PHYLOGENETIC DIVERSITY OF *nifH* GENES IN MARION ISLAND SOIL

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DECLARATION

I hereby declare that this thesis contains no material that has been accepted for an award or any other degree or diploma in any tertiary institution, and to the best of my knowledge contains no material previously written or published by another person, except where due acknowledgment in made in the text of the thesis.

Joanne Rapley

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ABSTRACT

The microbial life of sub-Antarctic islands plays a key role in the islands ecosystem, with microbial activities providing the majority of nutrients available for primary production.

Knowledge of microbial diversity "is still in its infancy", and this is particularly true regarding the diversity of micro-organisms in the Antarctic and sub-Antarctic regions.

One particularly important functional group of micro-organisms is the diazotrophs, or nitrogen-fixing bacteria and archaea. This group have not been-well studied in the sub-Antarctic region, but may play an important role in the nutrient cycling of the island.

This thesis explores the diversity of nitrogen-fixing organisms in the soil of different ecological habitats on the sub-Antarctic Marion Island. Results indicate that a wide variety of *nifH* genes are present in the soil, and may vary depending on ecological habitat. *nifH* fragments isolated in this study clustered with proteobacterial *nifH* fragments from Rhizobiacae, *Nostoc spp*. and fragments isolated from uncultured bacteria from similar cold environments such as the Qingzhang Plateau in Tibet and Canada's Ellesmore Island in the Arctic Circle.

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GLOSSARY AND ABBREVIATIONS

BLAST: Basic Local Alignment Search Tool

CTAB: Cetyl Trimethyl Ammonium Bromide

DGGE: Denaturing Gradient Gel Electrophoresis

PCR: Polymerase Chain Reaction

PVPP: polyvinyl polypyrolidone

RFLP: Restriction Fragment Length Polymorphism

TEMED: N,N,N',N'-Tetramethylethylenediamine

Tfb I: 100mM RbCl; 50mM $MnCl_2$-$4H_2O$; 30mM potassium acetate; 10mM $CaCl_2$-$2H_2O$; 15% glycerol.

Tfb II: 10mM MOPS; 10mM RbCl; $CaCl_2$-$2H_2O$; 15% glycerol.

CHAPTER 1 - LITERATURE REVIEW

1.1 Marion Island

1.1.1 Location, geology and climate

Marion Island (46°54'S, 37°57' E) lies approximately 2170km south east of Cape Town in the southern Indian ocean (Fig 1). It is a small island (290km^2) with a mostly cliff-face coastline and irregular topography.

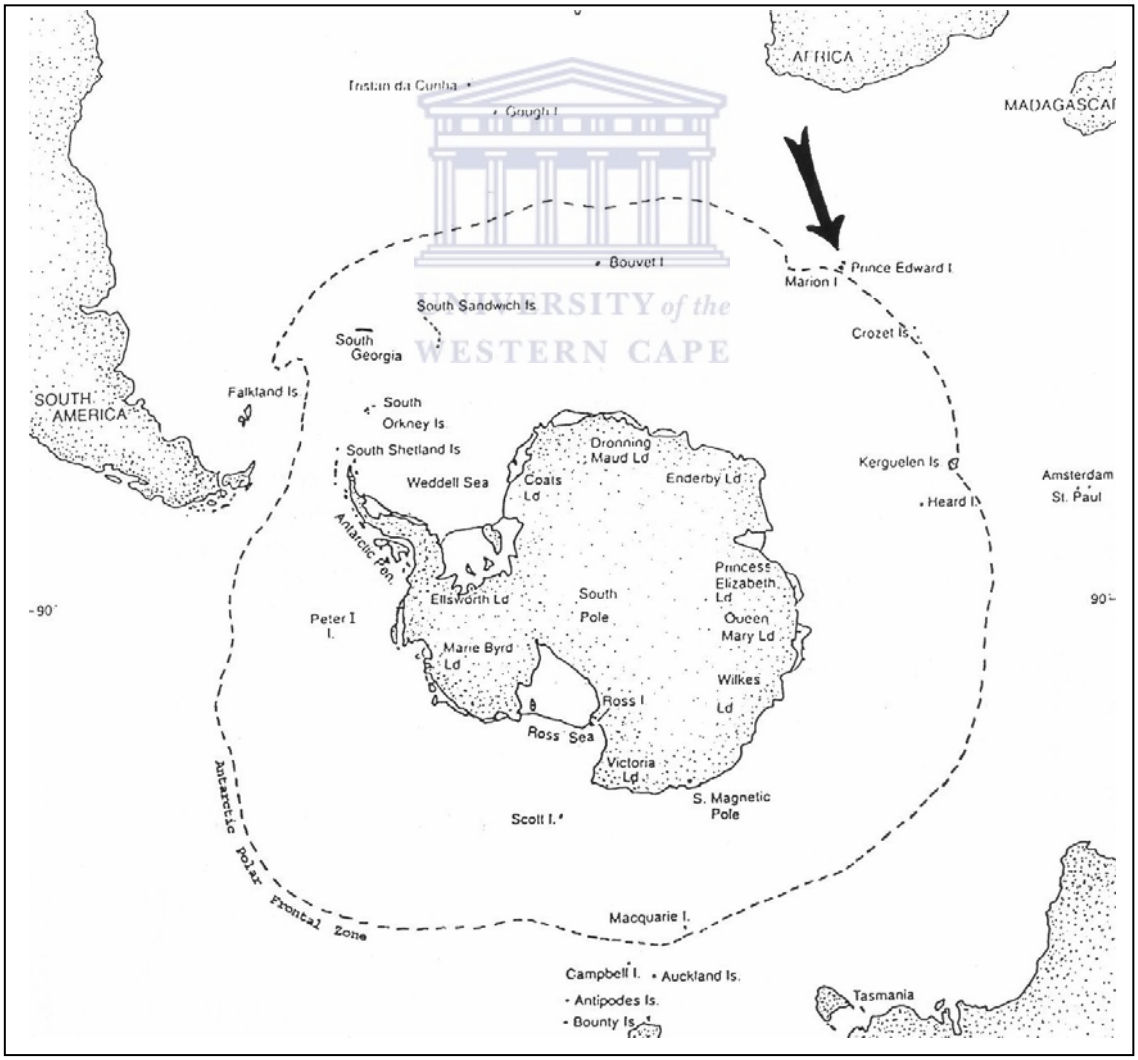

Fig 1.1: Location of Marion Island (from Hänel and Chown, 1998)

As one of the twin peaks of a (still active) coalescing shield volcano (the other being Prince Edward Island), Marion Island is probably between 0.5 to 1 million years old (Hänel and Chown 1998). The island was formed predominately from grey basalt lava, after which extensive glaciations occurred. Black basalt lava became the dominant rock type after the glaciers melted rapidly approximately 12 000 - 16 000 years ago. Red and black scoria cones dominate where main pyroclastic eruptions have occurred.

The southern ocean system is the main factor influencing the climate on the island, and conditions are characteristically sub-Antarctic, i.e. strong westerly winds; high precipitation (mean 2500mm per annum); relatively low air temperatures (mean 5.7°C); high humidity (mean 83%); high cloud cover and low incidence of sunshine (mean 3.6 hours a day).

Due to these relatively harsh climatic conditions and isolation, Marion Island has a typically sub-Antarctic ecosystem. These ecosystems occur in restricted spaces (i.e. small islands) and lack particular ecological functional groups such as mammalian herbivores and vertebrate and invertebrate predators. This results in an impoverished fauna and flora that relies heavily on a detritus nutrient chain rather than a grazing one (Smith, 1977; 2004). Although the diversity of higher plants is very low (e.g. only 22 vascular plant species are indigenous to Marion Island), primary productivity is very high (Smith, 1988).

Significant climate changes occurring in the sub-Antarctic over the last 3 decades, including temperature increases and reduced precipitation (Bergstrom and Chown,

1999), are likely to impact on island ecosystems in the region (Smith and Steenkamp, 1990; Chown and Smith, 1993; Bergstrom and Chown, 1999; Huyser *et al.*, 2000) and affect the local microbiota.

1.1.2 Habitats - physical, chemical and biological characteristics of soil

Smith and Steenkamp (2001) describe 23 different terrestrial habitats (in 7 community complexes) on Marion Island based on soil and vegetation properties. Classifying these habitats provides a useful guide to sub-Antarctic ecological communities and the classification published has been demonstrated to reflect ecological variables such as abiotic and biotic influences and different soil respiration rates (Smith, 2003). Some of the major aspects of each of the major communities are briefly described below.

Salt-spray communities occur on fibrous, black or dark brown peats. The vegetation is dominated by the erect dicot *Crassula moschata* and is co-dominated by the rosette dicot *Cotula plumosa* in 1.1 (Coastal Herbfield Habitat) and the cushion dicot *Azoerella selago* in 1.2 (Coastal Fellfield Habitat). Salt-spray communities are exposed to high levels of saline, and are influenced by birds and seals. Soils from these habitats are characterised by high soil salinity (all forms of Na, exchangeable and soluble forms of Mg).

Fellfield communities occur on exposed plateaus, volcanic ash and rocks. The sparse vegetation is dominated by the cushion dicot *Azorella selago*, as well as cushion and ball-forming mosses and lichens. Soils from these habitats are denser, drier and have

lower concentrations of N and P than the other habitats. They also have higher concentrations of Mg and Ca, and have a lower biotic influence than other habitats. The Xeric Fellfield (2.1) is drier, less organic and acidic than the Mesic Fellfield (2.2).

Slope communities contain an organic surface layer dominated by *Blechnum penna-marina* in 3.1-3.4, (Open Fernbrake, Closed Fernbrake, Mesic Fernbrake and Dwarf Shrub Fernbrake) or by *Acaena magellanica* and mosses or bryophytes in 3.5 and 3.6 (Slope Drainage Line and Spring and Flush).

Grassland communities are dominated by *Poa cookii.* 4.1 (Coastal Tussock Grassland) is strongly affected by petrels, penguins and seals, 4.2 (Inland Tussock) less so, 4.3 (Pedestalled Tussock) has tussock grass on tall peat pedestals, with *Callitriche* between the pedestals. Soils are acid (especially in 4.1) and have high levels of N and P (both organic and inorganic forms) associated with manuring.

Herbfield communities are strongly influenced by seabirds and seals, dominated by *Cotula plumosa* with *Poa cookii*. Three main types have been described. 5.1 (Cotula Herbfield) soils are highly saline, and have high levels of N and P. 5.2 (Biotic Mud) soils are heavily influenced by manuring and are very wet, anaerobic muds rich in inorganic N and P. 5.3 (Biotic Lawn) soil have much lower concentrations of N and P, but high levels of Ca and Mg and are thin peats covering scoria and volcanic ash.

Mire communities are defined by wet soils dominated by Mire graminoids (*Agrostis magellanica*, *Juncus scheuchzerioides*, *and Uncinia compacta*), mire bryophytes and/or *Bryum/Breutelia* mosses. There are 6 types: 6.1 (Dry Mire), 6.2 (Mesic Mire),

6.3 (Wet Mire), 6.4 (Mire Drainage Line), 6.5 (Biotic Mire), 6.6 (Saline Mire). Soils are very organic, have high moisture content, a low density and less Ca and Mg than the other habitats.

Polar desert communities occur at high altitudes (above 500m). These communities have no soils, but are covered by a thin, grit-like layer of volcanic ash under and between rocks. A very low cover (<1%) of moss and lichen also exists at some sites.

1.2 Microbial Biodiversity

Although the cold climate and excessively moist soil environment on Marion Island is likely to limit microbial activity (Wynn-Williams, 1980; Smith *et al.*, 1993), the importance of the microbial ecosystem must be emphasised. Activities such as decomposition are the predominant source of nutrients available for primary production (Smith, 1988; Smith and Steenkamp, 1992) and some research has been conducted in order to investigate and understand these systems.

Microbiological studies on the island (and in the sub-Antarctic in general) have been mostly limited to direct and plate counts of bacteria, yeast and fungi sampled from a small range of habitats, but results from these studies suggest wide variation between sites influenced by different environmental variables. For example, French and Smith (1986) report that more than three times as many bacteria occur in low altitude (≤50m) fellfields than in higher altitude (<500m) fellfields on Marion Island (161 ± 4 x 10^8 g^{-1} by direct count). These sites may be comparable to the Mesic and Xeric fellfield habitats described by Smith (2001), and differ in climatic severity (low

altitude sites generally being warmer and wetter) and nutrient concentrations, with Mesic fellfields slightly more organic than Xeric fellfields. Smith and Steyn (1982) correlate differences in aerobic soil bacteria and fungi counts to differences in N content and pH associated with animal manuring. Bacterial activity appears to be limited by nutrient concentration, and differs between different ecosystems, with biotically influenced sites having greater activity than fernbrakes, mires and fellfields (Grobler *et al.*., 1987). Grobler's pattern conforms to that exhibited by Smith's (1993) experiments with TSL (tensile strength loss of buried cotton strips) and suggests that the major ecological factor responsible for microbial distribution and soil respiration is the manuring influence of seals and seabirds (Smith, 2003).

1.2.1 Bacteria

Bacteria are important decomposers of organic material. In particular, they play a key role in fixing nitrogen and converting it to plant-utilisable forms (see 3.1.4 and 2.3). The abundance of bacteria in soil is influenced by environmental conditions, altitude and depth (see section 1.3) and their growth and activity are limited by nutrient concentrations (Grobler and Smith, 1987). Bacteria isolated from sub-Antarctic soil are generally more pyschotrophic than those from more temperate regions (Latter and Heal, 1971; see section 1.3.1).

The *Chlorisodontium* microbiota found in peat soils on the maritime Antarctic Signy Island are similar to cold peat soils elsewhere (Baker, 1970a; Baker and Smith, 1972). The most common bacteria found in soil here are gram-negative rods (Heal *et al.*, 1967) which are also the dominant terrestrial bacteria in other Antarctic maritime soils (Tearle and Richard, 1987).

Most of the bacteria found in sub-Antarctic habitats are aerobic, the only record of anaerobic bacteria (Vishniac, 1993) being Christie's (1987) report of *Desulfovibrio* and *Desulfotomaculum* in Signy Island peats.

Direct counts of bacteria from Signy Island fellfields are similar to those from Marion (French and Smith, 1986) ranging from 10^9 - 10^{10} dry soil (plate counts 10^4-10^6) (Smith and Tearle, 1985). Plate counts from ornithogenic soils on King George Island were 10^6-10^7 cfu g^{-1} dry soil (Pietr, 1986).

1.2.3 Eucarya

Fewer species of eucarya have been recorded in Signy Island soils than in tundras (Latter and Heal, 1971; Dowding and Widden, 1974). The psychrotrophic *Chrysosporium pannorum* was frequently isolated from Signy Island soil and seems to be associated with animal influences (feathers, nests, ornithogenic soil, elephant seal wallows) (Pugh and Alsopp, 1982). *Mortierella alpine* from Signy Island is more cold tolerant than isolates from temperate sites (Latter and Heal, 1971) and may be an important indicator genus in the maritime and sub-Antarctic (Wynn-Williams, 1985).

1.2.4 Diazotrophs – An Important Functional Group

Introduction

Biological nitrogen fixation is the reduction of atmospheric nitrogen gas to ammonium. This process plays an essential role in nutrient cycling (Blackburn, 1983)

and all ecological habitats depend on it to ensure the availability of biologically usable ammonium (Atlas and Bartha, 1997), as most organisms require combined forms of nitrogen to incorporate into cellular biomass (Brown and Johnson, 1977).

Nitrogen fixation is carried out by a limited number of phylogenetically diverse, proteobacteria, green sulfur bacteria, cyanobacteria, firmicutes, spirochaetes and archaea, both free-living and living in symbiotic associations. Such organisms are known as 'diazotrophs' and use the enzyme complex nitrogenase to mediate nitrogen fixation.

Nitrogenase is composed of two proteins, the heterotetramer metalloprotein dinitrogenase $\alpha_2\beta_2$, and the homodimer metalloprotein dinitrogenase reductase γ_2. Dinitrogen reductase couples ATP hydrolysis to interprotein electron transfer (Zehr *et al.*, 2003) while the α unit of dinitrogenase contains the active site for dinitrogen reduction. This is usually a FeMo-cofactor (encoded by the *nif* gene), although some diazotrophs use nitrogenases containing vanadium (encoded by the *vnf* gene) instead of molybedenum, or use iron only (encoded by *anf*). These three genetically distinct nitrogenase systems permit nitrogenase production and activation only in environments with low concentrations of ammonia and oxygen, as nitrogenase is sensitive to oxygen inactivation (Howard and Rees, 1996). One other system has also been identified, but not well-studied, a *nif*/*vnf*/*anf*-independent and oxygen-insensitive nitrogenase, occurring in *Streptomyces thermoautotrophicus* (Ribbe *et al.*, 1997). Raymond *et al.*, (1994) classifies genes encoding for nitrogenases into 5 distinct evolutionary clades:

I. Mo-Fe nitrogenases, mostly members of the proteobacteria and cyanobacteria
II. Anaerobic Mo-Fe nitrogenases from mostly anaerobic organisms such as clostridia, acetogenic bacteria and some methanogens
III. Alternative nitrogenases, including *anf* and *vnf* (excepting *vnfH*)
IV. Uncharacterised *nif* homologs detected only in methanogens and anoxygenic photosynthetic bacteria
V. Bacteriochlorophyll and chlorophyll biosynthesis genes in phototrophs.

The Mo-Fe nitrogenase is the most efficient system at binding and reducing dinitrogen (Joerger and Bishop, 1988) and alternative nitrogenases are generally expressed only when molybdenum concentrations are limiting, so this is the most commonly used, widely distributed and well-studied of the nitrogenase systems (Zhao *et al.*, 2006).

There are three fundamental reactions involved in nitrogen reduction by Mo-Fe nitrogenases. Firstly, the Fe protein is reduced by ferredoxins and/or flavodoxins. Secondly, single electrons are transferred from the Fe protein to the MoFe protein in a MgATP-dependent process and finally, electrons are transferred to the substrate at an active site within the MoFe protein. These processes can be summarised by the general equation (Simpson, 1984):

$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$$

The Mo-Fe nitrogenase system is regulated by a complex system incorporating a large number of different genes (Fischer, 1994), with dinitrogenase reductase being encoded by the *nifH* gene and dinitrogenase by the *nifD* (α) and *nifK* (β) genes. Many

nif operons also contain the genes *nifE* and *nifN* in conjunction with the *nifH*, *nifD* and *nifK* genes, often in the order nifHDKEN. *nifE* and *nifN* possibly function with the FeMo-cofactor or the FeV-cofactor, as they are not usually found in organisms relying solely on a Fe-only nitrogenase (Roll *et al.*, 1995).

The *nifH* gene is highly conserved, and has been used to investigate diazotroph diversity in a number of studies (see below).

Molecular diversity studies

Diazotrophs (nitrogen-fixing organisms) play a key role and studies assessing their diversity provide important information for assessing the productivity of an ecosystem organisms is very important for monitoring changes in community dynamics caused by environmental change, and is particularly important in environments that have not been well-studied (Deslippe and Egger, 2006).

Diazotrophs belonging to various groups of micro-organisms have been cultured, but the exacting conditions for successful growth puts some constraints on the usefulness of culture-dependent methods to easily and accurately evaluate diversity (Postgate, 1998). It is also highly likely that some diazotrophs are not culturable (Roszak and Colwell, 1987), making a culture-independent approach a more attractive option to assess diversity.

Culture-independent strategies have predominately focused on using the *nifH* gene to study diazotroph diversity, this having a couple of major advantages over using other

genes from the *nif* complex. Although all of the dinitrogen reductase genes *nifH*, *vnfH* and *anfH* are highly conserved, *nifH* is 'one of the oldest functional genes' (Ueda *et al.*, 1995) and phylogenetic trees based on analysis of *nifH* are generally consistent with those based on 16S rRNA analysis (Normund and Bousquet, 1989; Ueda *et al.*, 1995), making evolutionary analysis a reasonably simple process. In addition, the *nifH* gene has been relatively (as compared with other *nif* genes) well-studied, with a large number of sequences having been collected from environmental samples (Zehr *et al.*, 2003).

Culture-independent methods involving a variety of different molecular techniques have been explored, including DGGE, RFLP and clone library analysis (for details of these methods refer to Chapter 1.4). These methods require amplifying the *nifH* gene from a DNA sample, and a variety of primers have been published that target different *nifH* genes. Many of these primers target specific groups or genera, but primer bias has not been assessed in the majority of studies (Zehr and Capone, 1996). Designing 'universal' *nifH* primers to obtain a broad overview of diazotroph diversity has been the most common approach, however, this involves high degeneracy and reduced specificity (Widmer *et al.*, 1999). There may be vast phylogenetic differences between nitrogen-fixers (Zehr, 1989), which can be members of groups as diverse as the euryarchaea, proteobacteria, firmicutes and cyanobacteria (Raymond *et al.*, 2004). More sophisticated PCR approaches, such as nested PCR have been successfully applied to assist with these problems in a number of cases (e.g. Widmer *et al*., 1999).

Diazotroph diversity research has been largely biased towards aquatic environments (Izquierdo and Nüsslien, 2006), and a wide variety of oceanic (e.g. Zehr *et al.*, 1998;

Mehta *et al.*, 2003; Langlois *et al.*, 2005) and lake (e.g. Zani *et al.*, 2000; Steward *et al.*, 2004) samples have been collected and studied. Terrestrial research has described some diversity in salt marshes (Piceno *et al.*, 1999; Lovell *et al.*, 2001), in termite hindguts (Ohkuma *et al.*, 1996; Kudo *et al.*, 1998) and in microbial mats from various environments (reviewed by Zehr *et al.*, 2003)

Diazotroph diversity in soil environments have been less well-studied. Soil is a very heterogeneous medium, both physically and chemically (Ladd *et al.*, 1996) and diazotrophs can be distributed very differently from one cubic centimetre to the next (Vogel *et al.*, 2003; Izquierdo and Nüsslien, 2006;). Distribution of diazotrophs in soil may be influenced by soil texture (Riffkin *et al.*, 1999), nitrogen levels in the soil (Cejudo and Paneque, 1986; Limmer and Drake, 1998) and the vegetation of the environment (Bardgett *et al.*, 1999) as well as a host of other physical, chemical and biological factors (detailed in section 1.3).

Soil environments that have been examined for diazotrophs include a few forest soils (Widmer *et al*, 1999; Shaffer *et al.*, 2000; Poly *et al*., 2001), pastoral and agricultural soils (Poly *et al.*, 2001) rhizospheres (Ueda *et al.*, 1995; Piceno and Lovell, 2000; Hamelin *et al.*, 2002) and the dryland part of Senegal (Diallo *et al.*, 2004).

The molecular diversity of nitrogen-fixers in some polar and cold soils have also been briefly studied. Olson *et al.*, (1998) describe a fairly high diversity of diazotrophs in Antarctic dry valley soils within ice aggregates. These communities appeared similar to those described for temperate microbial mats, and were dominated by *Nostoc* or *Nostoc*-like cyanobacteria. Most recently, *nifH* diversity has been investigated in soil

from the Tibetan Plateau in China (Zhang *et al.*, 2006) and in soils associated with high Arctic dwarf shrubs on Ellesmere Island, Canada (Deslippe and Egger, 2006). Most of the clones from these studies were most closely related to the genes of uncultured bacteria, while the remainder belonged to proteobacteria (α, β, γ, δ). Nitrogenases were mostly type I, although type IV were also observed. The most interesting research outcome of these two studies was the high degree of similarity between many of their sequences from uncultured bacteria.

Diazotroph research on sub-Antarctic islands has been very limited. However, Line (1992) reports that cyanobacteria growing symbiotically with plants or lichen dominate the diazotroph assemblage on Macquarie Island, with important lichen species including *Pseudocyphellaria delisea* and *Bacillus sp* (*B. macerans or B. polymyxa*) being widespread across coastal, grassland slope and plateau habitats. Nitrogen-fixing cyanobacteria have also been documented on Signy Island (Horne, 1972), Marion Island (Croome, 1973; Smith, 1984), their distribution and nitrogen-fixing capabilities being influenced by a range of factors (see section 1.3), the most important limiting factor being light availability (Smith, 1984). No studies using molecular methods to investigate diazotroph diversity have been conducted in the sub-Antarctic to date.

1.3 Factors influencing the distribution of microbial communities in the sub-Antarctic

1.3.1 Abiotic influences

Temperature

Temperature has a very strong influence on microbial growth, survival and activity in all ecosystems, and all micro-organisms are adapted to survive within certain temperature ranges.

Micro-organisms exhibiting evolutionary adaptations to cold environments are known as "psychrophiles" or "psychrotrophs". Psychrophiles inhabit constantly cold environments, and grow between 0°C and 20°C, with optimum growth temperatures less than 15°C. Psychrotrophs or "psychrotolerant" organisms can grow at 0°C but have an optimal growth temperature between 20°C and 40°C.

Unsurprisingly, bacteria isolated from Signy Island soils have been found to exhibit a lower-growth-temperature maximum (*i.e.* be more pychrotolerant) than those from temperate soils (Latter and Heal, 1971), and in cold ecosystems such as alpine tundras, microbial communities often reach their maximum biomass during the winter months (Clein and Schimel, 1995; Brookes *et al.*, 1996; Lipson *et al.* , 1999).

It is generally accepted that increases in temperature are loosely correlated with increased microbial activity, and temperature is probably 'the key factor controlling

the cycling of carbon and nitrogen' in sub-Antarctic ecosystems (Huiskes *et al.*, 2004).

Only very limited soil temperature information is available for Marion Island. Some data from Blake's (1997) unpublished MSc thesis (University of the Free State) are reproduced by Smith (2003). These data give mean annual soil temperatures for four different habitats (Mesic mire, Closed fernbrake, Xeric fellfield and Polar desert). Unsurprisingly, the fellfield and polar desert are the coldest (mean 4.1°C and 4.5°C), with the mire and fernbrake at 6.3°C and 6.2°C, respectively). The polar desert and fellfield also exhibit much greater temperature fluctuations than the other two habitats, for example, the polar desert recorded a minimum temperature of –1.7°C with a maximum of 43.8°C. Smith (2003) reports that soil respiration did not differ along a temperature gradient and concludes that this implies the existence of a mixed microbial community with a diverse range of temperature dependent responses.

In the Antarctic, maritime and sub-Antarctic, the most significant temperature influences for micro-organisms are considered to be the frequency of freeze-thaw cycles (Visniac, 1993). Freeze-thaw cycles caused by rapid temperature fluctuations pose more challenges for survival than a constantly frozen environment (Macleod and Calcott, 1976). Occurring seasonally, their effects may be more pronounced in winter than summer and they encourage psychrotrophism in a variety of microbial life (Davey *et al.*, 1992). Communities are likely to change significantly as the large quantities of nutrients released after the initial melting of winter ice (Tearle, 1987; Lipson, 1999) results in a sharp increase in respiration rate and the growth of opportunistic micro-organisms such as psychrotrophic yeasts taking advantage of the

disruptive effects of the cycles (Wynn-Williams, 1980, 1982; Pugh and Allsopp, 1982). This has been demonstrated experimentally by Wynn-Williams (1982), who froze a peat core from Signy Island at -10° for 2 months. During this time, the bacteria and yeast biomass doubled. Additional storage at the same temperature resulted in a decrease in the yeast population to its original level, and a three-fold increase in the microfungi of the sample.

It is probable that gradual freezing or exposure to periods of cold temperatures will allow organisms to acclimatise and be less affected by freeze-thaw cycling (Levitt, 1980; Davey *et al.*, 1992) and such periods are common in wet sub-Antarctic ecosystems. Snow cover also lessens the effects of cycles by providing an effective layer of insulation against declining air temperatures.

Biological nitrogen fixation is sensitive to temperature, and diazotrophs are often considered to be mesophilic (Alexander, 1975), although studies demonstrate that fixation can occur at 0ºC (Alexander and Schell, 1978; Horne, 1972), although rate of fixation appears to be positively related to temperature, at least in cyanobacteria (Davey, 1983), and Davey and Marchant (1983) report a cessation of fixation at temperatures below -7˚C. Horne (1972) suggests that liquid water is a more important limiting factor for continued nitrogenase activation at low temperatures than temperature itself, and moisture levels are known to influence fixation (Davey, 1982). Deslippe *et al.*, (2005) reported changes in diazotroph community structure in response to warming, with subsurface nitrogen-fixers exhibiting the greatest changes.

Water

In an environment which receives 311 days of rain annually (Schulze, 1971), water is unlikely to be a limiting ecological factor on Marion Island, as soils are "close to field capacity moisture" (Smith 2003) most of the time. However, moisture content is probably the main determinant of guano decomposition and nutrient cycling in penguin rookeries (Wynn-Williams, 1990).

pH

Fungi usually represent an increasing proportion of the microflora as pH decreases and this is consistent with reports from sub-Antarctic islands (e.g. Dunt, 1965). Baker (1970a) suggests that acidity restricts bacteria, increasing availability of resources for acid-tolerant fungi. Marion island soils appear to be unusual for acidic peats, as they have more bacteria than fungi (Steyn and Smith, 1981; Smith and Steyn, 1982), compared to descriptions of tundra soils which have been estimated to contain 10 times as many fungi as bacteria (Holding, 1981).

pH may be an important factor in the distribution of diazotrophic cyanobacteria in the sub-Antarctic, as they prefer more basic sites and are not usually found in areas with a pH lower than 5 (Alexander, 1975). For example, *Nostoc commune* at Signy Island is capable of fixing nitrogen at pH 6.6, but maximum activity is observed at 8.7-9 (Horne, 1972).

