1 Continued.

Clone	Community Type	Accession Number	Closest BLASTn Match	% Identity	Phyla/Division
EUK134-E1	II	HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
EUK134-E8		X95935.1	*P. truncata* 18S rRNA gene	99	Bryophyta
EUK134-E12		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	100	Uncultured eukaryote
EUK134-H4		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	100	Uncultured eukaryote
EUK134-H6		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
EUK50-A4	III	X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
EUK50-A6		AJ239056.1	*Tortella tortuosa* 18S rRNA	99	Bryophyta
EUK50-B10		EF024417.1	*Eimeriidae* environmental sample clone Elev_18S_1076 18S rRNA	91	Apicomplexa
EUK50-C4		AJ239056.1	*Tortella tortuosa* 18S rRNA	99	Bryophyta
EUK50-D3		AJ239056.1	*Tortella tortuosa* 18S rRNA	99	Bryophyta
EUK50-D7		AJ239056.1	*Tortella tortuosa* 18S rRNA	99	Bryophyta
EUK50-D10		AF411268.1	*Cercomonas plasmodialis* 18S rRNA	99	Cercozoa
EUK50-E3		X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
EUK50-E4		X80985.1	*M. hornum* 18S rRNA	96	Bryophyta
EUK50-F3		AJ239056.1	*Tortella tortuosa* 18S rRNA	99	Bryophyta
EUK50-G5		AF023682.1	*Tortula ruralis* 18S ribosomal RNA	96	Bryophyta

Appendix B2 Fungal ITS rRNA gene sequences from the clone libraries. The percentage of similarity to the closest homologues in GenBank and the taxonomic affiliations are shown.

Clone	Community Type	Accession Number	Closest BLASTn Match	% Identity	Phyla/Division
ITS65-A1		AB540580.1	*Acremonium rutilum* strain: JCM 23088	82	Fungi, Ascomycota
ITS65-A2		AB540580.1	*Acremonium rutilum* strain: JCM 23088	84	Fungi, Ascomycota
ITS65-A5		AB540580.1	*Acremonium rutilum* strain: JCM 23088	85	Fungi, Ascomycota
ITS65-A7		HQ112288.1	*Stromatonectria caraganae* strain CBS 125579	76	Fungi, Ascomycota
ITS65-A8		AB540580.1	*Acremonium rutilum* strain: JCM 23088	78	Fungi, Ascomycota
ITS65-A9		AB540580.1	*Acremonium rutilum* strain: JCM 23088	74	Fungi, Ascomycota
ITS65-A12		HQ112288.1	*Stromatonectria caraganae* strain CBS 125579	78	Fungi, Ascomycota
ITS65-B2		AB540580.1	*Acremonium rutilum* strain: JCM 23088	93	Fungi, Ascomycota
ITS65-B4		AB540580.1	*Acremonium rutilum* strain: JCM 23088	73	Fungi, Ascomycota
ITS65-B6		AB540580.1	*Acremonium rutilum* strain: JCM 23088	88	Fungi, Ascomycota
ITS65-B12		HQ022967.1	*Colletotrichum* sp. FLS-2010 isolate FS-2.1(1)	80	Fungi, Ascomycota
ITS65-C2		JF951153.1	*Utrechtiana cibiessia* culture-collection CPC:18916	81	Fungi, Ascomycota
ITS65-C5	I	HQ112288.1	*Stromatonectria caraganae* strain CBS	73	Fungi, Ascomycota
ITS65-C12		AB540580.1	*Acremonium rutilum* strain: JCM 23088	87	Fungi, Ascomycota
ITS65-D2		AB540580.1	*Acremonium rutilum* strain: JCM 23088	80	Fungi, Ascomycota
ITS65-D9		AB540580.1	*Acremonium rutilum* strain: JCM 23088	89	Fungi, Ascomycota
ITS65-D10		HQ112288.1	*Stromatonectria caraganae* strain CBS 125579	76	Fungi, Ascomycota
ITS65-E7		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	81	Fungi, Ascomycota
ITS65-F1		FJ664884.1	*Verrucaria* sp. AO-2009b voucher A. Orange 17241 (NMW C.2007.001.102)	82	Fungi, Ascomycota
ITS65-F7		EU559734.1	*Polyblastia lutosa* isolate SS126	71	Fungi, Ascomycota
ITS65-F9		GU973834.1	*Bionectria* sp. ASR 301	74	Fungi, Ascomycota
ITS65-G6		FM200605.1	Fungal endophyte sp. AP358, isolate AP358	79	Eukaryota, Fungi
ITS65-G10		EU558521.1	*Arabidopsis lyrata* clone Gypsy20 transposon insertion display band	86	Viridiplantae, Streptophyta

