Frontpiece. *Pottia heimii* in the morning sun at Bratina Island, East Antarctica.

Dedication

For my mother Maree, who has always had faith in my abilities and encouraged me to be the best I can.

RAPD Analysis of East Antarctic *Pottia heimii* Populations

A thesis

submitted in partial fulfilment

of the requirements for the degree of

Master of Science in Biological Sciences

at the

The University of Waikato

by

Tracy Maree Dale

The University of Waikato

1997

ABSTRACT

Pottia heimii contributes significantly to the sparse terrestrial vegetation in East Antarctica, especially within the Dry Valley region. Using Randomly Amplified Polymorphic DNA (RAPD), levels of genetic variation were investigated in several East Antarctic *P. heimii* populations. The initial stage of the study optimised the RAPD protocol specifically for antarctic *P. heimii.* RAPDs gave similar profiles for genetically identical *P. heimii* tissue (shoots joined at the base) and were able to detect DNA differences between individual shoots within a moss clump, confirming the usefulness of RAPDs for *P. heimii* population studies.

Genetic distance matrices calculated from RAPD banding patterns were presented as dendrograms and used in an Analysis of Molecular Variance (AMOVA). Using single moss shoots, a significant level of genetic variation was detected within three 50 metre transects collected in the Miers Valley. The genetic spatial structure within these transects was random indicated by the distribution of genotypes. This suggested wind was the most likely agent for local propagule dispersal. No genetic subdivision existed between the transects, therefore individuals could be considered to be taken from a single population. Comparison of individuals from Miers Valley, East Garwood Valley, Bratina Island and Edmonson Point showed the first three Southern Victoria Land populations could be considered as a single larger population genetically distinct from the more northerly Edmonson Point population. The level of genetic variation within all the East Antarctic *P. heimii* populations analysed was considered significant.

ACKNOWLEDGMENTS

There are many people to thank who have been involved in many aspects of this study so to you all THANK-YOU. I would like to give individual acknowledgments, in no particular order go to the following people:

Dr. Mary Skotnicki, for continuing as my supervisor on returning to Canberra and particularly for her advise on RAPDs.

Dr. Allan Green for his invaluable advise during the writing of this thesis and for reading and re-reading all my draft copies.

Dr. Patricia Selkirk and Dr. Dieter Adam for their advise and company during the trip to Antarctica and Patricia's continued input at all stages of this study. Thanks also for providing the great photographs.

Dr Rodney Seppelt, Dr Tom Richardson and Prof. Dick Wilkens for their personal communications. Dr Barry O'Brian for photographing the *Pottia heimii* shoots under the microscope.

Thanks to the technicians and students in the Plant Physiology Lab. for providing a good atmosphere in which to work. Special thanks must go to Terrance Joe for passing on all his lab. and computer skills and Avi Holzapfel for his help and advise at many stages during this thesis.

Thanks to my family and friends, especially Grant for putting up with me and just being there, and last but by no means least, mum to whom this thesis is dedicated.

RAPD ANALYSIS OF EAST ANTARCTIC *POTTIA HEIMII* POPULATIONS

TABLE OF CONTENTS

CHAPTER THREE: DEVELOPMENT OF THE RAPD PROTOCOL FOR *POTTIA HEIMII*

CHAPTER FOUR: RAPD ANALYSIS OF *POTTIA HEIMII* POPULATIONS

LIST OF FIGURES

List of figures cont'd page

LIST OF TABLES

CHAPTER ONE

INTRODUCTION

The overall aim of this study was to investigate the genetic variability in East Antarctic populations of the moss species *Pottia heimii*. Specifically the aims were (1) to establish whether the Random Amplified Polymorphic DNA (RAPD) technique could be used to analyse genetic variation in antarctic *P. heimii,* (2) to establish whether genetic variation existed within and between populations of antarctic *P. heimii*, (3) if genetic variation existed, to determine whether there is any spatial structure to the variation within antarctic *P. heimii* and (4) if genetic variation existed, to determine whether patterns of variation gave any indication about the processes of dispersal/colonisation of *P. heimii* in East Antarctica.

1.1 Antarctica as a habitat

Antarctica is an area of approximately 14 million km^2, comprising one tenth of the land surface of the earth. Of this area less than 2% is ice free in summer; these locations are identified in Figure 1.1. The single largest ice free ground is in Southern Victoria Land at ca 77°S, with the Transantarctic Mountains providing significant rocky substrate towards the South Pole and smaller areas found around the Antarctic Peninsula (Campbell and Claridge 1987, Walton 1990). These ice free areas in Antarctica called, oases (Pickard 1986), are described as among the most extreme deserts in the world, being subject to low temperatures, limited precipitation, high salt accumulation and katabatic winds (Campbell and Claridge 1987, Longton 1988, Wharton 1994).

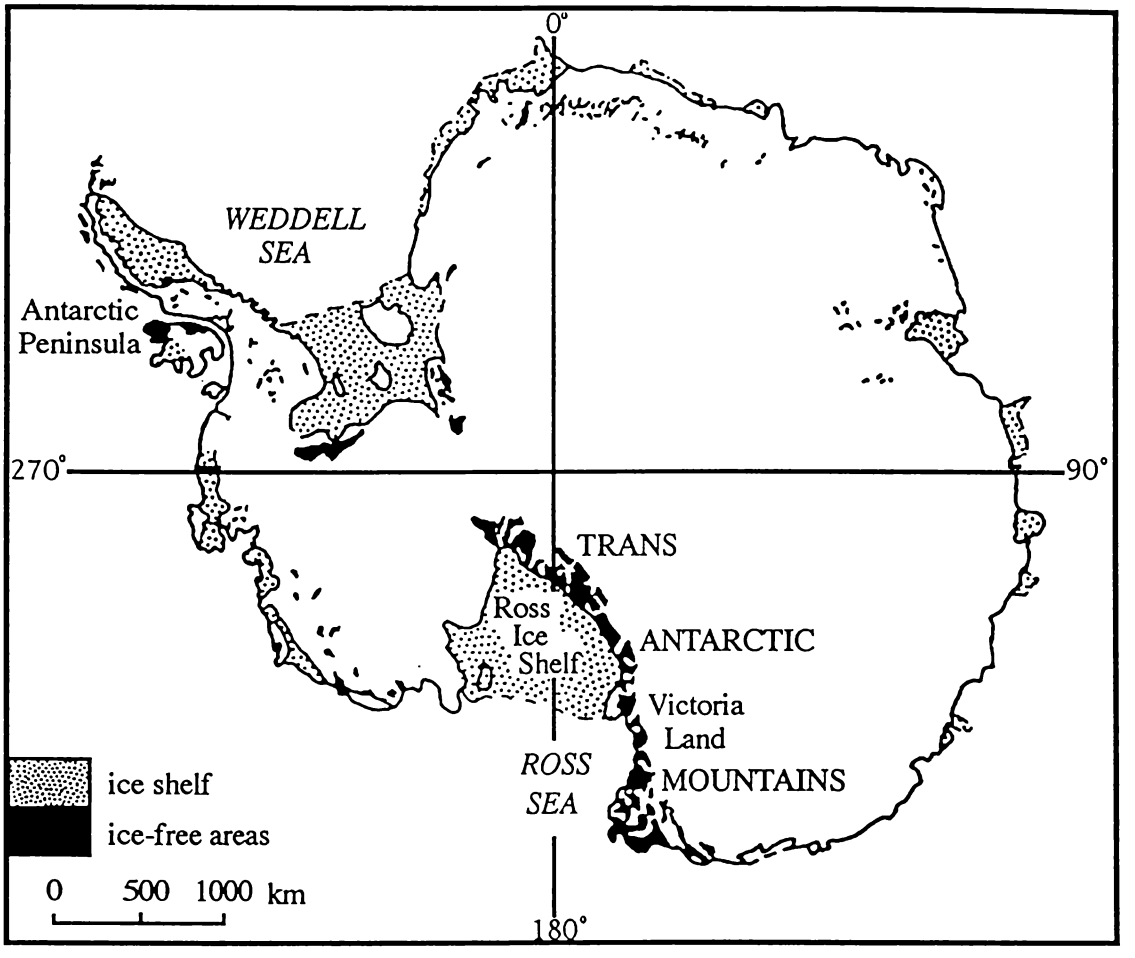

Figure 1.1 Distribution of ice-free areas in Antarctica. The black areas represent the localities where bare ground exists but the true extent is much less than indicated (Campbell & Claridge 1987)

Mosses, lichens and algae form the terrestrial communities in the ice free areas of continental Antarctica, with mosses forming a significant component of the terrestrial flora (Green 1985, Seppelt *et al.* 1995). The distribution of mosses in the severe climatic regime is dependent on the degree of shelter from strong winds and reflected radiation from surrounding ice fields, a northerly aspect, adequate liquid water supply in the growing season, direct solar radiation, stable substratum, ice free niches and adaptation to low temperature conditions (Holdgate 1970, Schwarz *et al.* 1992, Smith 1996)

Within continental Antarctica, species diversity of mosses is limited to approximately 25 species in 12 genera (*Bryum*, *Grimmia*, *Pottia*, *Plagiothecium*, *Ceratodon*, *Sarconeurum*, *Dicranella*, *Didymodon*, *Desmatodon*, *Tortula*, *Campylopus* and *Leptobryum)* (cf. Smith 1984, Seppelt and Selkirk 1984, Seppelt pers. comm.). *Pottia heimii,* which is the focus of this study, contributes significantly to the moss vegetation within Eastern Antarctica, being locally abundant in Victoria Land, the McMurdo Dry Valleys and Ross Island (Greene 1967, Seppelt *et al.* 1995). Within the McMurdo Dry Valley area, including the coastal site of Granite Harbour, only eight other moss species in addition to *P. heimii* have been recorded. These are *Bryum argenteum*, *Bryum pseudotriquetrum*, *Sarconeurum glaciale*, *Grimmia antarctici*, *Ceratodon purpureus*, *Didymodon gelidus* and two unidentified *Grimmia* species (Schwarz *et al.* 1992, Seppelt pers. comm.).

1.1.1 Miers Valley

The field work for this study was conducted between the 28 December 1994 and 12 January 1995. The most intensive sampling of *Pottia heimii* took place in the Miers Valley lying between the latitudes 78°06'S and 78°07'S and longitudes 163°44'E and 164°12'E (Figure 1.2). It is one of numerous ice free valleys making up a distinctive area known as the Dry Valleys region within Southern Victoria Land, Antarctica. The valley is approximately 11 km in length and varies in width from 1.5 to 2.5 km. It is glacially carved and the major part of the floor is between 80 to 150m above sea level. Miers Valley contains two glaciers, Miers Glacier on the northern side and Adams Glacier on the southern side of the valley. Both glaciers flow eastward from the Royal Society Ranges (Helm 1958). A permanently ice-covered lake, Lake Miers, lies 9 km inland from the western coast of McMurdo Sound. It is fed by the Miers and Adams glaciers during the summer months. The Miers river flows from Lake Miers to the Koettlitz Glacier along the floor of the valley during this time (Baker 1967). Figure 1.3 gives an illustration of the western end of Miers Valley.

Although climatic data for Miers Valley is not available, Campbell *et al.* (in press) describe these coastal valleys in general as having a mean annual temperature around -18°C, with summer maximum temperatures frequently at 0°C and occasionally as high as +7°C. During this period, surface soil temperatures may exceed 14°C for short durations. In winter however, surface soil temperatures may fall to as low as -38°C. Ground thawing ranges between 30-60cm depending on site conditions (Campbell *et al.* 1992, Wharton 1994, Campbell *et al.* in press). Precipitation occurs exclusively as snow with the area having an average of 7 falls of snow per month during the summer months, most of which is lost by sublimation. Liquid water is derived from the thawing of permanent ice and semi-permanent snow patches during summer, resulting in numerous small stream flows and small lakes (Campbell *et al.* in press). The region has 4 months of continuous sunlight and 4 months of darkness, each followed by transition periods of twilight (Wharton 1994)

The soils fall within a group defined by Campbell and Claridge (1987) as weathering stage I soils, showing negligible soil development and typically forming stone pavements. Sandy to silty till deposits are widespread and probably form the most extensive soil forming material (Campbell *et al.* in press). The floor of Miers Valley is covered with extensive lacustrine material containing silts, calcite and gypsum and glacial drift (Clayton-Greene *et al.* 1988).

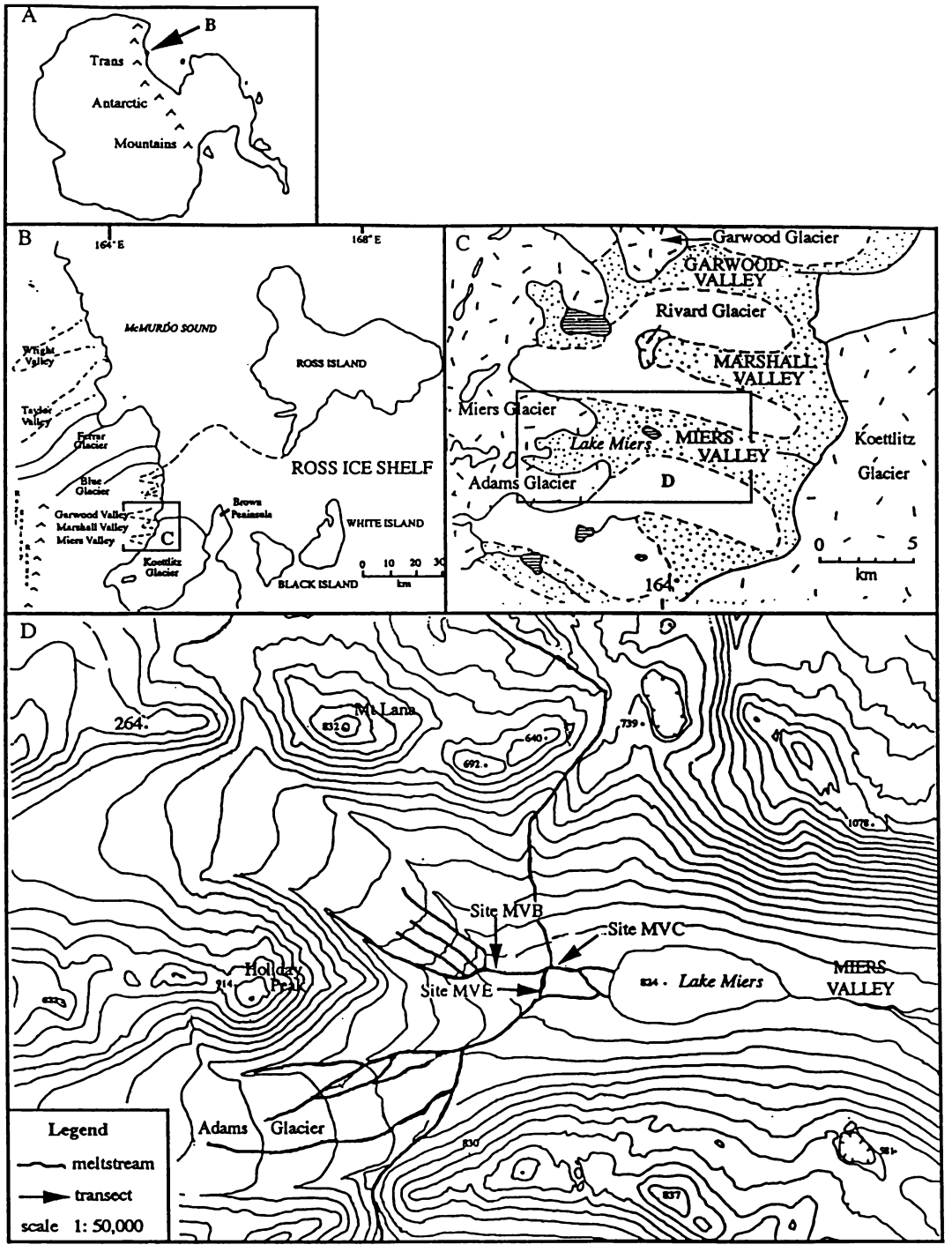

Figure 1.2 (**a**) Location of the Dry Valley region within East Antarctica, (**b**) & (**c**) location of Miers Valley within the Dry Valley region and (**d**) location of *P. heimii* transect sites MVB, MVE and MVC within the Miers Valley (adapted Clayton-Greene *et al.* 1988, Anon.(a) in preparation)

Figure 1.3 Miers Glacier (foreground) on the northern side and Adams Glacier on the southern side of Miers Valley, East Antarctica. Meltstreams flow from both glaciers in an easterly direction and eventually converge (photographed Selkirk).

1.2 Isozyme analysis of mosses

Using enzyme polymorphism, Cummins and Wyatt (1981) revealed that genetic variability exists within moss species. Using the temperate moss *Atrichum angustatum* they provided evidence that variation occurs not only among colonies but may also arise within colonies of this species. Subsequent studies of enzyme polymorphism in mosses show surprising overall high levels of variation within species, comparable in several cases to those found in flowering plants (Wyatt 1982, Wyatt *et al.* 1989b, Wyatt 1994, Soderstrom 1994).

Cummins and Wyatt (1981) state that the traditional view of mosses being "genetically impoverished" was based mainly on (1) the dominance of the bryophyte life cycle by the haploid generation and (2) the predominance of asexual reproduction over sexual reproduction. The dominance of the haploid gametophyte suggested the range of genotypes would be limited because only one allele of each gene is present, therefore, a given environment may select strongly against any deleterious alleles. Asexual populations in the past were presumed to have reduced or absent recombination events, suggesting low genetic variability within moss populations. However, novel genes may arise by mutation not only in sexual cells but also in vegetative cells. Indeed, levels of genetic variability in asexually reproducing populations are comparable to those found in sexually reproducing populations (Cummins and Wyatt 1981, Longton 1994, Newton and Mishler 1994, Soderstrom 1994, Wyatt 1994, Adam *et al.* in press, Selkirk *et al.* in press).