Nitrogen cycling

On Marion Island, the slow rate of decomposition of organic matter limits nutrient recycling (Smith, 1988) and as a result, the availability of nutrients such as nitrogen is very low compared to other ecosystems. The decomposition system is the predominant source of nutrients required by the plant communities (Smith, 1988; Smith and Steenkamp, 1992). This system has been the target of some research, and nutrient cycling has been extensively studied and described by Smith (Smith, 1977; Smith1987a; Smith, 1987b; Smith; 1987c; Smith, 1988).

The vegetation on Marion and other sub-Antarctic islands derive the majority of their nutrient requirements from the ocean ecosystem, with both the direct and indirect effects of manuring being the predominant source (Lindeboom, 1984; Smith, 2003). Lindeboom (1984) emphasizes the importance of penguin rookeries in nitrogen cycling on Marion Island, suggesting a pathway where 'penguins catch their food in the oceans, microorganisms degrade the penguin droppings, and rain precipitates the ammonia formed'. After deposition by penguins or seals, urea and uric acid hydrolyse, causing an increase in pH which favours ammonia formation (Erskine, 1998). Although it has been accepted that most of this ammonia is washed away (Gillham, 1961; Smith, 1978), Lindeboom (1984) suggests that more than 10% of this volatilized ammonia eventually spreads to the inland vegetation, and Erskine takes this into account for his model of nitrogen cycling in the sub-Antarctic Macquarie Island ecosystem (Fig. 2).

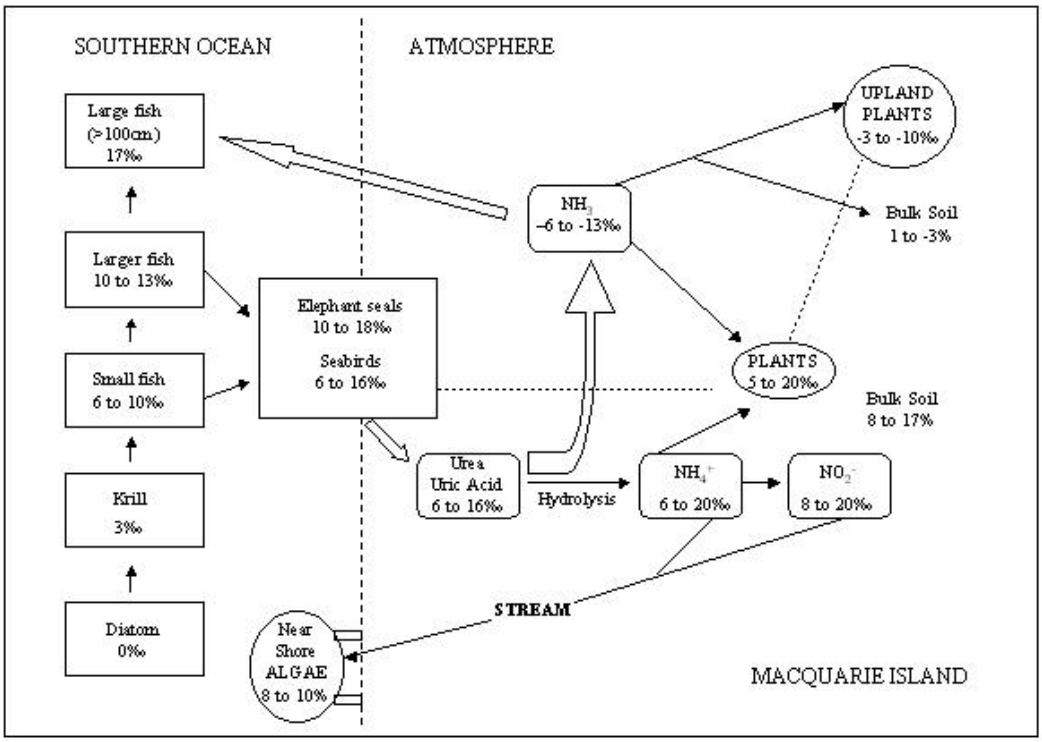

Fig 1.2: Model of nitrogen ($\delta^{15}N$) cycling in the sub-Antarctic Macquarie Island ecosystem (from Erskine *et al.*, 1998)

Biological nitrogen fixation on Marion Island is very low compared to arctic and sub-arctic tundras (Smith, 1988), but similar to the values reported for Signy Island by Horne (1972) and Christe (1987).

Diazotrophs provide their own nitrogen, and as such, may have a competitive advantage over non-diazotrophs in nitrogen-limited environments. Although diazotroph phylotypes have been reported in non-nitrogen-limiting environments (Zehr *et al.*, 2003), it is hypostheised that as the capacity to fix nitrogen becomes less important, such as in a more N-rich soil, the capacity to compete for carbon and other energy resouces may also be diminished (Kolb and Martin, 1988).

1.3.2 Biotic Influences

Vegetation

Bacteria usually have highest abundance in surface samples with dense plant covers (Bölter *et al.*, 1997). The vegetation gradient of Marion Island is significantly positively associated (r^2 = - 0.587) with bacterial counts (Smith and Steyn, 1982).

Diversity of vegetation may also influence microbial communities. Microbial biomass, activity levels (particularly regarding N mineralisation) and fungal abundance have been positively correlated with plant species richness (Zak *et al.*, 2002). Marion Island has a very low vegetation diversity, with relatively few vascular plants, mosses and lichen species (Smith *et al.*, 2001).

With respect to diazotrophs, no symbiotic relationships with vascular plants on Marion Island have been reported, nor have any of the genera endemic to the island ever been demonstrated to be capable of forming such associations (Smith, 1988). Nitrogen-fixing cyanobacteria do, however, occur epiphytically on mosses (Smith, 1988) and some species are believed to occur only with bryophytes in polar regions (Kanda and Inoue, 1994). Diazotrophic activity also appears to be strongly linked with plant primary production (Whiting *et al.*, 1986)

Animal influences

Deposits of guano and animal excrement on Marion Island contribute large quantities of organic nutrients such as urea and protein, as well as inorganic nutrients such as nitrogen and phosphates (Erskine, 1998; Smith, 2003; Tscherko *et al.*, 2003). Although some studies suggest that most the ammonia derived from these sources are washed away (Gillham, 1961; Smith, 1978), others suggest that enough are retained to have a significant impact on the soil type, microbial communities and vegetation (Lindeboom, 1984).

Some sub-Antarctic soil qualities have been quantitatively examined by Erskine (1998), who reports that Macquarie Island soils directly influenced by animal excrement contain almost 4 times the concentration of soluble nitrogen and 6 times as much protein than soils sampled away from animal influences. Manuring is also probably the major factor determining respiration rate in sub-Antarctic soils (Smith, 2003).

Higher nutrient contents caused by manuring are also associated with larger microbial communities and/or increased microbial activity (Smith and Steyn, 1982; Ramsey, 1983; Smith and Hilmer, 1984; Smith 2003; Tscherko *et al.*, 2003), but the type of excrement deposited also has certain implications for community activity. For example, penguin guano and seal excrement often contain the antibiotic acrylic acid, a compound commonly found in *Phaeocystis*, a phytoplankton consumed by krill (a major component in the Southern ocean food chain). This compound can inhibit the decomposition of fresh guano (Sieburth, 1963) and may explain Ramsey and

Stannard's (1986) report that a recently abandoned Adélie rookery at Ross Island contained 77 times as many colony-forming bacteria than a currently inhabited one. However, Tscherko *et al.* (2003) reported higher microbial biomass-to-enzyme activity ratios in soils influenced by guano than by excrement of other seabirds and, in general, many more bacteria are found in ornithogenic soils than in never-inhabited sites (Ramsey, 1983).

1.3.3 Seasonal cycles

Neither abiotic nor biotic influences exist statically and independently, but affect each other within dynamic seasonal and long-term cycles. The effects of these cycles on microbial populations have not been well documented in the sub-Antarctic. However, it is well established that seasonal changes in microbial populations can have significant effects on ecosystem nutrient cycling (Singh, 1989; Lipson, 1999), and some research has been conducted on the similar tundra ecosystem.

In alpine soils, microbial biomass reached its highest concentration in the winter and early spring (Fisk and Schmidt, 1996; Lipson *et al.*, 1999). This declined readily during the first month after the spring snow melt, during which a high level of extractable soil protein was observed and was presumed to have been released from the microbial biomass (Lipson *et al.*, 1999), including a rapid influx of available nitrogen which can be utilised by plants during their peak growing season (Lipson *et al.*, 1999; Lipson *et al.*, 2001). This has been suggested to be a temporal separation mechanism to reduce competition for nitrogen between micro-organisms and plants in this environment (Jaeger *et al.*, 1999). However, there are also occasional growth peaks during summer (Lipson *et al.*, 1999) and during the plant growing season,

microbial biomass is dynamic and seems to be associated with changes in substrate and temperature, with high temperatures having a negative effect on C availability (Lipson *et al.*, 2000). Differences in the summer and winter microbial communities of these soils have also been documented, with winter communities using more organic compounds such as cellulose and salicylate and having a higher fungal/bacterial ratio.

1.4 Molecular methods for detecting microbial populations

Development of molecular techniques in recent years has allowed culture-independent detection of microbial populations and their activities. These methods involve direct analysis of metagenomic DNA extracted from microbial communities. Analysis may use techniques such as PCR, denaturing gradient gel electrophoresis and/or restriction fragment polymorphism analysis to produce community fingerprints (Fig 1.3). Such techniques also permit phylogenetic (evolutionary) relationships between organisms to be examined (Pace, 1996).

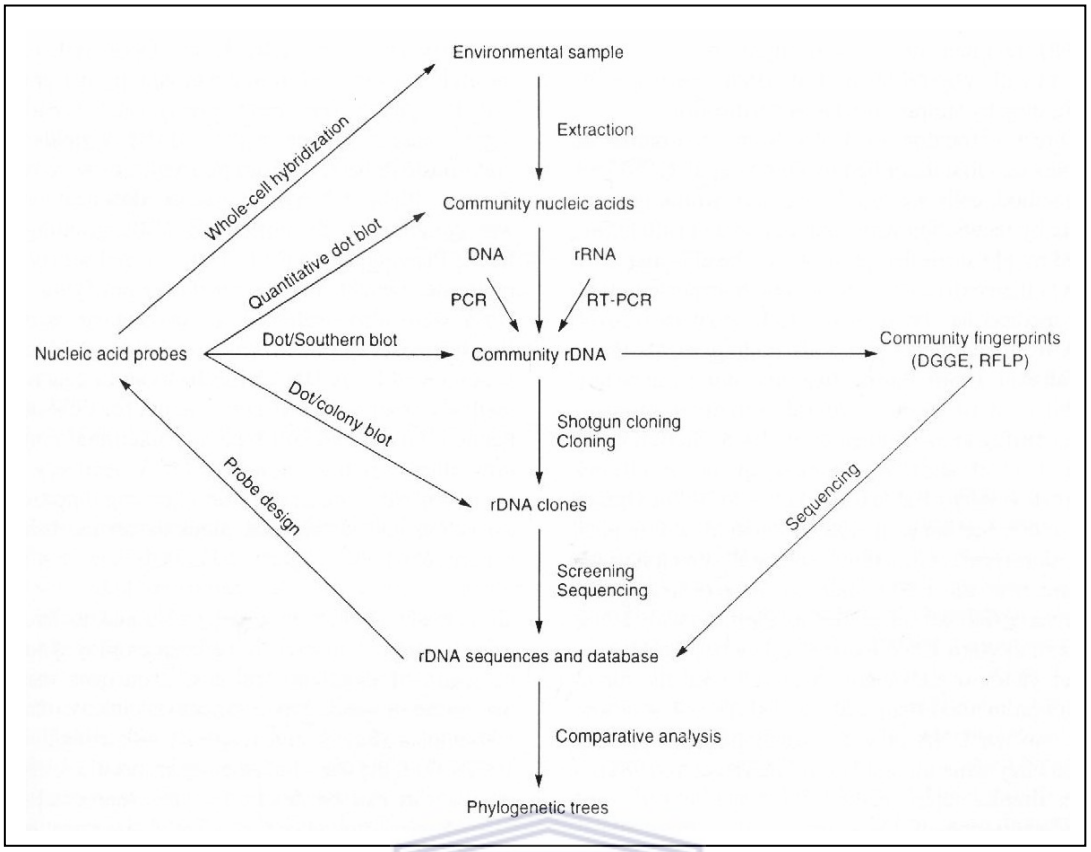

Fig 1.3: Procedures for phylogenetic analysis of microbial populations in soils (from Pace, 1996).

1.4.1 Sampling strategies

One of the most important, yet often ignored aspects of experimental design is the development of an appropriate sampling strategy (Wintzingerode *et al.*, 1997). Soil is a heterogeneous medium composed of many different microhabitats, and different micro-organisms may be found along transect sites only a few millimetres apart (Grundman and Debouzie, 2000). Using inappropriate sample sizes may result in data that are not representative of community structure.

Ellingsøe and Johnsen (2002) obtained significantly variable genetic structures of bacterial communities when using replicates of small samples (0.01g and 0.1g). These effects were not apparent when using replicates of larger samples (1g and 10g) and

Ranjard *et al.* (2003) proposes that samples of at least 1g are necessary to accurately assess fungal diversity.

1.4.2 DNA extraction

The first step in molecular analysis involves the extraction of nucleic acids (DNA and/or RNA) from the sample. DNA extracted from soil must be of a high molecular weight, be representative of the microbial community in the sample, and be pure enough (i.e., be free of inhibitory humic acids and proteins) to be used for additional analysis (such as PCR) (Yeates *et al.*, 1998). Most protocols published detail variations on two main approaches. The first involves the isolation of cells and subsequent lysis and purification (Balkwill *et al.*, 1975; Faegri *et al.*, 1977; Torsvik and Goksøyr, 1978), while the second involves the direct lysis of cells in the soil, after which the DNA is purified.

Higher yields of DNA are usually obtained using direct extraction (Steffan *et al.*, 1988), although more inhibitory compounds are often present in the final product (Ogram *et al.*, 1987; Steffan *et al.*, 1988; Tebbe *et al.*, 1993). Many procedures have been published for direct extraction (e.g. Ogram *et al.*, 1987; Miller *et al.*, 1999); but usually involve three main processes: physical disruption, chemical lysis, and enzymatic lysis.

Physical disruption may involve freeze-thawing, bead mill homogenization, ultrasonication or grinding under liquid nitrogen. Bead beating is the most common procedure as it is inexpensive, yields high concentrations of DNA, and is more likely

than other methods to lyse all micro-organisms effectively (Ogram *et al.*, 1987; Yeates *et al.*, 1998.)

Chemical lysis may use detergents such as sodium dodecyl sulfate (Ogram *et al.*, 1987) or CTAB. Enzymatic lysis is also used in some protocols, involving a variety of reagents such a lysozyme, proteinase K, achromopeptidase or pronase E.

One of the key problems involved in DNA extraction from soils is the presence of organic matter such as humic acids and proteins that inhibit PCR processes and other enzymatic manipulations of DNA (Holben, 1988). DNA may be purified by agarose gel electrophoresis, silica-based binding, ammonium acetate precipitation or Sephadex G-200 gel filtration. The simplest method, however, is the addition of polyvinylpolypyrollidone (PVPP) to the extracted acids. This removes most of the inhibitors by selective adsorption (Holben *et al.*, 1988).

Two simple and rapid direct extraction techniques have been developed by Zhou (1996) and Miller (1999). Zhou's method uses lysis with a high salt buffer and incubation for 2-3 hours in sodium dodecyl sulphate, and is suitable for a wide range of soil types. Miller's method involves bead-beating in a phosphate and SDS buffer, followed by precipitation in ammonium acetate and isopropanol is conducted. Both of these methods yield high molecular weight DNA of sufficient purity for PCR.

1.4.3 PCR and primer design

The polymerase chain reaction (Mullis *et al.*, 1986; Mullis and Faloona, 1987; Mullis, 1990) is routinely used to detect genes in microbial populations, and allows for the amplification of genes from viable, non-viable and dormant cells.

PCR permits the rapid, exponential amplification of DNA sequences using three processes repeated cyclically: denaturation, annealing and extension.

Selecting appropriate primers for PCR is crucial for valid and reliable results. Primers are designed to be complementary to the specific region of the DNA to be amplified and must be at least 70% homologous to the template in order for annealing and elongation to be successful (Steyn and Holland, 1993). "Universal" primers such as those used to amplify the conserved 16S rRNA gene are often used in phylogenetic studies, as DNA from many species is amplifiable. However, the sequence data available for designing primers is unlikely to reflect the natural diversity of organisms (Baker *et al.*, 2003).

1.4.4 DGGE

Denaturing gradient gel electrophoresis has recently been established as a useful tool for analysing microbial communities from 16S rRNA gene fragments (Muyzer *et al.*, 1993). This method allows small differences in gene sequence to be detected by exploiting the fact that different nucleotide sequences have slightly different melting points. The melting points are influenced by hydrogen bonds between complimentary

base pairs, as well as the attractive forces between neighbouring bases on the same strand ("stacking"). In general, sequences with a high proportion of GC pairs melt at higher temperatures than sequences with a high proportion of AT pairs.

DGGE involves electrophoresis of a DNA product (usually a PCR product) on a polyacrylamide gel through a gradient of increasing chemical denaturant (such as formamide and urea). As the product proceeds through the denaturant, portions of the lower melting strands dissociate and become separated from the higher melting strands by their increased mobility (due to their smaller size). The chemical denaturant gradient may be replaced by a gradient of increasing temperature in temperature gradient gel electrophoresis (TGGE).

Recently, DGGE has been used to successfully assess the diversity of functional groups of organisms. A popular method for examining diazotroph diversity uses PCR amplification of *nifH* genes followed by DGGE (e.g. Rosado *et al.*, 1998; Piceno *et al.*, 1999; Bagwell *et al*., 2000; Piceno and Lovell, 2000; Lovell *et al.*, 2001; Diallo *et al.*, 2004).

1.5 Objectives of study

To use culture-independent molecular methods (including PCR, DGGE and clone libraries) to investigate the diversity of nitrogen-fixing *nifH* genes in the soil of different ecological habitats on the sub-Antarctic Marion Island. As there is a paucity of information available concerning the microbiota of the island (excepting that obtained by classical techniques), this study will assist in answering a small part of some fundamental microbiological questions including:

- What is the qualitative and quantitative distribution of nitrogen-fixing micro-organisms in the soil of Marion Island?
- What factors are responsible for the distribution of diazotrophs in the soil of Marion Island, and does their distribution vary between different ecological habitats or altitudinal transects?
- Can new metagenomic strategies be used to access genes and gene products in the "uncultured' complement of soil micro-organisms?

CHAPTER 2 - MATERIALS AND METHODS

This chapter details the main methods and materials used for the experiments conducted during this study. For chemical names and constituents of buffers and medium, please refer to the Glossary.

2.1 Sampling

Sample sites representative of the 25 habitats described by Smith and Steenkamp (2001) were selected based on qualitative vegetation and soil properties. Site characteristics, including GPS positions (elevation, longitude and latitude), vegetation descriptions and potential biotic influences were recorded. Digital photographs of sample sites were taken. Samples were also collected at several points along 3 different established altitudinal transects (Table 3.2). These sites were identified using established stakes and GPS positions. In addition, samples were obtained from ecologically altered sites, including several abandoned albatross nests, penguin colonies, and habitats invaded by non-indigenous plant species.

Table 2.1: Some properties of the ecological habitats (from Smith and Steenkamp, 2001). Samples were collected from all habitats excepting those shaded grey, and locations of samples sites are given.

Habitat type		General	Dominant vegetation	Soil Chemistry	Location	Elevation	S	E
Coastal Herbfield	1.1	Shore-zone	Dicots, *Crassula moschata* and *Cotula plumosa*	Fibrous peat with very high Na and Mg	Archway	22m	46˚53.892'	37˚53.557'
Coastal Fellfield	1.2	Shore-zone, exposed	*Dicots, C. moschata and Azorella selago*	Fibrous peat, volcanic ash Very high Na and Mg	Archway	20m	46˚53.895'	37˚53.560'
Xeric Fellfield	2.1	Exposed, usually >100m altitude	*Sparse, A. selago, cushion and ball-forming moss, lichens*	Volcanic ash and rock. Basic and dry, high density, high Ca and Mg, low C and N, dry	Tafelberg	362m	46˚53.750'	37˚40.12'
Mesic Fellfield	2.2	Exposed, usually <100m altitude	*A. selago, Blechnum penna-marina, Agrostic magellanica, cushion and ball-forming moss, lichens*	Scoria with same characteristics as 2.1 but more moist	Scoria cone near Swartzkop	100m	46˚55.481'	37˚35.912'
Open Fernbrake	3.1	Succession between 2.2 and 2.3	*A. selago, B.penna marina, Ag. magellanica, Acaena magellanica.*	More organic and moist than 2.2				
Closed Fernbrake	3.2	Main habitat on slopes	*B. penna-marina, Ag. magellanica*	Deep, organic, moist, high organic C and P		23m	46˚52.877'	37˚51.927'
Mesic Fernbrake	3.3	Occurs on wetter, less steep slopes than 3.1	*B. penna-marina, Ag. magellanica*	Deep, highly organic, wet with high organic C, low organic P.	Archway	22m	46˚53.892'	37˚53.557'

Dwarf-Shrub Fernbrake	3.4	More sheltered and wet than 3.2	*Ac. magellanica, B. penna-marina, mosses*	Deep and organic similar to 3.3			
Slope Drainage Lines	3.5	Drainage lines on slopes and stream banks	*Ac. magellanica*, mosses	Deep, organic. with a higher pH and Ca/ Mg and lower C than 3.1 to 3.4			
Spring and Flush Habitat	3.6	Water tracks and springs	*Ac. magellanica*, mosses	A peat similar to 3.5 but with a higher pH		32m	46˚55.644 37˚35.66'
Coastal Tussock Grassland	4.1	Heavy influence of seabirds and seals	*P.cookii, C. plumosa, Callitriche antarctica, Montia fontana, Poa annua*	Compact acid organic peat with very high total N, inorganic N and P.	Archway	32m	46˚33.866'37˚53.485'
Inland Tussock	4.2	Inland slopes influenced by burrowing petrels	*P. cookii*, *Ac. magellanica, M. fontana, B. penna-marina*	Compact peat more basic than 4.1, high inorganic N and P			
Pedestalled Tussock Grassland	4.3		*P. cookii on tall peat pedestals, C. antarctica*	Compact peat Pedestals as in 4.1, mud as in 5.2	Archway	30m	46˚53.868'37˚53.421'
Cotula Herbfield	5.1	Heavy influence by seabirds and seals	*C. plumosa, P. cookii*	Compact peat high in total and inorganic N and P, high exchange of Na and Mg		11m	46˚53.018'37˚52.151
Biotic Mud	5.2	Close proximity to seal wallows and penguin rookeries	*C. Antarctica, M. fontana*	Very wet, organic mud, vey high inorganic N and P	Near Trypot Beach	2m	46˚53.131'37˚52.092'

Biotic Lawn	5.3	As 5.2	*P. annua, P. cookii, C. plumosa, C. antarctica, M. fontana*	Thin fibrous peat which is less organic and drier than 5.2, with higher Ca and Mg.		22m	46˚53.055'37˚52.044'
Dry Mire	6.1		*Ag. magellanica, B. penna-marina,* bryophytes	Dry oligotropic peat, drier and less organic with higher Ca and Mg than other mires	Ship's Cove	34m	46˚52.661'37˚51.567'
Mesic Mire	6.2	Boggy grassland	Graminoids, bryophytes	Wet, organic, dystrophic peat	Ship's Cove	64m	46˚51.532'37˚50.644'
Wet Mire	6.3	Bog	Bryophytes	Very wet, organic, waterlogged peat	Ship's Cove	91m	46˚51.616'37˚50.818
Mire Drainage	6.4	Bog in water tracks	Bryophytes	Waterlogged peat with very low inorganic P, highest pH for mires.	Ship's Cove	46m	46˚51.975'37˚51.037'
Biotic Mire	6.5	Influenced by seal and seabird manure	*Clasomatocolea vermicularis, Ag. magellanica, P.cookii*	Very wet peat with the highest recorded inorganic N and P for mires	Lake Edge near Swartzkop	148m	46˚55.665'37˚35.665'
Saline Mire	6.5	Influenced by salt-spray	*C. vermicularis, C. moschata, Ag. magellanica*	Very wet peat with high Na and Mg exchange, high inorganic N and P			
Polar Desert	7	Above 500m altitude	Cushion and ball-forming mosses, lichens	Rock and ash – no soil			

Table 2.2: Site information for transect sites.

			GPS Readings	
Sample	**Location**	**Elevation**	**S**	**E**
SKL	Swartzkop	218m	46˚52.574'	37˚36.481'
SKM	Swartzkop	415m	46˚55.795'	37˚37.222
SKH	Swartzkop	566m	46˚56.203'	37˚37.52'
MPL	Mixed Pickle	210m	46˚52.574'	37˚38.548'
MPM	Mixed Pickle	375m	46˚53.211'	37˚38.860'
MPH	Mixed Pickle	597m	46˚53.83'	37˚39.266'
TBL	Tafelberg	176m	46˚53.676'	37˚49.649'
TBM	Tafelberg	360m	46˚53.267'	37˚48.116'
TBH	Tafelberg	576m	46˚52.750'	37˚47.290'
SRL	Stoney Ridge	163m	46˚54.928'	37˚51.440'
SRM	Stoney Ridge	366m	46˚54.607'	37˚49.054'
SRH	Stoney Ridge	593m	46˚54.060'	37˚47.971'

Six 50g topsoil (1-10cm deep) samples from each site were collected using either sterile spatulas or wooden tongue depressors and placed in sterile 50ml plastic containers. Samples were homogenised by mixing in sterile plastic bags in the laboratory, and redistributed into sample containers. All samples were stored at -20ºC until processing.

2.2 DNA extraction

Several genomic DNA extraction methods were tested in order to determine the most suitable (in terms of yield and quality for PCR) for the particular soil types involved.

2.2.1 Basic 'Miller method'

Extractions based on the protocol of Miller (1999) used the following method: 0.5g of soil was placed in a 2ml screw-cap vial with 1g quartz and 300µl each of phosphate buffer (100mM NaH_2PO_4); SDS lysis buffer (100mM NaCl, 500mM Tris-HCl pH 8, 10% SDS) and chloroform-isoamyl alcohol (24:1). Vials were agitated for 1.5 min in a minishaker (IKA) and centrifuged for 5 min (13 000 rpm). 7M ammonium acetate was added to the supernatant to a final concentration of 2.5M and centrifuged for 5 min (13 000 rpm). The supernatant was transferred to a new tube and DNA was precipitated at room temperature for 15 min with 0.6 volumes isopropanol. After a further centrifugation of 5 min (13 000 rpm), the supernatant was removed and the pellet washed with 70% ethanol, centrifuged for 5 min, air-dried and resuspended in 20µl sterile water.

Modifications to the above protocol are detailed below:

2.2.2 Miller + PVPP

0.5g of PVPP was added to the soil samples before lysis.

2.2.3 Miller + Phenol-Chloroform

A phenol-chloroform extraction was used instead of an ammonium acetate precipitation. An equal volume of phenol-chloroform-isoamyl (24:1:1) was added, mixed and the tube centrifuged for 2 min at 13 000 rpm. An equal volume of chloroform isoamyl (24:1:1) was added to the aqueous phase, after which an equal

volume of chloroform was added and the mixture centrifuged for 5 min at 10 000rpm. 10% [wt/vol] sodium acetate was added. After mixing, 2.5 volumes of 100% ethanol were added and the tube incubated at -80°C overnight for precipitation. After a 20 min centrifugation at 13 000 rpm, the pellet was washed twice with 70% ethanol (5 min centrifuge, 13 000 rpm) and resuspended in 10µl sterile H_2O.

2.2.4 Miller + PVPP + Phenol Chloroform

0.5g PVPP was added to soil prior to lysis, after which a phenol-chloroform extraction was performed as detailed above.

2.2.5 FastPrep kit

The FastPrep system (BIO 101 – Vista, CA), based on a bead-beating method, was used to extract DNA according to the manufacturer's instructions.

2.2.6 Zhou method

DNA was also extracted according to Zhou (1996). 0.5g soil samples were placed in sterile 2ml tubes. 675µl of extraction buffer (0.1% CTAB, 100mM Tris-HCl pH 8, 100mM NaH_2PO_4, 100mM EDTA, 1.5M NaCl) and 2.5µl Protease K (20mg/ml) were added, and tubes were shaken horizontally at 37°C for 30 min (225 rpm). 75µl 20% SDS was added before a 2 hour incubation at 65°C, during which samples were gently mixed at intervals of 20 min. After a centrifugation (13 000 rpm), the supernatant was transferred to a new tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was mixed, centrifuged for 10 min (13 000 rpm) and the supernatant washed three times with an equal volume of chloroform (each centrifuged for 10 min at 13

000 rpm). DNA was precipitated with 0.6 volumes isopropanol for 20 min at room temperature, the pellet washed twice with 70% ethanol, air dried and resuspended in 50µl sterile water.