Appendix B2 Continued.

Clone	Community Type	Accession Number	Closest BLASTn Match	% Identity	Phyla/Division
ITS134-A8		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	80	Fungi, Ascomycota
ITS134-A2		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	77	Fungi, Ascomycota
ITS134-A3		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	79	Fungi, Ascomycota
ITS134-A6		FJ664858.1	*Verrucaria* sp. A. Orange 17054	89	Fungi, Ascomycota
ITS134-A7		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	80	Fungi, Ascomycota
ITS134-A1		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	81	Fungi, Ascomycota
ITS134-A10		FJ664858.1	*Verrucaria* sp. A. Orange 17054	89	Fungi, Ascomycota
ITS134-A11		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	78	Fungi, Ascomycota
ITS134-B3	II	FJ664858.1	*Verrucaria* sp. A. Orange 17054	89	Fungi, Ascomycota
ITS134-B6		FJ664858.1	*Verrucaria* sp. A. Orange 17054	90	Fungi, Ascomycota
ITS134-B7		FJ664858.1	*Verrucaria* sp. A. Orange 17054	92	Fungi, Ascomycota
ITS134-B9		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	80	Fungi, Ascomycota
ITS134-C1		EU559734.1	*Polyblastia lutosa* isolate SS126	93	Fungi, Ascomycota
ITS134-C11		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	81	Fungi, Ascomycota
ITS134-D4		FJ664858.1	*Verrucaria* sp. A. Orange 17054	90	Fungi, Ascomycota
ITS134-D7		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	81	Fungi, Ascomycota
ITS134-D8		FJ664858.1	*Verrucaria* sp. A. Orange 17054;	92	Fungi, Ascomycota
ITS134-E4		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673).	82	Fungi, Ascomycota
ITS134-E5		FJ664858.1	*Verrucaria* sp. A. Orange 17054	89	Fungi, Ascomycota

Appendix B2 Continued.

Clone	Community Type	Accession Number	Closest BLASTn Match	% Identity	Phyla/Division
ITS134-E6		EU559734.1	*Polyblastia lutosa* isolate SS126	92	Fungi, Ascomycota
ITS134-E8		FJ664858.1	*Verrucaria* sp. A. Orange 17054	92	Fungi, Ascomycota
ITS134-E9		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	81	Fungi, Ascomycota
ITS134-E11		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	81	Fungi, Ascomycota
ITS134-F1		AM229059.1	Uncultured soil fungus clone F47 (S2)	100	Fungi, Ascomycota
ITS134-F3		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	77	Fungi, Ascomycota
ITS134-F5		FJ664858.1	*Verrucaria* sp. A. Orange 17054	91	Fungi, Ascomycota
ITS134-F8	II	FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	81	Fungi, Ascomycota
ITS134-F10		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	81	Fungi, Ascomycota
ITS134-F12		GU327440.1	Uncultured *Nectriaceae* clone R81p4	75	Fungi, Ascomycota
ITS134-G1		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	82	Fungi, Ascomycota
ITS134-H5		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	81	Fungi, Ascomycota
ITS134-H8		FJ664831.1	*Verrucaria anziana* voucher A. Orange 16377 (NMW C.2005.001.673)	80	Fungi, Ascomycota
ITS50-A2		AB540580.1	*Acremonium rutilum* strain: JCM 23088	94	Fungi, Ascomycota
ITS50-A4		AB540580.1	*Acremonium rutilum* strain: JCM 23088	95	Fungi, Ascomycota
ITS50-A8		AB540580.1	*Acremonium rutilum* strain: JCM 23088	94	Fungi, Ascomycota
ITS50-A12	III	AB540580.1	*Acremonium rutilum* strain: JCM 23088	90	Fungi, Ascomycota
ITS50-B4		AB540580.1	*Acremonium rutilum* strain: JCM 23088	94	Fungi, Ascomycota
ITS50-B6		AB540580.1	*Acremonium rutilum* strain: JCM 23088	78	Fungi, Ascomycota
ITS50-B11		AB540580.1	*Acremonium rutilum* strain: JCM 23088	84	Fungi, Ascomycota

Appendix B2 Continued.