1.3 DNA level analysis

Molecular techniques using DNA markers allow efficient detection of differences between the DNA of individual organisms. While allozyme polymorphism is still a popular approach for population studies (Adam *et al.* in press, Selkirk *et al.* in press) it deals with a limited group of functional enzymes and concerns only variation in the coding sequences of genes and their subsequent expression.

Using DNA markers, DNA polymorphism may be found anywhere in the genome including coding and non coding, single copy or repetitive DNA. They potentially provide a more powerful technique for identifying levels of variability (Burke *et al.* 1992, Bachmann 1994). Random amplified polymorphic DNA (RAPD) is one DNA marker technique tool that is fast becoming prevalent in population biology (Bachmann 1994) because no knowledge of the target DNA sequence is needed and only a small amount of both tissue and ultimately DNA is required (15-25ng per reaction). RAPDs are inexpensive, have an uncomplicated protocol and can scan a large number of loci for a large number of samples. It is a non radioactive assay with a large number of random primers commercially available.

1.4 RAPDS as a molecular tool

RAPDs detect nucleotide sequence polymorphisms in a DNA amplification-based assay (Tingey and del Tufo 1993). RAPD amplification conditions are essentially the same as for the polymerase chain reaction (PCR) (Arnau and Cooper 1991), however in each RAPD assay only a single primer is annealed to the genomic DNA. The arbitrary primer, usually 10 base pairs (bp) in length, is mixed with genomic DNA, a thermostable DNA polymerase, deoxyribonucleotides (dNTPs) and a suitable buffer containing $MgCl_2$. The mix is subjected to temperature cycling consisting of 3 steps, denaturation (94°C), annealing (35°C) and extension (72°C), resulting in an exponential amplification of the target DNA. Figure 1.4 gives a diagrammatic summary of the events occurring during temperature cycling.

During the annealing step, the arbitrary primer binds to sites with many homologous or near homologous sequences in the DNA template. There will be a finite probability that some of these sites will be arranged in opposite orientation, on opposite strands so that the 3' ends of the primer point to each other and are within an amplifiable distance i.e. within a few thousand nucleotides. Those segments of DNA will produce PCR amplified DNA, which can be separated on an agarose gel and visualised under UV

light after ethidium bromide staining (Howland and Arnau 1991, Rafalski and Tingey 1993, Tingey and del Tufo 1993, Williams *et al.* 1993). A given DNA template (genome) will produce a series of such PCR products which are, in effect, the RAPD profile for that particular genome.

Polymorphisms in RAPD profiles arise as a result of genomes having either base changes within the primer binding site or an insertion or deletion within an amplified nucleotide sequence. They are detected by the presence or absence of the amplified product of a particular size in the RAPD profile of individual samples (Tingey and del Tufo 1993).

RAPD markers are inherited in Mendelian fashion (Adam *et. al.* in press) and therefore are dominant because profiles are scored for the presence or absence of a specific DNA product. This technique is particularly well suited to *P. heimii* because mosses are haploid, therefore, the dominant expression masking heterozygotes in diploids is avoided (Rafalski and Tingey 1993, Boisselier-Dubayle *et al.* 1995, Adam *et al.* in press, Selkirk *et al.* in press). RAPDs have been selected for *P. heimii* as there is no genetic information available for this species and because of the small amount of DNA in a single *P. heimii* shoot.

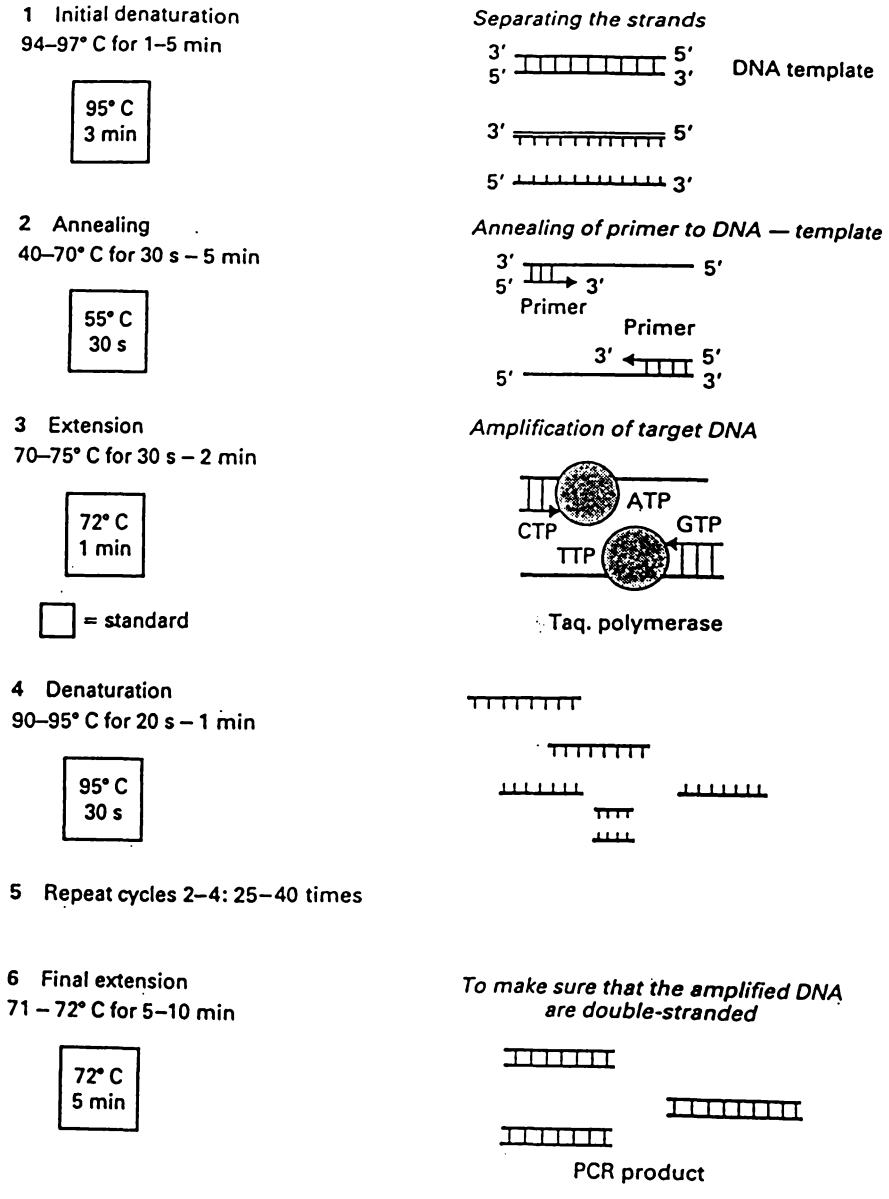

Figure 1.4 Diagrammatic summary of the polymerase chain reaction (Arnau and Cooper 1991)

1.5 RAPD analysis of antarctic moss

There has, until recently, been only limited population studies of antarctic moss at a DNA level. The species studied to date are the cosmopolitan moss *Bryum argenteum* (Adam *et al.* in press) and the less widespread moss *Sarconeurum glaciale* (Selkirk *et al.* in press). Using RAPDs these investigations have shown that genetic variability exists within and between several antarctic populations from Ross Island, Southern Victoria Land and the Vestfold Hills in East Antarctica. At the initiation of the present study no RAPD data for *P. heimii* was available. During the course of this study, it has been established that RAPDs can be used as a taxonomic tool to differentiate between six moss species from East Antarctica, including *P. heimii* (Skotnicki *et al.* in press).

1.6 Taxonomy of *Pottia heimii*

Pottia heimii (Hedw.) Feurnr. is a member of the moss family Pottiaceae. Zander (1993), in a taxonomic review of Pottiaceae, changed the name of *P. heimii* to *Hennediella heimii.* However in recent literature (Seppelt *et al.* 1995) this species is still referred to as *P. heimii* and this name will be used here. Kanda (1981) established that the antarctic *P. heimii* is synonymous with the species recorded earlier as *Bryum antarcticum.* Seppelt *et al.* (1995) give a comprehensive description of the habitus of antarctic *P. heimii.* Kanda's (1981) sketches of *P. heimii* collected at Syowa Station show examples of leaf, capsule and spore structures.

1.7 Reproduction of *Pottia heimii*

Sporophytes are rare on mosses growing in continental Antarctica. Most fail to produce spores due to environmental constraints on the development of gametangia and sporophytes. Antarctic moss species are forced to rely on asexual methods as their major mode of reproduction (Longton 1988). However, *P. heimii* has the highest frequency of sporophyte recorded among moss species in Eastern Antarctica, with numerous immature sporophyte occurrences having been recorded at two locations

within the McMurdo Dry Valleys: Canada Glacier flush in Taylor Valley and Cape Chocolate (Seppelt *et al.* 1992). Cape Chocolate is the southern-most record of sporophyte occurrence. Seppelt *et al.* (1992) carried out microtopographic studies of the Canada Glacier flush, concluding that *P. heimii* sporophyte formation was associated with favourable microsites in hollows or adjacent to rocks. The main factor contributing to the favourable nature of these sites was protection from high speed winds which facilitated a warmer and more humid environment. Fruiting material of *P. heimii* has also been reported at Cape Bernacchi and Marble Point (Greene 1967), Syowa Station (Kanda 1981) and at Cape Geology within Granite Harbour (Seppelt *et al.* 1995).

1.8 Habitat of *Pottia heimii*

Transect studies by Schwarz *et al.* (1992) at the Canada Glacier flush provide some general conclusions about the niche occupied by *P. heimii* in the antarctic environment. *Pottia heimii* generally dominates the edges of flowing water zones or the higher regions which tend to be drier and salt encrusted, as opposed to the wetter sites occupied by the other predominant moss species *Bryum argenteum.* Figures 1.5 a and b show examples of the typical habitat of *P. heimii* in Miers Valley.

1.9 Dispersal of *Pottia heimii*

Mosses are not found as single individuals but as a large number of individual shoots that form a clump (Mägdefrau 1982). Dispersal and establishment of mosses is via propagules, potentially including both spores and vegetative propagules i.e. fragments of moss gametophyte (Longton 1988). *Pottia heimii* propagules in Antarctica are presumed to be dispersed by abiotic means, primarily wind and water (Wharton 1994). The direction of transport is therefore determined by topography and wind direction.

Dispersal may be local, i.e. dispersal within a population, or distant, i.e. dispersal between populations or localities (Soderstrom 1994). To date it is unclear whether water or wind is the predominant mode of dispersal used by *P. heimii.* (Selkirk pers. comm.).

1.10 Arrival of *Pottia heimii* in Antarctica

It is not known how long *P. heimii* has been present on the continent of Antarctica. Either it is a remnant from when climates in continental Antarctica were less extreme, having survived in ice-free refugia e.g. nunataks during periods of glaciation or *P. heimii* is a post glacial immigrant establishing on the continent within roughly the last 18, 000 years (Kappen and Straka 1988, Longton 1988). Distribution of *P. heimii* within the Southern Hemisphere includes the Falkland Islands and southern South America (Greene 1986). *Pottia heimii* is documented as rare in New Zealand (Beever *et al.* 1992).

Figure 1.5a Typical habitat of *P. heimii* in Miers Valley, with Adams Glacier providing free water for mosses in the summer months (photographed Adam)

Figure 1.5b Typical microsite of *P. heimii* located on the drier regions at the edge of the Adams Glacier meltstream (photographed Adam).

1.11 RAPD analysis of *Pottia heimii*

RAPD analysis has the potential to contribute valuable information for a large number of questions pertaining to antarctic *P. heimii* at a population level. The aims of this study were:

(1) to develop a method for application of RAPDs to *P. heimii*, establishing whether:

(a) RAPD profiles were producible for antarctic *P. heimii*

(b) RAPD profiles for *P. heimii* were reproducible

(c) genetically identical *P. heimii* tissue (joined shoots) gave identical RAPD profiles

(d) RAPDs were able to detect differences between individual *P. heimii* shoots.

(2) If all the above points could be positively answered, then to determine whether there was:

(a) within-clump genetic variation in *P. heimii*

(b) genetic variation within *P. heimii* transects collected in Miers Valley

(c) genetic variation between *P. heimii* transects collected in Miers Valley.

(d) genetic variation between geographically isolated *P. heimii* populations collected within Eastern Antarctica.

This could aid in the understanding of local colonisation events where:

(a) low levels of genetic variation would suggest a single source of propagules

(b) higher levels of genetic variation would infer multiple sources of propagules

(3) If all the above points could be positively answered, to determine whether there was spatial structure in *P. heimii* transects in Miers Valley. There were two possible scenarios for a population structure:

(a) if individuals in close proximity (1cm apart) were more genetically similar than individuals some distance apart (10m apart) then a structured distribution of genotypes could be assumed;

(b) if genetic variability was as great between individuals in close proximity (1cm apart) as between individuals some distance from each other (10m apart) then random distribution of genotypes could be assumed.

(4) If all the above points could be positively answered, to determine whether the pattern of variation within and between transects revealed anything about the processes of genotypic dispersal of *P. heimii* in Miers Valley. This might contribute to understanding the major local dispersal mechanisms of *P. heimii* propagules in Miers Valley where:

(a) a random pattern of genotypic distribution within a local area would suggest wind dispersal of propagules;

(b) a directional distribution of genotypes within a local area would suggest water dispersal of propagules.

CHAPTER TWO

MATERIALS AND METHODS

2.1 *Pottia heimii* sampling sites

All collection sites in East Antarctica are indicated in Figure 2.1. Within Miers Valley three 50m transect sites were chosen for *P. heimii* collection and designated by the codes MVB, MVC and MVE. Each transect was considered to represent one of three *P. heimii* subpopulations within Miers Valley. The pattern of distribution of the individual subpopulations each followed the course of a separate glacial meltstream within the valley (Figure 1.2d). Transect MVB (Figures 2.2 a & b) was beside the melt stream from Miers Glacier, upstream of its confluence with the stream from Adams Glacier. The start of the transect was approximately 500m east from the tip of Miers glacier and 350m east of a cairn on the north side of the stream. Most of the stream bed was dry, with a small amount of flowing water on its northern side.

Transect MVE (Figure 2.3) was on the southern side of the valley, beside the meltstream from Adams Glacier. The start of the transect was approximately 1000m down from the tip of Adams Glacier. The Adams Glacier stream is branched with site MVE approximately 80m downstream from this junction but upstream of the Miers and Adams confluence. Transect MVC (Figure 2.4) started approximately two metres downstream of the Miers and Adams meltstreams confluence along a dry stream bed. *Pottia heimii* patches at MVC were sheltered by a small moraine approximately one metre in height. Several small streams ran into this section from the south facing slope of the valley.

Pottia heimii was also collected from East Garwood Valley (Figure 2.5), Bratina Island (Figure 2.6) and Edmonson Point (Figure 2.7). East Garwood Valley is located approximately 10 km north east of Miers Valley at 78°00'S 164°05'E. Bratina Island is a small island located approximately 30 km east of Miers Valley, within the Ross Ice Shelf off the tip of Brown Peninsula at 78°00'S 165°35'E. Edmonson Point is a coastal site located approximately 12 km east of Mt Melbourne (74°20'S 164°30'E) and approximately 500 km north of Miers Valley.

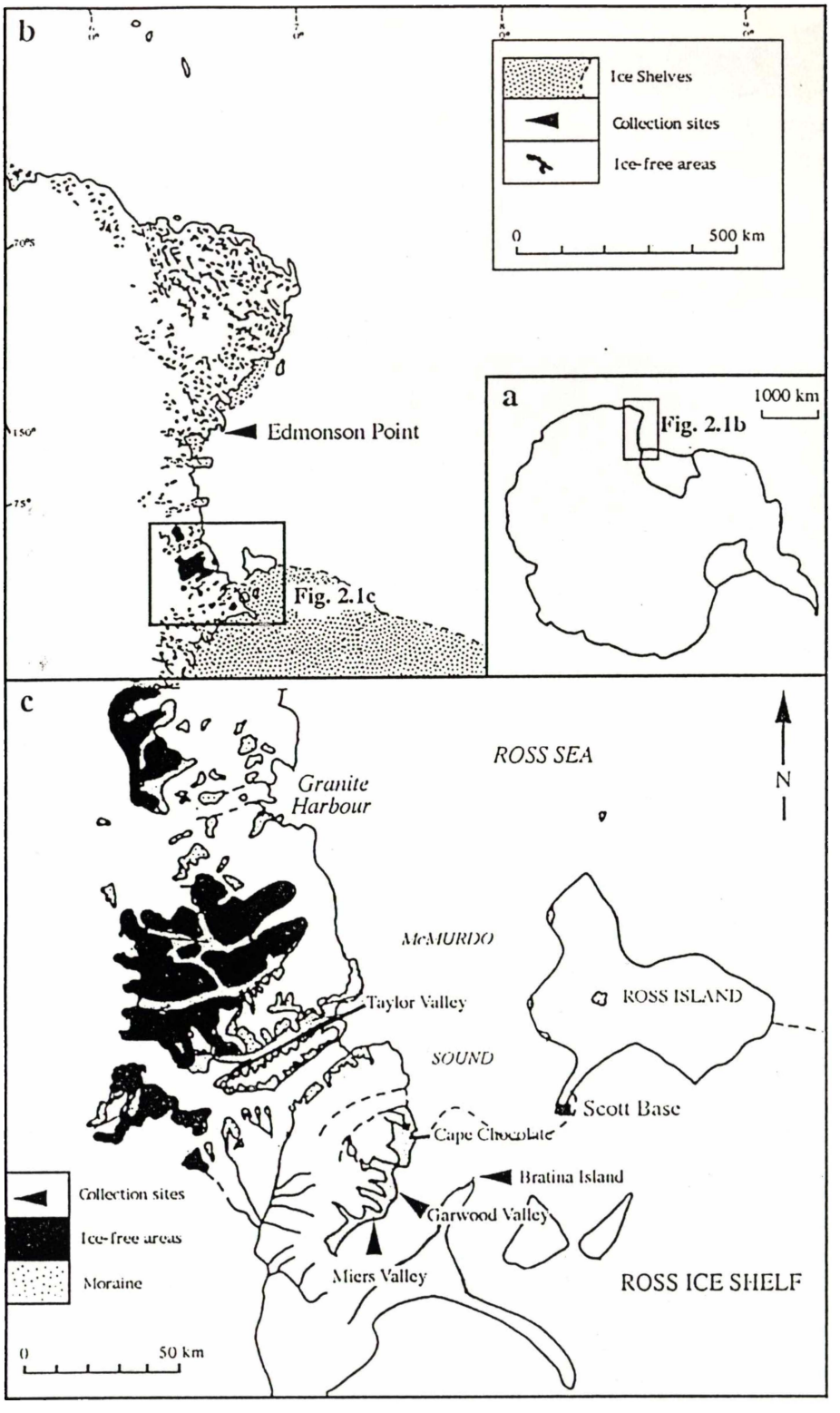

Figure 2.1 Locations of *P. heimii* collection by the field party K022 in East Antarctica. The collection sites included Miers Valley, East Garwood Valley, Bratina Island and Edmonson Point (adapted Campbell and Claridge 1987).