All DNA extracts were confirmed by electrophoresis (100V, 20 min) using 1% TBE agarose gels containing ethidium bromide in 0.5×TBE buffer, and were visualized with a UV transilluminator. DNA concentration was measured by comparison with lambda *Pst*I markers, and/or with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, USA).

2.3 DNA purification

2.3.1 PVPP

PVPP spin columns were constructed using a P20 tip (end of tip cut) placed inside a 0.6 ml PCR tube (with end and lid cut) enclosed in a 1.5 ml tube (lid removed). A solution of 50% PVPP in TE was deposited inside the filter tip with 50µl DNA. Columns were centrifuged for 20 min at 3300 rpm, DNA was collected from the base of the tubes and the process was repeated until a clear DNA solution was recovered (typically twice, but up to 4 times for very dark samples).

2.3.2 Gel purification methods

Several gel purification methods were used, including silica binding and commercial kits.

2.3.3 Silica binding (based on Boyle and Lew, 1995)

DNA bands were excised from 1% agarose gels and incubated at 65ºC in 3 volumes 6M NaI until the agarose dissolved. 10µl of fine silica in suspension was added, the solution vortexed and allowed to bind on ice for 20 min. After a 30 sec centrifugation, the supernatant was removed and washed 4 times with 500µl New Wash (50mM NaCl, 10mM Tris-HCl pH 8, 2.5mM EDTA and 50% ethanol). The pellet was air-dried and eluted in 10µl sterile water at 55ºC for 10 min. The supernatant was recovered after a centrifugation of 2 min.

The Fermentas DNA extraction kit #K0513, GeneClean III, the Talent Cleanmix kit and GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences product code 27-9602-01) were used according to the manufacturer's directions.

2.2.4 Sephacryl

Mini spin columns were constructed as described above for PVPP purification. 200µl of Sephacryl S-500 HR suspension (Amersham Biosciences 17-0613-10) was placed inside the P20 tip, centrifuged for 2 min (3000 rpm), the elute was discarded and another 200µl loaded and recentrifuged. The column was washed three times with 150µl TE, centrifuged firstly for 2 min at 3000 rpm, then finally for 3 min at 3500 rpm. The column was placed in a fresh 1.5ml tube, DNA was added, incubated on the column for 3 min and finally eluted by centrifugation (2 min, 3000 rpm).

2.4 PCR

The universal 16S bacterial primers E9F 5'-GAGTTTGATCCTGGCTCAG-3' (Hansen *et al.*, 1998) and 1510R 5'GGTTACCTTGTTACGACTT-3' (Reysenbach and Pace, 1995) were used to amplify the 16S rRNA gene to assess DNA purity. The 50µl reactions contained 0.5µM of each primer, 1×PCR amplification buffer (100mM Tris-HCl pH 8.3, 15mM $MgCl_2$, 0.01% [w/v] gelatin), 200µM dNTPs, 2µl template DNA and 2.5U *Taq* DNA polymerase. After an initial denaturation step of 3 min at 94°C, 30 PCR cycles were run with the program: 1 min denaturation 94°C, 1 min annealing at 55°C and 2 min extension at 72°C in a Techne thermocycler (Techne, Cambridge). A 'hotstart' technique was used for all experiments, where all reactions were set up on ice, transferred directly from ice to the thermocycler at 94°C and *Taq* DNA polymerase was added after the initial denaturation. PCR products (5-20µl) were separated using electrophoresis on a 1% TBE agarose gel with ethidium bromide (100V, 30 min) and visualised using a UV transilluminator. Series of dilutions of the template DNA (1/10; 1/25; 1/50; 1/100; 1/200; 1/500; 1/1000) were tested in order to establish the optimal concentration.

Universal *nifH* primers FGpf19 5'-TACGGCAARGGTGGNATHG-3' and FGpH273 5'-CTCCGEGCCRCCNGAYTC-3' (Simonet *et al.*, 1991) were used to amplify *nifH* genes from extracted genomic DNA. The 50µl reactions in 0.2ml tubes contained 0.5µM of each primer, 1×PCR amplification buffer (100mM Tris-HCl pH 8.3, 15mM $MgCl_2$, 0.01% [wt/vol] gelatin), 200µM dNTPs, 100ng template DNA and 2.5U *Taq* DNA polymerase. To establish optimal conditions, in some experiments, $MgCl_2$ concentration was varied over the range 1.5 to 6mM, and BSA (bovine serum

albumin) was also added at concentrations ranging from 50ng/µl – 400ng/µl. PCR conditions were as follows: 3 min initial denaturation at 94°C, followed by 40 cycles of 1 min denaturation 94°C, 1 min annealing at 50°C and 1 min extension at 72°C. Initial experiments tested programs from 30-40 cycles, with annealing temperatures ranging from 45-65°C. Experiments varying annealing temperature were conducted on a gradient PCR machine, while all other amplifications were carried out using a Techne thermocycler (Techne, Cambridge).

Amplified products (20µl) were viewed on a UV transilluminator after electrophoresis (100V, 45 min) on a 2.5% TBE agarose gel with ethidium bromide.

For optimisation experiments, DNA extracted from the root nodules of clover and bean plants was used as the template, and also served as a control for remaining experiments. This DNA was extracted using the protocol of Huget *et al.* (2003). Briefly, approximately 40 nodules were washed with distilled water and crushed with a mortar and pestle into a thick paste, which was then added to 300µl of filter sterilized extraction buffer (100mM Tris pH 8, 20mM EDTA, 1.4M NaCl, 1% [wt/vol] polyvinyl polypyrolidone, 2% [wt/vol], cetyltrimethyl ammonium bromide) in a sterile 1.5ml tube. The homogenate was incubated for 60 min at 65°C, after which it was centrifuged for 10 min at 7000g and the supernatant extracted with an equal volume of chloroform-isoamyl (24:1). After a 20 min centrifuge at 13 000g, DNA from the aqueous phase was precipitated overnight at -20°C. DNA was centrifuged for 30 min at 13 000g, the pellet washed twice with 300µl 70% ethanol (5 min centrifuge at 13 000g) and suspended in 10µl sterile water. This DNA was

checked for purity by PCR with universal bacterial primers E9F and 1510R, and was used directly in all experiments without further purification.

2.5 Denaturing Gradient Gel Electrophoresis

2.5.1 Primer design

The computer program RNAFold was used to predict melting domains of the primers FGph19 and FG pl273 with a variety of 40bp GC-clamps attached to either end of either primer.

2.5.2 PCR

The primers *nifH* primers FGpf19 5'-TACGGCAARGGTGGNATHG-3' and FGpH273 5'-CTCCGEGCCRCCNGAYTC-3' (Simonet *et al*., 1991) were used to amplify *nifH* genes from extracted genomic DNA. The 50µl reactions in 0.2ml tubes contained 0.5µM of each primer, 1×PCR amplification buffer (100mM Tris-HCl pH 8.3, 15mM $MgCl_2$, 0.01% [wt/vol] gelatin), 200µM dNTPs, 100ng template DNA and 2.5U *Taq* DNA polymerase. PCR conditions were as follows: 3 min initial denaturation at 94°C, followed by 40 cycles of 1 min denaturation 94°C, 1 min annealing at 50°C and 1 min extension at 72°C, followed by a final extension of 10 min at 72ºC.

2.5.3 DGGE

DGGE was performed using the Bio-Rad Vertical Electrophoresis system (Bio-Rad, Hercules, USA), essentially as described by Muyzer *et al*., (1993). Two 50µl PCR

samples were pooled and column purified with the Amersham Bioscience GFX PCR DNA and Gel Band Purification kit. The samples were concentrated by using a final elution volume of 30µl, which was loaded directly onto 9% polyacrylamide gels in 0.5×TAE buffer. Denaturant gradients varied in the range of 10% -70% urea and formamide. APS and TEMED were added to the gels as a catalyst for polymerization. Acrylamide gels were cast with a gradient maker and allowed to polymerize for 2 h before loading of samples. Electrophoresis was performed at a constant voltage (between 100 and 200V) for 12-23 h at a temperature of 60°C. Gels were stained for 20 min in TAE buffer containing 0.5µg/ml ethidium bromide, destained in TAE buffer for 15 min, and photographed on a UV transilluminator (AlphaImager® Imaging System**,** AlphaInnotech)

2.6 Clone Libraries of *nifH* genes

Rubidium chloride competent *E. coli* cells were prepared by Dr. Bjarne Faurholm according to the following protocol: 5-10ml of *E. coli* culture was grown overnight in LB and was used to inoculate 500ml autoclaved LB medium. After growing at 37ºC to an OD_{595} of 0.5, the culture was incubated on ice for 15 min and transferred to two autoclaved 250ml centrifuge bottles and centrifuged for 10 min (4500 rpm) in a GSA rotor. The supernatant was removed and pelleted cells suspended in 30ml total of Tfb I. Suspensions were combined and incubated on ice for 15 min, centrifuged for 5 min at 4000 rpm and resuspended in 6ml Tfb II on ice. Aliquots were placed in crushed dry ice and stored at –80ºC until required for transformations.

LB ampicillin plates were prepared by adding 15g agar to 1 litre of autoclaved LB medium, microwaved until the agar dissolved and incubated at 55ºC for 3 h.

Ampicillin was added to a final concentration of 100µg/ml, and approximately 25ml was poured into petri plates. Plates were prepared 3-4 days prior to transformation and stored at 4°C.

2 h before transformation, the plates were inoculated with 40µl X-gal (20mg/ml in N,N-dimethylformamide) and 40µl IPTG (0.1M), and incubated at 37°C for at least 1.5 h before plating.

Template DNA was purified from *nifH* PCR reactions before ligation. Three reactions (150µl) were loaded into one well of a 2.5% TBE agarose gel, and electorophoresed at 100V for 45 min, after which the product band was excised from the gel and purified using the GFX PCR DNA and Gel Band Purification kit.

Overnight DNA ligations (at room temperature) were performed using the Fermentas InsT/Aclone™ PCR Product Cloning Kit (#K1214), in accordance with the manufacturer's instructions.

Ligated DNA was transformed into competent *E. coli* cells, using a chemical transformation procedure as follows: competent cells were thawed on ice, inoculated with 5µl of ligation mixture and incubated on ice for 30 min, after which they were heat shocked in a 42°C water bath for 45 sec. Cells were then incubated on ice for a further 5 min. 900µl of prewarmed (37°C) LB was added to the tube and cells were then streak plated onto LB containing 100µM ampicillin. Plates were incubated at 37ºC overnight.

Libraries were screened by adding very small quantities of randomly selected white colonies to 25µl PCR reactions containing 0.5µM each of primer M13F and M13R, 1×PCR amplification buffer (100mM Tris-HCl pH 8.3, 15mM $MgCl_2$, 0.01% [w/v] gelatin), 200µM dNTPs, 2µl template DNA and 2.5U *Taq* DNA polymerase. After an initial denaturation step of 3 min at 94°C, 30 PCR cycles were run with the program: 1 min denaturation 94°C, 1 min annealing at 64°C and 1 min extension at 72°C in a Techne thermocycler (Techne, Cambridge). PCR products were analysed on a 2.5% agarose gel. Those fragments of the expected size for a product containing a *nifH* insert (450bp) were diluted (1:1000) in sterile water, and 2µl was used as a template in a further PCR reaction using *nifH* primers FGpf19 and FGpH273 and the conditions described above. Products of 254bp or similar were subjected to RFLP analysis.

2.7 RFLP analysis

1µl of M13 PCR product was digested in a 20µl restriction enzyme mixture containing 16.5µl sterile water, 2µl of 10× restriction enzyme buffer and 0.5µl restriction enzyme. Each product was digested separately with both *Hpa*II (in buffer Tango) and *Hae*III (in buffer R) (Fermentas) overnight at 37ºC. RFLPs were analysed on 3% agarose gels (10µl loaded and run at 80-100V for 2 h). Restriction patterns were used to differentiate between clones, and single clones representing different patterns were sequenced (University of Cape Town).

2.8 DNA sequence analysis

Sequences were used to confirm RFLP patterns using theoretical fragmentation with the online tool WatCut. Vector and primer sequences were removed, and inserts were checked for nucleotide alignments against the GeneBank database using NCBI Blast. Sequences were also 6-frame translated into protein sequences (using Vector NTI V.8) and BLAST was used to assess alignments with translated GeneBank database files (using protein-translation query). A neighbour-joining phylogenetic tree was constructed using CLUSTAL W (Thompson *et al.,* 1994) from amino-acid sequences (using the translation frames that produced highest scoring BLAST alignments) of clones and selected nifH sequences obtained from Genebank. Bootstrap values were calculated using CLUSTAL W and were based on 1000 replicates.

CHAPTER 3 – RESULTS AND DISCUSSION

3.1 DNA Extraction

DNA extraction methods vary significantly in efficiency when applied to different soil types (Miller *et al.*, 1999), and it is therefore necessary to optimise protocols for individual soils to ensure high recovery of DNA of sufficient purity for further analysis. High recovery is believed to enhance accuracy of representation of diversity (Miller *et al.,* 1999).

A bead beating method such as that proposed by Miller *et al*. (1999) is assumed to introduce less bias into extractions, as a high concentration of DNA is recovered and lysis is predicted to be equally effective for all micro-organisms (Ogram *et al.*, 1987; Yeates *et al.*, 1998). This method was used in all DNA extraction optimisation experiments, with modifications as detailed in Chapter 2 and in comparison with a commercially available kit (Bio 101 Fast Prep).

All modifications gave a yield of high-molecular weight DNA in a similar fragment range (Fig. 3.1), which vary somewhat in concentration. Concentration readings on a spectrophotometer imply that the Miller+PVPP and the FastPrep kits yield DNA of a considerably lower concentration (Table 3.3). However, these samples were lighter in

colour than those obtained using the other methods (which produced very dark brown to black samples). This may have influenced spectrophotometer readings.

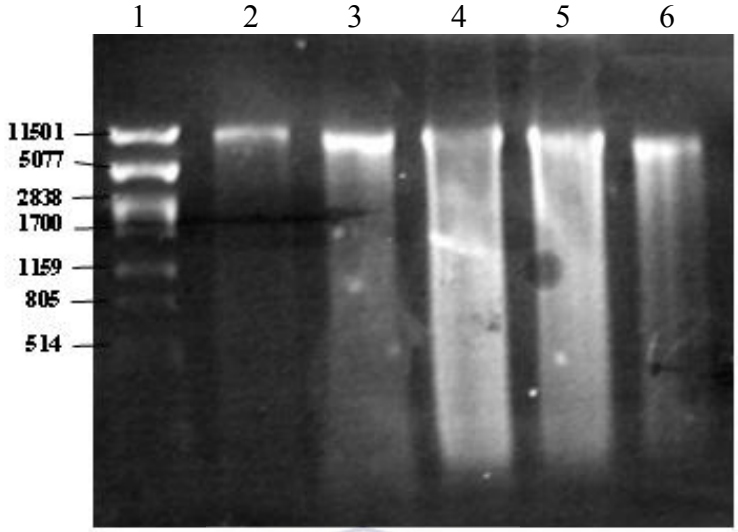

Fig. 3.1: Example of DNA extracts from sample C (Coastal Fellfield 2.2). Lane 1 is *Pst*I lambda marker (values indicate size of fragments in bp); Lane 2 = extract using the Miller method; Lane 3 = extract using the Miller method with 0.5g PVPP added before lysis; Lane 4 = extract using Miller method with a phenol-chloroform extraction, Lane 5 = extract using Miller method with PVPP and phenol-chloroform extraction, Lane 6 = extract using Bio101 Fastprep kit.

Contrary to the results obtained by Miller *et al.* (1999), addition of a phenol-chloroform step did not improve yields. Instead, DNA bands were less distinct and exhibited more smearing (shearing) than those obtained by other methods (Fig. 3.1).

3.2 DNA Purification

Crude DNA extracts were rarely of sufficient purity to permit successful PCR amplification, and it was necessary to dilute all samples up to 1/1000 before any amplification was possible. Even with these dilutions, amplification bands were faint or exhibited considerable smearing.

Difficulties in obtaining PCR products during and after optimisation experiments were attributed to inhibitory substances in DNA extracts, as positive controls in these experiments amplified readily and extracts were the characteristic dark colour indicative of humic acid contamination. 260/230 and 260/280 ratios measured on the NanoDrop spectrophotometer also suggested the presence of these contaminants, as humic acids exhibit absorbance at 230nm and 260nm. A low 260/230 ratio (<2) indicates their presence in a sample, while a low 260/280 ratio indicates protein contamination (Yeates *et al.*, 1998).

Inhibitory substances are very commonly reported in genomic DNA extracted from soil (Wilson, 1997) and include humic and fulvic acids (Torsvik, 1980; Holben *et al.*, 1988; Steffan *et al.*, 1988; Tsai and Olson, 1991); proteins and other inorganic and organic compounds. Humic and fulvic acids are complex polyphenolic compounds produced by the decomposition of plants, animals and microbes, are ubiquitous in soil and are somewhat resistant to biological and chemical degradation (Schnitzer, 1991). Their three-dimensional chemical structures are responsible for their capacity to bind many other compounds, including most natural organic compounds (Stevenson, 1976), and as they are similar to DNA in terms of size and charge properties (Holben, 1994), co-extraction with DNA may be almost unavoidable. Humic acids can interfere with enzymatic manipulations of DNA, including restriction enzyme analysis (Holben *et al*., 1988; Tsai and Olson, 1991, Theron and Cloete, 2000) and are also reported to inhibit *Taq* polymerase (Tsai and Olson, 1992; Tebbe and Vahjen, 1993; Zhou *et al.*, 1996) to a

significant degree. As little as 1µl of humic acid extract may cause significant inhibition (Tsai and Olson, 1992).

Direct lysis DNA extraction procedures such as those used in this study have been reported to result in increased co-extracted PCR inhibitory substances in comparison to cell extraction (Ogram *et al.*, 1987; Tsai and Olson, 1992; Tebbe and Vahjen, 1993). However, their increased comparative yield and the fact that they are generally regarded to introduce less bias in analysis of microbial diversity (Holben *et al.*, 1988; Steffan *et al.*, 1988) contributed to their selection as an appropriate method for this study.

All purification methods excepting gel purification kits improved the success of PCR reactions as compared with crude extracts (Figs 3.3-3.8), despite considerably reducing DNA concentration (Fig 3.2, Table 3.3). Interestingly, their effects on DNA concentrations varied slightly depending on the extraction method used, and the combination purification using PVPP and Sephacryl permitted more DNA to be retained than Sephacryl alone. This might be due to PVPP binding large humic acids initially so that competition for elution space in the matrix was limited to DNA only. In the process using Sephacryl alone, large humic acids might be co-eluted with DNA, or even eluted first.

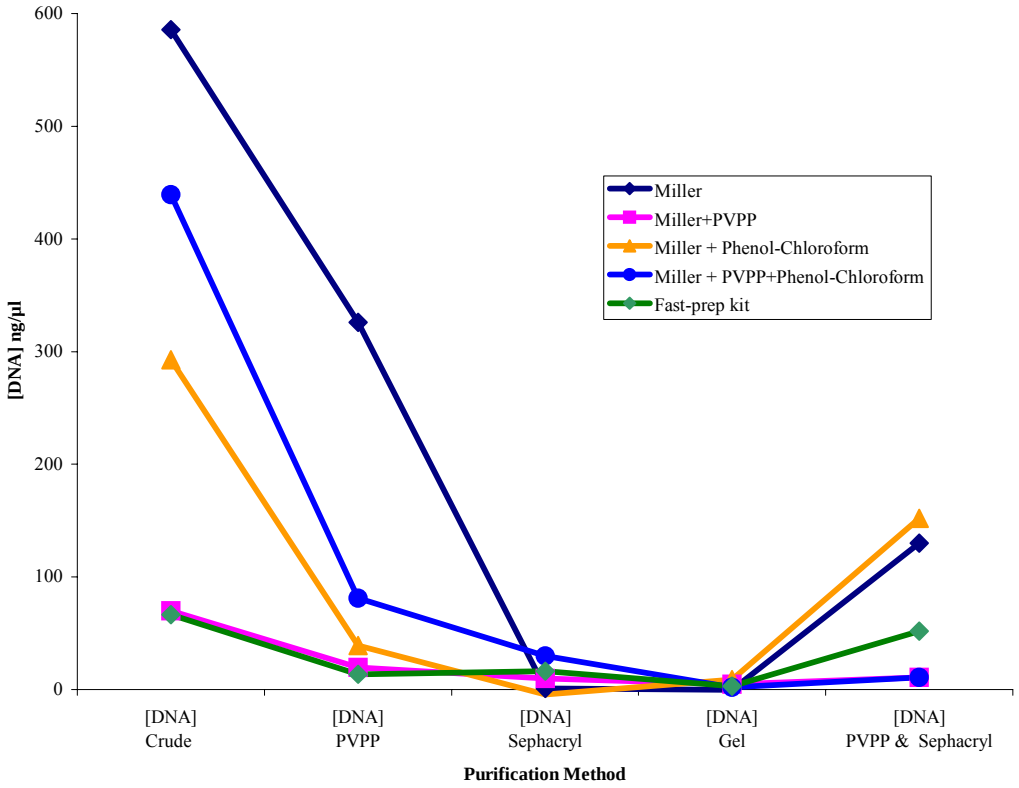

Fig. 3.2: Effect of purification procedures on DNA concentration.

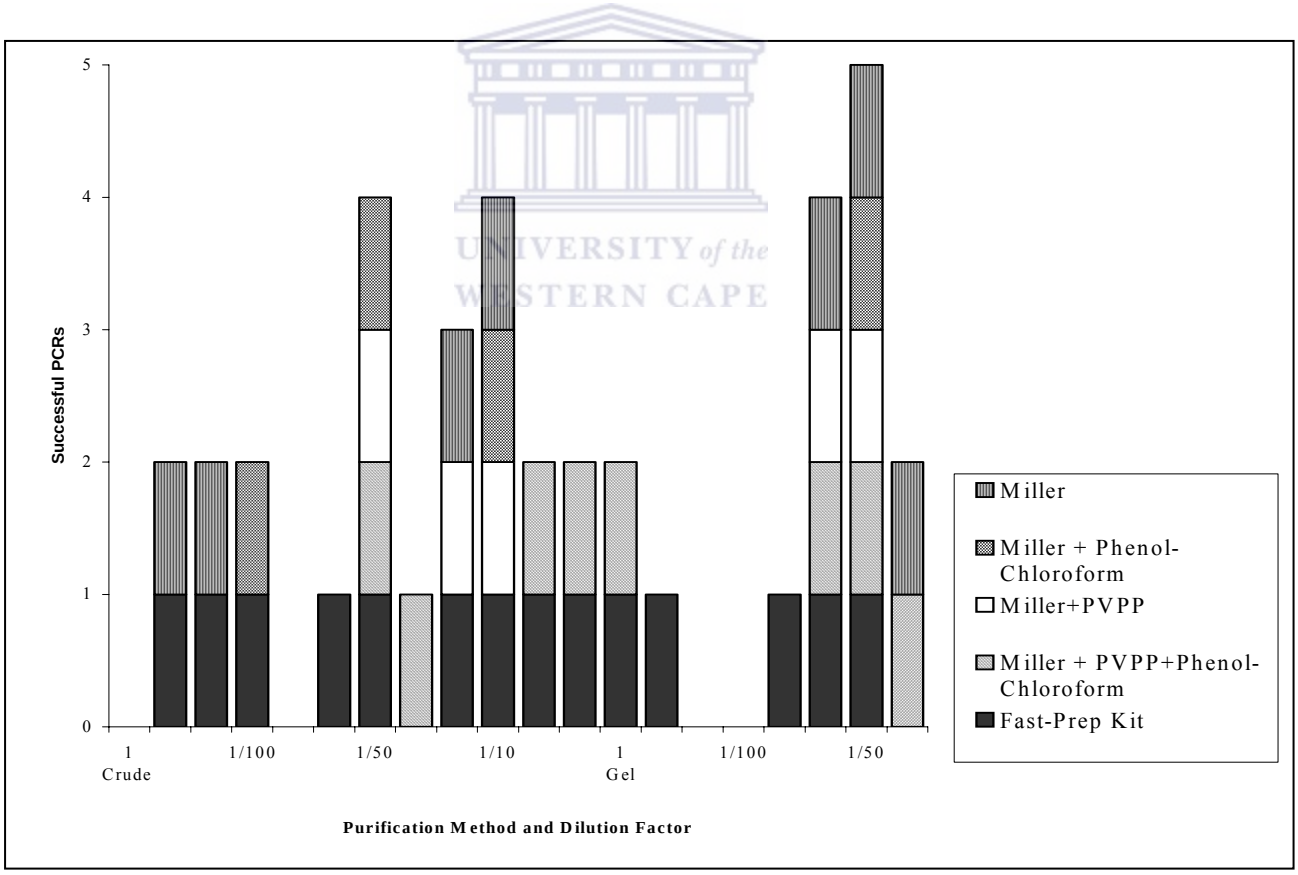

Fig. 3.3: PCR perfomance as a measure of DNA purity. A score of 1 denotes a successful amplification using the extraction method shown in the legend. The purification method is shown on the x-axis.

		Miller	Miller+PVPP	Miller + Phenol-Chloroform	Miller + PVPP+Phenol-Chloroform	Fast-prep kit
Crude	**[DNA] (ng/µl)**	585.60	69.80	292.70	439.40	66.50
	260/280	1.54	1.56	1.53	1.48	1.72
	260/230	1.01	0.94	1.06	0.71	0.09
PVPP	**[DNA] (ng/µl)**	326.00	19.60	39.10	80.9	13.60
	260/280	1.72	1.95	1.68	1.78	1.88
	260/230	1.41	0.40	0.65	0.72	0.35
Sephacryl	**[DNA] (ng/µl)**	0.90	10.00	-4.50	29.9	16.20
	260/280	-1.32	1.63	0.70	1.83	2.13
	260/230	0.03	0.17	-0.13	0.49	0.34
Gel	**[DNA] (ng/µl)**	-0.50	4.80	8.70	2.1	3.20
	260/280	0.69	2.63	1.50	1.79	1.51
	260/230	-0.99	0.38	0.36	0.04	0.12
PVPP & Sepacryl	**[DNA] (ng/µl)**	129.80	10.90	152.00	10.9	51.8
	260/280	1.80	1.67	1.77	1.67	1.86
	260/230	1.29	0.30	1.26	0.30	0.82

Table 3.3: DNA concentrations and spectral properties for DNA extracted and purified using different methods.

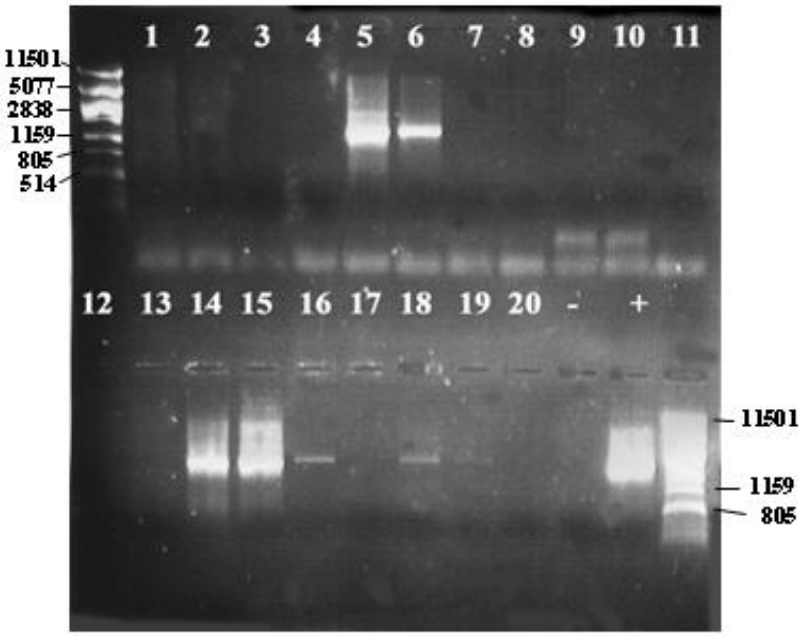

Fig. 3.4: PCR amplifications (universal bacterial primers E9F and 1510R) from DNA extracted using the Miller method and various purification techniques. First lane = *Pst*I lambda marker (values indicate sizes in bp); Lanes 1-4 = PVPP purifications with dilution series (1=1/1, 2 =1/10, 3 = 1/50, 4 = 1/100); Lanes 5-8 = Sephacryl purifications with dilution series (5=1/1, 6=1/10, 7= 1/50, 8=1/100); Lanes 9-12 = Gel purifications (Talent kit) with dilution series (9=1/1, 10=1/10, 11=1/50, 12=1/100); Lanes 13-16 = PVPP + Sephacryl purifications with dilution series (13=1/1, 14=1/10, 15=1/50, 16=1/100); Lanes 17-20 = crude DNA (no purification) with dilution series (17=1/1, 18=1/10, 19=1/50, 20=1/100); - is negative control (sterile water); + is positive control (*E. coli*).