Clone	Community Type	Accession Number	Closest BLASTn Match	% Identity	Phyla/Division
ITS50-C3		AB540580.1	*Acremonium rutilum* strain: JCM 23088	91	Fungi, Ascomycota
ITS50-C5		AB540580.1	*Acremonium rutilum* strain: JCM 23088	91	Fungi, Ascomycota
ITS50-C12		AB540580.1	*Acremonium rutilum*	99	Fungi, Ascomycota
ITS50-D2		AB540580.1	*Acremonium rutilum* strain: JCM 23088	80	Fungi, Ascomycota
ITS50-D3		AB540580.1	*Acremonium rutilum* strain: JCM 23088	71	Fungi, Ascomycota
ITS50-D8		AB540580.1	*Acremonium rutilum* strain: JCM 23088	94	Fungi, Ascomycota
ITS50-D11		AB540580.1	*Acremonium rutilum*, strain: JCM 23088	94	Fungi, Ascomycota
ITS50-E1		AB540580.1	*Acremonium rutilum* strain: JCM 23088	94	Fungi, Ascomycota
ITS50-E3		AB540580.1	*Acremonium rutilum* strain: JCM 23088	85	Fungi, Ascomycota
ITS50-E10		AB540580.1	*Acremonium rutilum* strain: JCM 23088	94	Fungi, Ascomycota
ITS50-E12		AB540580.1	*Acremonium rutilum* strain: JCM 23088	94	Fungi, Ascomycota
ITS50-F1		AB540580.1	*Acremonium rutilum* strain: JCM 23088	91	Fungi, Ascomycota
ITS50-F4	III	AB540580.1	*Acremonium rutilum* strain: JCM 23088	91	Fungi, Ascomycota
ITS50-F5		AB540580.1	*Acremonium rutilum* strain: JCM 23088	91	Fungi, Ascomycota
ITS50-F7		AB540580.1	*Acremonium rutilum* strain: JCM 23088	93	Fungi, Ascomycota
ITS50-F11		AB540580.1	*Acremonium rutilum* strain: JCM 23088	91	Fungi, Ascomycota
ITS50-G3		AB540580.1	*Acremonium rutilum* strain: JCM 23088	91	Fungi, Ascomycota
ITS50-G5		AB540580.1	*Acremonium rutilum* strain: JCM 23088	90	Fungi, Ascomycota
ITS50-G6		AB540580.1	*Acremonium rutilum* strain: JCM 23088	92	Fungi, Ascomycota
ITS50-G7		AB540580.1	*Acremonium rutilum* strain: JCM 23088	91	Fungi, Ascomycota
ITS50-G8		AB540580.1	*Acremonium rutilum* strain: JCM 23088	94	Fungi, Ascomycota
ITS50-G9		AB540580.1	*Acremonium rutilum* strain: JCM 23088	91	Fungi, Ascomycota
ITS50-H4		FJ762512.1	Uncultured fungus clone Singleton_612970_3069	97	Uncultured Fungi
ITS50-H7		AB540580.1	*Acremonium rutilum* strain: JCM 23088	90	Fungi, Ascomycota
ITS50-H9		AB540580.1	*Acremonium rutilum* strain: JCM 23088	95	Fungi, Ascomycota

Appendix B3 Microalgal 18S rRNA gene sequences from the clone libraries. The percentage of similarity to the closest homologues in GenBank and the taxonomic affiliations are shown.