Figure 2.2a The start of *P. heimii* transect MVB, approximately 50m from the tip of Miers Glacier in Miers Valley, East Antarctica (photographed Adam).

Figure 2.2b Transect MVB, showing approximately 70% ground coverage of *P. heimii* (photographed Adam).

Figure 2.3 *Pottia heimii* transect sites MVE beside the northern arm of the Adams Glacier meltstream in Miers Valley, East Antarctica (photographed Adam).

Figure 2.4 *Pottia heimii* transect site MVC, below the confluence of the Miers and Adams Glacier in Miers Valley, East Antarctica (photographed Adam).

Figure 2.5 Western end of East Garwood Valley, with Garwood Glacier in the background and two moss hunters in the foreground (photographed Selkirk).

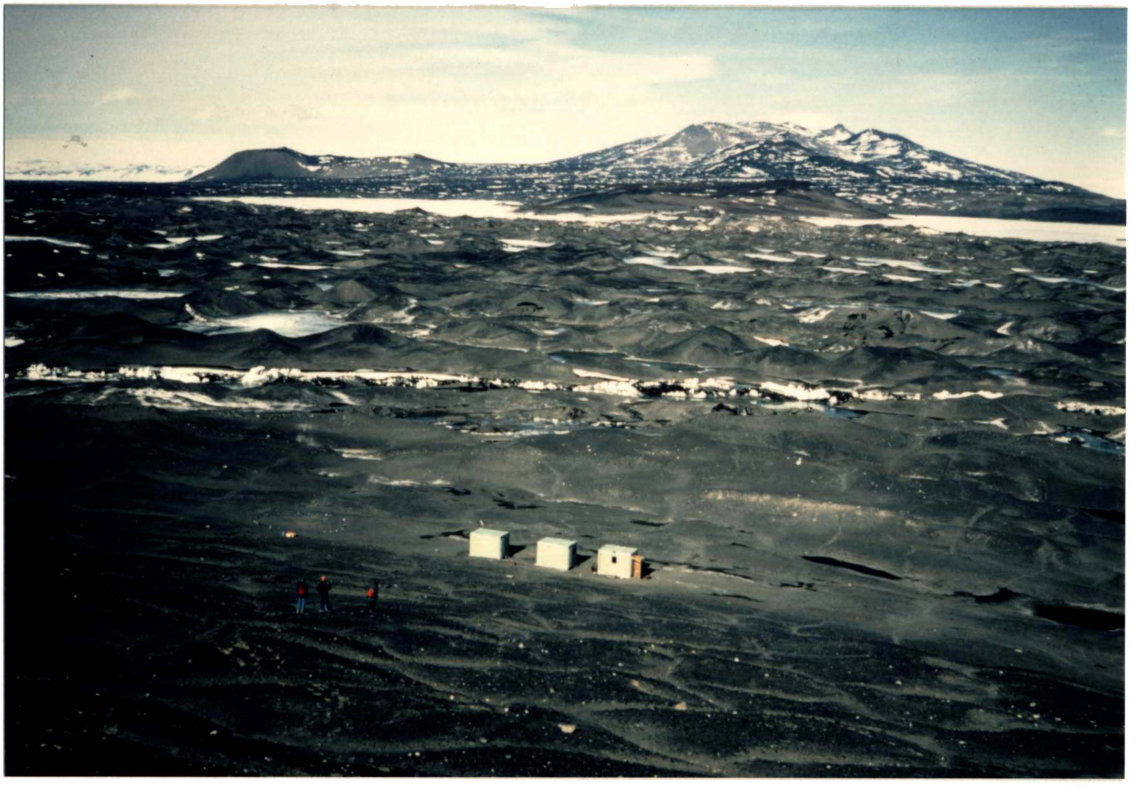

Figure 2.6 The view from Bratina Island facing south towards Brown Island, with unusual pond formations on the ice shelf and Bratina Island huts in the foreground. (photographed Selkirk).

Figure 2.7 The coastal beach of Edmonson Point in Northern Victoria Land, East Antarctica (photographed Selkirk).

2.2 *Pottia heimii* collection

Pottia heimii plants were identified for collection initially by the distinctive dark brownish green colour of its leaves. However observations in the field and under the microscope show that sun and shade forms of *P. heimii* are frequently found. Plants that are snow covered or under rocks, generally in low light situations, have a green appearance to their leaves. Plants subjected to high light levels produce leaves that are reddish/brown in colour. Figures 2.8 a and b show these different forms.

Samples from Miers Valley and East Garwood were collected along 50m transects. Within each transect, samples were collected as follows; 10 samples 1cm apart, 10 samples 10cm apart, 10 samples 1m apart and 5 samples 10m apart. When *P. heimii* was absent from the planned sample points, material was collected from the nearest available position. Figures 2.9 and 2.10 show diagrams of the sampling strategies used for transects MVB and MVE respectively. Samples from East Garwood and Bratina Island were collected along approximately 10m transects in a similar manner. Samples were collected from several sites from Edmonson Point. Each sample was approximately 1cm square in size, which proved more than adequate for genetic analysis and allowed for minimum impact on the environment.

Permission to collect plant specimens and importation of plant material into New Zealand was granted by the New Zealand Antarctic Programme (NZAP) and the Ministry of Agriculture and Fisheries respectively (NZAP Operations Manual 1994).

Samples were collected in paper bags and on return to New Zealand each sample was designated a single number e.g. MV101 and stored in the freezer at -20°C for later use in the laboratory.

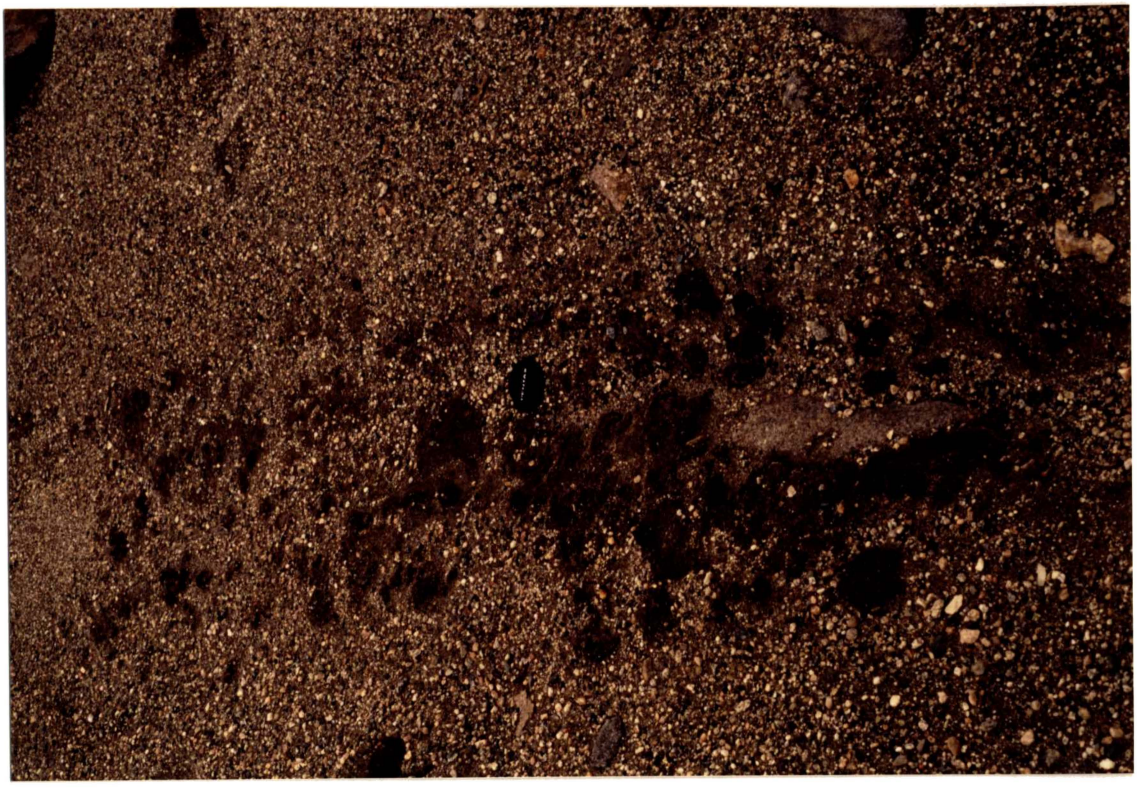

Figure 2.8a Sun form of *P. heimii* photographed in Miers Valley, East Antarctica (photographed Adam).

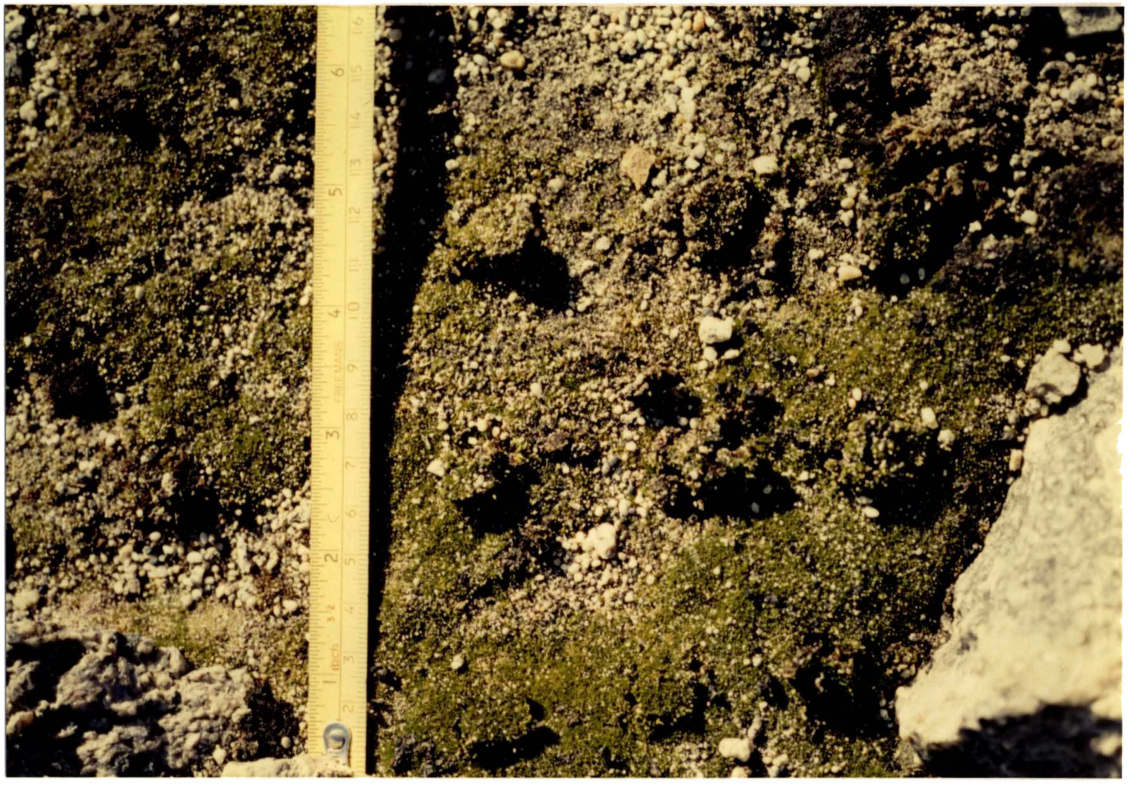

Figure 2.8b Shade form of *P. heimii* photographed in Garwood Valley, East Antarctica (photographed Adam).

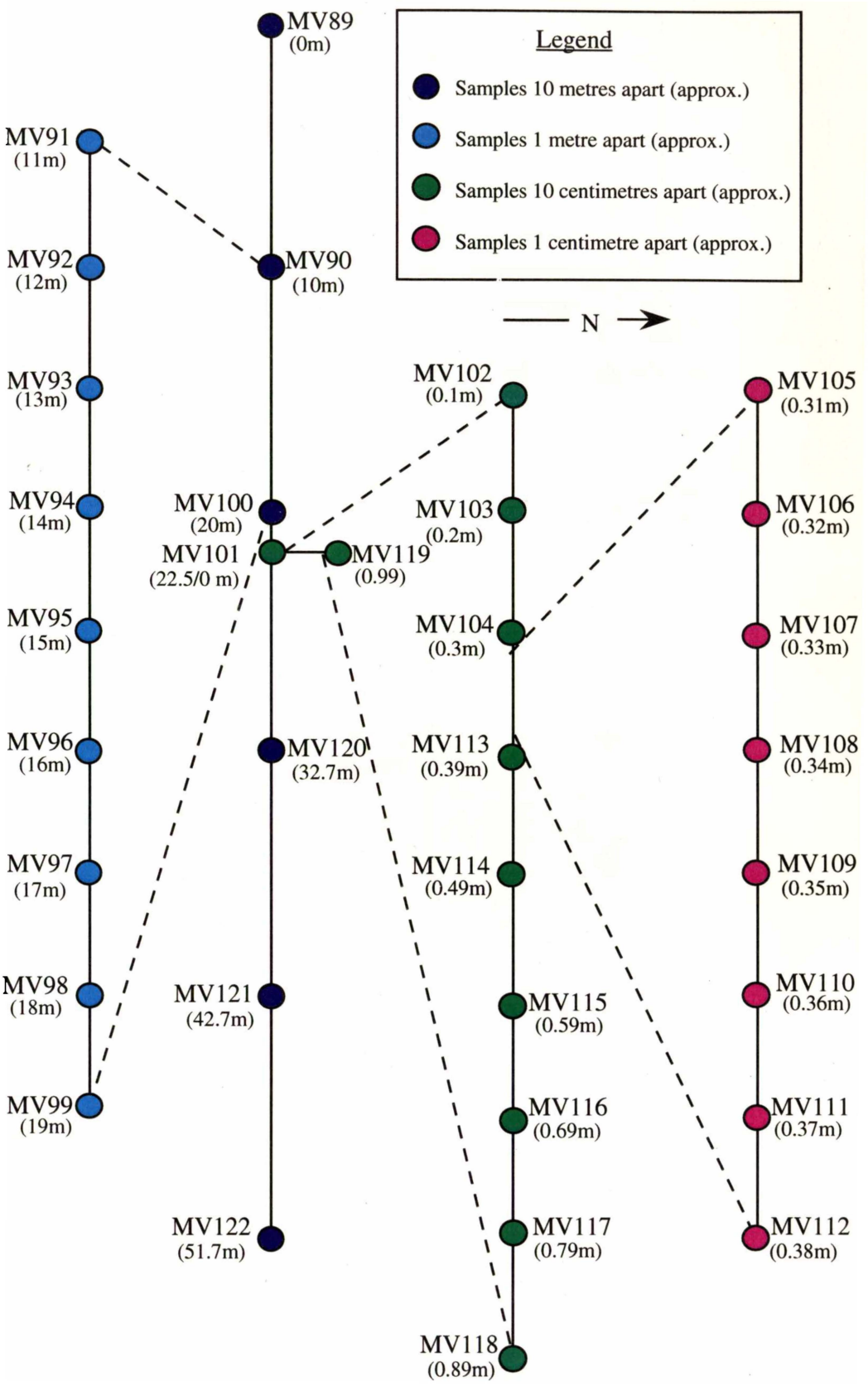

Figure 2.9 Miers Glacier Meltstream Transect MVB (NB. not to scale).