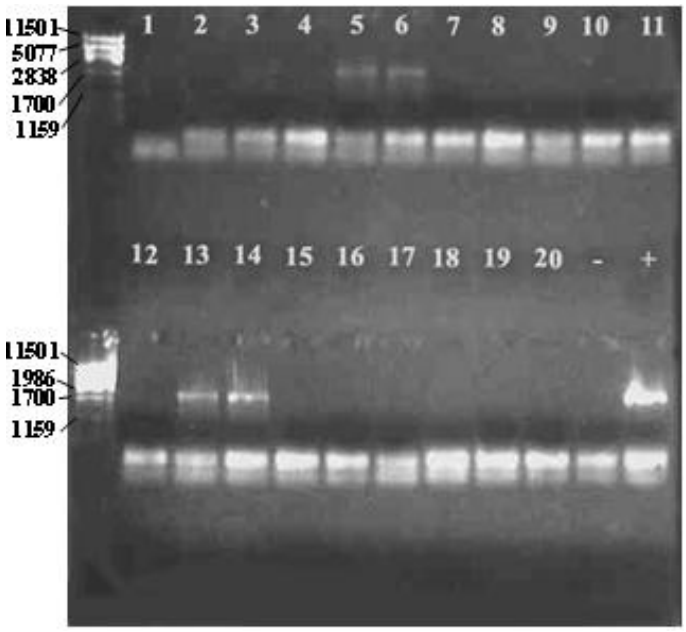

Fig. 3.5: PCR amplifications (universal bacterial primers E9F and 1510R) from DNA extracted using the Miller method with 0.5g PVPP added prior to lysis and various purification techniques. First lane = *Pst*I lambda marker (values indicate sizes in bp); Lanes 1-4 = PVPP purifications with dilution series (1=1/1, 2 =1/10, 3 = 1/50, 4 = 1/100); Lanes 5-8 = Sephacryl purifications with dilution series (5=1/1, 6=1/10, 7= 1/50, 8=1/100); Lanes 9-12 = Gel purifications (Talent kit) with dilution series (9=1/1, 10=1/10, 11=1/50, 12=1/100); Lanes 13-16 = PVPP + Sephacryl purifications with dilution series (13=1/1, 14=1/10, 15=1/50, 16=1/100); Lanes 17-20 = crude DNA (no purification) with dilution series (17=1/1, 18=1/10, 19=1/50, 20=1/100); - is negative control (sterile water); + is positive control (*E. coli*).

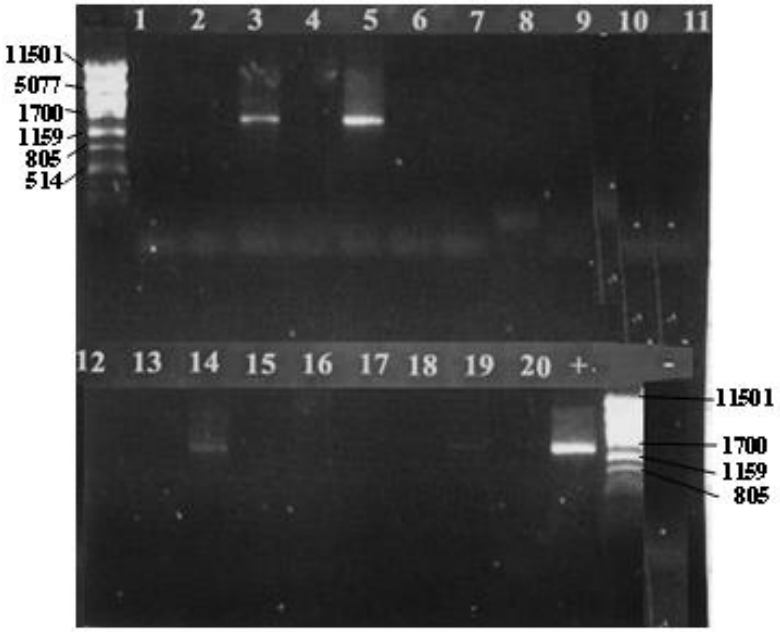

Fig. 3.6: PCR amplifications (universal bacterial primers E9F and 1510R) from DNA extracted using the Miller method with a phenol-chloroform extraction and various purification techniques. First lane = *Pst*I lambda marker (values indicate sizes in bp); Lanes 1-4 = PVPP purifications with dilution series (1=1/1, 2 =1/10, 3 = 1/50, 4 = 1/100); Lanes 5-8 = Sephacryl purifications with dilution series (5=1/1, 6=1/10, 7= 1/50, 8=1/100); Lanes 9-12 = Gel purifications (Talent kit) with dilution series (9=1/1, 10=1/10, 11=1/50, 12=1/100); Lanes 13-16 = PVPP + Sephacryl purifications with dilution series (13=1/1, 14=1/10, 15=1/50, 16=1/100); Lanes 17-20 = crude DNA (no purification) with dilution series (17=1/1, 18=1/10, 19=1/50, 20=1/100); - is negative control (sterile water); + is positive control (*E. coli*).

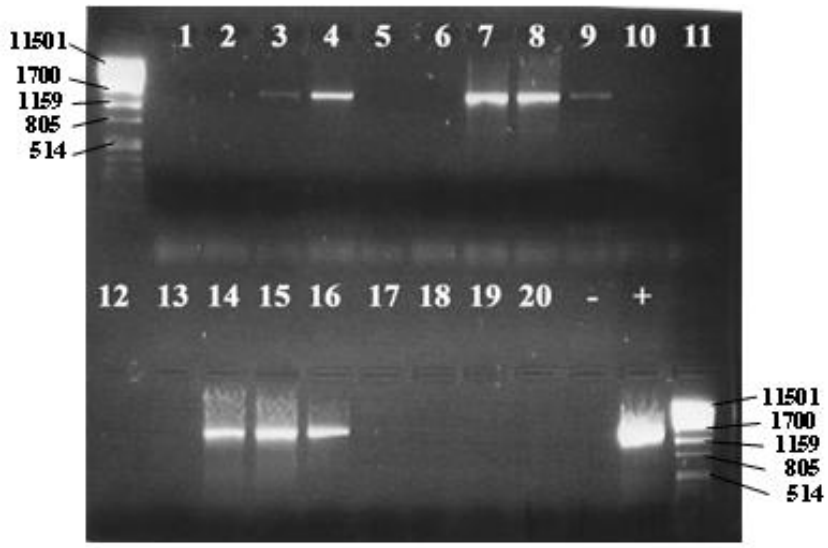

Fig. 3.7: PCR amplifications (universal bacterial primers E9F and 1510R) from DNA extracted using the Miller method with 0.5g PVPP added before lysis, a phenol-chloroform extraction and various purification techniques. First lane = *Pst*I lambda marker (values indicate sizes in bp); Lanes 1-4 = PVPP purifications with dilution series (1=1/1, 2 =1/10, 3 = 1/50, 4 = 1/100); Lanes 5-8 = Sephacryl purifications with dilution series (5=1/1, 6=1/10, 7= 1/50, 8=1/100); Lanes 9-12 = Gel purifications (Talent kit) with dilution series (9=1/1, 10=1/10, 11=1/50, 12=1/100); Lanes 13-16 = PVPP + Sephacryl purifications with dilution series (13=1/1, 14=1/10, 15=1/50, 16=1/100); Lanes 17-20 = crude DNA (no purification) with dilution series (17=1/1, 18=1/10, 19=1/50, 20=1/100); - is negative control (sterile water); + is positive control (*E. coli*).

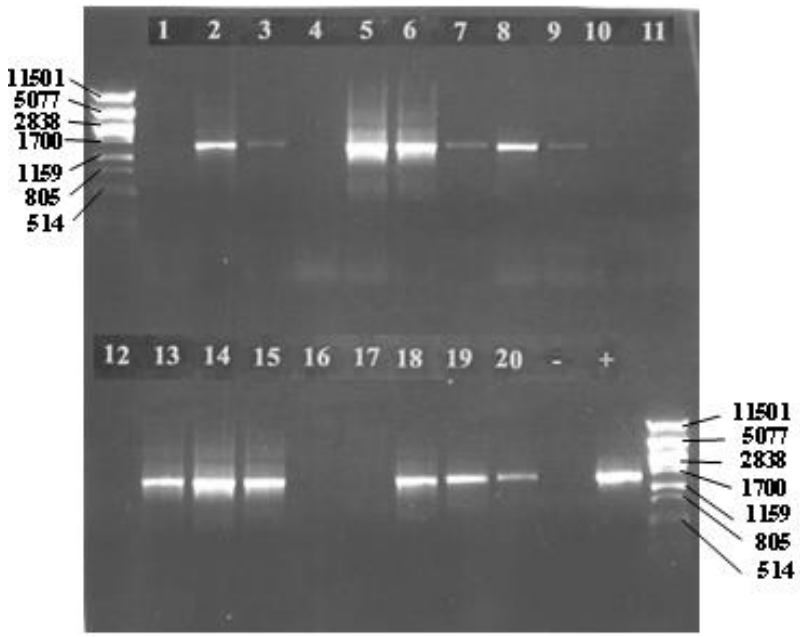

Fig. 3.8: PCR amplifications (universal bacterial primers E9F and 1510R) from DNA extracted using the Fast-prep kit and various purification techniques. First lane = *Pst*I lambda marker (values indicate sizes in bp); Lanes 1-4 = PVPP purifications with dilution series (1=1/1, 2 =1/10, 3 = 1/50, 4 = 1/100); Lanes 5-8 = Sephacryl purifications with dilution series (5=1/1, 6=1/10, 7= 1/50, 8=1/100); Lanes 9-12 = Gel purifications (Talent kit) with dilution series (9=1/1, 10=1/10, 11=1/50, 12=1/100); Lanes 13-16 = PVPP + Sephacryl purifications with dilution series (13=1/1, 14=1/10, 15=1/50, 16=1/100); Lanes 17-20 = crude DNA (no purification) with dilution series (17=1/1, 18=1/10, 19=1/50, 20=1/100); - is negative control (sterile water); + is positive control (*E. coli*).

3.2.1 PVPP purification

The addition of polyvinylpolypyrrolidone (PVPP) to soil before lysis is a commonly used technique to improve DNA purity (Young *et al.*, 1993; Malik *et al.*, 1994; Zhou *et al.*, 1996). PVPP hydrogen bonds to phenolic compounds, forming a precipitate that may be removed by centrifugation (Young *et al.*, 1993). PVPP may also be used in mini-spin columns to remove humic acids after DNA extraction. In this study, centrifuging crude DNA passed through several PVPP columns removed the dark colour of the extracts and

in some cases permitted PCR amplification. In all experiments, purification with PVPP resulted in a small loss of DNA. However, when viewed on an agarose gel, bands from PVPP purified extracts were less smeared. The addition of PVPP to soil before lysis did not result in an appreciable difference in the DNA yield or in the number of successful PCR amplifications (Table 3.3), although amplification bands were clearer and more distinct using DNA extracted with this method (Fig. 3.5).

3.2.2 Sephacryl™

Sephacryl™ (Amersham Biosciences, 27-5120-01) is a porous medium that can be used to purify DNA based on size exclusion chromatography. Molecules such as DNA, that are larger than the largest pores of the medium, are eluted rapidly, while smaller molecules enter the medium to different degrees (depending on size) and are eluted more slowly. Although Harry *et al.* (1999) propose that humic acid contamination persists after Sephacryl™ purification, due to competition with DNA for adsorption sites, mini-spin columns packed with Sephacryl™ were highly effective in this study compared to the other purification methods (Figs 3.4-3.8.). This may be due to the large size of the DNA fragment extracted or the comparative size differences between the DNA fragment size and the size of the humic acid molecules.

The similar Sepharose 4B and Sephadex G-200 matrices have also been documented to be highly effective for DNA purification in spin-columns (Jackson *et al.*, 1997; Miller *et al.*, 1999).

3.2.3 Gel purification

DNA bands excised from agarose gels were purified using silica-binding and a variety of different commercial kits, including the Fermentas DNA extraction kit #K0513, GeneClean III , Talent Cleanmix kit and GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences product code 27-9602-01)

Silica-binding resulted in a severe reduction in DNA yield, such that, in many cases, no DNA could be observed after electrophoresis. No PCRs using template DNA from this method were successful. This loss was also observed when using the Fermentas kit. The Talent Cleanmix kit also resulted in a noticeable loss, although DNA was still observable in gels, and some PCRs were successful when DNA preparations were diluted 1/10 – 1/50.

3.3 *nifH* PCR optimisation

The 'universal' degenerate *nifH* primers FGpf19 and FGpH273 (Simonet *et al.*, 1991) targeting the conserved *nifH* gene region in diazotrophic species, were selected to explore the diversity of nitrogen-fixing genes in Marion Island soil. DNA extracted using the FastPrep kit with the combined PVPP and Sephacryl purification (Fig. 3.9) was first tested for amplifiability using PCR with universal bacterial primers (Fig. 3.10). The PCR conditions proposed by Simonet *et al.* (1991) for use with the universal *nifH* primers were found to be inappropriate for successful amplifications. Number of cycles,

annealing temperatures, annealing and extension times and magnesium concentrations were adjusted until suitable conditions were established (Chapter 2). Optimal annealing temperature was decided using gradient PCR (Fig 3.12). Amplifications were successful and in most cases gave a product of the expected size (256bp), although some products were slightly smaller or larger, possible due to deletions and insertions in the *nifH* genes of different species. Some PCRs also resulted in double-banded products. These were attributed to non-specific binding due to the degeneracy of the primers, and increasing the annealing temperature to 60ºC often resolved this problem.

Fig. 3.9: DNA extracts using Fast-prep soil extraction kit and purified once through a PVPP column and then once through a Sephacryl column. Lane 1 is *Pst*I lambda marker (values denote size of fragments in bp); letters denote habitats as follows: C = Mesic Fellfield 2.2; E = Biotic Lawn 5.3; N = Closed Fernbrake 3.2; PT = Pedestalled Tussock Grassland 4.1; Q = Dry Mire 6.1; U = Wet Mire, 6.3; V = Coastal Fellfield 1.2.

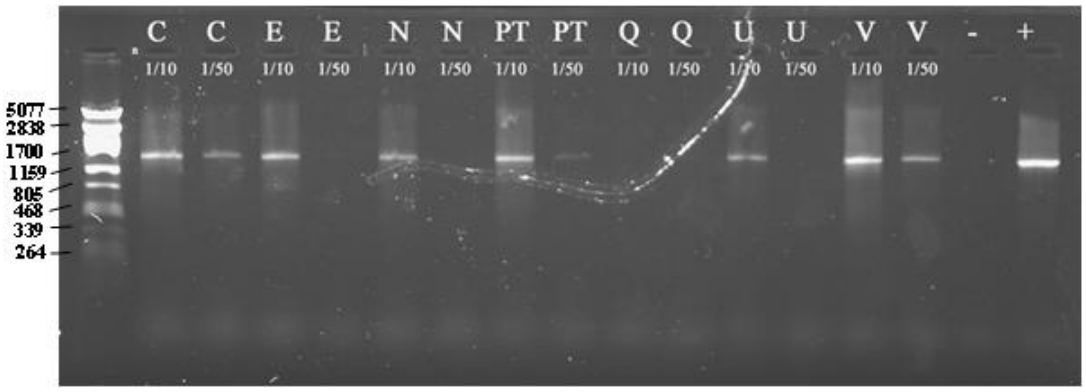

Fig. 3.10: PCR products for samples from different habitats (numbers indicate dilutions) using universal bacterial primers E9F and 1510R. Lane 1 contains *Pst*I lambda marker (values indicate size of fragment in bp), DNA extracts used are as in Fig. 3.9; - denotes negative control (sterile water); + denotes positive control (*E. coli*).

Fig. 3.11: PCR products for samples from different habitats (numbers indicate dilutions) using universal *nifH* primers FGph19 and FGpl273. Lane 1 contains *Pst*I lambda marker (values indicate size fragments in bp), DNA extracts used are as in Fig. 3.9; - denotes negative control (sterile water); + denotes positive control (DNA extracted from root nodules of bean plant).

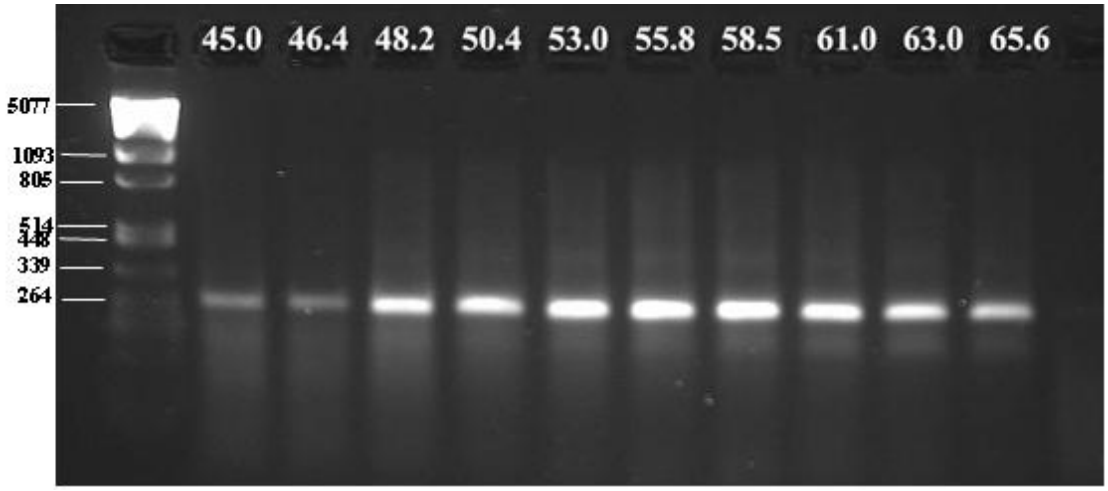

Fig. 3.12: Gradient PCR using DNA extracted from sample C (Mesic Fellfield 2.2) using the Fastprep kit, purified with PVPP and Sephacryl. Lane 1 show *Pst*I lambda marker (values indicate sizes of fragments in bp); numbers shown on other lanes denote annealing temperatures (°C); the final lane is a negative control (sterile water). PCR using universal *nifH* primers FGph19 and FG pl273, gave the expected size of 254kb.

Addition of BSA

Bovine serum albumim has been shown to minimise inhibition of PCR by contaminating substances (Hoss *et al.*, 1992; Romanowski *et al.*, 1993; Kreader, 1996), and presumably acts by forming hydrogen bonds with the peptide bonds oxygens in phenolic compounds (Kreader, 1996). In this study, the addition of BSA to PCR reactions in the quantities (200-400ng/µl) proposed by Kreader (1996) did not improve the success of any reactions with universal bacterial or *nifH* primers. This may be due to excessively high contamination of crude (i.e. unpurified) DNA samples, as although Kreader reports that adding BSA in these concentrations permits the accommodation of up to 10ng/µl humic acid (as opposed to inhibition at 0.1ng/µl in unmodified PCRs) for a successful reaction,

humic acid was not quantified in extracts from this study and may well have been above this level.

Although the *nifH* gene is highly conserved at the amino acid level, the degeneracy of the genetic code has resulted in significant variability at the nucleotide level (Zehr and McReynolds, 1989), and as such, for effective 'universal' amplification, primers must be highly degenerate (Zehr and McReynolds, 1989; Widmer *et al.,* 1999).

Despite this, PCR amplification using such primers has been used as a basis for investigating diazotroph diversity in various different environmental samples (Zehr and MacReynolds, 1989; Ben-Porath and Zehr, 1994; Olson *et al.*, 1999; Lovell *et al.*, 2001), and *nifH* phylogeny derived from such studies is usually congruent with phylogeny obtained from 16S PCR studies (Hennecke, 1985; Young, 1992; Young, 1993; Ueda, 1995; Zehr *et al.*, 2003).

On many occasions, extra bands were observed after gel electrophoresis of PCR products amplified with *nifH* PCRs, most likely caused by non-specific binding (e.g. Fig. 3.16). This occurred even after optimisation experiments involving increased annealing temperatures and magnesium concentration adjustments. Non-specific binding is a common problem encountered when using degenerate primers, and has also been reported with *nifH* primers by Simonet *et al.*, (1991). To reduce this, several approaches have been used, including nested PCRs using different *nifH* primer sets (e.g. Widmer *et al.,* 1999; Shaffer *et al.,* 2000; Bürgmann *et al.*, 2004) or *nifH* primers sets targeting

smaller communities of diazotrophs (e.g. Piceno *et al.*, 1999; Poly *et al.,* 2001; Rösch *et al.*, 2002).

3.4 DGGE analysis

nifH fragments from genomic DNA were successfully amplified with the primers Fgpf19 and FgpH273gc (Fig 3.13), with bands of the expected size (256kb) being obtained in all samples.

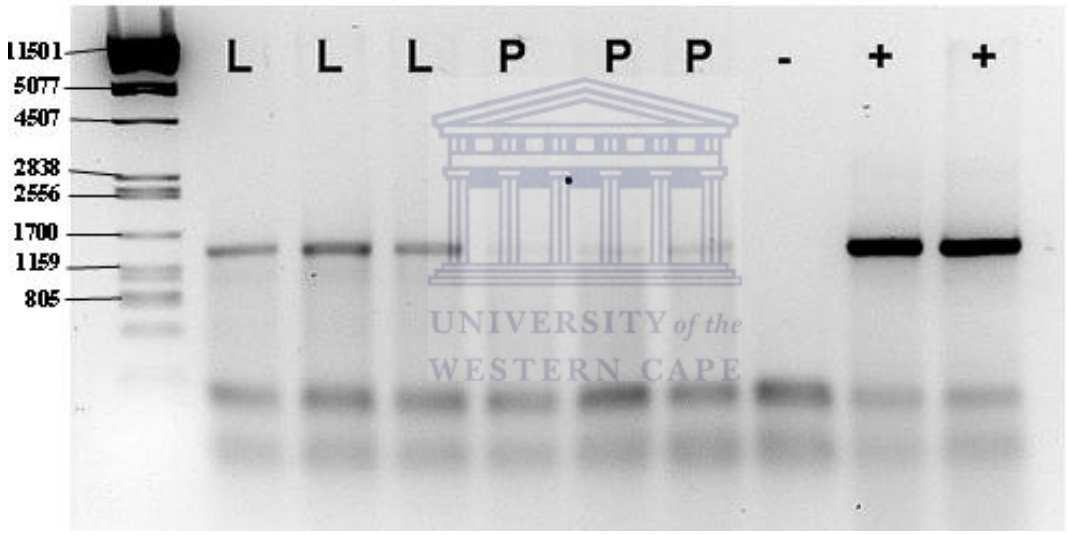

Fig 3.13: PCR products obtained using *nifH* primers with a GC clamp.

DGGE analysis was performed on *nifH*gcPCR products obtained from genomic DNA extracted from a variety of habitats, C (Mesic Fellfield 2.2); L (Biotic Mire .5); V (Coastal Fellfield 1.2); E (Biotic Lawn 5.3); PT (Pedestalled Tussock Grassland 4.1) and U (Wet Mire 6.3). DGGE products consisted of a single band (size) for each sample (Fig 3.14), which was unusual, and did not agree with RFLP results (section 3.6). These results are most likely to have been caused by some experimental/operational error and

their lack of conclusive data does not rule out DGGE as a potentially useful method to study *nifH* diversity in sub-Antarctic soil, indeed it has been sucessfully used to examine microbial community structure in a number of other studies (see section 1.4.4).

Fig 3.14: DGGE analysis of *nifH* fragments obtained by PCR of genomic DNA from soil habitat samples. Letters indicate samples from habitats as follows: V= Coastal Fellfield 1.2; U = Wet Mire 6.3; PT=Pedestalled Tussock Grassland 4.1; L=Biotic Mire 6.5; C = Mesic Fellfield 2.2; E = Biotic Lawn 5.3.

3.5 Clone Libraries

nifH fragments were cloned as described in Chapter 2 and 20 white clones were randomly selected and screened for inserts using M13 PCR. 65% of clones from sample C (Mesic Fellfield 2.2), 100% of clones from sample L (Biotic Mire 6.5), 80% of clones from

sample V (Coastal Fellfield 1.2), 85% of clones from sample E (Biotic Lawn 5.3) and 80% of clones from sample PT (Pedestalled Tussock Grassland 4.3) gave products of an appropriate size (560bp) for a plasmid with a correctly sized insert (Fig. 3.15) - only first 10 colonies screened from C,L and V shown). These products were diluted 1/1000 and used as a template in a PCR with *nifH* primers to confirm the insert was a *nifH* gene. In many cases, more than one band was observed on agarose gels after *nifH* PCR.

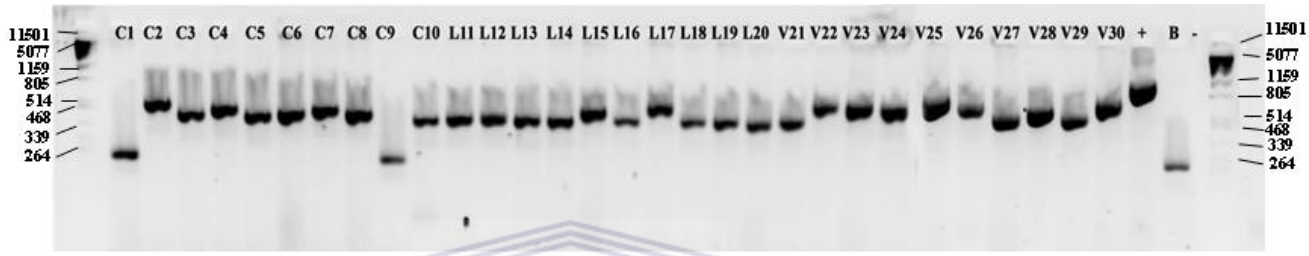

Fig 3.15: Colony PCR (M13) of randomly selected white colonies from samples C, L and V. B = blue colony selected from control plate (vector without insert).

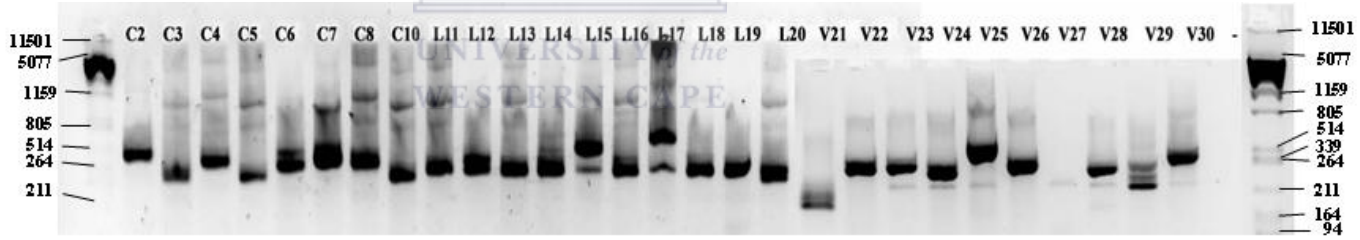

Fig 3.16: *nifH* PCR using diluted M13 PCR (above) as template.

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3.6 RFLP analysis

RFLP analysis was performed on M13 PCR products obtained from clone libraries from samples C (Mesic Fellfield 2.2), L (Biotic Mire 6.5) and V (Coastal Fellfield 1.2). Samples from these habitats were selected for analysis as they represent significant ecological differences and are very common habitats on Marion Island.

Analysis suggested considerable variability within and between samples (Figs. 3.17-3.21), with identical patterns being observed only once (L11 and L14). All clones representing different patterns from Set 1 were sequenced for phylogenetic analysis (Figs. 3.17, 3.18, 3.19). Patterns from sample V cannot be compared visually with those from samples C and L as a different cloning vector was used for this transformation.