Clone	Community Type	Accession Number	Closest BLASTn Match	% Identity	Phyla/Division
MA73-A1		HQ910365.1	Uncultured eukaryote clone P-13_E4 18S rRNA	77	Uncultured eukaryote
MA73-A2		AJ243346.1	Uncultured eukaryote clone P-13_E4 18S rRNA	77	Uncultured eukaryote
MA73-A12		AF023707.1	*Cyrtomnium hymenophyllum* 18S rRNA	74	Bryophyta
MA73-B1		AF023682.1	*Tortula ruralis* 18S rRNA	75	Bryophyta
MA73-E3		HQ190191.1	Uncultured fungus clone FN12 18S rRNA	84	Uncultured Fungus
MA73-E5	I	HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	87	Uncultured eukaryote
MA73-E8		AF023682.1	*Tortula ruralis* 18S rRNA	84	Bryophyta
MA73-F5		HM490263.1	Uncultured eukaryote clone EUK1A D1 18S rRNA	75	Uncultured eukaryote
MA73-F9		HM490268.1	Uncultured eukaryote clone EUK1A E4 18S rRNA	75	Uncultured eukaryote
MA73-H2		GU297788.1	Uncultured eukaryote clone SL.E15# 18S rRNA	81	Uncultured eukaryote
MA73-H5		AF023707.1	*Cyrtomnium hymenophyllum* 18S rRNA	66	Bryophyta
MA134-A1		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
MA134-A2		FN394778.1	Uncultured eukaryote clone 01DLS110600064 18S rRNA	99	Uncultured eukaryote
MA134-A8		AJ243346.1	*Cyathophorum bulbosum*18S rRNA	73	Bryophyta
MA134-A9		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
MA134-A11		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	100	Uncultured eukaryote
MA134-B3		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
MA134-B4	II	X80985.1	*M. hornum* 18S rRNA	94	Bryophyta
MA134-B9		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
MA134-C1		DQ629410.1	*Selaginella wildenowii* 18S rRNA	79	Lycopodiophyta
MA134-C6		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
MA134-C11		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
MA134-C12		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
MA134-D1		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
MA134-D4		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	100	Uncultured eukaryote

Appendix B3 Continued.

Clone	Community Type	Accession Number	Closest BLASTn Match	% Identity	Phyla/Division
MA134-D5	II	HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	100	Uncultured eukaryote
MA134-D6		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
MA134-F6		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
MA134-G12		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
MA134-H3		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
MA134-H7		EF024819.1	Uncultured eukaryote clone Elev_18S_1332 18S rRNA	73	Uncultured eukaryote
MA134-H10		HM490274.1	Uncultured eukaryote clone EUK1A H10 18S rRNA	99	Uncultured eukaryote
MA50-A1	III	X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
MA50-A4		X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
MA50-A5		X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
MA50-A7		X80985.1	*M. hornum* 18S rRNA	94	Bryophyta
MA50-A8		Y17871.1	*Encalypta streptocarpa* 18S rRNA	94	Bryophyta
MA50-A11		X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
MA50-A12		X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
MA50-B3		EF024819.1	Uncultured eukaryote clone Elev_18S_1332 18S rRNA	99	Uncultured eukaryote
MA50-B4		X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
MA50-B6		Y17871.1	*Encalypta streptocarpa* 18S rRNA	90	Bryophyta
MA50-B7		X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
MA50-C2		X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
MA50-C6		X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
MA50-C9		X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
MA50-D1		X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
MA50-E7		EF526889.1	Uncultured marine eukaryote clone NA2_1H8 18S rRNA	99	Uncultured eukaryote
MA50-E8		EF024819.1	Uncultured eukaryote clone Elev_18S_1332 18S rRNA	99	Uncultured eukaryote

Appendix B3 Continued.

Clone	Community Type	Accession Number	Closest BLASTn Match	% Identity	Phyla/Division
MA50-E10		X80985.1	*M. hornum* 18S rRNA	94	Bryophyta
MA50-G8		X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
MA50-G12		X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
MA50-H1	III	X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
MA50-H8		X80985.1	*M. hornum* 18S rRNA	95	Bryophyta
MA50-H12		X80985.1	*M. hornum* 18S rRNA	95	Bryophyta