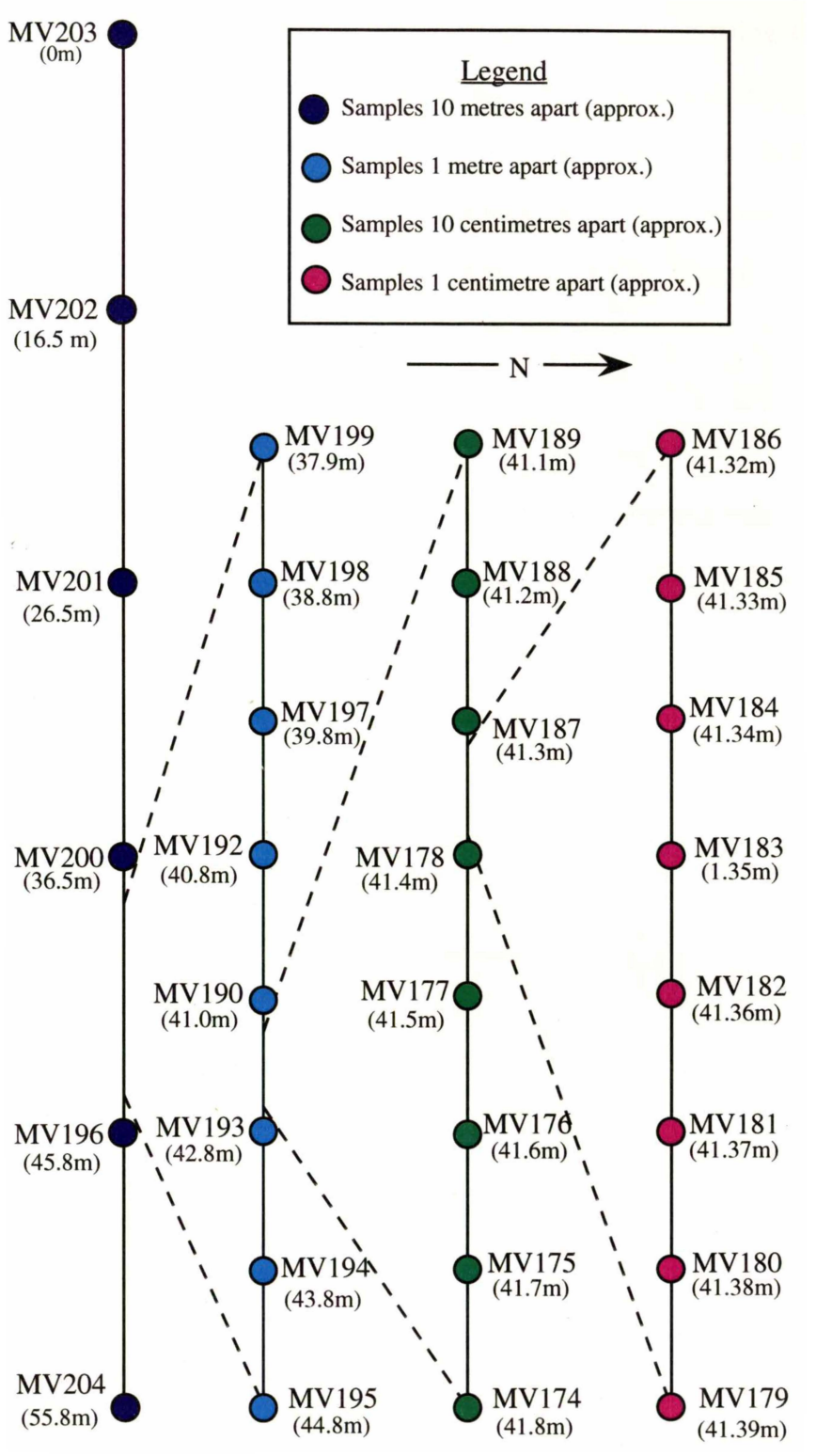

Figure 2.10 Adams Glacier Meltstream Transect MVE (NB. not to scale).

2.3 *Pottia heimii* identification and preparation

Field samples were identified as *P. heimii* under a microscope using 100x to 200x magnification. *Pottia heimii* leaves were broadly obovate, acute, with the margins weakly denticulate towards the apex and the costa ceasing in the apex. The upper laminal cells were irregular hexagonal to rhombic-hexagonal, becoming somewhat rectangular towards the base (cf. Seppelt *et al.* 1995). Figure 2.11 shows a photomicrograph of a *P. heimii* shoot.

Care was taken with those field samples that were a mixture of species to ensure that only *P. heimii* was selected. A single moss shoot was removed using fine forceps and cleaned thoroughly with distilled water to remove any soil. Healthy shoots were selected in preference to shrivelled, dry shoots to ensure the highest possible DNA yield. Shoots covered in salt or with visible algal contamination were avoided in order to decrease the chance of DNA contamination. The average length of a single shoot of *P. heimii* was 2-3mm.

Figure 2.11 Photomicrograph of a single *P. heimii* shoot (x150) (photographed O' Brian).

2.4 RAPD protocol for *Pottia heimii*

The RAPD protocol described here for *P. heimii* was developed over several months. The process of its development is described in Chapter Three.

2.4.1 DNA isolation

Isolation of DNA from a single *P. heimii* shoot was considered important for two reasons. Firstly, during sample collection mixed clumps of *P. heimii* and *Bryum argenteum* were seen, where propagules of both species had either established in the same area over a similar period of time or an already established clump of one species has been a favourable site for colonisation by the other species (Figure 2.12). This situation might well be occurring within clumps of *P. heimii* with different genotypes, but no visible differences would then be present. Secondly Adam *et al.* (in press) had reported within-clump variation in antarctic *B. argenteum* using RAPD analysis.

Adam *et al.* in press and Selkirk *et al.* in press isolated DNA from the antarctic moss species *B. argentum* and *Sarconeum glaciale* for RAPD analysis following the methods Wang *et al.* (1993). Although this method of DNA extraction proved successful for these species it was a very crude method and was not satisfactory for *P. heimii.* Therefore, three more intensive DNA isolation methods were used.

Figure 2.12 A mixed clump of *P. heimii* and *B. argenteum* in Miers Valley, East Antarctica (photographed Adam).

2.4.1.1 Modified methodology of Edwards *et al.* (1991)

This isolation method was used in the initial optimisation of the RAPD reaction mix and thermocycler programme (Chapter 3.3) but replaced in further experimental work for more intensive DNA isolation methods.

(1) A glass drill bit was used to grind a single moss shoot in 80µl of DNA extraction buffer (1M Tris pH 7.5, 1M NaCl, 0.5M EDTA and 10% SDS) in a 1.5ml Eppendorf tube. The suspension was incubated at room temperature for 10-60 minutes.

(2) 80µl of Tris-saturated 1:1 phenol:chloroform (pH 8.0) were added, the suspension was vortexed briefly and spun in a Biofuge 13 centrifuge (Heraeus sepatech) at 13000 rpm for 3 minutes.

(3) The supernatant was transferred into a clean Eppendorf tube, 150µl of ice-cold 100% ethanol were added and the sample incubated at -20°C for 10-30 minutes.

(4) The DNA was pelleted by centrifuging for 5 minutes at 13000 rpm and vacuum dried using a Hetrovac VR-1 (High Technology of Scandinavia).

(5) The dry DNA pellet was resuspended in 250µl of sterilised TE buffer pH 8.0 (1mM Tris-HCl, 0.5M EDTA). The DNA sample was stored at 4°C.

2.4.1.2 Modified methodology of Doyle and Doyle (1990)

This isolation method was used in the analysis of *P. hemii* populations (Chapter Four).

(1) 100µl of CTAB isolation buffer (2% CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris-HCl pH 8.0 and 1% polyvinylpyrollidone) and 5µl proteinase K (1mg/ml) were preheated to 60°C.

(2) A glass drill bit was used to grind a single moss shoot in the CTAB isolation buffer in a 1.5ml Eppendorf tube, and 0.16µl of the oxidising agent mercaptoethanol were added. The tube was inverted several times and incubated at 60°C for 30 minutes, with occasional swirling.

(3) 133µl chloroform : isoamyl alcohol (24:1) was added, the suspension was thoroughly mixed by vortexing briefly and centrifuged for 5 minutes at 13, 000 rpm in a Biofuge 13 centrifuge (Heraeus sepatech) to separate the phases. The aqueous phase was removed and transferred to a clean tube. Step three was repeated.

(4) An equal volume of ice-cold isopropanol was added and the tube was gently inverted several times and incubated at -20°C for 30 minutes to precipitate the nucleic acids.

(5) The precipitate was pelleted by centrifuging for 15 minutes at 13, 000 rpm. The supernatant was removed and the pellet was resuspended in 83µl 1M NaCl at 37°C. Difficult pellets were dislodged with a blunted 200µl pipette tip.

(6) The resuspended nucleic acid solution was centrifuged for five minutes at 13000 rpm to remove the precipitated polysaccharides. The supernatant containing the DNA was transferred to a clean tube and incubated at 95°C for 30 minutes to denature any remaining protein.

(7) The nucleic acids were precipitated in an equal volume of ice-cold isopropanol by incubating at -20°C for 20 minutes. The DNA precipitate was pelleted by centrifuging for 15 minutes at 13000 rpm. The DNA pellet was washed in 83µl 70% ethanol by centrifuging for 3 minutes. This was repeated using 95% ethanol.

(8) The supernatant was removed by inverting gently on tissue paper and the pellet was vacuum dried using a Hetrovac VR-1 (High Technology of Scandinavia). The nucleic acid pellet was resuspended in 25µl of sterile TE pH 8.0 (1mM Tris-HCl, 0.5M EDTA) and RNase treated with 0.5µl of RNase (1 mg/ml) at 37°C overnight. The DNA sample was stored at 4°C.

2.4.1.3 Skotnicki DNA isolation method (unpublished)

This isolation method was used as a quick and easy method towards the end of this study (Chapter Four) to add in additional samples for RAPD analysis. Although the DNA concentration and purity were not measured, RAPD profiles for samples isolated using this method were comparable in quality and reproducibility to samples isolated by the modified methodology of Doyle and Doyle (1990).

(1) A glass drill bit was used to grind a single moss shoot in 50µl of CTAB isolation buffer (2% CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris-HCl pH 8.0 and 1% polyvinylpyrollidone) in a 1.5ml Eppendorf tube. A further 99.5µl of CTAB isolation buffer plus 0.5µl of beta-mercaptoethanol were added, the suspension was vortexed briefly and incubated in a 55°C waterbath for 20 minutes.

(2) 75µl of 24:1 chloroform : isoamyl were added, the suspension was vortexed and centrifuged at 13, 000 rpm for 5 minutes in a Biofuge 13 centrifuge (Heraeus sepatech) to separate the phases. The aqueous phase was removed and transferred to a clean tube.

(3) 15µl of ammonium acetate (7.5M) and 150µl ice-cold isopropanol were added, the tube gently inverted several times and incubated at -20°C for 10 minutes to precipitate the nucleic acids.

(4) The precipitate was pelleted by centrifuging for 8 minutes at 13, 000 rpm. The supernatant was removed and the pellet rinsed with 150µl of 75% ethanol by centrifuging for three minutes. The ethanol was removed by inverting the tube gently on tissue paper and the step repeated with 95% ethanol.

(5) The DNA pellet was vacuum dried using a Hetrovac VR-1 (High Technology of Scandinavia). The dry nucleic acid pellet was resuspended in 25µl of sterile TE pH 8.0 (1mM Tris-HCl, 0.5M EDTA) and RNase treated with 0.5µl of RNase (1mg/ml). The DNA sample was stored at 4°C.

2.4.2 RAPD amplification of *Pottia heimii* DNA

The components of a single RAPD reaction were template DNA, PCR reaction buffer (1M Tris-HCl, 500mM KCl, pH 8.3), $MgCl_2$, deoxyribonucleotide-triphosphates (dATP, dCTP, dGTP, dTTP Boehringer Mannheim, Mannheim, Germany), a random primer (Operon Technologies Inc., Alameda, California), *Taq* DNA polymerase (Boehringer Mannheim, Mannheim, Germany) and template DNA. Table 2.1 shows (1) the order in which the reagents were added (a-f were combined to make a master mix which was finally added to the DNA template), (2) the volume of the reagent added and (3) the final concentration of the reagent.

Table 2.1 Components of a single RAPD reaction for *P. heimii* DNA

(1) Reagent	(2) Volume	(3) Final Concentration.
(a) sterilised H_2O	4µl	-
(b) 10x PCR buffer	1µl	1x
(c) $MgCl_2$ (25mM)	1µl	2.5mM
(d) dNTPs (2.5mM each)	1µl	0.25mM
(e) primer (2mM)	1.5µl	0.3mM
(f) Taq DNA polymerase	0.5µl	0.5 units
(g) template DNA	1µl	4-12ng
final volume	10µl	

The final reaction mix was transferred to a capillary tip (Corbett Research) using a CP-1 Auto Pipetter (Corbett Research), sealed using a HS-2 Heat Sealer (Corbett Research) and placed directly into a FTS-4000 Capillary Thermal Sequencer (Corbett Research). This air-cooled thermocycler allowed rapid heating and cooling between ambient and 94°C, minimising temperature differences between samples. The thermocycler programme is shown in Table 2.2. Ramp rates for heating and cooling were two degrees per second, therefore the programme took approximately two hours to complete.

Table 2.2 FTS-4000 Capillary Thermal Sequencer (Corbett Research) Programme B.

Cycle#	Step #	Temperature (°C)	Time
1	1	94	1 min.
	2	35	1 min.
	3	72	2 min.
2-44	1	94	10 sec.
	2	35	10 sec.
	3	72	50 sec.
45	1	72	3 min.
	2	25	1 min.

Once the reaction was complete the capillary tip was removed from the thermocycler, the end was cut and the plunger was depressed to release the product. Five microlitres of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (w/v) sucrose in water (Sambrook *et al.* 1989)) were added to the RAPD sample. DNA amplification fragments were separated in 1.5% agarose gel (multi purpose agarose, Boehringer Mannheim, Mannheim, Germany) (two lanes contained 100bp ladder (Operon Technologies Inc.)) in 1 x Tris-acetate (TAE) buffer (0.04M Tris-acetate, 1mM EDTA (pH 8.0) (Sambrook *et al.* 1989)) at 100 volts for three hours. Fragments were then visualised by staining the gel in ethidum bromide (60µl EthBr (10mg/ml) in 800ml of distilled water) for 15 minutes and destaining under running tap water for 20 minutes. The DNA bands were illuminated under UV light and recorded on 667 film (Polaroid).

Sixty (Operon Technologies Inc.) primers were evaluated for suitability, of these, six were selected for the final study (Table 2.3). These primers gave reproducible results, with informative and easily scored bands. The number of bands per primer ranged from 6 to 27, with the fragments between 400bp to 1.2 kb in length. Reproducibility was checked for each sample using the primers OPP-15 and OPA-03. Duplicates were run for at least half of the samples with the remaining four primers. Control samples, containing all reagents except DNA, were used in order to check that no self-amplification or DNA contamination occurred. If products were observed in these lanes, it was checked that they did not correspond to any polymorphic loci scored. For the purpose of this study all bands were scored. Each individual DNA sample was represented by a vector of 1 or 0, 1 for the presence of any particular band and 0 for its absence. The 100bp ladder allowed the comparison of bands across several gels.

Table 2.3 The six primers (Operon Technologies, Inc., Alameda, Calif.) selected for RAPD analysis of *P. heimii.*

Primer	Nucleotide sequence (5'-3')
OPA-03	AGTCAGCCAC
OPA-09	GGGTAACGCC
OPA-18	AGGTGACCGT
OPA-19	CAAACGTCGG
OPP-06	GTGGGCTGAC
OPP-15	GGAAGCCAAC

2.5 Analysis of RAPD bands

2.5.1 Pairwise distance calculations

The RAPDistance package (Armstrong *et al.* 1995) was used to record and analyse the relatedness of RAPD generated DNA fragments between samples. The package was chosen on the grounds that it is the best available package and is well tried, with over 6000 users world wide (Skotnicki pers. comm.). The scored data (1 for the presence of any particular band and 0 for its absence) was entered into RAPDistance and converted into a numerical form to calculate distances between pairs of samples, and to produce a distance matrix, as follows:

n = the number of bands positions

nx = the number of bands present in x

ny = the number of bands present in y

n11 = the number of positions where x=1 AND y=1

n00 = the number of positions where x=0 AND y=0

n01 = the number of positions where x=0 AND y=1

n10 = the number of positions where x=1 AND y=0

Different algorithms were used to calculate the similarity of pairs of samples. RAPDistance offers a choice of 18 algorithms; 5 were chosen for this study (Table 2.4). Metrics 1, 2, and 8 give similarity values (s) in the range 0 to +1. These are converted to distances as (1-s). Metrics 5 and 18 give values (s-) in the range -1 to +1. These are converted to the same range by the transformation (s+1) / 2. The results were stored as triangular matrices (Armstrong *et al.* 1995).

Table 2.4 *Pottia heimii* samples in the RAPDistance package (Armstrong *et al.* 1995).

Algorithm	**Calculation from numerical data**
Dice (1)	2*n11 / ((2*n11) + n01 + n10)
Jaccard (2)	n11 / (n-n00)
Sokal and Sneath (5)	(n11*n00) - (n01*n10)) /sqrt ((n11+n01) *(n11+n01)* (n01+n00) * (n11+n10) * (n10+n00))
Sokal and Sneath 2 (8)	0.25*((n11/(n11+n10)) + (n11/(n11+ n01)) + n00/(n00 + n10)) + (n00/(n00 + n01))
Hamman (18)	(n11- (n10+n01) + n00 / n

2.5.2 Dendrogram analysis

An unrooted dendrogram was created from the pairwise distances using the computer programs NJTREE (Jin 1988) and TDRAW (Ferguson 1990), based on the neighbour-joining method of Saitou and Nei (1987) and Studier and Keppler (1988). Node values showed the genetic similarity between isolates, a small node value indicating that isolates where genetically very similar. A Permutation Tail Probability (PTP) test (Faith and Cranston 1991) was used to assess the significance of the trees generated by the neighbour-joining method. This calculated the degrees of freedom from the average of 20 randomly generated trees, and tested whether a tree calculated from a set of distances reflected a tree-like signal in the data. A standard deviation above four indicated a high probability that the tree was not an artefact of the algorithm (Skotnicki pers. comm.).