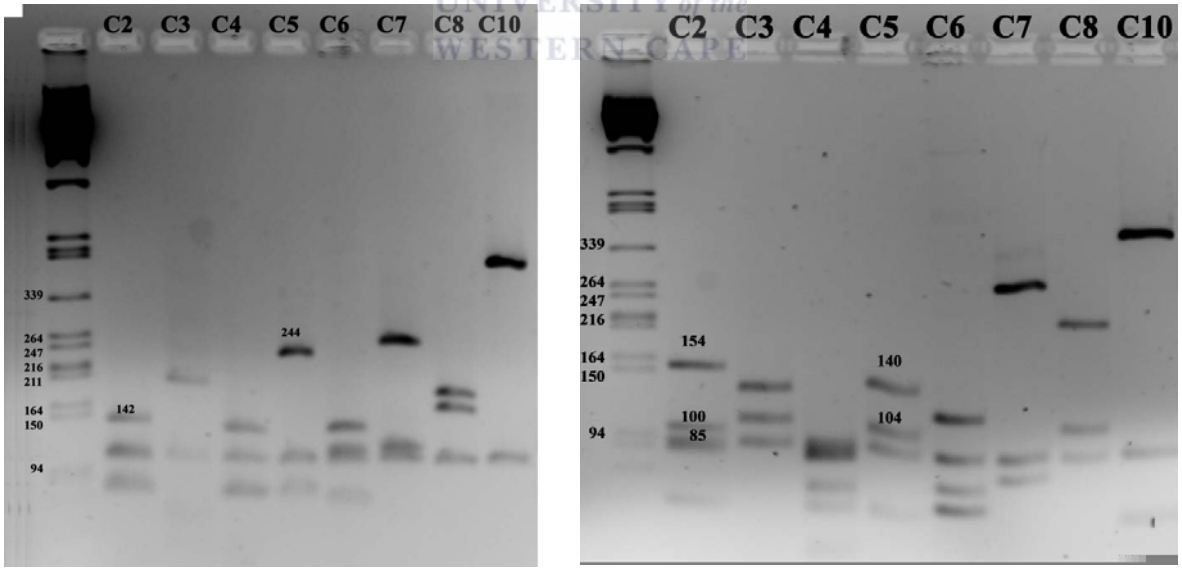

Fig 3.17: *Hpn*II (left) and *Hae*III (right) RFLP patterns for clones from sample C (Mesic Fellfield, 2.2)

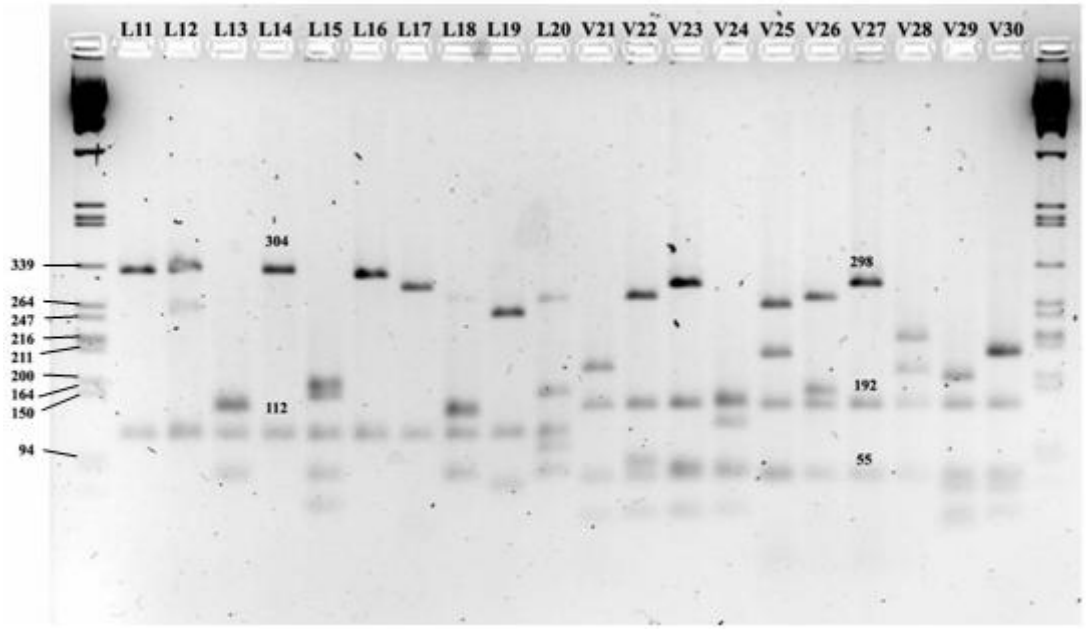

Fig 3.18: *Hpn*II RFLP patterns for clones from samples L (Biotic Mire 6.3) and V (Coastal Fellfield 1.2)

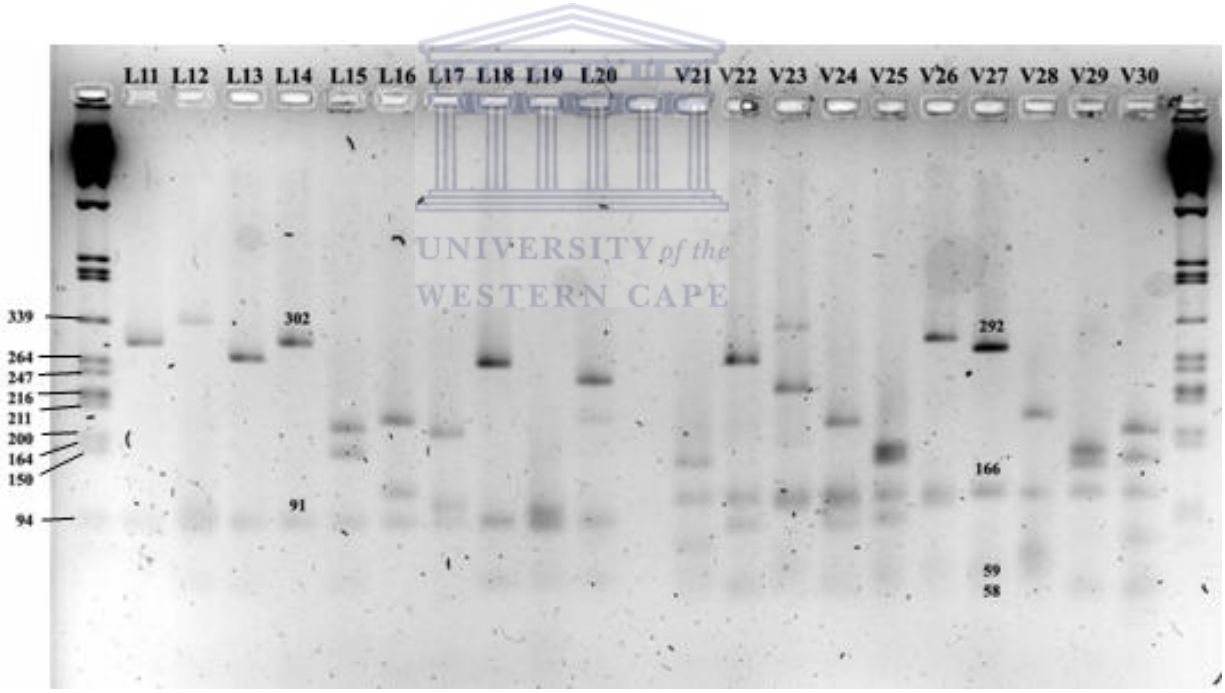

Fig 3.19: *Hae*III RFLP patterns for clones from samples L (Biotic Mire 6.3) and V (Coastal Fellfield 1.2)

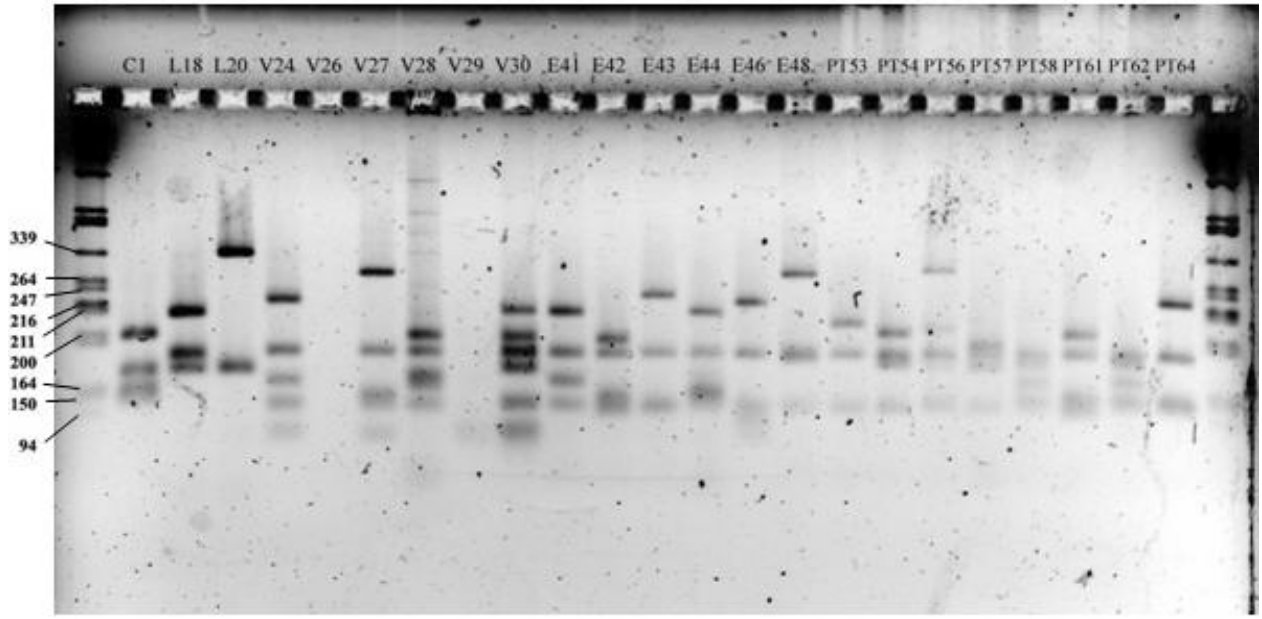

Fig 3.20: *Hpn*II RFLP patterns for clones from samples C (Mesic Fellfield 2.1), L (Biotic Mire 6.3), V (Coastal Fellfield 1.2) and PT (Pedestalled Tussock Grassland 4.3).

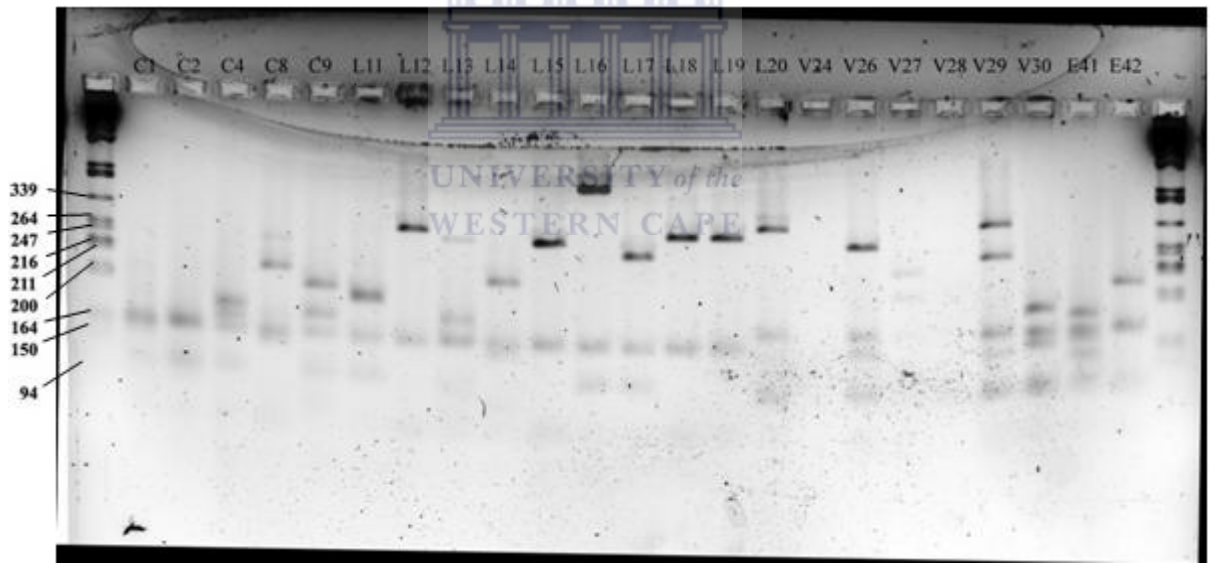

Fig 3.21: *Hae*III RFLP patterns for clones from samples C (Mesic Fellfield 2.1), L (Biotic Mire 6.3), V (Coastal Fellfield 1.2) and E (Biotic Lawn 5.3)

The lack of diversity indicated by DGGE analysis (Fig. 3.14) is is stark constrast to the high level suggested by RFLP and sequence analysis (Figs. 3.17-3.22). The reasons for this discrepency are unclear.

RFLP analysis of *nifH* genes is a valuable tool for investigating diversity of diazotrophs in soil (Lepo *et al.*, 1995; Widmer *et al.*, 1999) despite it being time-consuming and rather expensive, as many clones must be sequenced in order to provide a reasonable representation of microbial diversity. The high level of diversity observed in this study with RFLP analysis is consistent with that documented in many studies of environmental samples. For example, Felske *et al.*, (1997) sequenced 165 clones from an acid peat sample, and reported that only 37 of these were redundant.

3.7 Sequence analysis

Nucleotide sequence alignments (Table 3.4) showed some odd similarities to known sequences, although it was clear that *nifH*-like sequences were amplified in several clones (L14, L16, L18, L19, Clone 3, Clone 10, V29). Because of the high degeneracy of *nifH* genes, amino acid alignments were thought to provide a more accurate basis for phylogenetic analysis, and 6-frame translations of nucleotide sequences were compared to the protein and translated BLAST databases (Table 3.5). From these alignments, definite *nifH* and *nifH*-like sequences were identified in a number of clones (Table 3.5).

A phylogenetic tree based on amino acid analysis (Fig. 3.22) places clone sequences and selected BLAST sequences into 6 main groups, or clusters (see appendix for sequences) for ease of discussion. These are divided into two main superclusters: *nifH* genes, and *nifH*-like genes on the tree (Fig. 3.22). Sequence alignments indicate that definite *nifH* genes were amplified in clones L16, Clone 10, L18 and L19, as sequences from these

clones aligned closely with *nifH* sequences from proteobacteria, cyanobacteria and uncultered bacteria from cold environments (Fig 3.22 – Clusters I and II). The majority of clones, however, fell into the *nifH*-like supercluster, which including protochlorophyllidae reductase genes and hypothetical and transporter proteins, although these may in fact be members of a divergent, 'type IV' nitrogenase group which may or may not be capable of active nitrogne fixation. All clusters are discussed separately in the following section. Only clones that were related to *nifH* or *nifH*-like sequences were included in the tree.

From the phylogenetic tree (Fig. 3.22), clones from some habitats seem to cluster together. All clone samples from the mesic fellfield (C library) are in clusters IV and VI, while those from the coastal fellfield are in clusters III and IV. The highest phylogenetic diversity is found in the biotic mire, clones from this habitat are distibuted over all clusters, excepting cluster VI. Differences between sequences from different habitats are discussed in more detail in section 3.7.2.

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Table 3.4: Highest scoring BLAST nucleotide-nucleotide alignments. Bold font indicates significant alignment with *nifH* or *nifH* related gene fragments.

Clone	Fragment size	Highest scoring nucleotide BLAST alignments	Gene	% identity
C2	265	No significant similarity found.		
C3	193	*Sinorhizobium meliloti* 1021 (AL591985.1)	Hypothetical protein	91% (31/34)
C4	180	*Erwinia carotovora* subsp. *Atroseptic* (AM260479.1)	MFS transporter	96% (24/25)
C5	210	*Rattus norvegicus* protein (CP000112.1)	3-deoxy-D-manno-octulosonate cytidylytransferase	92% (26/28)
C6	233	*Geobacter metallireducens* GS-15, comp (CP000148.1)	GGDEF protein	93% (30/32)
C7	273	*Hordeum mosaic* virus strain ATCC PV81 (AY623627.1)		96% (24/25)
C8	244	Human DNA sequence NT_007592.14		100% (21/21)
L14	**213**	***Rhodopseudomonas palustris* CGA009 protochlorophyllide reductase** (BX572597.1)	***BchL***	**84% (176/209)**
L15	225	*Pan troglodytes* BAC clone RP43-16G9 f (AC188167.4)		95% (23/24)
L16	**215**	**Uncultured bacterium *nifH* gene for dinitrogenase reductase (AB184942.1)**	***nifH* gene**	**91% (194/211)**
L17	359	*Homo sapiens* chromosome 5 (NT_029289.10)	ubiquitin-conjugating enzyme	100% (21/21)
L18	**232**	***Anabaena variabilis* ATCC 29413 chromosome (CP000117.1)**	**Nitrogenase iron protein**	**83% (140/167)**

L19	**215**	**Uncultured nitrogen-fixing bacterium mRNA for dinitrogenase reductase (AB208364.1)**	**Nitrogen reductase**	**94% (197/209)**
Clone 3	**204**	***Fremyella diplosiphon* clone 3109A9 light-independent protochlorophyllide reductase (AY548448.1)**	**DPOR**	**87% (115/!32)**
Clone 10	**230**	***Anabaena variabilis* ATCC 29413 (CP000117.1)**	**Nitrogenase iron protein**	**85% (140/164)**
V21	161	*Rhodopseudomonas palustris* CGA009 protochlorophyllide reductase (BX572601.1)	unknown protein1	100% (21/21)
V22	257	*Yersinia pestis* biovar *Medievalis* str. 91001 (AL590842.1)	Pyruvate kinase II	92% (38/41)
V23	207	*Burkholderia mallei* ATCC 23344 chromo (CP000546.1)	Homogentisate 1,2-dioxygenase	100% (22/22)
V24	285	No significant similarity found.		
V25	471	Zebrafish DNA sequence from clone. (BX649547.7)		100% (22/22)
V26	272	*Photorhabdus luminescens* (BX571875.1)	segment 17/17 of sequence	96% (25/36)
V27	**213**	***Synechococcus sp.* CC9605, light-independent protochlorophyllide reductase (CP000110.1)**	**DPOR**	**88% (120/136)**
V28	259	*Mesorhizobium loti* MAFF303099 plasmid pMLb D (CP000248.1)	Acyl-CoA dehydrogenase-like	100% (20/20)
V29	208	*Streptomyces coelicolor* (AL939116.1)	Putative precorrin-8X methylmutase	100% (24/24)
V30	282	*Aspergillus fumigatus (AC186974.5)*		100% (21/21)

Table 3.5: Highest scoring BLAST protein-translation alignments. Letters in brackets after clone name indicate translation frame, i.e. D= direct and C=complementary frames. Bold font indicates significant alignment with *nifH* or *nifH* related gene fragments.

Clone	Amino acids	Highest scoring BLAST protein-translation alignments	Gene	% identity
C2 (+3)	86	*Deinococcus geothermalis* DSM 11300 (CP000359.1)	glycosyl transference, group 1	34% (30/86)
C3 (-1)	**63**	***Sinorhizobium meliloti* hypothetical protein (AL591985)**	Hypothetical protein	**85%** (35/41)
C4 (-1)	**60**	***Chromobacterium violaceum***	probable competence protein	55% (21/38)
C5 (+3)	60	*Pelobacter propionicus* DSM 2379 (CP000482.1)	3-deoxy-D-manno-octulosonate cytidylyltransferase	45% (27/60)
C6 (+3)		*Dechlororamonas aromatica* RC13	GGDEF protein	62% (18/29)
		Burkholderia mallei NCTC 10229 chromosome II	GGDEF protein	62% (15/24)
C7 (+1)	**91**	***Agrobacterium tumefaciens* str. C58 garD CD (AE009229.1)**	**Section 255 of complete seq**	**50% (45/90)**
C8		No significant similarity found		
L14 (+2)	70	***Rhodopseudomonas palustris* BisB18 light-independent protochlorophyllide reductase (CP000301.1)**	**DPOR**	**91% (64/70)**
		***Rhodopseudomonas palustris* BisB5 light-independent protochlorophyllide reductase (CP000283.1)**	**DPOR**	**90% (63/70)**

L15		No significant similarity found		
L16 (+3)	**71**	**Uncultured bacterium clone Qinglin-8 dinitrogenase reductase (AY601072.1)**	***nifH***	**98% (355/461)**
		Uncultured bacterium clone Qinglin-5 dinitrogenase reductase (AY601069)	***nifH***	**98% (355/461)**
L17 (+2)	119	*Pseudomonas fluorescens* Pf-5 +1 (CP000076.1)	Nicotinamide mononucleotide transporter PnuC	**50% (56/111)**
L18 (-1)	**77**	***Nostoc*. Commune nitrogen fixation protein (L23514.1)**	***nifU***	**79% (61/77)**
L19 (+3)	**71**	**Uncultured bacterium clone SE2 dinitrogenase reductase (AF414643.1)**	***nifH***	**97% (69/71)**
		Uncultured nitrogen-fixing bacterium mRNA for dinitrogen reductase (AB208365.1)	***dinitrogen reductase***	**97% (69/71)**
		***Burkholderia xenovorans* LB400 chromosome II (CP000271.1)**	***nifH***	**97% (69/71)**
		Uncultured bacterium clone Yushu-5 (AY601041.1)	***nifH***	**97% (69/71)**
		Uncultured nitrogen-fixing bacterium mRNA for dinitrogenase reductase (AB208364.1)	***nifH***	**97% (69/71)**
Clone3 (-1)	**61**	***Fremyella diplosiphon* clone 3109A9 for light-independent protochlorophillide reductase (AY548448.1)**	**DPOR**	**63% (29/46)**
Clone10 (-1)	**77**	***Nostoc* commune nitrogen fixation protein (L23514.1)**	***nifU***	**82% (63/76)**

		Uncultured bacterium clone Yushu-11	***nifH***	**82%(63/76)**
V21		No significant similarity found		
V22 (+3)	84	*Xylella fastidiosa* 9a5c, section 68 (AE003849.1)	Pyruvate kinase type II	73% (62/84)
V23 (+3)	69	*Agrobacterium tumefaciens* C58 (AY523972S5)		40% (20/49)
V24 (+1)	95	*Photorhabdus luminescens* subsp. (AE007940.1)		58% 10/17
V25 (+1)		*Mycobacterium sp. KMS* (CP000518.1)	Conserved hypothetical	57% (20/35)
		Mycobacterium sp. MCS (CP000384.1)	protein	57% (20/35)
V26 (+2)	88	*Polaromonas sp.* JS666 (YP 550199.1)	oxidoreductase FAD/NAD	39% (34/87)
V27 (+3)	**70**	***Erythrobacter sp.* NAP1 protochlorophyllide reductase (AY672001.1)**	***bchL***	**90% (63/70)**
		***Erythrobacter sp.* CV29 protochlorophyllide reductase (AY672000.1)**	***bchL***	**90% (63/70)**
		***Erythrobacter sp.* BS140 protochlorophyllide reductase (AY671999.1)**	***bchL***	**90% (63/70)**
		***Citromicrobium sp.* CV44 protochlorophyllide reductase (AY671998.1)**	***bchL***	**90% (63/70)**
V28		No significant similarity found		
V29 (+1)	**69**	***Rhodopseudomonas palustris* BisB18 (CP000301.1)**	**DNA methylase N-4/N-6**	**81% 21/66**
V30 (+3)	93	*Methylococcus capsulatus* str. Bath, complete genome (AE017282.2)	dethiobiotin synthetase	(31%) 21/66

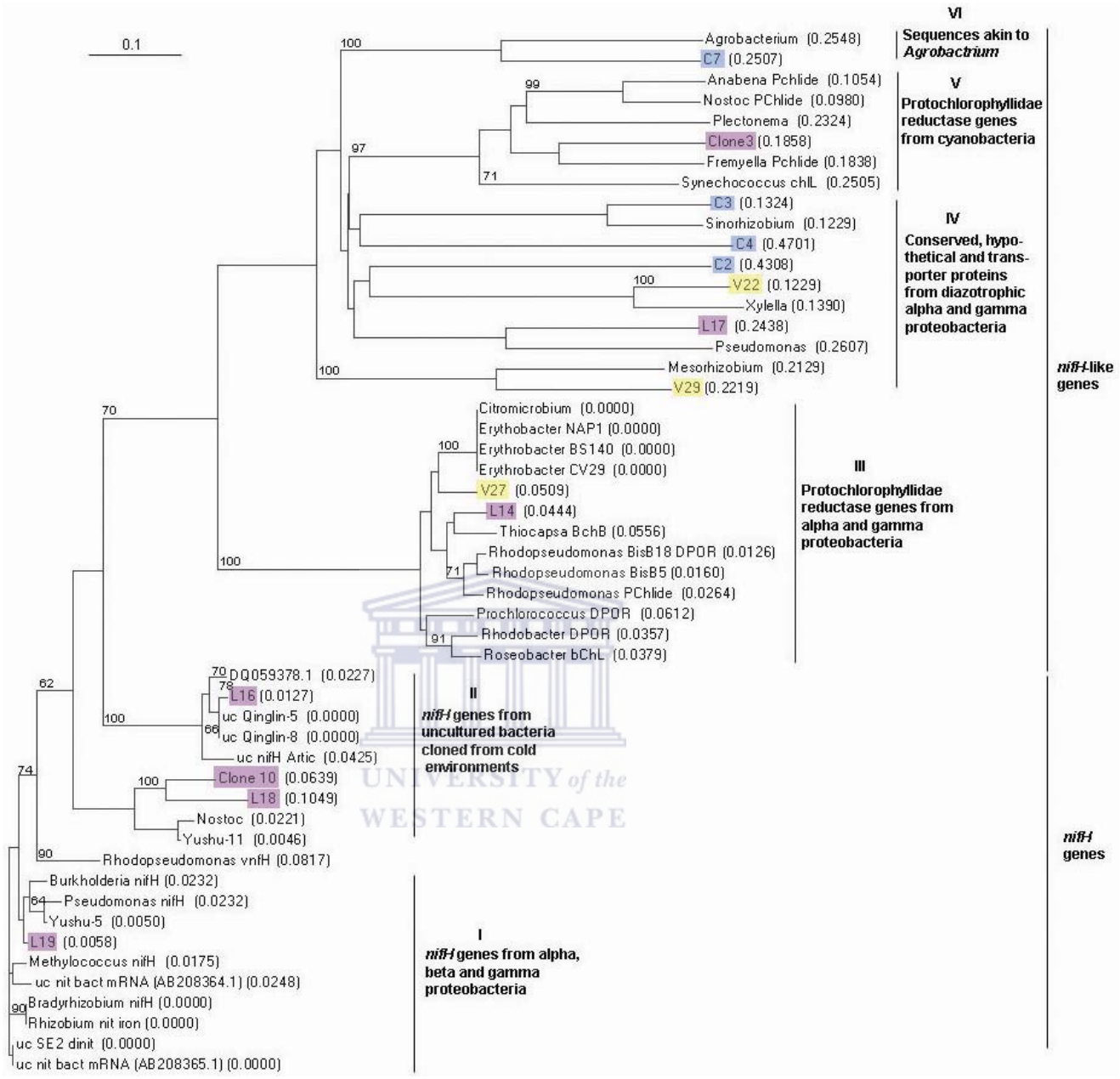

Fig. 3.22: Phylogenetic tree based on amino acid sequences of clones and translated published *nifH* sequences. Highlights indicate sequences from samples of different habitats; blue=clones from C library, Mesic Fellfield 2.2; purple= clones from L library, Biotic Mire 6.5; yellow= clones from V library, Coastal Fellfield 1.2. Bootstrap values greater than 50 are given and are based on 1000 replicates.

3.7.1 Discussion of the different phylogenetic clusters

Cluster I - *nifH* genes from proteobacteria

Cluster 1 contains a variety of *nifH* sequences from a selection of α-, β- and γ-proteobacteria. These are divided into 4 sub-clusters, which have not been distinguished by separate labels on the phylogenetic tree due to their comparatively short evolutionary distances (all less that 0.03 sequence divergence). Only one clone, L19, fell into this cluster and showed a high sequence identity to a number of *nifH* genes from different organisms (Table 3.5). A *vnfH* gene (vanadium nitrogenase gene) from *Rhodopseudomonas palustris* falls between clusters I and II, both *nifH* clusters. These alternative nitrogenases are often unable to be distinguished from *nifH* genes, and their inclusion in *nifH* phylogenies have been reported in other studies (Kessler *et al.*, 1997), suggesting that *nifH* phylogenies do not indicate metal type.

Cluster II – *nifH* genes from uncultured bacteria from cold environments

The low identies of clones to known sequences in this study is comparable to the novelty observed by Deslippe and Egger (2006) and Zhang *et al.* (2006) in samples collected from Ellesmore Island and the Tibetan Plateau, and reflects the paucity of data availiable on soil diazotroph diversity in isolated polar environments. One of the most interesting outcomes of this study is the high degree of similarity observed between several of the biotic mire clones and the *nifH* sequences from uncultured bacteria generated by Zhang *et*

al. (2006) from samples collected from alpine prairies on the Tibetan Plateau. These clones included Clone 10 (82% similarity to Yushu-11), clone L19 (97% similarity to Yushu-5) and clone L16 (97% similarities to both Qinglin-5 and Qinglin-8). These clones are probably pyschrotrophic or psychrotolerant organisms, given their similarity to the Artic and Tibetan samples. Deslippe and Egger (2006) also observed a high degree of similarity between their Artic *nifH* sequences and those from the Tibetan samples, and two of their clones DQ059378.1 and DQ059343.1 (uc *nifH* Artic) were included in the phylogenetic tree (Fig. 3.22) of this study.

The fact that very similar *nifH* genes were found in all three of these studies (high Arctic, Tibetan Plateau and Marion Island) is evidence to support the hypothesis that the distribution of diazotrophs may be predicted on the basis of habitat charcteristics (Zehr *et al.,* 2003). All three sites are characterised by low temperature, poor substrate quality and relative isolation. The Quigzang Plateau typically has very low temperatures, low moisture and oxygen levels, high winds, strong solar radiation and great temperature differences between day and night. Ellesmere Island is located near Canada in the Arctic Circle, and is generally covered by glaciers and ice. Few studies have been published regarding diazotrophs in cold environments. However, ssu rDNA has been used to construct phylogenies suggesting relatedness between bacteria from Antarctic ice and from other cold, non-polar locations (Priscu and Christner 2002). Zehr and Priscu (unpub. data reported by Priscu and Christner, 2002) analysed 16S rDNA sequences from samples taken at Lake Bonney, Antarctica. 70% of clones from this site exhibited high identity with other marine or lake species, with most showing greatest similarity to those

isolated from polar environments. Broad geographic distribution of related species from similar cold environments indicates evolution of similar adaptations to survive and grow at low temperatures (National Academy of Sciences, 2004).

Clusters III and V - Protochlorophyllidae reductase type genes

Sequences of several clones (L14, Clone 3, V27) exhibited closer similarity to light-independent protochlorophyllide reductase than with *nifH* genes (Table 3.5). These formed 2 distinct clusters on the amino acid tree (Fig 3.22), the first containing sequences from proteobacteria and the second from cyanobacteria, and are represented on the tree with abbreviations DPOR, PChlide, BchB, BChL and chlL. Cluster III contains protochlorophyllidae genes from protobacteria, predominately α-proteobacteria, although one γ-proteobacteria, *Thiocapsa spp*. is also included. Cluster V contains protochlorophyllidae reductase genes from cyanobacteria (also see section 3.7.1 Cluster V).

Clone 3 (biotic mire) aligned most closely with protochlorophyllidae reductase from cyanobacteria (*Synechococcus spp.*, *Nostoc* commune and *Anabaena spp.* - see section 3.7.1 Cluster V), while L14 and V27 aligned more closely with protochlorophyllidae reductase genes from various protobacteria. V27 exhibited the closest similarity to *Erythrobacter* and *Citrobacterium* strains, two closely related spihingomondales (α-proteobacteria) usually found in organic rich environments. Although L14 aligned most closely with *Rhodopseudomonas palustris* in the BLAST analysis, tree analysis also

places it close to *Thiocapsa* protochlorophillidae reductase, *Thiocapsa* being a γ-proteobacteria. L18 also falls into cluster IV, although the tree shows it at a great distance from the other sequences, and BLAST analysis suggests it is more closely related to a *Nostoc*-type nitrogen fixing protein (Table 3.5).

Protochlorophyllide reductase is a protein involved in the synthesis of chlorophyll (Chl) and bacteriochlorophyll (BChl), the essential photochemically active reaction centre pigments involved in photosynthesis. In this process, protochlorophyllide is reduced to a clorin, which may be used for direct synthesis of Chl, or which is further reduced in a subsequent reaction into bacteriochlorin, a precursor of bacteriochlorophyll. The protochlorophyllide reduction reaction requires an enzyme catalyst, the light-dependent protochlorophyllide oxidoreductase LPOR, or the light-independent protochlorophyllide reductase DPOR. While LPOR requires that protochlorophyllide absorbs light before the enzyme will promote reduction, DPOR permits reduction regardless of light conditions. Primitive anoxygenic photosynthetic bacteria contain only DPOR (Xiong *et al.,* 1998), while angiosperms contain only LPOR (Fujita, 1996; Armstrong, 1998). Cyanobacteria, algae and gymnosperms contain both (Adamson, 1997).

Strong structural similarities between the three protein subunits that constitute dark protochlorophyllidae reductase and the three subunits of nitrogenase have been reported (Burke *et al.,* 1993; Fujita *et al.,* 1993; Fujita, 1996; Fujita *et al.,* 2000), with BchL/ChlL, BchN/ChlN, and BchB/ChlB subunits of DPOR corresponding to *nifH*, *nifD*, and *nifK* respectively. Similarities are particularly evident between the *nifH* and BchL/ChlL

proteins, with 33% identity and 50% similarity (Fujita *et al.*, 1991; Suzuki and Bauer, 1992; Burke *et al.*, 1993; Fujita and Bauer, 2000). Functional homologies have also been observed; both nitrogenase and DPOR are dependent on ATP and dithionite (Fig 4.1) (Fujita and Bauer, 2000).

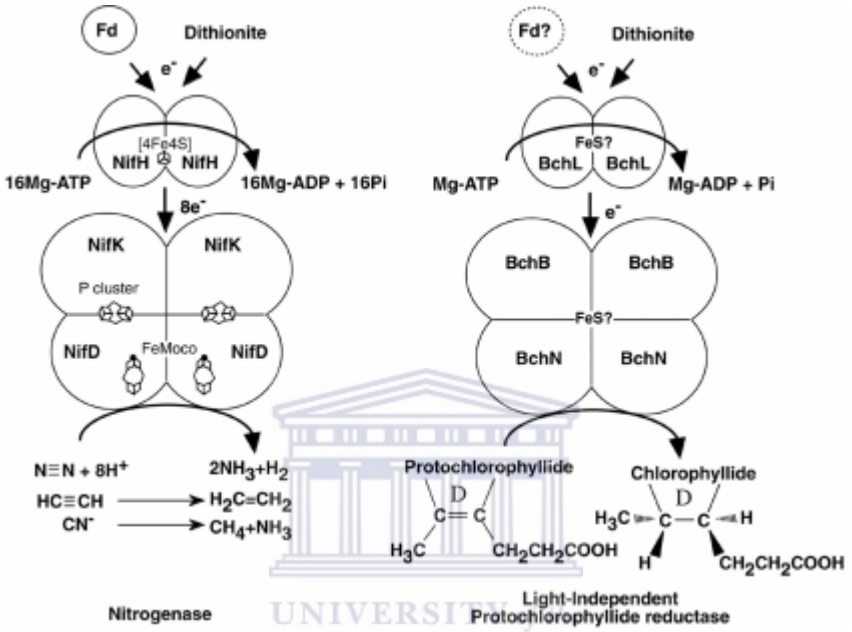

Fig 4.1: Similarities between nitrogenase and DPOR. Electrons from *nifH* (BchL) ATPase flow to *nifDK* (BchB/BchN), where subsequent reactions occur to fix nitrogen or synthesise chlorophyllide (from Fujita and Bauer, 2000).

Phylogenetic analysis of these genes suggests their evolution from an ancient gene duplication event (Xiong *et al.*, 2000). Gene duplication and recruitment have probably occurred several times during the evolution of nitrogenase, the current distribution of which has been influenced by gene loss and horizontal gene transfer (Raymond *et al.*, 2004).

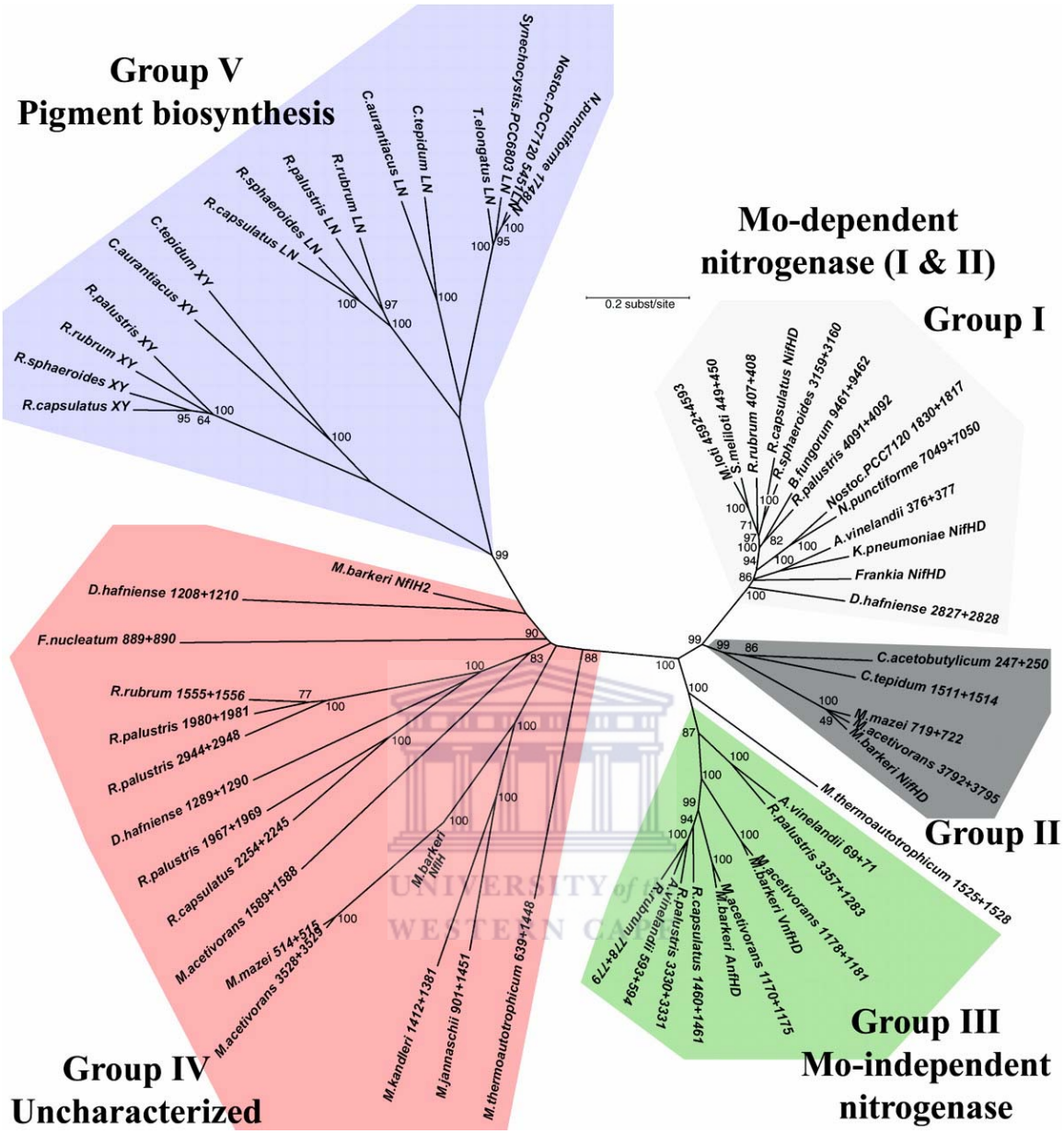

Fig. 4.2: Phylogenetic relationships between nitrogenases and Pchlide (pigment biosynthesis), (from Raymond *et al.*., 2004).

These similarities account for the close alignment observed in this study between clones containing gene fragments amplified using degenerate universal *nifH* primers and protochlorophyllide reductase CDs from various proteobacteria, particularly as such primers may target the same conserved regions on both *bchL* and *bchX* (Mehta *et al.*,

2003). Some *nifH* primers (Mehta *et al.*, 2003) have even been used purposely for targeting *bchL* and *bchX* genes (Oz *et al.*, 2005).

The clone L14 aligned most closely (91%) with the *bchL* gene of *Rhodopseudomonas palustoris*. Some of the clones produced by Deslippe and Egger (2006) from DNA extracted from soil in the high arctic also aligned fairly closely (85%) with this gene, they suggest that this probably indicates that their clones more likely belong to the type IV 'nitrogenases' (Group IV 'uncharacterized' in Fig. 4.2). This group of deeply branching, highly divergent genes includes sequences of *nif*-like genes, homologous chlorophyllidae reductase genes and sequences from the archaea (Zehr *et al.*, 2003). Many have been obtained from organisms which are not known to be photosynthetic or diazotrophic, and are thought to be relic, ancestral proteins to both the nitrogenase and bacterichlorophyll genes (Raymond *et al.*, 2004), that are not likely to be involved in nitrogen fixation if actively transcribed (Deslippe and Egger, 2006). It is possible that some of the sequences obtained in this study that aligned more closely to DPOR genes do in fact fall into this category of type IV nitrogenases.

Cluster IV

Cluster IV contains clones and sequences that are not neccessarily *nifH* sequences, but may be related to *nif*-like genes, photochlorophildae reductase-like genes or genes distantly related to *nifH*. Clones included in the cluster were obtained from all three habitats - three from the mesic mire (C2, C3, C4) and one each from the coastal fellfield

(V22) and biotic mire (L17). The clones in this cluster generally exhibited low similarities to known sequences, with the exception of C3, which exhibited an 85% similarity to a 'hypothetical protein' from *Sinorhizobium meliloti* (Table 3.5), with which it unsurprisingly aligns most closely with on the phylogenetic tree. BLAST analysis places the other clones most closely with various conserved transporter proteins, although none of them closely, and it is probably more likely that these clone sequences fall into the divergent type IV nitrogenases, or are perhaps not nitrogenases at all.

Clusters V and II - Cyanobacteria

Several clones (Clone 10, clone L18 and clone 3) from the biotic mire most closley resembled DPOR and *nifH* genes from cyanobacteria (Table 3.5). Clone 3 most closely aligned with a DPOR gene from *Fremyella displosiphon* (63% identity, Table 3.5), and these sequences fell into cluster V on the phylogenetic tree (Fig 3.22), which was phylogentically distinct from DPOR genes from proteobacteria (cluster III). Clones L18 and clone 10 most closely (82% and 79% respectively) resembled *nifH* proteins from *Nostoc spp.* (Table 3.5) and aligned together in a separate cluster with *Nostoc* and Yushu-11 (uncultured *nifH* gene from the Tibetan Plateau), grouped with cluster 2 for the purposes of discussion (Fig. 3.22).

Line (1992) reported cyanobacteria to be the dominant N_2-fixing organisms on Maquarie Island and his study suggested that they grew symbiotically or epiphytically with plants or lichens, resulting in widespread N_2 reduction in wet habitats. Smith and Russell (1982)

investigated acetylene reduction in epiphytic cyanobacteria on Marion Island, and report that the highest reduction rates occurred 'at a highly mineraltrophic mire receiving nutrient-rich runoff from an adjacent bird-occupied slope', i.e. a biotic mire similar to the one sampled in this study. This suggests that cyanobacteria may play an important role in the soil diazotrophic communities of Marion Island, at least in the biotic mire habitat. Smith and Russell (1982) observed only low rates of acetylene reduction by cyanobacteria in exposed fellfields, and indeed, no genes from cyanobacteria were generated in this study from the fellfield site. This is not surprising, given that bryophytes on Marion Island seem to be positively related with water content (Russell, 1987) and the cyanobacteria appear to be occur mostly with bryophyes and other vegetation growing at wetter sites in polar areas (Kanda and Inoue, 1994).

Nostoc is a fairly ubitiqous organism, found in many environments, and various aspects of its biology and ecology have been described for Antarctic (Horne, 1972; Davey, 1983; Kashyap *et al.*, 1991; Kanda and Inoue, 1994; Novis and Smissen, 2006) and maritime Antarctic (Wirtz *et al.*, (2003) including Signy Island (Christe, 1987) strains. In Antarctic soil, *Nostoc* is a dominant primary producer in Antarctic habitats (Vincent and Quesada, 1994; Olson *et al.*, 1998) and an important source of nitrogen fixation (Seppelt and Broady, 1988; Olson, 1998). Its success in cold habitats is a result of an ability to withstand repeated freeze-thaw cycles (Hawes *et al.*, 1992) and to continue to fix nitrogen at low temperatures (Davey and Marchant, 1983).

In polar regions, it appears to be always associated with bryophytes (Davey, 1982), and probably contributes significant quantities of ammonium for bryophyte growth (Seppelt and Broady, 1988).

Cluster VI

Cluster VI only contains two sequences, clone C7 and a section (255) of the sequence of *Agrobacterium tumefaciens* C58 Genebank Accession number AE009229, which C7 aligned most closely to, although not with a high similarity (50%, Table 3.5). *A. tumefaciens* is believed to have nitrogen-fixing abilities (Kanvinde and Sastry, 1990), and a fragment of the sequence of this organism has been amplified by Simonet *et al.* (1991), using the same primers as in this study, although the very low similarity observed in this study does not suggest a high degree of relatedness between this organism and clone C7.

3.7.2 Diazotroph communities in the different habitats

Mesic Fellfield

BLAST analysis showed that the majority of clones from the mesic fellfield aligned most closely with various proteins from alpha and gamma-proteobacteria (Table 3.5). However, all of these alignments had quite low identities with known sequences, excepting C3, which had a fairly high (85%) sequence similarity with a 'hypothetical protein' of *Sinorhizobium meliloti*. As it is not clear what function this 'hypothetical

protein' might have, it is probably reasonable to hypothesise that it might be a *nif*, or *nif*-like protein sequence, although this may not be the case. *Sinorhizobium* is well-known to have nitrogen-fixing capabilities and sequences from this organism have been amplified with in other studies Simonet *et al.*, 1991, using the same primers as were used in this study.

Only 4 sequences from this habitat were included on the phylogenetic tree, and all except for C7 (see 3.7.1 Cluster VI) fell into cluster IV of *nifH*-like divergent proteins. C3 and C4 aligned most closely with Rhizobiacae species (α-proteobacteria), and C2 with gamma-proteobacteria.

The lack of sequence similarity observed between clones from the mesic fellfield and known sequences is probably not surprising, given that very few studies have been conducted on diazotroph diversity in comparable habitats. Given the physiochemical and biological properties of this habitat, i.e. the highly basic scoria-derived soil with a high Ca and Mg level, the sparse vegetation, relatively high altitude and no biotic influence, it is probably the least attractive (of the three habitats investigated) to diazotrophs, or micro-organisms in general for that matter. The low mean soil temperature (4.1ºC) and high temperature fluctuations (Blake, 1997), mean that micro-organisms inhabiting this habitat would need to be psychotrophic or at least psychotolerant, and be capable of surviving freeze-thaw cycles. The lack of bryophytes excludes many epiphytic cyanobacteria from the community and indeed, Smith and Russell (1982) observed only low rates of acetylene reduction by cyanobacteria in exposed fellfields.

The lack of vascular plants in this habitat might be associated with the poor soil chemistry and lack of usable nitrogen. The low N, P and C levels in the soil may make this habitat unattractive to many micro-organisms. Although low N levels should give diazotrophs a selective advantage (Olson *et al.,* 1998), Zhang *et al.*, 2006 reported that diazotroph diversity on the Tibeatan Plateau was negatively correlated with C and N levels in the soil and were the main factor influencing diversity, along with altitude. Samples from higher altitude sites on the Tibetan plateau had the smallest number of clones, and also exhibited lower diversity of diazotrophs (Zhang *et al.*, 2006). These factors might also play and important role in the distribution of diazotrophs in the soil of Marion Island, as the lowest number of clones containing *nifH* genes (65% compared to 80% for the coastal fellfield and 100% for the biotic mire) and definite *nifH* genes were observed in the mesic fellfield habitat, which is found in high altitudes and has very low N and C levels.

Coastal Fellfield

No *nifH* genes were amplified from the coastal fellfield but one sample (V27) showed high similarity to protochlorophyllidae reductase (see 3.7.1 Clusters III and V). Three clones from this habitat were aligned on the phylogenetic tree and fell into clusters III and IV (see 3.7.1 Clusters III and V and Cluster IV). The coastal fellfield had an intermediate diversity between the mesic fellfield and the biotic mire, and could be said (with some risk of over-simplification) to have an intermediate environment between the two, with some biotic influence from animals, a fibrous peat soil with a high Na and Mg

content which may limit microbial communities to reasonably salt-tolerant organisms. No temperature data is available for this habitat and would prove useful in additional analysis of microbial communities. The vegetation of the coastal fellfield is more complex than the mesic fellfield, but less so than the biotic mire, and consists primarily of dicots, *Crassula moschata* and *Azorella selago*.

Biotic Mire

A wide variety of *nifH* sequences from diazotrophic organisms were obtained from the biotic mire, including α-proteobacteria, cyanobacteria, γ-proteobacteria and uncultured bacteria similar to Artic and Tibetan diazotrophs; a much higher diversity than from the mesic and coastal fellfield habitats. 100% of clones contained *nifH* or *nifH*-like sequences and clones from this habitat were distributed over all clusters excepting VI, and the physiochemical and biological properties of the soil can probably explain this comparatively high diversity. With an average annual temperature of 6.3ºC (Blake, 1997), mire habitats are the warmest on the island and also have the fewest major temperature fluctuations, which makes them much more attractive to micro-organisms than other habitats. With a very wet environment enriched by N input from penguin colonies and seal wallows, the soil has high inorganic N and P levels and supports a variety of vegetation, including bryophytes. Cyanobacteria commonly associated with bryophytes are able to grow and *Nostoc*-type cyanobacteria are probably a fairly important component of the diazotroph community (see section 3.7.1, Clusters V and II.). Purple bacteria (α-protobacteria) similar to *Rhodopseudomonas paulstris* are probably

also a fairly common component. Two identical clones (L11 and L14) showed a sequence most similar to DPOR genes of this species, and genes amplified from clone L14 may represent either DPOR genes or type IV nitrogenases of this nitrogen-fixing organism. *Rhodopsedomonas paulstris*-type nitrogenases were also common in genes amplified from soil diazotrophs in the high Artic (Deslippe and Egger, 2006), so it is likely that related organisms may be important in cold soil microbial communities, although it is also possible that if these genes are in fact type IV nitrogenases, the organisms may not necessarily be active nitrogen-fixers.

CHAPTER 4 – CONCLUSIONS

This is the first, preliminary study of diazotroph diversity in the sub-Antarctic to employ modern molecular techniques. RFLP analysis suggested a very high diversity of *nifH* genes in the soil, as only two clones were identical, and sequence analysis revealed a variety of *nifH*-like genes in the soil of Marion Island. Further RFLP analysis of additional clones would be useful for establishing a truer picture of overall diversity, and it is apparent from this short study that RFLP and clone library analysis of PCR-amplified *nifH* genes from genomic DNA soil extracts are useful genetic tools for assessing diazotroph diversity in the sub-Antarctic. A combination column purification method using PVPP and Sephacryl is useful for removing inhibitory humic acids and increasing PCR yields. Non-specific amplification may cause problems, particularly when using degenerate, universal primers, however, a strategy such as nested PCR may be effective in increasing specificity but was not found to be necessary in this study. It is probable that DGGE might also prove to be a useful method for investigating diazotroph diversity, although it was unsuccessful in this study, it is likely that this is simply the result of experimental or operator error, and time constraints prohibited a more thorough investigation into its effectiveness.

The universal *nifH* primers amplified genes other than specific *nifH* genes, in particular they amplified protochlorophyllide reductase genes used in photosynthesis and sequences derived from clones imply the presence of such proteins in diazotrophic organisms inhabiting Marion Island, and provide additional evidence for their strong structural

similarities. Using *nifH* primers that target more specific groups, such as cyanobacteria would provide useful information regarding the presence of specific groups of diazotrophs in the soil. Of course, not all diazotrophs necessarily contain *nifH* genes, as some use alternative genetic systems to code for nitrogenase, and the use of only one gene to analyse phylogenetic relationships can be misleading (Zehr *et al.*, 2003). Using the *nifH* gene for diversity studies does have many advantages, however. As the highly conserved, 'ideal molecular marker' for nitrogen fixing organisms (Deslippe and Egger, 2006), the majority of studies focus on *nifH.* At least until a more substantial database of diazotroph sequences from sub-Antarctic soils has been established, it is most sensible to concentrate research efforts on this gene, although it would also be interesting to investigate the diversity of nitrogen-fixers using alternative nitrogenases.

Sequence analysis of clones in this study contributes evidence to support Zehr *et al*. (2003) hypothesis that nitrogen-fixing organisms are not randomly distributed but occur in relation to habitat type, even over geographically distant locations. In this case, some of the sub-Antarctic diazotrophs were most closely related to diazotrophs isolated from uncultured bacteria from two other cold, remote environments including the Qingzhang Plateau in Tibet and Canada's Ellesmore Island in the Arctic Circle. The lack of sequence similarity exhibited by many of the clones may reflect the paucity of data on nitrogen-fixing organisms in the sub-Antarctic, a location where diazotrophs probably play a very important role in nutrient cycling.

Given that the biotic mire has the highest soil temperature (Blake, 1997), high moisture content, high organic content (influence from seabird and seal manure) and a bryophyte community capable of supporting nitrogen-fixing cyanobacteria, it is perhaps not surprising that this habitat harbours a greater diversity of diazotrophs than either the coastal fellfield (which is more exposed and has a high Na and Mg content) or the mesic fellfield (with its high altitude, basic soil low in nutrients and sparse vegetation). Cyanobacteria are probably and important (possibly dominant) component of the nitrogen-fixing biota in the Biotic Mire, while α-protobacteria seems to be the most important in the mesic fellfield (from which the fewest *nifH* clones and sequences were obtained). Although additional research is required to confirm results, sequence and phylogenetic analysis from this study indicates that different diazotrophic communities are present in the different ecological habitats on Marion Island.

REFERENCES

Adamson, H.Y.; R. G. Hiller, R.G.; Walmsley, J. (1997). Protochlorophyllide reduction and greening in angiosperms: an evolutionary perspective, *Journal of Photochemistry and Photobiology* **41**:201-221.

Affourtit, J.; Zehr, J.P.; Paerl, H.W. (2001) 'Distribution of nitrogen-fixing organsims along the Neuse River Estuary, North Carolina', *Microbial Ecology* **41**:114-123.

Alexander, V. (1975) 'Nitrogen fixation by blue-green algae in polar and sub-polar regions', In: Stewart, W.D.P. (ed.) *Nitrogen fixation by free-living micro-organisms*, Cambridge University Press, Cambridge, England. pp. 175-188.

Alexander, V.; Schell, D.M. (1973) 'Seasonal and spatial variation of nitrogen fixation in the Barrow Alaska tundra', *Arctic and Alpine Research* **5**: 77-88.

Armstrong, GA (1998) Greening in the dark: light-independent chlorophyll biosynthesis from anoxygenic photosynthetic bacteria to gymnosperms, *Journal of Photochemistry and Photobiology*, **43**, 87-100.

Atlas, R.M.; Bartha, R. (1997) *Microbial Ecology: Fundamentals and Applications 4th Edition*, Benjamin Cummings, California, 306 pp.

Auman, A.; Speake, C.C.; Lidstrom, M.E. (2001) '*nifH* sequences and nitrogen fixation in type I and type II methanotrophs', *Applied and Environmental Microbiology* **67**: 4009-4016.

Bagwell, C.E.; La Rocque, J.R.; Smith, G.W.; Polson, S.W.; Friez, M.J.; J. W. Longshore, J.W.; C. R. Lovell, C.R. (2002) 'Molecular diversity of diazotrophs in oligotrophic tropical seagrass bed communities', *FEMS Microbial Ecology* **39**:113-119.

Bailey, A.D.; Wynn-Williams, D.D. (1982) 'Soil microbiological studies at Signy Island, South Orkney Islands', *British Antarctic Survey Bulletin* **51**: 167-191.

Balkwill, D.L.; Labeda, D.P.; Casida, L.E. Jr. (1975) 'Simplified procedure for releasing and concentrating microorganisms from soil for transmission electron microscopy viewing as thin-section and frozen-etched preparations', *Canadian Journal of Microbiology* **21**: 252-262.

Baker, G.C.; Ah Tow, L.; Cowan, D.A. (2003) 'PCR-based detection of non-indigenous microorganisms in "pristine" environments', *Journal of Microbiological Methods* **53**: 157-164.

Baker, J.H. (1970a) 'Quantitative study of yeasts and bacteria in a Signy island peat', *British Antarctic Survey Bulletin* **23**: 51-55.

Baker, J.H. (1970b) 'Yeasts, moulds and bacteria from an acid peat on Signy Island', In: Holdgate, M. (ed.) *Antarctic Ecology* **2**, Academic Press, London, pp. 717-722.

Baker, J.H.; Smith, D.S. (1972) 'The bacteria in an Antarctic peat', *Journal of Applied Bacteriology* **35**: 589-596.

Bardgett, R.D.; Mawdsley, J.L.; Edwards, S.; Hobbs, P.J.; Rodwell, J.S.; Davis, W.J. (1999) 'Plant species and nitrogen effects on soil biological properties of temperate upland grasslands', *Functional Ecology* **13**: 650-660.

Ben-Porath, J.; Zehr, J.P. (1994) 'Detection and characterization of cyanobacterial *nifH* genes', *Applied and Environmental Microbiology* **60**: 880-887.

Bergstrom, D.M.; Chown, S.L. (1999) 'Life at the front: history, ecology and change on southern ocean islands', *Tree* **14**: 472-477.

Blackburn, T.H. (1983) 'The microbial nitrogen cycle', In: Krumbein, C.W.E. (ed.) *Microbial Geochemistry*, Blackwell, Oxford pp. 63-89.

Block, W. (1984) 'Terrestrial microbiology, invertebrates and ecosystems', In: Laws, R.M. (ed.) *Antarctic Ecology* **1**, Academic Press, London, pp. 163-236.

Blume, H.P; Schneider, D.; Bölter, M. (1996) 'Organic matter accumulation in and podzolisation of Antarctic soils', *Zeitschrift für Pflanzenernährung und Bodenkunde* **159**:411-412.

Bölter, M., Blume, H.P.; Schneider, D.; Beyer, L. (1997) 'Soil properties and distributions of invertebrates and bacteria from King George Island (Arctowski Station), maritime Antarctic', *Polar Biology* **18**: 295-304.

Boyle, J.S. and Lew, A.M. (1995) 'An inexpensive alternative to glassmilk for DNA purification', *Trends in Genetics* **11**:8.

Brookes, P.D.; Williams, M.W, Schmidt, S.K. (1996) 'Microbial activity under alpine snowpacks, Niwot Ridge, Colorado', *Biogeochemistry* **32**: 93-113.

Brown, C.M. and Johnson, B. (1977) 'Inorganic nitrogen assimilation in aquatic microorganisms', *Advances in Aquatic Microbiology* **1**: 49-114.