2.5.3 Analysis of Molecular Variance (WINAMOVA)

WINAMOVA is a statistical analysis package that uses the pairwise distance matrix calculated by RAPDistance to perform an Analysis of Molecular Variance (AMOVA). While AMOVA was developed specifically for Restriction Fragment Length Polymorphism (RFLP) (Excoffier *et al.* 1992), Huff *et al.* (1993) showed that RAPD data could easily be accommodated. WINAMOVA allowed for the calculation of molecular variance within and between groups of individuals as a percentage value.

CHAPTER THREE

DEVELOPMENT OF THE RAPD PROTOCOL FOR *POTTIA HEIMII*

3.1 Introduction

The development of a RAPD protocol specifically for *Pottia heimii* was an integral component of this study. Over the months of protocol development it became apparent that *P. heimii* was a more difficult species to work with, with respect to RAPDs, than the antarctic species of *Bryum argenteum* and *Sarconuem glaciale* (Adam *et al.* in press, Selkirk *et al.* in press). To allow for the investigation of genetic variability of *P. heimii* populations in East Antarctica, particularly in the Miers Valley, the development of the protocol involved (1) obtaining an amplifiable product, (2) optimising PCR conditions for clear and uncontaminated profiles and (3) obtaining reproducible RAPD profiles. The following summarises the chronological order of experimental work.

3.2 Materials and methods

Pottia heimii samples used during the protocol development are described in Chapter 2.1. DNA isolation and RAPD reactions followed the protocols described in Chapters 2.4.1 and 2.4.2 respectively. However, thermocycler programmes and specific combinations of reagents change at various stages and are referred to in the text. Pairwise distance matrices (described in Chapter 2.5.1) were calculated from the RAPD banding patterns obtained in section 3.5.3 and this data was presented as a dendrogram (described in Chapter 2.5.2).

3.3 Optimisation of reaction mix and thermocycler programme

3.3.1 Original DNA isolation method

DNA was isolated using single moss shoots from field samples MV17, BI643 and EP798 using the modified methodology of Edwards *et al.* (1991). This method is described in Chapter 2.4.1.1 and is referred to here as the Edwards method. The Edwards method was chosen initially as it is designed to rapidly extract small amounts of plant genomic DNA specifically for PCR. The DNA was resuspended in 250µl TE buffer following the procedure of Selkirk *et al.* (in press) on *S. glaciale.*

3.3.2 Original RAPD protocol and primer trial one

In an attempt to find random primers that yielded an amplifiable product a trial was conducted using the 20 primers from Operon primer kit P. A volume of 0.5µl DNA per 10µl reaction was chosen based on the RAPD protocol described by Selkirk *et al.* (in press) for *S. glaciale* which uses 1µl of DNA per 25µl RAPD reaction. The DNA was amplified with 1µl PCR-reaction buffer (Chapter 2.4.2), 0.8µl $MgCl_2$ (25mM), 1µl dNTPs (2.5mM each), 1.5µl primer (20mM), 0.5µl Taq polymerase and 4.7µl sterilised water and using the designated thermocycler programme A (Table 3.1) (Skotnicki pers. comm.). From the primer trial OPP-01, OPP-05 and OPP-06 were chosen for further experimental work because they amplified *P. heimii* DNA and produced the clearest profiles with the brightest bands.

Table 3.1 FTS-4000 Capillary Thermal Sequencer (Corbett Research)

Programme A.

Cycle#	Step #	Temperature (C°)	Time
1	1	94	3 min.
	2	40	2 min.
	3	72	3 min.
2-44	1	94	10 sec.
	2	40	10 sec.
	3	72	50 sec.
45	1	72	5 min.
	2	25	1 min.

3.3.3 DNA concentration

DNA titrations were performed to determine what volume of DNA per RAPD reaction was optimal for *P. heimii*, i.e. what volume produced clear bands with minimal background. DNA was isolated, using the Edwards method, from field samples MV17, MV22, MV41, MV47, MV76, MV88, MV206, EG352 and BI645. Initial working stocks of each isolate were presumed to have similar DNA concentrations because the size of starting tissue was approximately the same (a single 2-3mm moss shoot). These isolates were titrated at volumes of 5µl, 2.5µl, 1.5µl and 1µl per RAPD reaction. One microlitre of each isolate was diluted to 1/2, 1/5, 1/10 using sterile water and 1µl was used per RAPD reaction. The different DNA volumes and dilutions were amplified (1µl PCR-reaction buffer, 0.8µl $MgCl_2$ (25mM), 1µl dNTPs (2.5mM each), 1.5µl primer (20mM), 0.5µl of Taq polymerase and thermocycler programme A) with primers OPP-01, OPP-05 and OPP-06. The volume of sterile water was varied to accommodate the different volumes of DNA samples.

RAPDs were repeated at least twice using all three primers because a large number of the reactions yielded no amplification product. When amplification did occur only a few smeary bands could be seen. Repeating the PCR reactions several times showed there was no consistency in results and DNA volumes that looked promising in one PCR experiment were unable to give the same quality of results in later PCR experiments.

3.3.4 DNA quantification

Quantification of DNA in the isolates amplified in section 3.3.3 was attempted by electrophoresing 30µl of genomic DNA in 1.5 % agarose but no bands were visible at the position where genomic DNA would be expected. This indicated that the amount of DNA was below 10ng/µl, the lower detection limit of ethidium bromide (Wilkins pers. comm.). The same samples were tested using a UV-VIS Recording Spectrophotometer UV-160 (Shimadza) but this also lacked the sensitivity needed for the low DNA concentrations and consequently no DNA was detected. However, DNA concentrations of between 5-40ng per reaction are in the range considered to be acceptable for RAPD analysis (Richardson pers. comm.). Therefore, it was possible DNA concentrations could still be above the lower threshold. At this stage a more sensitive technique of quantification was not available.

The volume of 1µl DNA per RAPD reaction was eventually chosen for further experimental work because, at that time, it appeared to give the most consistent results from section 3.3.3.

3.3.5 Primer trial two

Further primers were evaluated for suitability using 1µl of DNA isolated from field samples MV101, MV105 and MV107 using the Edwards method. The isolates were amplified (1µl PCR-reaction buffer, 0.8µl $MgCl_2$ (25mM), 1µl dNTPs (2.5mM each), 1.5µl primer (20mM), 0.5µl Taq polymerase, 4.2µl sterilised water and thermocycler programme A) with 40 primers from Operon primer kits A and B. OPA-03, OPA-05, OPA-12, OPA-17, OPA-18, OPA-19, OPA-20, OPB-04, OPB-10, and OPB-19 were amplified with additional isolates since it appeared they could potentially produce RAPD profiles with clear, well separated bands that would be easy to score. As in the previous trial (cf. section 3.3.2), the DNA did not amplify with any consistency with the primers chosen. This suggested that other parameters within the RAPD reaction mix and thermocycler programme needed to be optimised.

3.3.6 Magnesium concentration

Titration of $MgCl_2$ concentration is important, because magnesium is the metallic cofactor for Taq polymerase and forms soluble complexes with dNTPs (and impurities in the DNA) (Sambrook *et al.* 1989, Anon.(b) 1995). However, too much magnesium may adversely effect the PCR reaction and produce a non-specific smear of PCR product (Wilkins pers. comm.). Increased concentrations of $MgCl_2$ could be required when using capillary tips (Corbett research) as the polypropylene material is able to bind magnesium (Anon.(c) 1995).

One microlitre of DNA isolated (using the Edwards method) from field samples MV206, EG352 and BI645 was amplified (1µl PCR-reaction buffer, 1µl dNTPs (2.5mM each), 1.5µl primer (20mM), 0.5µl Taq polymerase and thermocycler programme A) with 1.5mM, 2mM, 2.5mM, 3mM and 3.5mM of $MgCl_2$, using the primers OPP-01 and OPP-05. The volume of sterile water was varied to accommodate the different volumes of $MgCl_2$ solution.

The $MgCl_2$ concentration that produced the clearest RAPD profiles with both primers was 2.5mM per RAPD reaction (Figure 3.1), although profiles still looked slightly smeary. This $MgCl_2$ concentration was used in all subsequent RAPD reactions.

3.3.7 Primer concentration

High primer concentrations can give smeary bands while too low concentrations may make it difficult to detect amplification products (Williams *et al.* 1993). Because most profiles had appeared smeary using 1.5µl of undiluted primer stock (final concentration of 3µM per reaction) per RAPD reaction, a 1 in 10 primer stock dilution was trialed (final concentration of 0.3µM per reaction) (Skotnicki pers. comm.).

One microlitre of DNA, isolated (using the Edwards method) from field samples MV206, EG352 and BI645 was amplified (1µl 10x PCR-reaction buffer, 1µl $MgCl_2$ (25mM), 1µl dNTPs (2.5mM each), 0.5µl Taq polymerase, 4µl sterilised water and thermocycler programme A) with a 1/10 dilution and undiluted stock of primers OPP-01 OPP-05 and OPP-06. In all cases, primers showed that the diluted primer gave clearer profiles although there was a slight decrease in the number of bands amplified (Figure 3.1). Diluting the primer also economised on the amount used per reaction. The primer concentration of 0.3 µM per reaction was used in all subsequent RAPD reactions.

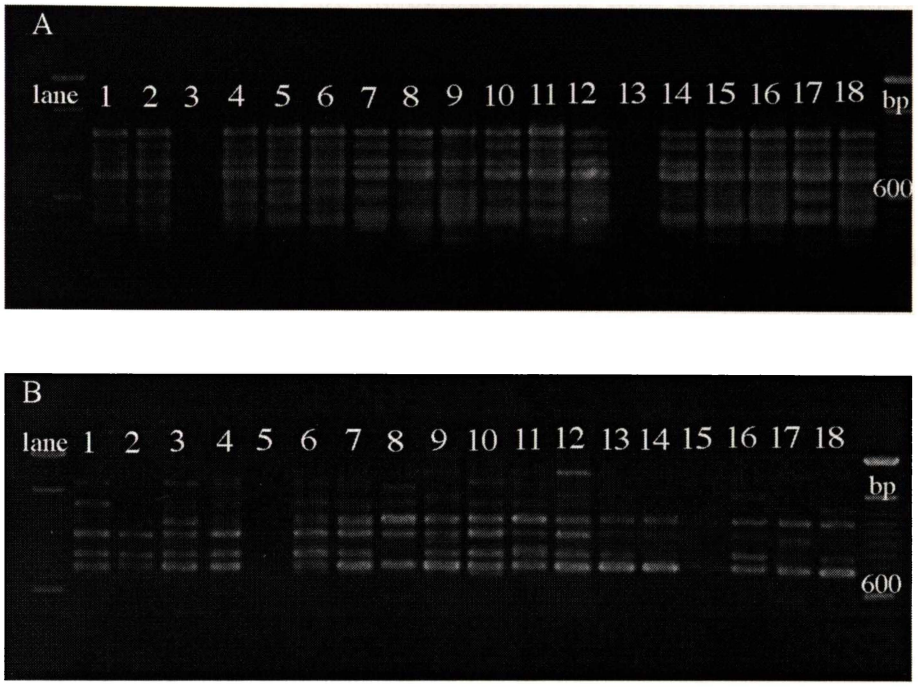

Figure 3.1a & b. Duplicate RAPD banding pattern obtained on an agarose gel for *P. heimii* isolates. For both gels, lanes 1-6 reaction mix contained 2.0mM $MgCl_2$ (MV91 (lanes 1-2), MV100 (lanes 3-4) and MV122 (lanes 5-6)), lanes 7-12 reaction mix contained 2.5mM $MgCl_2$ (MV91 (lanes 7-8), MV100 (lanes 9-10) and MV122 (lanes 11-12)) and lanes 13-18 contained 3.0mM $MgCl_2$ (MV91 (lanes 13-14), MV100 (lanes 15-16) and MV122 (lanes 17-18)) (cf. section 3.3.6). All reaction mixes in A contained an undiluted stock of the primer OPP-06 and all reaction mixes in B contained a 1/10 dilution of the same primer (cf. section 3.3.7). DNA was isolated via the modified methodology of Edwards *et al.* (1991), resuspended in 250µl TE buffer and amplified using the primer OPP-01. The control reaction with no DNA added (not shown) showed no amplification product.

3.3.8 Annealing temperature

Decreasing the annealing temperature during the thermocycling might facilitate increased amplification of the template DNA by lowering the stringency of binding between the random primer and homologous and near-homologous nucleotide sequences on the DNA template (Williams *et al.* 1993).

The thermocycler programme for the amplification of *P. heimii* DNA was modified by lowering the annealing temperature from 40°C to 35°C. The length of time at each stage in cycle one (cf. Table 3.1) was also increased to ensure all stages were completed to their full potential. This thermocycler programme, designated programme B, is shown in Table 2.2. One microlitre of DNA isolated from field samples MV107, MV108 and MV110 (using the Edwards method) was amplified (1µl 10x PCR-reaction buffer, 1µl $MgCl_2$ (25mM), 1µl dNTPs (2.5mM each), 1.5µl primer (2mM), 0.5µl Taq polymerase and 4µl sterilised water) with primers OPP-01 and OPP-06, using the original and new thermocycler programmes. The results showed (photographs not shown due to poor quality) there were more bands per RAPD profile, i.e. greater amplification, and that the clarity of profiles was improved using the new thermocycler programme. This critical point in the development of the RAPD protocol for *P. heimii* suggested that the original programme was not optimal for amplification of *P. heimii* DNA. The altered thermocycler programme was used in all subsequent reactions.

3.3.9 Summary

The quality of RAPD profiles had greatly improved with the altered RAPD protocol. Suitable primers gave clear bright bands that were well separated and could be easily scored. In summary, the protocol now differed from the original protocol described in section 3.3.2 by having an increased $MgCl_2$ concentration (2.5mM instead of 2.0mM), a decreased random primer concentration (0.3µM instead of 3µM), a decreased annealing temperature (35°C instead of 40°C) and an increased volume of DNA template per PCR reaction (1µl instead of 0.5µl), although the DNA concentration was still unknown. The reaction mix used in further experiments contained 1µl 10x PCR-reaction buffer, 1µl $MgCl_2$ (25mM), 1µl dNTPs (2.5mM each), 1.5µl primer (2mM), 0.5µl Taq polymerase, 4µl sterilised water and an annealing temperature of 35°C.

3.4 Optimisation of reproducibility

3.4.1 Within-clump variation

The aim of this experiment was to determine the extent of genetic variability within a single *P. heimii* clump. Five single shoots were extracted from ten field samples i.e. MV90, MV91, MV92, MV93, MV94, MV95, MV96, MV97, MV98 and MV99 using the Edwards method, giving a total of 50 DNA isolates. The ten field samples were collected along a 10m transect. The isolates were amplified with primers OPP-01 and OPP-05. The RAPD profiles for these isolates were extremely polymorphic, a typical example for isolates amplified with OPP-01 being given in Figure 3.2. Although a certain degree of variability was anticipated between individual shoots within a single moss clump, the extreme polymorphism found for these isolates was unexpected.

To determine whether these results were reproducible i.e. if a single DNA sample would produce identical RAPD profiles when amplified in duplicate, DNA isolated from samples MV91a, MV91b, MV91c, MV98a, MV98b and MV98c were amplified in duplicate.

The results, seen in Figure 3.3, show many band differences between all duplicate reactions with both primers OPP-01 and OPP-05 and, as a result, it was concluded that the results were irreproducible.

3.4.2 Primer trial three

In order to find primers that not only gave scorable bands but more importantly also gave reproducible RAPD profiles, trials were repeated on twelve primers from Operon primer kits A, B and P that had shown potential in primer trials 1 and 2 (cf. 3.3.2 and 3.3.5). Isolates MV92, MV93 and MV94 were amplified with all twelve primers. The results showed only primer OPP-06 possessed the required attributes of reproducibility and scorable RAPD profiles. Several other primers e.g. OPA-03, OPP-19 and OPP-15, gave good profiles but were not reproducible. The large number of primers unable to give reproducible results was considered to be unusually high, suggesting that the quality of the template DNA could be responsible for the variability.

3.4.3 DNA purity

One possible explanation for the lack of reproducibility was that DNA isolates could have been contaminated by impurities such as polysaccharides or RNA. An opportunity arose to quantify the DNA and purity of several isolates using a Biotech GeneQuant II RNA/DNA Calculator (Pharmacia) and contamination was identified. The wavelengths 260nm and 280nm were used for quantifying and purity checking calculations. The advantage of this system was that it could detect low levels of DNA (less than 10ng/µl) and only 5µl of DNA sample was required per calculation. DNA concentration and purity readings for a number of DNA samples are shown in Table 3.2. The purity readings (260/280) were below 1.5 which is the lower purity limit recommended for RAPD analysis (1.8 is considered pure). DNA concentration readings at such a low purity level could furthermore not be regarded as accurate. (GeneQuant II RNA/DNA Calculator user manual, Richardson pers. comm.).

To establish if contamination was the cause of the unreproducible results, DNA from field samples MV90, MV100 and MV122 was isolated using the modified methodology of Doyle and Doyle (1990). This method is described in Chapter 2.4.1.2 and is referred to here as the CTAB method. The method was chosen because of its many additional clean-up stages in comparison to the Edwards method, including the salting out of polysaccharides and a RNase treatment. CTAB methods of DNA isolation are also widely used in plant DNA isolation procedures throughout the literature. However, there was concern that the additional clean-up steps might decrease the final yield of DNA. At the time no method was available to quantify the purity of these samples but it was assumed that levels of contamination would be less than in DNA isolated by the Edwards method. The DNA samples were amplified in duplicate with primers OPP-01 and OPP-15, both giving irreproducible profiles in previous trials (cf. section 3.4.2). The results showed that the higher purity of the DNA isolates increased the clarity of RAPD profiles but did not increase reproducibility between duplicates.