Bürgmann, H.; Widmer, F.; Sigler, W.V.; Zeyer, J. (2004) 'New molecular screening tools for analysis of free-living diazotrophs in soil', *Applied and Environmental Microbiology* **70**: 240-247.

Burke DH, Hearst JE, Sidow A (1993) Early evolution of photosynthesis: clues from nitrogenase and chlorophyll iron proteins. *Proceedings of the National Academy of Science USA*, **90**: 7134-7138.

Cejudo, F.J.; Paneque, A. (1986) 'Short-term nitrate (nitrite) inhibition of nitrogen fixation in *Azotobacter chroococcum*', *Journal of Bacteriology* **165**:240-243.

Chown, S.L.; Smith, V.R. (1993) 'Climate change and the short-term impact of feral house mice at the sub-Antarctic Prince Edward Islands', *Oecologia* **96**: 508-516.

Christie, P. (1987) 'Nitrogen in two contrasting Antarctic bryophyte communities', *Journal of Ecology* **75**: 73-94.

Clein, J.S.; Schimel, J.P. (1995) 'Microbial activity of tundra and taiga soils at sub-zero temperatures', *Soil Biology and Biochemistry* **27**: 1231-1234.

Collins, N.J.; Baker, J.H. and Tillbrook, P.J. (1975) 'Signy Island, maritime Antarctica', In: Rosswell, T. and Heal, O.W. (eds.) *Structure and Function of Tundra Ecosystems Ecological Bulletin* **20**, Swedish Natural Science Research Council, Stockholm, pp. 345-374.

Croome, R.L. (1973) 'Nitrogen fixation in the algal mats on Marion Island', *South African Journal of Antarctic Research* **3**: 64-67.

Davey, A. (1982) 'In situ determination of nitrogen fixation in Antarctica using a high sensitivity portable gas chromatograph', *Australian Journal of Ecology* **7**: 395-402.

Davey, A. (1983) 'Effects of abiotic factors on nitrogen fixation by blue-green algae in Antarctica', *Polar Biology* **2**: 95-100.

Davey, A.; Marchant, H.J. (1983) 'Seasonal variation in nitrogen fixation by *Nostoc* commune Vaucher at the Vestfold Hills, Antarctica', *Phycologica* **22**: 377-385.

Davey, M.C. (1988) 'Ecology of terrestrial algae of the fellfield ecosystems of Signy island, South Orkney Islands', *British Antarctic Survey Bulletin* **81**: 69-74.

Davey, M.C.; Pickup, J.; Block, W. (1992) 'Temperature variation and its biological significance in fellfield habitats on a maritime Antarctic island', *Antarctic Science* **4**: 383-388.

Deslippe, J.R.; Egger, K.N.: Henry, G.H.R. (2005) 'Impacts of warming and fertilization on nitrogen-fixing microbial communities in the Canadian High Arctic', *FEMS Microbiology and Ecology* **53**: 41-50.

Deslippe, J.R.; Egger, K.N. (2006) 'Molecular diversity of *nifH* genes from bacteria associated with High Arctic Dwarf Shrubs', *Microbial Ecology* **51**: 516-525.

Diallo, M.D.;Willems, A.; Vlomans, N.;Cousin, S.; Vandekerckhove, T.T.; de Lajudie, P.; Neyra, M.; Vyverman, W.; Gillis, M.; Van der Gucht, K. (2004) 'Polymerase chain reaction denaturing gradient gel electrophoresis analysis of the N_2-fixing bacterial diversity in soil under *Acacia tortilis* ssp. *raddiana* and *Balanities aegyptiaca* in the dryland part of Senegal' *Environmental Microbiology* **6**: 400-415.

Döbereiner, J.; Day, J.M. (1974) 'Nitrogen fixation in the rhizosphere of tropical grasses', In: Stewart, W.D.P. (ed.) *Nitrogen Fixation by Free-Living Bacteria*, Cambridge University Press, Cambridge, England.

Dowding, P.; Widden, P. (1974) 'Some relationships between fungi andtheir environment in tundra regions', In: Holding, A.J.; Heal, O.W.; Maclean, S.F. Jnr and Flanagan, P.W. (eds.*), Soil Organisms and Decompostion in Tundra*, Tundra Biome Steering Committee, Stockholm.

Dunt, J.S. (1965) 'Observations on the fungi of Macquarie Island', *ANARE Scientific Reports Series B* **78**: 22 pp.

Ellingsøe, P.; Johnsen, K. (2002) 'Influence of soil sample sizes on the assessment of bacterial community structure', *Soil Biology and Biochemistry* **34**: 1701-1707.

Ellis-Evans, J.C.; Wynn-Williams, D.D. (1985) 'Interaction of soil and lake microflora at Signy Island', Siegfried, W.R.; Condy, P.R. and Laws, R.M. (eds.) *Proceedings from the 4th Scientific Committee on Antarctic Biology*, Springer-Verlag, Berlin.

Erskine, P.D.; Bergstrom, D.M.; Schmidt, S.; Stewart, G.R.; Tweedie, C.E. and Shaw, J.D. (1998), 'Subantarctic Macquarie Island – a model ecosystem for studying animal-derived nitrogen sources using ^{15}N natural abundance', *Oecologia* **177**: 187-193.

Falcón, L.I.; Cipriano, F.; Chistoserdov, A.Y.; Carpenter, E.J. (2002) 'Diversity of diazotrophic unicellular cyanobacteria in the tropical North Atlantic Ocean', *Applied and Environmental* Microbiology **68**: 5760-5764.

Faegri, A.; Torsvik, V.L.; Goksøyr, J. (1977) 'Bacterial and fungal activities in soil: separation of bacteria by a rapid centrifugation technique', *Soil Biology and Biochemistry* **9**: 105-112.

Felske, A.; Wolterlink, A.; Van Lis, R.; Akkermans, A.D.L. (1997) 'Phylogeny of the main 16S rRNA sequences in Drentse A grassland soils (the Netherlands)', *Applied and Environmental Microbiology* **64**: 871-879.

Fischer, H.M. (1994) 'Genetic regulation of nitrogen fixation in rhizobia', *Microbiological Reviews* **58**: 352-386.

Fisk, M.C.; Schmidt, S.K. (1996) 'Microbial responses to nitrogen additions in alpine tundra soils', *Soil Biology and Biochemistry* **28**: 751-755.

French, D.D.; Smith, V.R. (1986) 'Bacterial populations in soils of a sub-Antarctic Island', *Polar Biology* **5**: 5-21.

Frost, P.G.H. (1979) 'Seabird distribution and transport of nutrients from marine to terrestrial ecosystems', *South African Journal of Antarctic Research* **9**: 20-27.

Fujita, Y., Takahashi, Y. Shonai, F. Ogura, Y., H. Matsubara, H. (1991) 'Cloning, nucleotide sequence and differential expression of the *nifH* and *nifH*-like (*frxC*) genes from filamentous nitrogen-fixing cyanobacterium, *Plectonema boryanum*', *Plant Cell Physiology* **32**:1093-1106.

Fujita Y (1996) 'Protochlorophyllide reduction: a key step in the greening of plants', *Plant Cell Physiology* **37**:411-421.

Fujita Y., Bauer C.E. (2000) 'Reconstitution of light-independent protochlorophyllide reductase from purified Bchl and BchN-BchB subunits', *The Journal of Biological Chemistry* **275**: 23583-23588.

Fujita Y., Matsumoto H., Takahashi Y., Matsubara H. (1993) 'Identification of a *nifDK*-like genes (ORF467) involved in the biosynthesis of chlorophyll in the cyanobacterium *Plectonema boryanum*', *Plant Cell Physiology* **34**: 305-314.

Gillham, M.E. (1961) 'Modification of sub-Antarctic flora on Macquarie Island by sea birds and sea elephants', *Proceedings from the Royal Society of Victoria* **74**: 1-12.

Grobler, D.C.; Toerien, D.F.; Smith, V.R. (1987) 'Bacterial activity in soils of a sub-Antarctic island', *Soil Biology and Biochemistry* **19**: 483-490.

Grundmann, G.L.; Debouzie, D. (2000) 'Geostatistical analysis of the distribution of NH_4^+ and NO_2^- oxidizing bacteria and serotypes at the millimeter scale along a soil transect', *FEMS Microbiology Ecology* **34**: 57-62.

Guerinot, M.L.; Colwell, R.R. (1985) 'Enumeration, isolation and characterization of N_2-fixing bacteria from seawater', *Applied and Environmental Microbiology* **50**: 350-355.

Hamelin, J.; Fromin, N., Tarnawski, S.; Teyssier-Cuvelle, S.; Aragno, M. (2002) '*nifH* gene diversity in the bacterial community associated with the rhizooosphere of *Molinia coerulea*, an oligotrophic perennial grass', *Environmental Microbiology* **4**: 477-481.

Hänel, C.; Chown, S. (1998) *An introductory guide to the Marion and Prince Edward Island Special Nature Reserves*, Department of Environmental Affairs and Tourism, Pretoria.

Hansen, M.C., Tolker-Neilson, T., Givskov, M.; Molin, S. (1998) 'Biased 16S rDNA PCR amplification caused by interference from DNA flanking the template region', *FEMS Microbiology Ecology* **26**: 141–149.

Harry, M.; Gambier, B.; Bourezgui, Y.; Ganier-Sillam, E. (1999) 'Evaluation of purification procedures for DNA extracted from organic rich samples: interference with humic substances', *ANALUSIS*, **27**: 439-442.

Hawes, I.; Howard-Williams, C.; Vincent, W.F. (1992) 'Dessication and recovery of Antarctic cyanobacterial mats', *Polar Biology* **12**: 587-594.

Heal, O.W.; Bailey, A.D.; Latter, P.M. (1967) 'Bacteria, fungi and protozoa in Signy island soils compared with those from a temperate moorland', *Philosophical Transactions of the Royal Society (London) Series B* **252**: 191-197.

Hennecke, H.; Kaluza, K.;Thöny, B.; Fuhrmann, M.; Ludwig, W.; Stackebrandt, E. (1985) 'Concurrent evolution of nitrogenase genes and 16S rRNA in *Rhizobium* species and other nitrogen-fixing bacteria', *Archaea Microbiology* **142**: 342-348.

Holben, W.E.; Jansson, J.K.; Chelm, B.K. and Tiedje, J.M. (1988) 'DNA probe methods for the detection of specific microorganisms in the soil bacterial community', *Applied and Environmental Microbiology* **33**: 1225-1228.

Holben, W.E. (1994) 'Isolation and purification of bacterial DNA from soil', *Methods of Soil Analysis, Part 2 – Microbiological and Biochemical Properties.* Madison, USA, Soil Science Society of America. pp. 727-751.

Holding, A.J. (1981) 'The microflora of tundra', In: Bliss, L.C.; Heal, O.W.; Moore, J.J. (eds.) *Tundra ecosystems: a comparative analysis*, Cambridge University Press, Cambridge, pp. 561-586.

Holdgate, M.W. (1977) 'Terrestrial ecosystems in the Antarctic', In: Fuchs, V.E.; Laws, R.M (eds.) Scientific research in the Antarctic: *Philosophical Transactions of the Royal Society of London* **B279**:5-25.

Holdgate, M.W.; Allen, S.E.; Chambers, M.J.G. (1967) 'A preliminary investigation of soils of Signy Island, South Orkney Islands', *British Antarctic Survey Bulletin* **53**: 57-75.

Horne, A.J. (1972) 'The ecology of nitrogen fixation on Signy Island, South Orkney Islands', *British Antarctic Survey Bulletin*: **27**: 1-18.

Hoss, M.; Kohn, M.; Paabo, S.; Knauer, F.; Schroder, W. (1992) 'Excrement analysis by PCR', *Science* **359**: 199.

Howard, J.B. and Rees, D.C. (1996) 'Structural basis of biological nitrogen fixation', *Chemical Reviews* **96**: 2965-2982.

Huget, V.; McGray Batzli, J.; Zimpfer, J.F.; Normand, P.; Dawson, J.O.; Fernandez, M.P. (2001) 'Diversity and specificity of *Frankia* strains in nodules of sympatric *Myrica gale*, *Alnus incana* and *Shepherdia Canadensis* determined by rrs gene polymorphism', *Applied and Environmental Microbiology* **67**: 2116-2122

Huiskes, A.H.L.; Aerts, R.; Boschker, H.T.S.; Cornelissen, J.H.C. ; Pel, R.; Rozema, J. and Moerdijk, T.C.W. (2004) 'Effects of environmental change on carbon and nitrogen fluxes in Antarctic terrestrial ecosystems', *Regional Sensitivity to Climate Change in Antarctic Terrestrial and Limnetic Ecosystems Projects*, http://www.riscc.aq/ , accessed 5 June, 2004.

Huyser, O.; Ryan, P.G.; Cooper, J. (2000) 'Changes in population size, habitat use and breeding biology of lesser sheathbills at Marion Island: impact of cats, mice and climate change?', *Biological Conservation* **92**: 299-310.

Izquierdo, J. and Nüsslein, K. (2005) 'Nitrogen fixation in Arctic soil environments', http://www.bio.umass.edu/micro/nusslein.nifasme/data.htm

Izquierdo, J.A.; Nüsslein, K. (2006) 'Distribution of extensive *nifH* gene diversity across physical soil environments', *Microbial Ecology* **51**: 441-452.

Jackson, C.R.; Harper, J.P.; Willoughby, E.; Rosen, E.; Churchhill, P.F. (1997) 'A simple, efficient method for separation of humic acids and DNA from environmental samples', *Applied and Environmental Microbiology* **63**: 4993-4995.

Jaeger, III C.H.; Monson, R.K.; Fisk, M.C.; and Schmidt, S.K. (1999) 'Seasonal partitioning of nitrogen by plants and soil microorganisms in an alpine ecosystem', *Ecology* **80**: 1883-1891.

Joerger, R.D. and Bishop, P.E. (1988) 'Bacterial alternative nitrogen fixation systems', *Critical Reviews in Microbiology* **16**:1-14.

Kanda, H.; Inoue, M. (1994) 'Ecological monitoring of moss and lichen vegetation in the Syowa station area, Antarctica', *Polar Biology* **7**: 221-231.

Kanvinde, L.; Sastry, G.R.K. (1990) '*Agrobacterium tumefaciens* is a diazotrophic bacterium', *Applied and Environmental Microbiology* **56**: 2087-2092.

Kashyap, A.K.; Pandey, K.D.; Guptar, K. (1991) 'Nitrogenase activity of the Antarctic cyanobacterium Nostoc commune: influence of temperature', *Folia Microbiologica* **36**: 557-560.

Kessler, P.S.; Mclarnan, J.; Leigh, J.A. (1997) 'Nitrogenase phylogeny and the molybdenum dependence of nitrogen fixation in *Methanococcus maripaludis*', *Journal of Bacteriology* **179**: 541-543.

Kolb, W.; Martin, P. (1988) 'Influence of nitrogen on the number of N_2-fixing and total bacteria in the rhizosphere', *Soil Biology and Biochemistry* **20**: 221-225.

Kreader, C. (1996) 'Relief of amplification inhibition in PCR with bovine serum albumim or T4 gene 32 protein', *Applied and Environmental Microbiology* **62**: 1102-1106.

Ladd, J.N.; Forster, R.C.; Nannipieri, P.; Oades, J.M. (1996) 'Soil structure and biological activity', *Soil Biochemistry* **9**:23:78.

Langlois, R.J.; LaRoche, J.; Raab, P.A. (2005) 'Diazotrophic diversity and distribution in the tropical and subtropical Atlantic Ocean', *Applied and Environmental Microbiology* **71**: 7910-7919.

Latter, P.M.; Heal, O.W. (1971) 'A preliminary study of the growth of fungi and bacteria from temperate and Antarctic soils in relation to temperature', *Soil Biology and Biochemistry* **3**: 365-379.

Levitt, J. (1980) *Responses of plants to environmental stresses. Volume 1: Chilling, freezing and high temperature stress*. Academic Press, New York. 497 pp.

Lindeboom, H.J. (1984) 'The nitrogen pathway in a penguin rookery', *Ecology* **65**: 269-277.

Line, M.A. (1992) 'Nitrogen fixation in the sub-Antarctic Macquarie Island', *Polar Biology* **11**: 601-606.

Limmer, C.; Drake, H.L. (1998) 'Effect of carbon, nitrogen, and electron acceptor availiablity on anerobic N2 fixation in beech forest soil', *Soil Biology and Biochemistry* **30**: 153-158.

Lipson, D.A.; Raab, T.K.; Schmidt, S.K.; Monson, R.K. (2001) 'Microbial and environmental controls over amino acid turnover in an alpine soil', *Soil Biology and Biochemistry* **33**: 189-198.

Lipson, D.A.; Schadt, C.W.; Schmidt, S.K. (2002) 'Changes in soil microbial community structure and function in an Alpine dry meadow following spring snow melt', *Microbial Ecology* **43**: 307-314.

Lipson, D.A.; Schmidt, S.K. (2004) 'Seasonal changes in an alpine soil bacterial community in the Colorado Rocky Mountains', *Applied and Environmental Microbiology* **70**: 2867-2879.

Lipson, D.A.; Schmidt, S.K.; Monsoon, R.K. (1999) 'Links between microbial population dynamics and nitrogen availability in an alpine ecosystem', *Ecology* **8**: 1623-1631.

Lipson, D.A.; Schmidt, S.K.; Monson, R.K. (2000) 'Carbon availability and temperature control the post-snowmelt decline of microbial biomass in an alpine soil', *Soil Biology and Biochemistry* **32**: 441-448.

Lovell, C.R.; Friez, M.J.; Longshore, J.W.; Bagwell, C.E. (2001) 'Recovery and phylogenetic analysis of *nifH* sequences from diazotrophic bacteria associated with dead aboveground biomass of *Spartina alterniflora*', *Applied and Environmental Microbiology* **67**: 538-5314.

Macleod, R.A.; Calcott, P.H. (1976) 'Cold shock and freezing damage to microbes', In: Gray, T.R.G.; Postgate, J.R. (eds.) *The survival of vegetative microbes*, Cambridge University Press, Cambridge.

Madigan, M.T.; Markinko, J.M.; Parker, J. (2000) *Brock Biology of Microorganisms Ninth Ed.* Prentice Hall International Inc, London.

Malik, M.; Kain, J.; Pettigrew, C.; Ogram, A. (1994) 'Purification and molecular analysis of microbial DNA from compost', *Journal of Microbiological Methods* **20**: 183-196.

Maxam A.M., Gilbert W. (1977) 'A new method for sequencing DNA', *Proceedings of the National Academy Science* **74**: 560-4

Mehta, M.P.; Butterfield, D.A.; Baross, J.A. (2003) 'Phylogenetic diversity of nitrogenase (*nifH*) genes in deep-sea and hydrothermal vent environments of the Juan de Fuca ridge', *Applied and Environmental Microbiology* **69**: 960-970.

Miller, D. N.; Bryant, J.E.; Madsen, E.L.; Ghiorse, W.C. (1999) 'Evaluation and Optimization of DNA Extraction and Purification Procedures for Soil and Sediment Samples', *Applied and Environmental Microbiology* **65**: 4715-4724.

Mullis, K.B. (1990) 'The unusual origin of the polymerase chain reaction', *Scientific American* **262**: 56-65.

Mullis, K.B.; Faloona, F.A. (1987) 'Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction', *Methods in Enzymology* **155**: 335-351.

Mullis, K.; Faloona, F.; Scharf, S.; Saiki, R.; Horn, G.; Ehrlich, H. (1986) 'Specific enzymatic amplification of DNA in vitro: The polymerase chain reaction', *Cold Spring Harbour Symposium on Quantitative Biology* **51**: 263-273.

Muyzer, G.; deWaal, E.D.; Vitterlinden, A.G. (1993) 'Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain

reaction amplified genes coding for 16S rRNA', *Applied and Environmental Microbiology* **59**: 695-700.

National Academy of Sciences (2004) 'Chapter 2: Important Questions in Polar Biology', *Frontiers in Polar Biology in the Genomic Era*, National Academies Press, pp. 25-81.

Normund, P.; Bousquet, J. (1989) 'Phylogeny of nitrogenase sequences in *Frankia* and other nitrogen-fixing microorganisms', *Journal of Molecular Evolution* **29**: 436-447.

Novis, P.M.; Smissen, R.D. (2006) 'Two genetic and ecological groups of Nostoc commune in Victoria Land, Antarctica, revealed by AFLP analysis', *Antarctic Science* **18**: 573-581.

Ohkuma, M.; Noda, S.; Kudo, T. (1999) 'Phylogenetic diversity of nitrogen fixation genes in the symbiotic microbial community in the gut of diverse termites', *Applied and Environmental Microbiology* **65**: 4926-2934.

Ogram, A.; Sayler, G.S.; Barkay, T. (1987) 'The extraction and purification of microbial DNA from sediments', *Journal of Microbiological Methods* **7**: 57-66.

Oz, A.; Sabehi, G.; Koblízek, M.; Massana, R.; Béjà, O. (2005) '*Roseobacter*-like bacteria in Red and Mediterranean Sea aerobic anoxygenic photosynthetic populations', *Applied and Environmental Microbiology* **71**: 344-353.

Pace, N.R. (1996) 'New perspectives on the natural microbial world: molecular microbial ecology', *ASM News* **62**: 463-470.

Piceno, Y.M.; Lovell, C.R. (2000) 'Stability in natural bacterial communities: I. Nutrient addition effects on rhizosphere diazotroph assemblage composition', *Microbial Ecology* **39**: 32-40.

Piceno, Y.M.; Noble, P.A.; Lovell, C.R. (1999) 'Spatial and temporal assessment of diazotroph assemblage composition in vegetated salt mash sediments using denaturing gradient gel electrophoresis', *Microbial Ecology* **38**: 157-167.

Pietr, S.J. (1986) 'The physiological groups of microorganisms in different soils at Admiralty Bay region (King George Island, South Shetland Islands, Antarctica)', *Polish Polar Research* **7**: 385-405.

Poly, F.; Ranjard, L.; Nazaret, S.; Gourbiere, F.; Monrozier, L.J. (2001) 'Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen-fixers in soil', *Research in Microbiology* **152**: 95-103.

Postgate, J.(1998) *Nitrogen Fixation*, 3rd Edition, Edward Arnold Publishers, Baltimore, p 108.

Priscu, J.C.; Christner, B.C. (2002) 'Earth's icy biosphere', In: Bull, A. (ed.) *Microbial Diversity and Bioprospecting*, ASM Press, pp. 130-146.

Pugh , G.J.F.; Allsopp, D. (1982) 'Micro-fungi on Signy Island, South Orkney Islands, South Atlantic Ocean', *British Antarctic Survey Bulletin* **57**: 55-68.

Ramsay, A.J. (1983), 'Bacterial biomass in ornithogenic soils of Antarctica', *Polar Biology* **1**: 221-225.

Ramsey, A.J.; Stannard, R.E. (1986) 'Numbers and viability of bacteria in ornithogenic soils of Antarctica', *Polar Biology* **5**: 195-198.

Ranjard, L.; Lejohn, D.P.H.; Mouogel, C.; Schehrer, L.; Merdinoglu, D.; Chaussod, R. (2003) 'Sampling strategy in molecular microbial ecology: influence of soil sample size on DNA fingerprinting analysis of fungal and bacterial communities', *Environmental Microbiology* **5**: 1111-1120.

Raymond, J.; Siefert, J.L.; Staples, C.R.; Blankenship, R.E. (2004) 'The natural history of nitrogen fixation', *Molecular Biology and Evolution* **21**: 541-554.

Reysenbach, A.L.; Pace, N.R. (1995) in *Archaea: A Laboratory Manual* – Thermophiles (Robb, F.T. and Place, A.R., eds.), Cold Spring Harbour Press, Cold Spring Harbour, pp. 101–107.

Ribbe, M.; Gadkari, D.; Meyer, O. (1997) 'N_2 fixation by *Streptomyces thermoautotrophicus* involves a molybdenum-dinitrogenase and a manganese-superoxide oxidoreductase that couple N_2 reduction to the oxidation of superoxide produced from O_2 by a molybdenum-CO dehydrogenase', *Journal of Biological Chemistry* **272**: 26627-26633.

Riffkin, P.A.; Quigley, P.E.; Kearney, G.A.; Cameron, F.J.; Gault, R.R.; Peoples, M.B.; Thies, J.E. (1999) 'Factors associated with biological nitrogen fixation in dairy pastures in south-western Victoria', *Australian Journal of Agricultural Research* **50**: 261-272.

Roll, J.T.; Shah, V.K.; Dean, D.R.; Roberts, G.P. (1995) 'Characteristics of NIFNE in *Azotobacter vinelandii*: implications for the synthesis of the iron-molybdenum cofactor of dinitrogenase', *Journal of Biological Chemistry* **270**: 4432-4437.

Romanowski, G.; Lorenz, M.G.; Wackernagel, W. (1993) 'Use of polymerase chain reaction and electroporation of *Escherichia coli*. to monitor the persistence of extracellular plasmid DNA introduced into natural soils', *Applied and Environmental Microbiology* **59**: 3438- 3446.

Rosado, A.S.; Duarte, G.F.; Seldin, L.; Dirk Van Elsas, J. (1998) 'Genetic diversity of *nifH* gene sequences in *Paenibacillus azotofixans* strains and soil samples analyzed by

denaturing gradient gel electrophoresis of PCR-amplified gene fragments', *Applied and Environmental Microbiology* **64**: 2770-2779.

Rösch, C., Mergel, A.; Bothe, H. (2002) 'Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil', *Applied and Environmental Microbiology* **68**: 3818-3829.

Roszak, D.B.; Colwell, R.R. (1987) 'An improved method for purifying DNA from soil for polymerase chain reaction amplifications and molecular ecology applications', *Molecular Ecology* **6**: 787-791.

Roszak, D.B.; Colwell, R.R. (1987) 'Survival strategies of bacteria in the natural environment', *Microbiology Review* **51**: 365-379.

Russell, S. (1987) 'Water relations and nutrient status of bryophytes communities at Marion Island (sub-Antarctica), *Symposium Biologica Hungrica* **35**: 39-56.

Sanger F.; Coulson, A.R., 'A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase'. *Journal of Molecular Biology* **94**: 441-448.

Seppelt, R.D.; Broady, P.A. (1988) 'Antarctic terrestrial ecosystems: the Vestfold Hills in context', *Hydrobiologia* **165**: 177-184

Schulze, V.R. (1971) 'The climate on Marion Island', In: van Zinderen Bkker, E.M.; Winterbottom, J.M.; Dyer R.A. (eds.) *Marion and Prince Edward Islands*, A.A. Balkema, Cape Town, pp. 16-31.

Schnitzer, M. (1991) 'Soil organic matter – the next 75 years', *Soil Science* **151**: 41-58.

Shaffer, B.T. Widmer, F.; Porteous, L.A.; Seidler, R.J. (2000) 'Temporal and spatial distribution of the *nifH* gene of N_2-fixing bacteria in forests and clearcuts in Western Oregon', *Microbial Ecology* **39**:12-21.

Sieburth J. M. (1963) 'Effect of the nematicides oxamyl and phenamiphos on numbers of bacteria and algae in soil under pasture and on foliage', *New Zealand Journal of Science* **27**: 197-207.

Singh, J.S.; Raghubanshi, A.S.; Singh,R.S.; Srivastava, S.C. (1989) 'Microbial biomass acts as a source of plant nutrients in dry tropical forest and savanna', *Nature* **338**: 499-500.

Simonet, P.; Grosjean, M.; Misra, A.K.; Nazaret, S.; Cournoyer, B.; Normand, P. (1991) '*Frankia* genus-specific characterization by polymerase chain reaction', *Applied and Environmental Microbiology* **57**: 3278-3286.

Simpson, F.B.; Burris, R.H. (1984) ‘A nitrogen pressure of 50 atmospheres does not prevent evolution of hydrogen by nitrogenase’, *Science* **224**:1095–1097.

Smith, H.G.; Tearle, P.V. (1985) 'Aspects of microbial and protozoan abundances in Signy island fellfields', *British Antarctic Survey Bulletin* **66**: 83-90.

Smith, O.W. (1982) ‘Nitrogen fixation’, In: Burns, R.G.; Slater, J.H. (eds.) *Experimental Microbial Ecology*, Blackwell, Oxford, pp. 212-220.

Smith, V.R. (1977) ‘A qualitative description of energy flow and nutrient cycling in the Marion Island terrestrial ecosystem’, *Polar Record* **18**: 361-370.

Smith, V.R. (1978) ‘Standing crop and production estimates of selected Marion Island plant communities’, *South African Journal of Antarctic Research* **8**: 102-105.

Smith, V.R. (1984) ‘Effects of abiotic factors on acetylene reduction by cyanobacteria epiphytic on moss at a sub-Antarctic island’, *Applied and Environmental Microbiology* **48**: 594-600.

Smith, V.R. (1985) *Seasonal dynamics of standing crop and chemical composition in Marion Island (sub-Antarctic) vegetation*, unpublished Ph.D. thesis, University of Orange Free State, Bloemfontein.

Smith, V.R. (1987a) ‘Production and nutrient dynamics of plant communities on a sub-Antarctic island. 1. Standing crop and primary productivity of mire-grasslands’, *Polar Biology* **7**: 57-75.

Smith, V.R. (1987b) ‘Production and nutrient dynamics of plant communities on a sub-Antarctic island. 2. Standing crop and primary productivity of fjaeldmark and fernbrakes’, *Polar Biology* **7**: 125-144.

Smith, V.R. (1987c) ‘Production and nutrient dynamics of plant communities on a sub-Antarctic island. 3. Standing stocks, uptake and loss of nutrients in mire-grasslands’, *Polar Biology* **8**: 135-153.

Smith, V.R. (1988) 'Production and nutrient dynamics of plant communities on a sub-Antarctic Island. 5. Nutrient budgets and turnover times for mire-grasslands, fieldmark and fernbrakes', *Polar Biology* **8**: 255-269.

Smith, V.R. (1993) ‘Climate change and ecosystem functioning: a focus for sub-Antarctic research in the 1990s’, *South African Journal of Science* **89**: 69-71.

Smith, V.R. (2003) ‘Soil respiration and its determinants on a sub-Antarctic island’, *Soil Biology and Biochemistry* **35**: 77-91.

Smith, V.R. (2004) *The biology and ecology of sub-Antarctic islands as carried out on Marion and Prince Edward Islands*, Department of Botany, Stellenbosch University, http://www.sun.ac.za/botany/smithresearch.html.

Smith, V.R.; Hilmer, T. (1984) 'Bacterial numbers in the freshwater bodies of a sub-Antarctic island', *South African Journal of Antarctic Research* **14**: 23-26.

Smith, V.R.; Russell, S. (1982) 'Acetylene reduction by bryophyte-cyanobacteria associations on a Subantarctic island', *Polar Biology* **1**: 153-157.

Smith,V.R.; Steenkamop, M. (1990) 'Climatic change and its ecological implications at a sub-Antarctic island', *Oecologia* **85**: 14-24.

Smith, V.R.; Steenkamp, M. (1992) 'Soil nitrogen transformations on a sub-Antarctic island', *Antarctic Science* **4**: 41-50.

Smith, V.R.; Steenkamp, M. (2001) 'Classification of the terrestrial habitats on Marion Island based on vegetation and soil chemistry', *Journal of Vegetation Science* **12**: 181-198.

Smith, V.R.; Steyn, M.G. (1982) 'Soil microbial counts in relation to site characteristics at a Subantarctic island', *Microbial Ecology* **8**: 253-266.

Smith, V.R.; Steenkamp, M., French, D.D. (1993) 'Soil decomposition potential in relation to environmental factors on Marion Island (sub-Antarctic)', *Soil Biology and Biochemistry* **25**: 1619-1633.

Steffan, R.J.; Goksoyr, J.; Bej, A.K.; Atlas, R.M. (1988) 'Recovery of DNA from soils and sediments', *Applied and Environmental Microbiology* **54**: 2908-2915.

Stevenson, F.J. (1976) 'Organic matter reactions involving pesticides in soils', In: Kaufman, D.D.; Still, G.G.; Paulson, G.D.; Bandal, S.K. (eds.), Bound and Conjugated Pesticide Residues, ACS Symposium Series vol. 29, American Chemical Society, Washington D.C. pp. 180-207.

Steward, G.F.; Zehr, J.P.; Jellison, R.; Montoya, J.P.; Hollibaugh, J.T. (2004) 'Vertical distribution of nitrogen-fixing phylotypes in a meromictic hypersaline lake', *Microbial Ecology* **47**: 30-40.

Steyn, C.D.; Holland, P.W. (1993) *Essential Developmental Biology: A Practical Approach*, Oxford University Press, Oxford, England.

Steyn, M.G.; Smith, V.R. (1981) 'Microbial populations in Marion Island soils', *South African Journal of Antarctic Research* **10/11**: 14-18.

Suzuki, J.Y.; Bauer, C.E. (1992) 'Light-independent chlorophyll biosynthesis: involvement of the chloroplast gene chlL (*frxC*)', *Plant Cell* **4**: 929-940.

Tearle, P.V. (1987) 'Cryptogamic carbohydrate release and microbial response during spring freeze-thaw cycles in Antarctic fellfield fines', *Soil Biology and Biochemistry* **19**: 381-390.

Tearle, P.V.; Richard, K.J. (1987) 'Ecophysiological grouping of Antarctic environmental bacteria by APU 20 NE and fatty acid fingerprints', *Journal of Applied Bacteriology* **63**: 497-503.

Tebbe, C. C.; Vahjen, W. (1993). 'Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast', *Applied Environmental Microbiology* **59**: 2657-2665

Theron, J.; Cloete, T.E. (2000) 'Molecular techniques for determining microbial diversity and community structure in natural environments', *Critical Reviews in Microbiology* **26**: 37-57.

Thompson, J.D., Higgins, D.G.; Gibson, T.J. (1994) 'CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice', *Nucleic Acids Research* **22**: 4673-4680.

Torsvik, V.L. (1980) 'Ísolation of bacterial DNA from soil', *Soil Biology and Biochemistry* **12**: 15-21.

Torsvik, V.L.; Goksøyr, J. (1978) 'Determination of bacterial DNA in soil', *Soil Biology and Biochemistry* **10**: 7-12.

Tsai, Y.L.; Olson, B.H. (1991) 'Rapid method for direct extraction of DNA from soil and sediments', *Applied and Environmental Microbiology* **57**: 1070-1074.

Tsai, Y.L.; Olson, B.H. (1992) 'Detection of low numbers of bacterial numbers in soils and sediments by polymerase chain reaction', *Applied and Environmental Microbiology* **58**: 754-757.

Tscherko, D.; Bölter, M.; Beyer, L.; Chen, J.; Elster, J.; Kandeler, E.; Kuhn, D. and Blume, H.P. (2003) 'Biomass and enzyme activity of two soil transects at King George Island, maritime Antarctica', *Arctic, Antarctic and Alpine Research* **35**: 34-47.

Ueda, T.; Suga, Y.; Yashiro, N.; Matsuguchi, T. (1995) 'Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences', *Journal of Bacteriology* **177**: 1414-1417.

Vincent, W.F.; Quesada, A. (1994) 'Ultraviolet radiation effects on cyanobacteria: implications for Antarctic microbial ecosystems', In: Vincent, W.F.; Quesada, A. (eds) *Ultraviolet Radiation in Antarctica, Antarctic Research Series* **62**, AGU, Washington DC, pp 111-124.

Vishnaic, H.S. (1993) 'The microbiology of Antarctic soils', In: Friedmann, E.I. *Antarctic Microbiology,* pp. 297-341. Wiley-Liss Inc.

Vogel, J.; Normand, P.; Thioulouse, J.; Nesme, X.; Grundmann, G.L. (2003) 'Relationship between spatial and genetic distance in *Agrobacterium* spp. in 1 cubic centimeter of soil', *Applied and Environmental Microbiology* **69**: 1482-1487.

Whiting, G.J.; Gandy, E.L.; Yoch, D.C. (1986) 'Tight coupling of root-associated nitrogen fixation and plant photosynthesis in the salt marsh grass Spartina alterniflora and carbon dioxide enhancement of nitrogenase activity', *Applied and Environmental Microbiology* **52**: 108-113.

Widmer, F.; Shaffer, B.T.; Porteous, L.A.; Seidler, R.J. (1999) 'Analysis of *nifH* gene pool complexity in soil and litter at a Douglas Fir Forest site in the Oregon Cascade Mountain range', *Applied and Environmental Microbiology* **65**: 374-380.

Williams, A.J.; Berruti, A. (1978) 'Mineral and energy contributions of feathers moulted by penguins, gulls and comorants to the Marion Island terrestrial ecosystem', *South African Journal of Antarctic Research* **8**: 71-74.

Wilson, L.G. (1997) 'Inhibition and facilitation of nucleic acid amplification', *Applied and Environmental Microbiology* **63**: 3741-3751

Wintzingerode, F.V.; Göbel, U.B.; Stakebrandt, E. (1997) 'Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis', *FEMS Microbiology Reviews* **21**: 213-229.

Wirtz, N.; Thorsten, LO.H.; Green, T.G.; Türk, R.; Pintado, A.; Sancho, L.; Schroeter, B. (2003) 'Lichen fungi have low cyanobiont selectivity in maritime Antarctica', *New Phytologist* **160:** 177-183.

Woese, C.R. (1993) 'The Archea: their history and significance', In: Kates, M.; Kushner, D.J.; Matherson, A.T. (eds.) *The Biochemistry of Archaea (Archaebacteria),* Elsevier, Amsterdam, pp. vii-xxix.

Wynn-Williams, D.D. (1980) 'Seasonal fluctuations in microbial activity in Antarctic moss peat', *Biological Journal of the Linnean Society* **14**: 11-28.

Wynn-Williams, D.D. (1982) 'Stimulation of seasonal changes in microbial activity of maritime Antarctic peat', *Soil Biology and Biochemistry* **14**: 1-12.

Wynn-Williams, D.D. (1990) 'Ecological aspects of Antarctic Microbiology', In: Marshall, K.C. (ed.) *Advances in Microbial Ecology* **11**: 71- 146.

Xiong J.; Inoue K.; Bauer C.E. (1998) 'Tracking molecular evolution of photosynthesis by characterization of a major photosynthesis gene cluster from *Heliobacillus mobilis.' Proceedings of the National Academy of Science USA* **95**: 14851-14856.

Xiong, J.; W. M. Fischer, K. Inoue, M. Nakahara, and C. E. Bauer. (2000) 'Molecular evidence for the early evolution of photosynthesis', *Science* **289:**1724-1730.

Xiong, J.; Bauer, C.E. (2002) 'Complex evolution of photosynthesis', *Annual Review of Plant Biology* **53**: 503-521.

Yeager, C.M.; Kornosky, J.L.; Housman, D.C.; Grote, E.E.; Belnap, J.; Kuske, C.R. (2004) 'Diazotrophic community structure and function in two successional stages of biological soil crusts from the Colorado plateau and Chihuahuan desert', *Applied and Environmental Microbiology* **70**:973-983.

Yeates,C.; Gillings, M.R.; Altavilla, N.; Veal, D.A. (1998) 'Methods for microbial DNA extraction from soil for PCR amplification', *Biological Procedures Online* **1**: 40-47.

Young, C.C.; Burghoff, R.L.; Keim, L.G.; Minak-Bernero, V.; Lute, J.R.; Hinton, S.M. (1993) 'Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soil', *Applied and Environmental Microbiology* **59**: 1972-1974.

Young, J. P.W. (1992) 'Phylogenetic classification of nitrogen-fixing organisms', In: Stacy, G.; Burris, R.H.; Evans, H.J. (eds.) *Biological Nitrogen Fixation*, Chapman and Hall, New York pp. 43-86.

Young, J.P.W. (1993) 'Molecular phylogeny of rhizobia and their relatives', In: Palacios, R.; Mora, J. and Newton, W.E. (eds.) *New Horizons in Nitrogen Fixation*, Kluwer Academic Publishers, London, pp.587-592.

Zak, D.R.; Holmes, W.E.; White, D.C.; Peacock, A.D.; Tilman, D. (2002) 'Plant diversity, soil microbial communities and ecosystem function: are there any links?', *Ecology* **84**: 2042–2050.

Zani, S.; Mellon, M.T.; Collier, J.L.; Zehr, J.P. (2000) 'Expression of nifH genes in natural microbial assemblages in Lake George, New York, detected by reverse transcriptase PCR', *Applied and Environmental Microbiology* **66**: 3119-3124.

Zehr, J.P.; Capone, D.G. (1996) 'Problems and promises of assaying the genetic potential for nitrogen fixation in the marine environment', *Microbial Ecology* **32**: 263-281.

Zehr, J.P.; Jenkins, B.D.; Short, S.M.; Steward, G.F. (2003) 'Nitrogenase gene diversity and microbial community structure: a cross-system comparison', *Environmental Microbiology* **8**: 539-554.

Zehr, J.P.; McReynolds, L.A. (1989) 'Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*', *Applied and Environmental Microbiology* **55**: 2522-2526.

Zehr, J.P.; Mellon, M.; Braun, S.; Litaker.W.; Steppe, T.; Paerl, H.W. (1995) 'Diversity of heterotrophic nitrogen fixation genes in a marine cyanobacterial mat', *Applied and Environmental Microbiology* **55**: 2522-2526.

Zehr, J.P.; Mellon, M.T.; Zani, S. (1998) 'New nitrogen-fixing microorganisms detected in oligotrophic oceans by amplification of nitrogenase (*nifH*) genes', *Applied and Environmental Microbiology* **64**: 3444-3450.

Zhao, Y.; Bian, S.M.; Zhou, H.N.; Huang, J.F. (2006) 'Diversity of nitrogenase systems in diazotrophs', *Journal of Integrative Plant Biology* **48**: 745-755.

Zhou, J.; Bruns, M.A.; Tiedje, J.M. (1996) 'DNA recovery from soils of diverse composition', *Applied and Environmental Microbiology* **62**: 316-322.

APPENDIX: SEQUENCES FROM CLONES

Sequences from *nifH* clone library C – Mesic Fellfield

Clone C2

```
1       CAGGCATGCC  GTCAGGCGCT  TGGCTTGCCT  ATCAATGCTC  GTTTCATCGG
51      CTATATGGGC  AGCTGGGCCA  GGAATCGCGG  CACTAACGTG  CTGATCGATG
101     CATTCCGGCG  CGTGCGTGCA  ACGCAACCCA  ATTTGCAACT  CCTGCTCAGT
151     GGCCGACCGC  CGCAGCGTGT  ACTGTCCGAG  CCGGGCGTAA  TCGCTACCGG
201     CTATGTCGCT  GACGCACAAC  TGCCTACATT  GATCAACGCG  CTTGATGTTG
251     CCTGTGTGAT  TACC
```

Clone C3

```
1       GAGCGGACAA  TGGGATAGGA  AAAAGAAAGC  CGAGTTTACT  GGTTGTTTGC
51      GTGCGATTCT  TAGCGGCCCA  TCCAGCCTCC  ATCCACGGTG  AGGATGGTTC
101     CGGTGACATA  GTCGCTGGCG  GGTGATGCGA  GAAAGACGGC  GGCTCCGGCG
151     AGATCGTGCG  GTTCTCCCCA  ACGGCCTGCA  GGGATGCGCT  CAA
```

Clone C4

```
1       TGGCCGGCTC  GAACGCGGTG  CCAAGCGCAG  CCTGCGCCAC  GATCACGACA
51      TAGAGTAGCG  GCAGATCGGC  CTTGGTGTGC  ACGAACAGCA  GCAGGACAAG
101     CGCGAGGGCC  TGTAGCACGT  TGATCGCCAC  CATGAGCAGG  CGCCGGTCAA
151     TGCGATTGAC  CAGTCGCGCC  GCAACCGGTG
```

Clone C5

```
1       CTCCGGGCCA  CCAGATTCCC  TGCCACATGA  CCTCGCCCGA  GCACCCCAGC
51      GGCACCGACS  GCGTCTGGCA  GGTGGCGAGC  GAGTTGGGCG  CCGCCGCGGC
101     GGTCAACATC  CAGGGCGATG  AACCCTTGGT  CCGTCCTGAG  ATGCTGGCCA
151     CCTTGATCGA  GACGCTGTTT  GCCGCACCCG  ACACCGAGGT  GGCGACCATC
201     TACACGTCCG
```

Clone C6

```
1       GTAAGGCGCC  AGACCAGTGC  ATTGCTGCGG  CGGACCGCGC  CCTCTACCGG
51      GCCAAGGAAG  AGGGGAGGGA  CAAGGTCGTG  GCGGTCGGGG  TCACGATGCC
101     CACCCAGGCG  CTAGGGAGTC  CGGCCCCCGA  TGACCACGAG  CCGAAGATCA
151     TGTTCCAAGA  GAGCCGCCGC  TAGGCACGGG  AGGCCCAGCC  CCGCACCTAG
201     ACTGTGGYTC  CTATGGATTC  CCTGTTTAGC  CCC
```

Clone C7

```
1       CAAATTGCGG  CCAGCTTTAA  TTATCCGGGC  GCATTAGATG  CCTATGACAA
51      TATCGATGGC  ATTGTCGCGC  TTACTCACGA  AGGTGGCTGC  GGCATGCGCT
101     CGGCTGGCGA  AGGCTATGAA  ATGCTCGAAC  GCGTACTAAC  TGGTTACGCA
151     AGGCACCCTA  ATTTTGGCGG  CATATTGGTG  GTTGGAATAG  GTTGCGAAAC
201     GATGCAATTG  CATCGCCTTA  CCACCGAAAA  CAACCTCAAA  GAAACTACGT
251     TTTTTAAATC  GCTTAATATT  CAA
```

Clone C8

```
1       GCATTATGGT  CGATGATCTG  TTGGCAGGTG  CCTTTGCGGC  GGCCTGTTTA
51      TGGTTGCTTT  ATGCTTTTTA  TCCGGTGATG  TTTAACTGGA  TACCCCTGTA
101     ACACCCTTAA  TTTTCCCCGA  TGAATCTGGT  CTCACAAGAG  CCAGCAGTCT
151     CCTAGAGTCT  CTTCGTGGGG  CTAGATTCAT  CCGAAGCATT  TTTTCAACGA
201     ATCAAAGCCC  ACAACAGCCT  GAGCTTCCTA  ATGATTGAAG  CGTT
```

Sequences from *nifH* clone library L – Biotic Mire

Clone L14

```
1       CAAAAGCACC  ACGTCGTCCA  ATCTGTCGGT  GGCGTTTTCA  AAACTGGGCA
51      AGCGCGTGTT  GCAGATCGGT  TGCGATCCCA  AGCACGACTC  CACATTTACG
101     CTGACCAAGC  GGCTGGTGCC  CACGGTGATC  GACATCCTGG  AGACGGTCGA
151     ATTTCACGCC  GAAGAACTGC  GCCCTGAAGA  TTTTCTTCTC  GAGGGCTACA
201     ACGGTGTGAT  GTG
```

Clone L15

```
1       GGAGGGAATG  GAGTCAACGC  AGGGATACTC  GTGGACGCGG  CCAGGCCTTG
51      ACGCGCCTCC  GGGGCGAGAC  ATAATCTCCC  GCATGAACGC  AGTCGAGCAC
101     TCATCCAACT  CGCCTGGTGC  AGATGCAGGA  AGGTGGGTTC  TCGTCACTCG
151     CAGGAACTAT  TGACAGTCGC  CAGAGAAGCC  AATCCCACCT  TTGCCGTAAC
201     ATATGGATCG  GATCCCGGGC  CCGTC
```

Clone L16

```
1       GCAAGTCAAC  CACAACCCAG  AATACGGTGG  CAGGACTGGC  ATACCTTGGC
51      AAGAAGGTCA  TGATCGTCGG  ATGCGATCCC  AAGGCAGACT  CCACCCGCCT
101     GATCCTGCAC  GCCAAGGCCC  AGAATACGGT  TATGGACCTG  GTGCGCGAAC
151     GCGGTACCGT  TGAGGACCTG  GAGCAGGAAG  ATGTCATGAA  GGTCGGTTAC
201     GGCGATGTCA  AATGC
```

Clone L17

```
1       GTGGCGCGGG  GTAAAGGAAG  AAGGCGCCGT  CAGGGTGGAA  CGGCTGGCGG
51      TGCGAGGACT  GGCGTTGGGA  ATAGTCGTTG  GCACGGCGGG  CAGTTTTTTG
101     CTGGGTTACT  TGGTGGCGCG  CTACACGGAT  GCGGCGCTCC  CGCACATTGA
151     CGCCGCGCTG  ACGAGTTTTA  GTTTGGTCGC  GCAATGGTGG  TCAACGCGCA
201     AGCACATTGC  CAATTGGTGG  CTCTGGATTG  TGGTCGACGC  CCTGGAAATA
251     GGCGTCTTCC  TTTATAAGCG  TCTCTATCTA  ACTTCCGTCC  TGTTCGCGTC
301     CCTTATCTTT  CTGGCCGTGC  TGGGGCTGCG  CGCATGGCAC  AAAGCGCTTC
351     GCGAGCAA
```

Clone L18

```
1       GGCCGCCGGA  TTCTACSCWT  CTWATATTGC  GRAAGCCAGK  GATCMCCMCC
51      YCTTCRAGTT  CAATATCTTC  CACTGCACCG  CGTTCRGCAG  CCAATTGGAG
101     AACGCTTGTT  TGAGCTTTGC  TGTGGAGAAT  CAACCGGGKG  GAATCAGCTT
151     TGGGGTCACA  ACCCACAATT  AGAATGSGTT  GACCTACTTC  TGCCATTGCA
201     GCTAGGGTAT  TTTGGGAGGK  GGTARATTTA  CC
```

Clone L19

```
1       GCAAGAGCAC  CACCAGCCAG  AACACGCTGG  CCGCGCTGTC  TGAAATGGGC
51      CAGAAGATCC  TCATCGTCGG  CTGCGACCCC  AAGGCCGACA  GCACCCGCCT
101     GATCCTGCAC  GCCAAGGCGC  AGGACACCAT  CCTGTCGCTG  GCCGCGGAAG
151     CCGGCAGCGT  GGAGGACCTG  GAGCTCGAAG  ACGTCATGAA  GATCGGCTAC
201     CGCGACATCC  GTTGC
```

Clone 3

```
1       CCTGACCGAA  AGCGGGTAGA  TAGATTCTCC  AATAGGGTGT  GCACCCAATA
51      CACGATAGTA  CTTTTACCTT  GACCGGGTTT  TTGATTCCGA  CAATTATCGA
101     CACCTTGCAA  GAAAAAGACT  ATCACTACGA  GGATGTCTGG  CCCGAAGATG
151     CAATCTATAA  AGGCTACGGC  GGTGTCGATT  GTGTTGAGTC  TGGCGGCCCG
201     GAGA
```

Clone 10

```
1       GGGACGCCAG  ATTCTACGCA  TCTGATATTG  CGGAACCAGT  GATCACCAGC
51      GTCTTCGAGT  TCAATATCTT  CCACTGCACC  GCGTTCAGCA  GCCAATTGGA
101     GAACGCTTGT  TTGAGCTTTG  CTGTGGAGAA  TCAACCGGGT  GGAATCAGCT
151     TTGGGGTCTC  AACCCACAAT  TAAAATGCGT  TGACCTACTT  CTGCCATTGC
201     AGCTAGGGTA  TTTTGGGAGG  TGGTAGATTT
```


Sequences from *nifH* clone library V – Coastal Fellfield

Clone V21

```
1       CAGGGACTCG  GCCCCGACGA  CTCGGGACCT  GGTTCGCTTA  TCGACGACGC
51      GGACGCGCTC  GTTGGACGTG  ATCGAGATGG  TCAGCTGCTG  GATGCTGCGT
101     ACGCCGGTTC  GGTGGTACGT  CGCACCGCCA  CCGCGCTTCA  TGAGTCGGGC
151     GGCCCGGAGA
```

Clone V22

```
1       ACCAAGCGCG  CCGCCTGTTG  CGCGAGGCCG  GTGGCGACGC  CGCAATCGTG
51      GCCAAGATCG  AACGCACCGA  AGCAATTGCC  GCACTCGGCG  AAATCATCGA
101     CGCCAGTGAT  GTGGTCATGG  TCGCCCGTGG  TGATCTTGGT  GTTGAAATTG
151     GCGATGCCGA  ATTGCCTGGT  CTGCAAAAAA  AGATCATCCG  CGAAACGCTG
201     GAGCGCAGCC  GCGTGGTTAT  CACCGCCACC  CAGATGATGC  GATCAATGGT
251     CGAATC
```

Clone V23

```
1       GTAGAGGTAG  TCTTCCCGCG  TGCTGGGGGT  CACGGCCAGC  AACTCGCGGC
51      CTGGCGTGTG  CACCACACCC  CGCGCAGCCG  ACCGATTTCG  GAGGAGACGT
101     GTACGGGCAT  GCCCAAAGCT  AACCAGCAGT  CCGTGGGTGG  TGTCCGAACG
151     CCCGCCGCGA  TATCTTGTGA  ATCAATTCAC  AAGCCGCTTA  TACCTGTGAA
201     ACGTATC
```

Clone V24

```
1       GAGTTCAGTC  CGCTCGACAA  GCAGGCATGC  CGTCAGGCGC  TTGGCTTGCC
51      TATCAATGCT  CGTTTCATCG  GCTATATGGG  CAGCTGGGCC  AGGAATCGCG
101     GCGCTAACGT  GCTGATCGAT  GCATTCCGGC  GCGTGCGTGC  AACGCAACCC
151     AATTTGCAAC  TCCTGCTCAG  TGGCCGACCG  CCGCAGCGTG  TACTGTCCGA
201     GCCGGGCGTA  ATCGCTACCG  GCTATGTCGC  TGACGCACAA  CCGCCTACAT
251     TGATCAACGC  GCTTGATGTT  GCCTGTGTGA  TTACC
```

Clone V25

```
1       GTAAAACCGG  CGTTGTCGTT  GGTCTGGCCG  AGTGGTTTGC  GGCGAATGAG
51      ATTCCCGTCG  CGTTAGCTCG  ATCTTCATAC  GGAGAATAAA  GCCCGTGGCT
101     CGGTGAAGCA  TTTCTTCGAT  GGCACCGTGA  CCAAAGTCGA  TATTCATACC
151     GTCGATCTGC  CAATTCAAAT  TCAAGCTGGC  CTTGATCCGA  AACAAATTGC
201     CAAAATGCTC  GGCGAAAGTC  TACGTCAGCT  GTGACACTGT  CGACGCCATC
251     TGCRATGCAA  TTCGTTCCGG  AGTCCGTGCC  ACAGGCTAAC  GGCGTGCCGG
301     CTGGCAGTCC  AAAGCCGAAA  GGCCGCGGCC  GCGAGAAGGC  AGCGGCCACT
351     ATCCGTGAGC  GATGAAGGTG  ATTTCTATCG  AGCATGAGCA  CCCTGGAGGG
401     ATGGTGTCTT  GCTCGAAAAC  ACGCGCAGCG  AAACGGCAAT  GCGGCACTGC
451     CCTTGCGAAT  TGACGATTGC  A
```


Clone V26

```
1       GCAGGAAAAG  CTTCCACAGC  GCATCTTCCT  CTTCTATGCA  AACAAACGAC
51      CAGAAGATGC  TCCATTTCTT  GATGAGTTA   AACAGCCTTG  CGGCACGAAA
101     TTCTAACTTT  ACATTTGTTC  CGACAATGA   ATCACATGAA  TGATTCTGAG
151     CAGGAGTGGA  AAGGAGAGAC  CGGACATAT   CGACCATCAG  CTTGTGTAGA
200     AATATTTAAA  GCTGGCCAAT  TTATCCGAA   GAAGCTATAT  CAACCGCCAT
251     TTATTATATA  GCGGGTCCTT  C
```

Clone V27

```
1       GCAAATCCAC  CACCTCATCA  AATCTGTCAG  CCGCCTTCTC  GCTGCTGGGC
51      AAGCGCGTGC  TCCAGATCGG  CTGCGATCCC  AAGCATGACA  GCACCTTCAC
101     CCTGACCAAG  AAGCTGATGC  CCACGGTTAT  CGACATTTTG  GAAACAGTAG
151     ACTTCCACCA  TGAAGAGCTG  CGTGCCGAAG  ACTATATGTT  TGAAGGCTTC
201     AACGGCGTTA  TGT
```

Clone V28

```
1       GACTGCGCCA  GTCCTGTGCA  CCACTGGATC  ACTGCRACCA  GACCCAAGAC
51      ATGGGCAATA  CCGGCGCACA  ACCCTCCATT  GGCCCACTTC  CCCGCTGGAC
101     CAGCGTTTAT  ATCGTCCATC  CCGACGTGCG  TGCCTACCAC  TGGATGCTGG
151     CCAATACGGA  CGCCCTCGGT  ACTTACGGCA  CCCACTATCG  TGACGCCGAG
201     ACGGGATGGC  CCGTCAGTAT  TCAGCAGCAC  CCCTATGTAA  CCCTTACCAA
251     CTGGGCATA
```

Clone V29

```
1       GCCTTTGAAG  ACACCTGGGA  GTGGAATCCG  GATGCGATGC  GCGCCTACGA
51      GGAGATCGTC  GAGCGCGGTG  GGCGCGTTGC  CGACACCATG  CGCGCCTTTT
101     GGACGTTTCT  CCACGGCACC  GACATGATGG  CCTATCTCGC  CATGATGGCC
151     CCGCGCCTGG  TCGAACTCCG  CCGCGTCCTC  AAGGAAACCG  GCTCCATCTA
201     TCTTCACT
```

Clone V30

```
1       GAGCATGAGT  CAAGCCAAAG  CATGTAAAAC  TATAATGGTC  AGAAGCAATC
51      AGAGATGTAC  GTCATGAAAC  TTCAAGCGAA  ATTGTTCTTC  ACTGGGATTT
101     GCCTCCTTGC  AAGCCCACTC  CTCTGGGCCG  CCACACCGAC  AGCCGCGGCA
151     CTCCCCACAC  CTCCGGCCGC  TTCCACTGCG  GGGACTCAGA  CCCAGGAACT
201     CGGCAAAGTG  AATGTGGGTG  GAGTTGCGGT  CATCAAGGCG  GCCGTGCGGG
251     CGCTGCAGAC  AGTCAAGGTA  GCACTCAAGG  AA
```