Table 3.2 DNA concentration and purity readings for isolates prepared using the modified methodology of Edwards *et al.* (1991) and resuspended in 250µl TE buffer.

Sample number	DNA concentration (ng/µl)	DNA purity (260/280 nm)
MV100a	66	0.8
MV100b	83	0.8
MV100c	74	0.8
MV91a	18	0.8
MV91b	21	0.8
MV91c	35	0.8
MV90a	17	0.8
MV90b	22	0.8
MV90c	18	0.8

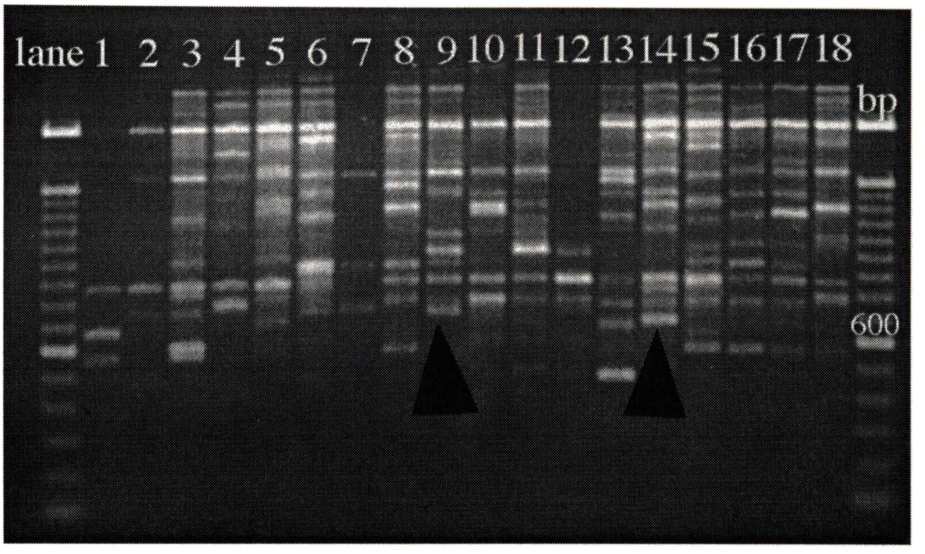

Figure 3.2 RAPD banding pattern obtained on an agarose gel for *P. heimii* isolates MV90a-e (lanes 1-5), MV91a-e (lanes 6-10), MV92a-e (lanes 11-15) and MV93a-c (lanes 16-18). Arrows show examples of the unexpected high levels of polymorphism seen within *P. heimii* clumps. DNA was isolated via the modified methodology of Edwards *et al.* (1991), resuspended in 250µl TE buffer and amplified using the primer OPP-01. The control reaction with no DNA added (not shown) showed no amplification product.

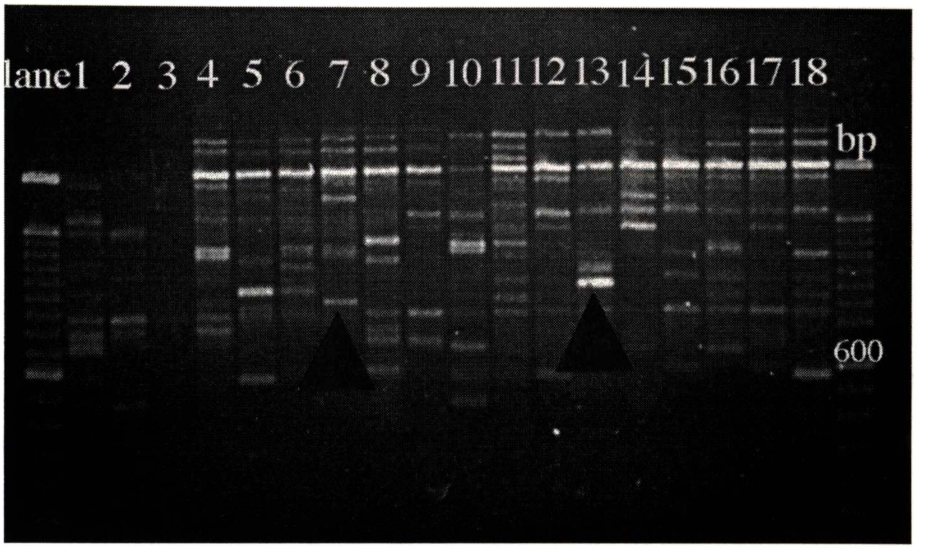

Figure 3.3 Duplicate RAPD banding pattern obtained on a agarose gel for *P. heimii* isolates MV96a (lanes 1-2), MV96b (lanes 3-4), MV96c (lanes 5-6), MV96d (lanes 7-8), MV96e (lanes 9-10), MV97a (lanes 11-12), MV97b (lanes 13-14), MV97c (lanes 15-16) and MV97d (lanes 17-18). Arrows show examples of irreproducible bands between duplicate samples. The DNA was isolated via the modified methodology of Edwards *et al.* (1991), resuspended in 250µl TE buffer and amplified using the primer OPP-01 The control reaction with no DNA added (not shown) showed no amplification product.

3.4.4 DNA concentration

Williams *et al.* (1993) state that a lack of reproducibility is most likely due to low concentrations of DNA. Therefore, it was possible that DNA concentrations in the trials conducted so far were less than 5ng per RAPD reaction.

DNA titrations were performed using DNA isolated from field samples MV91, MV100 and MV122, using the CTAB method. One, two, four and five microlitres of DNA were used per PCR reaction. The different DNA volumes were amplified with primers OPP-01, OPP-05, OPP-09, OPP-14 and OPP-15. All samples showed band differences between duplicates at all DNA concentrations. However, these differences did decrease slightly at the higher DNA concentrations, suggesting that increasing the DNA concentration could lead to reproducible results.

3.4.5 Increasing DNA concentration

The DNA concentrations were increased by isolating DNA from field samples MV91, MV100 and MV122 (using the CTAB method) and resuspending the pellet in 100µl TE buffer rather than 250µl. This would more than double the concentration of DNA per isolate. One, two and four microlitres of these isolates were amplified with primers OPP-01, OPP-05, OPP-09 OPP-13, OPP-14, and OPP-15. Primers OPP-05, OPP-13 and OPP-14 showed poorer reproducibility between duplicate samples than OPP-01, OPP-09 and OPP-15 but all primers showed a lack of reproducibility to varying degrees, at all DNA concentrations. These results led to the suspicion that the amount of starting tissue was inherently too low to yield enough DNA for reproducible results.

3.4.6 Increased tissue volume and DNA isolation

An increased volume of *P. heimii* tissue during DNA isolation should increase the concentration of DNA per isolate and might provide reproducible results. To determine at what tissue volume reproducible RAPD results could be achieved DNA was isolated (using the CTAB method) from field samples BI634 and BI666 using a single shoot, 1mg (approximately ten shoots), 10mg and 100mg of *P. heimii* tissue. The DNA pellets were resuspended in 100µl TE buffer. These isolates were amplified with the primer OPP-05, which in section 3.4.2 had given irreproducible results. The results (Figure 3.4) showed that all single shoot duplicates have bands present in one RAPD profile that are absent in the other. Samples prepared from 1mg of tissue gave increased but not perfect reproducibility between duplicates, as if some of the missing bands from the single shoot samples had been 'filled in'. Preparations from 10mg and 100mg tissue generally showed perfect reproducibility. The profiles of the latter samples were slightly smeary compared to the former, possibly because these samples contained too much DNA (cf. Williams *et al.* 1993). From these results it was concluded that DNA concentration did affect reproducibility and that low DNA concentrations (isolated from a single shoot) were responsible for the previous lack of reproducibility between duplicates. In addition, too much DNA (isolated from 100mg of tissue) might contribute to smeary RAPD profiles.

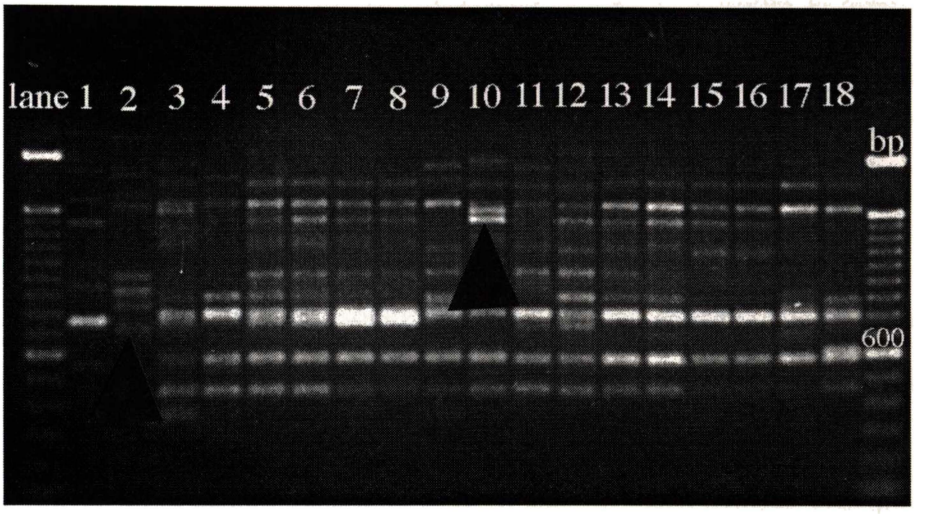

Figure 3.4 Duplicate RAPD banding patterns obtained on an agarose gel for *P. heimii* isolates BI634 and BI666. DNA was isolated from a single shoot (lanes 1-2 & 9-10, respectively), 1mg (lanes 3-4 & 11-12) 10mg (lanes 4-6 & 13-14) and 100mg (lanes 7-8 & 15-16) of tissue The banding patterns show that increased DNA concentrations increase reproducibility between duplicate reactions. Arrows indicate examples of irreproducible bands. The DNA was isolated via the modified methodology of Doyle and Doyle (1990), resuspended in 100µl TE buffer and amplified using the primer OPP-05. The control reaction with no DNA added (not shown) showed no amplification product.

3.4.7 One milligram of tissue and decreased TE buffer volumes

DNA isolated from 10mg and 100mg of tissue was not appropriate for use in this study because the presence of a large number of individual shoots per isolate could mean a mixture of different genotypes, possibly masking any potential polymorphisms between sample sites. As demonstrated in Figure 3.4, DNA samples prepared from 1mg of tissue (approximately ten shoots) gave results that bordered on being reproducible, therefore, that volume of tissue was therefore used for experimental work during the next phase.

The CTAB method was used to isolate DNA using 1mg of moss tissue from field samples BI634 and BI666. The DNA was resuspended in 100µl, 50µl and 25µl TE buffer. This could potentially increase the DNA per reaction to a point where it would facilitate reproducible results. One microlitre of each of these isolates was amplified with the primer OPP-05. Those isolates resuspended in 100µl and 50µl TE buffer showed irreproducible profiles between duplicates. However, isolates resuspended in 25µl TE buffer showed good reproducibility between all duplicates.

3.4.8 RAPD analysis using ten shoots

DNA was isolated using ten shoots from field samples MV104, MV105, MV106, MV107, MV108, MV109, MV110, MV111, MV112 and MV113 and the pellets resuspended in 25µl TE buffer. The ten field samples were collected within a one metre transect. One microlitre of each isolate was amplified with primers OPP-01, OPP-05, OPP-06, OPP-13 and OPP-19. Reproducibility was excellent for all samples with all primers. Although these field samples were collected in close proximity to each other, a small degree of variability was expected between samples but the extent of polymorphism was extremely low, as shown for the primer OPP-19 in Figure 3.5. This again raised concerns about the mixing of individual moss shoots leading to the masking of any polymorphisms that could potentially occur between field samples.

3.4.9 Decreasing shoot numbers

DNA was isolated, using the CTAB method, using ten shoots, five shoots and a single shoot from field samples MV109, MV110, MV112 and MV113. The pellet was resuspended in 25µl TE buffer. These isolates were amplified with primers OPP-01, OPP-13 and OPP-19. DNA isolated from ten and five shoots gave reproducible profiles between all duplicates, while the single shoot isolates showed reproducibility for 80% of the duplicate profiles. An example of reproducibility between single shoots is given in Figure 3.6. From these results it was concluded DNA isolated from a single shoot resuspended in 25µl TE buffer was able to give reproducible duplicate profiles for the majority of DNA isolates.

The above experiment was corroborated by the quantification of DNA using the GeneQuant II RNA/DNA Calculator (Pharmacia Biotech) (Table 3.3). As expected DNA concentrations were approximately ten times higher for ten shoots than a single shoot. Purity values were considered borderline with increased tissue volumes giving slightly increased readings. DNA concentrations for single shoot isolates ranged between 4-12ng/µl. Some values were below 5ng/µl which would explain why 20% of the isolates in the above experiment were not reproducible.

In hindsight, DNA extracted from a single moss shoot, resuspended in 100µl and 4µl used per RAPD reaction (cf. section 3.4.5) should contain approximately the same quantity of DNA if extracted from a single moss shoot, resuspended in 25µl and 1µl used per RAPD reaction. However, when experimental work in section 3.4.5 was reached, the decision was made to continue with the optimisation of reproducibility because the potential existed to further improve the results. This decision was also made using primers that did not give reproducible results at any stage during experimental work.

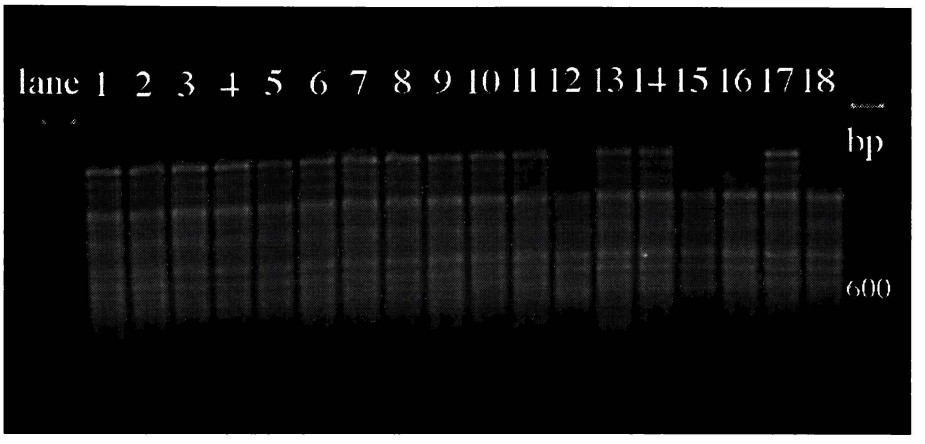

Figure 3.5 Duplicate RAPD banding pattern obtained on a agarose gel for *P. heimii* isolates MV104 (lanes 1-2), MV105 (lanes 3-4), MV106 (lanes 5-6), MV107 (lanes 7-8), MV108 (lanes 9-10), MV109 (lanes 11-12), MV110 (lanes 13-14), MV111(lanes 15-16) and MV112 (lanes 17-18). The banding patterns show an example of the low degree of polymorphism seen between isolates when DNA was isolated from ten shoots per field sample. The DNA was isolated via the modified methodology of Doyle and Doyle (1990), resuspended in 25µl TE buffer and amplified using the primer OPP-15. The control reaction with no DNA added (not shown) showed no amplification product.

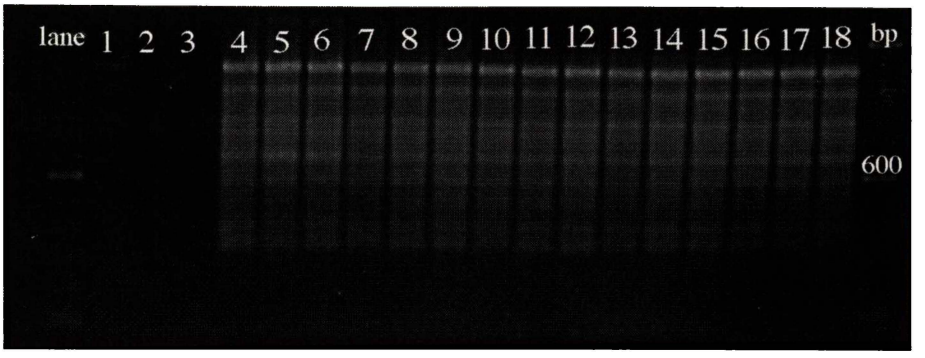

Figure 3.6 Duplicate RAPD banding pattern obtained on a agarose gel for *P. heimii* the isolate MV109 and MV110 where reaction mixes contained DNA isolated from 10 shoots (1/2 dilution) (lanes 1-2 & 9-10 respectively), 5 shoots (lanes 3-4 & 11-12), 2 shoots (lanes 5-6 & 13-14) and a single shoot (lanes 7-8 & 15-16). The banding patterns show an excellent example of reproducibility using DNA extracted from a single *P. heimii* shoot. The DNA was isolated via the modified methodology of Doyle and Doyle (1990), resuspended in 25µl TE buffer and amplified using the primer OPP-19. The control reaction with no DNA added (not shown) showed no amplification product.

Table 3.3 DNA concentration and purity values for samples prepared using the modified methodology of Doyle and Doyle (1991) and resuspended in 25µl TE buffer.

Sample Number	Extraction method	Number of shoots	Concentration of DNA (ng/µl)	DNA purity 260/280 nm
MV104	CTAB	10	47	1.7
MV105	CTAB	10	26	1.5
MV106	CTAB	10	19	1.5
MV109	CTAB	10	23	1.6
MV110	CTAB	10	19	1.6
MV112	CTAB	10	15	1.5
MV113	CTAB	10	24	1.6
MV109	CTAB	1	6	1.1
MV110	CTAB	1	5	1.2
MV112	CTAB	1	12	1.4
MV113	CTAB	1	7	1.2
MV94	CTAB	1	6	1.3
MV95	CTAB	1	4	.99
MV96	CTAB	1	4	1.1
MV97	CTAB	1	5	1.3
MV98	CTAB	1	8	1.3
MV99	CTAB	1	5	1.1
MV633	CTAB	1	5	1.1
MV786	CTAB	1	9	1.2

3.4.10 Summary

These results suggest that DNA isolated from a single shoot and resuspended in 25µl TE buffer provided the absolutely lowest limit of DNA concentration viable for RAPD analysis with enough volume to carry out a complete series of reactions. Due to the small volume of working stock only 1µl of sample per RAPD reaction could be used, as most samples were amplified in duplicate for all the primers used.

3.5 Testing the protocol and its usefulness for population studies of *Pottia heimii*

3.5.1 Introduction

RAPDS were used (1) to determine whether they gave similar profiles for genetically identical *P. heimii* tissue and (2) to establish whether they were able to detect DNA differences between individual P. *heimii* shoots within a clump.

3.5.2 Joined shoots

Nine sets of two joined shoots (Figure 3.7) were taken from field samples MV94 and EP789. RAPDs were performed in duplicate using the primers OPA-03, OPA-19, OPP-06 and OPP-15. Of the nine joined shoots analysed eight gave identical profiles for all four primers. The remaining joined shoots that did not give identical profiles differed by two bands out of a total of 64 bands scored. Figure 3.8 shows an example of RAPD banding patterns for genetically identical tissue.

3.5.3 Individual shoots within a clump

DNA was isolated from ten individual shoots taken randomly across field samples approximately $1cm^2$ in size. The field samples included MV94, MV95 and EP798. The isolates were amplified with the primers OPA-03, OPA-19, OPP-06 and OPP-15. Figure 3.9 shows the neighbour joining tree or dendrogram generated using the algorithm of Jaccard. A PTP test on this dendrogram gave a result of 92.26 standard deviations from random. All five algorithms used in the analysis gave similar dendrograms that differed only in minor details. The dendrogram showed that although many isolates taken from within a single moss clump were identical i.e. branch lengths were equal to zero, there was some definite within-clump variation. Of the 72 bands scored, 12 were polymorphic for field sample MV95, ten were polymorphic for field sample MV94 and eight were polymorphic for field sample EP578. Figure 3.10 shows examples of polymorphism seen between individual shoots.

3.5.4 Summary

The results in sections 3.5.2 and 3.5.3 concluded that (1) RAPDs were reproducible for *P. heimii* because RAPDs gave similar profiles for genetically identical *P. heimii* tissue, (2) RAPDs could detect genetic variation between *P. heimii* shoots and (3) genetic variation existed within a single *P. heimii* clump.

Figure 3.7 Photomicrograph of two joined *P. heimii* shoots (x150) (photographed O'Brian).

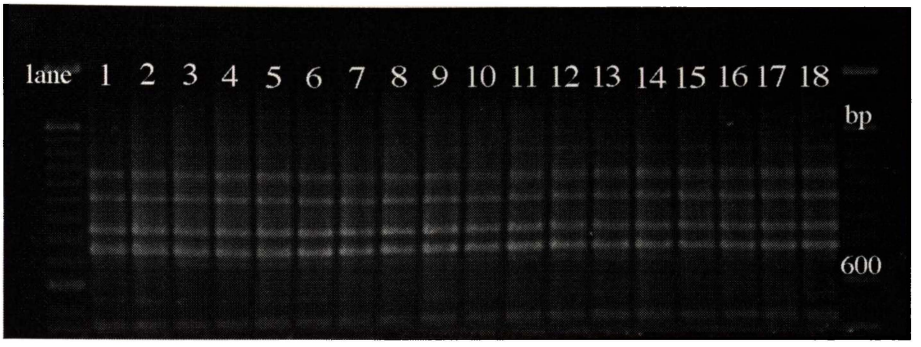

Figure 3.8 Duplicate RAPD banding patterns obtained on an agarose gel for joined *P. heimii* shoots taken from field sample MV94. Identical banding patterns were found for genetically identical tissue. The DNA was isolated via the modified methodology of Doyle and Doyle (1990), resuspended in 25µl TE buffer and amplified using the primer. The control reaction with no DNA added (not shown) showed no amplification product.

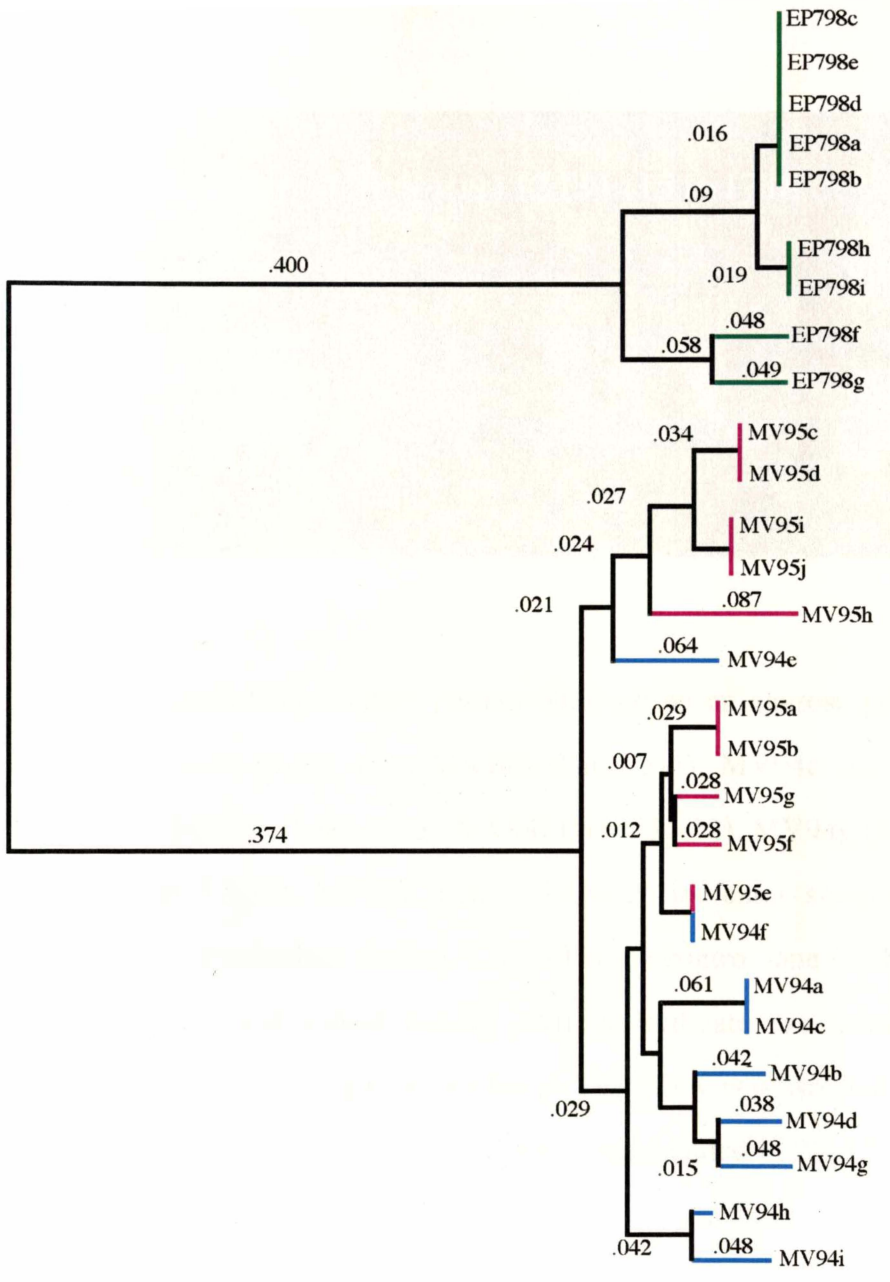

Figure3.9
Neighbour joining tree obtained using the algorithm of Jaccard. Approximately ten isolates from field samples MV94 (———), MV95 (———) and EP798 (———) were used in RAPD reactions with four different primers.

Figure 3.10 Duplicate RAPD banding pattern obtained on an agarose gel for *P. heimii* isolates MV94a* (lanes 1-2), MV94b (lanes 3-4), MV94c (lanes 5-6), MV94d (lanes 7-8), MV94e (lanes 9-10), MV94f (lanes 11-12), MV94g (lanes 13-14), MV94h* (lanes 15-16), MV94i (lanes 17-18) (* indicates samples were reextracted due to unreproducible results). Lane 19 is the control lane (no bands in this lane correspond to the isolate bands) . Arrows indicate examples of the polymorphism seen within a single moss clump. The DNA was isolated via the modified methodology of Doyle and Doyle (1990), resuspended in 25µl TE buffer and amplified using the primer OPA-03.

CHAPTER FOUR

RAPD ANALYSIS OF *POTTIA HEIMII* POPULATIONS

4.1 Introduction

This chapter will analyse *Pottia heimii* populations in East Antarctica to answer the questions put forward in Chapter 1.10. In summary, these were to establish whether genetic variation existed within and among populations of antarctic *P. heimii* (2) if genetic variation existed, to determine whether there was any spatial structure to the variation within antarctic *P. heimii* and (3) if genetic variation existed, to determine whether patterns of variation gave us any indication about the processes of dispersal/colonisation of *P. heimii* in East Antarctica

4.2 Materials and methods

The samples used for this analysis are described in Chapter 2.1 and were analysed using the RAPD protocol for *P. heimii* described in Chapter 2.6. Pairwise genetic distance matrices were calculated from RAPD banding patterns (described in Chapter 2.5.1) and these data were presented as dendrograms (described in Chapter 2.5.2). Results of an Analysis of Molecular Variance (WINAMOVA) (described in Chapter 2.5.3) using the same data were also presented.

4.3 Analysis within transect sites in Miers Valley

Samples collected from transect MVB and transect MVE (Figure 2.1) were analysed to determine whether there was genetic variation within each transect and to ascertain whether there was any genetic spatial structure to these transects. A total of 87 polymorphic bands were scored using primers OPA-03, OPA-09, OPP-06 and OPP-15. Figure 4.1 shows an example of these RAPD banding patterns.

4.3.1 Dendrogram analysis

Figures 4.2 (transect MVB) and 4.3 (transect MVE) show the neighbour joining trees or dendrograms generated using the algorithm of Jaccard. The PTP tests on these dendrograms gave results of 10.22 and 17.49 standard deviations from random. All five algorithms used in the analysis gave similar dendrograms that differed only in minor details. Small values of 0.056 in Figure 4.2 and 0.029 in Figure 4.3 were seen at node A from which the majority of the isolates were grouped. Within both transects, no two isolates showed the same RAPD profile for all four primers. Two Edmonson Point isolates were used as a comparison group and were found on a branch separate from the Miers Valley isolates. Both dendrograms showed field samples collected ten metres or one metre apart were clustered on the same branches similar to field samples collected ten centimetres or one centimetre apart.

Figure 4.1 Duplicate RAPD banding pattern obtained on an agarose gel for *P. heimii* isolates MV90 (lanes 1-2), MV91 (lanes 3-4), MV92 (lanes 5-6), MV93 (lanes 7-8), MV94 (lanes 9-10), MV95* (lanes 11-12), MV96 (lanes 13-14), MV97 (lanes 15-16), MV98 (lanes 17-18), MV99 (lanes 19-20) (* indicates samples were reextracted due to unreproducible results). Arrows indicate examples of polymorphisms seen between isolates from transect MVB. The DNA was isolated using the modified methodology of Doyle and Doyle (1991), resuspended in 25µl TE buffer and amplified using the primer OPA-03. The control reaction with no DNA added (not shown) showed no amplification product.

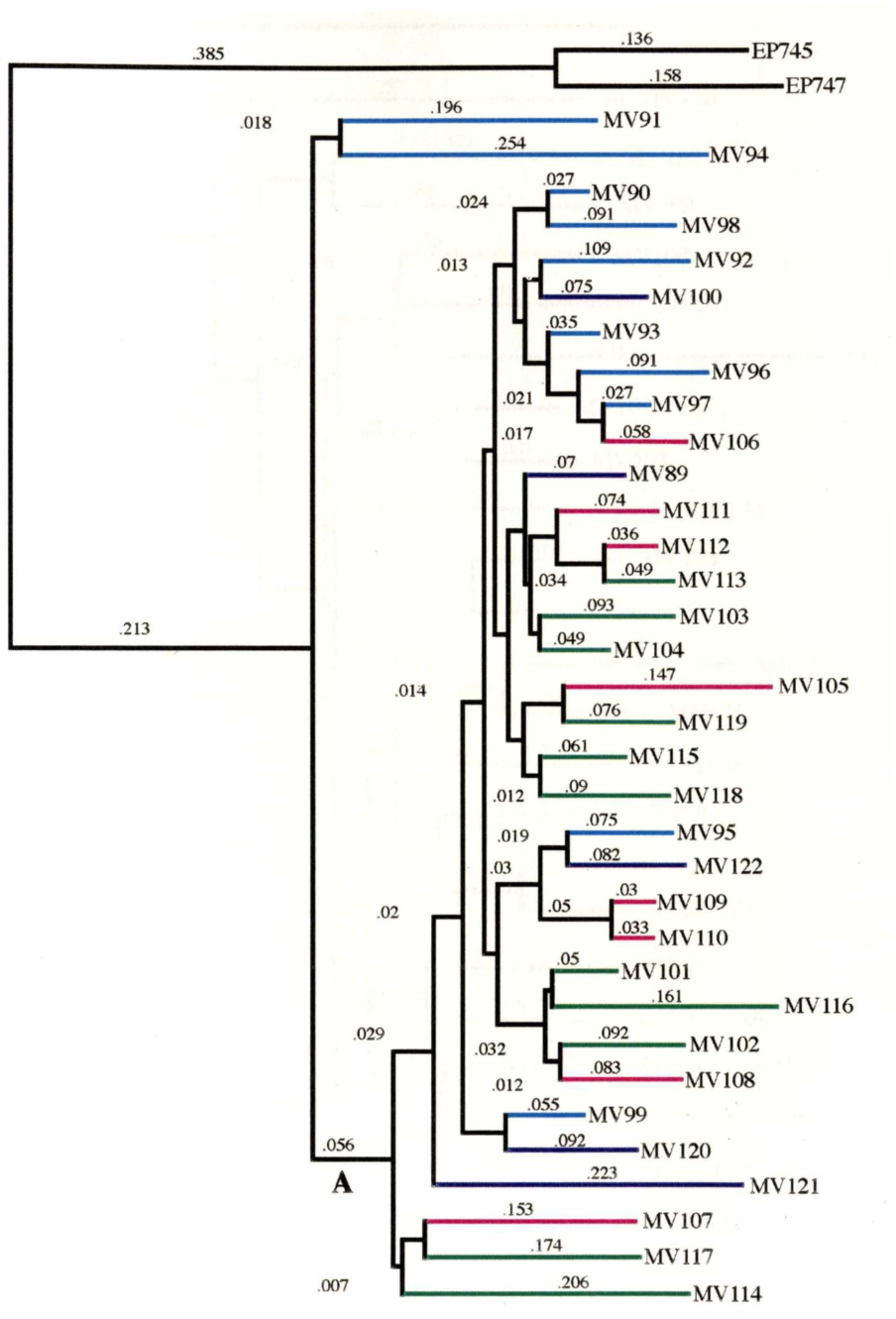

Figure 4.2

Neighbour joining tree obtained using the algorithm of Jaccard. Isolates from *P. heimii* samples collected 1cm (——), 10cm (——), 1m (——) and 10m (——) apart along transect MVB (Miers Valley meltstream) were used in RAPD reactions with four different primers. Two samples from Edmonson Point (EP) were compared as an outgroup. Note the small value at node A (see text).

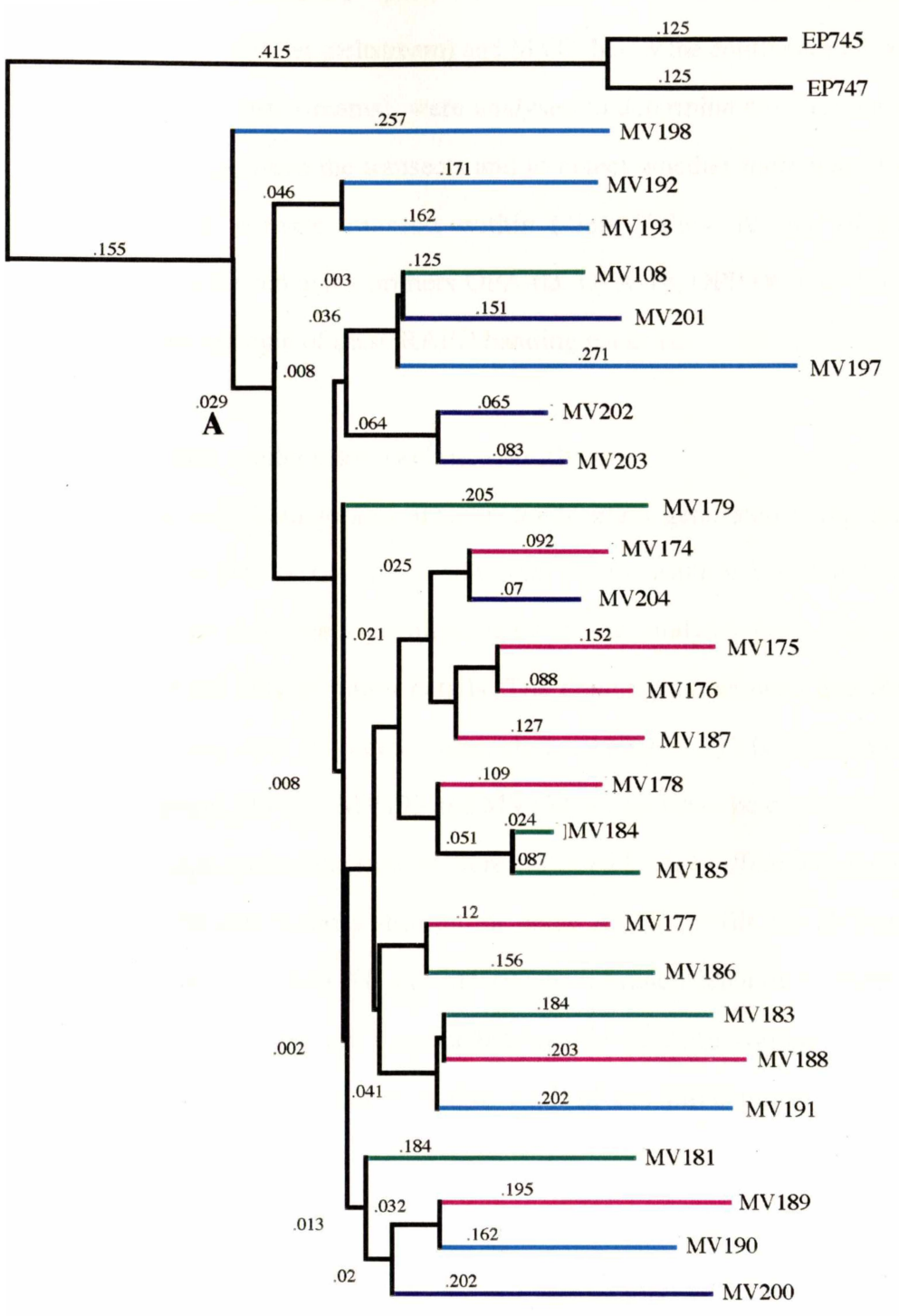

Figure 4.3

Neighbour joining tree obtained using the algorithm of Jaccard. Isolates from *P. heimii* samples collected 1cm (——), 10cm (——), 1m (——) and 10m (——) apart along transect MVE (Adams Glacier meltstream) were used in RAPD reactions with four different primers. Two samples from Edmonson Point (EP) were compared as an outgroup. Note the small value at Node A (see text).

4.4 Analysis between transect sites in Miers Valley

Field samples, collected one metre apart, from transects MVB (Miers Glacier meltstream), MVE (Adams Glacier meltstream) and MVC (below the confluence of the Miers and Adams Glaciers meltstreams), were analysed to determine whether there were genetic differences between the transects and to detect whether there was any genetic spatial structure to these transects within Miers Valley. A total of 92 polymorphic bands were scored using primers OPA-03, OPA-18, OPP-06 and OPP-15. Figure 4.4 shows an example of these RAPD banding patterns.

4.4.1 Dendrogram and molecular variance analysis

Figure 4.5 shows the neighbour-joining tree or dendrogram generated using the algorithm of Jaccard. The PTP test on this dendrogram gave a result of 5.63 standard deviations from random. All five algorithms used in the analysis gave similar dendrograms that differed only in minor details. The dendrogram showed that the isolates did not group into their respective transects i.e. transects MVB, MVC and MVE. For example isolates MV121, MV195 and MV134 are shown to be clustered on the same branch, although each of the isolates were collected from a different transect within Miers Valley. No two isolates showed the same RAPD profile for all four primers, with isolates MV124, MV190 and MV197 on a branch separate from the majority of the isolates. The molecular variance between the transects was calculated as 4.21% while the molecular variance within each of the three transects was calculated as 95.79%.

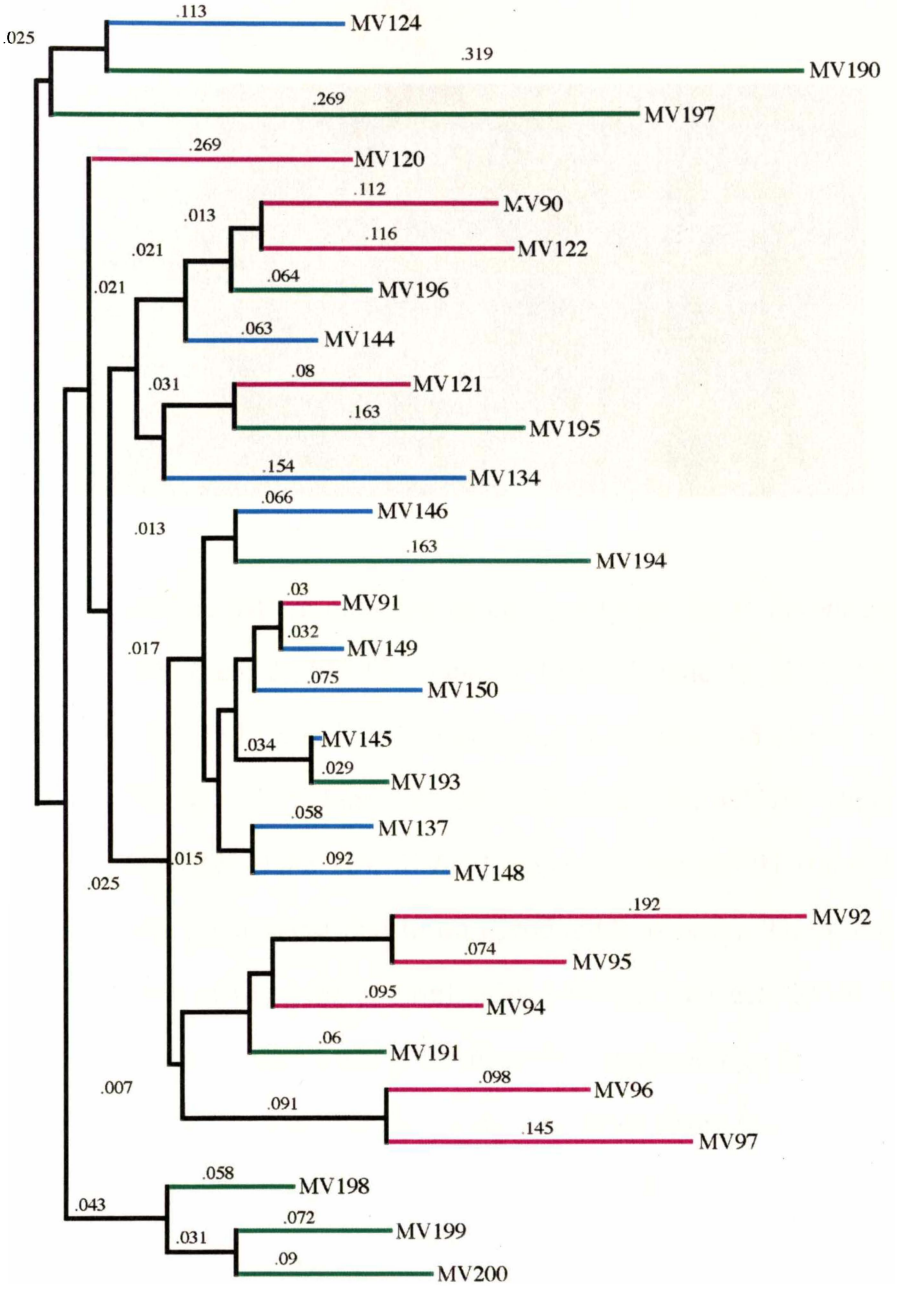

Figure 4.5

Neighbour joining tree obtained using the algorithm of Jaccard. Approximately ten *P. heimii* isolates from transects MVB (——), MVC (——) and MVE (——) were used in RAPD reactions with four different primers.

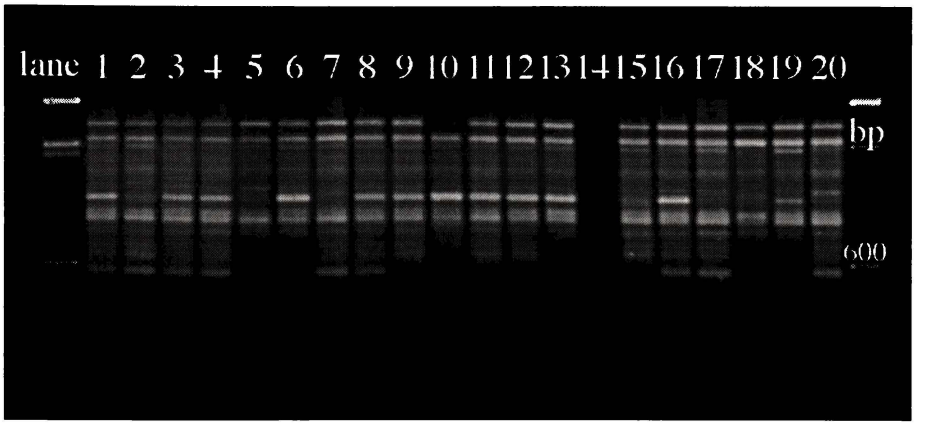

Figure 4.4 RAPD banding pattern obtained on an agarose gel for *P. heimii* isolates MV90 (lane 1), MV120 (lane 2), MV121 (lane 3), MV122 (lane 4), MV124* (lane 5), MV134 (lane 6), MV137 (lane 7), MV144 (lanes 8-9). MV 145 (lanes 10-11), MV146 (lanes 12-13), MV147* (lane 14), MV148 (lane 15), MV149 (lane 16), MV150 (lane 17), MV190* (lane 18), MV191 (lane 19) and MV91 (lane 20) (* indicates samples were reextracted due to unreproducible results). These banding patterns show examples of the genetic similarities between transects MVB, MVC and MVE. The DNA was isolated using the modified methodology of Doyle and Doyle (1990), resuspended in 25µl TE buffer and amplified using the primer OPA-03. The control reaction with no DNA added (not shown) showed no amplification product.

4.5 Analysis between isolated populations in East Antarctica

Field samples selected randomly from Miers Valley (29 samples), East Garwood Valley (7 samples), Bratina Island (8 samples) and Edmonson Point (6 samples) were analysed to determine whether genetic differences existed between geographically isolated populations. A total of 118 polymorphic bands were scored using primers OPA-03, OPA-18, OPP-06 and OPP-15. Figure 4.6 shows an example of these RAPD banding patterns.

4.5.1 Dendrogram and molecular variance analysis

Figure 4.7 shows the neighbour-joining tree or dendrogram generated using the algorithm of Jaccard. The PTP test on this dendrogram gave a result of 58.38 standard deviations from random. All five algorithms used in the analysis gave similar dendrograms that differed only in minor details. The dendrogram showed that the Edmonson Point isolates clustered on a branch separate from the Miers Valley, East Garwood Valley and Bratina Island isolates. A small value of 0.036 was seen at node A from which the majority of the isolates were grouped. However, except for isolates EP787 and EP788, no two isolates showed the same RAPD profile for all four primers, with the isolate EG355 being on a branch separate from the other Southern Victoria Land isolates.

The first molecular variance analysis compared the Miers Valley group (transects 1, 2 and 3) with the East Garwood group and gave a molecular variance of 14.90% between the groups and 85.10% within each of the groups. The second analysis compared the Miers Valley, East Garwood and Bratina Island as a single group to the Edmonson Point group. The molecular variance between the groups was 62.37% and the molecular variance within the groups was 37.63%.

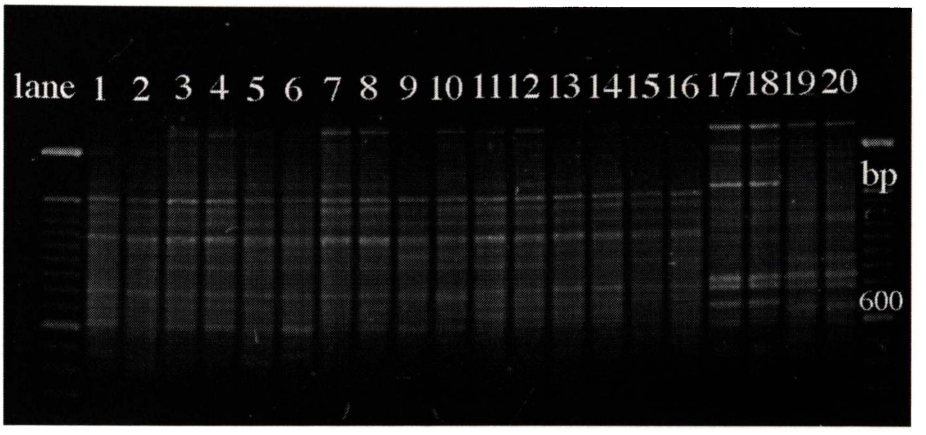

Figure 4.6 Duplicate RAPD banding pattern obtained on an agarose gel for *P. heimii* isolates MV96 (lanes 1-2), BI581 (lanes 3-4), BI590 (lanes 5-6), BI591 (lanes 7-8), BI592 (lanes 9-10), BI593 (lanes 11-12), BI594 (lanes 13-14), BI595 (lanes 15-16), EP731 (lanes 17-18) and EP732 (lanes 19-20). These banding patterns show examples of the polymorphisms seen between geographically isolated populations. The DNA was isolated using the modified methodology of Doyle and Doyle (1990), resuspended in 25µl TE buffer and amplified using the primer OPP-15. The control reaction with no DNA added (not shown) showed no amplification product.

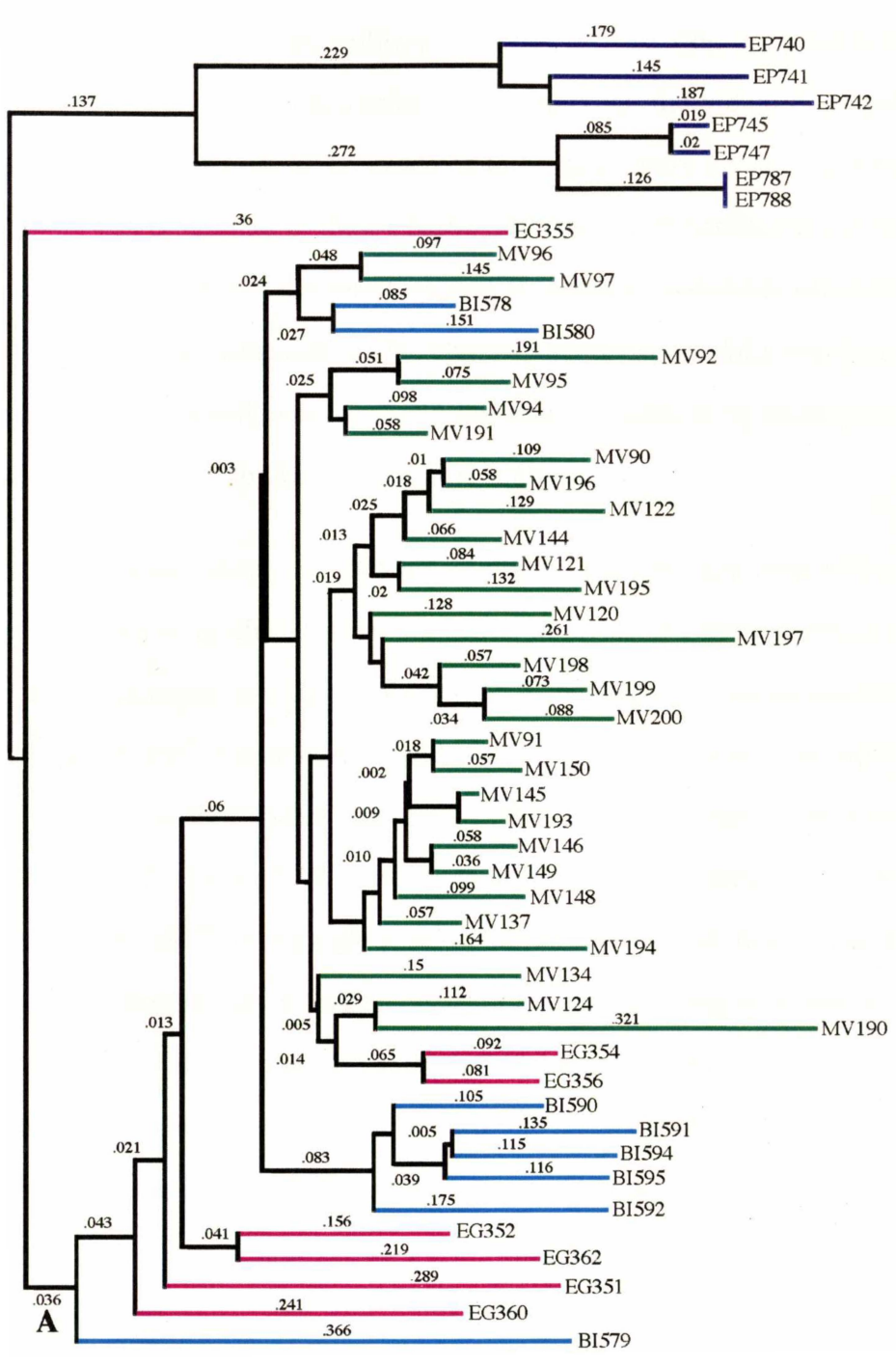

Figure 4.7
Neighbour joining tree obtained using the algorithm of Jaccard. *Pottia heimii* isolates from Miers Valley (——), East Garwood Valley (——), Bratina Island (——) and Edmonson Point (——) were used in RAPD reactions with four different primers. Note the small value at Node A (see text).

4.6 Discussion

4.6.1 Within-clump variation in *Pottia heimii*

The results in Chapter 3.5.3 showed within-clump genetic variation occurred in a species where reproduction is presumed to be entirely asexual. Individual *P. heimii* shoots in such close proximity would be expected to be genetically identical if they were purely clonal. The occurrence of these genetic differences between shoots can be explained by mutation rates that were very high and produced considerable variation even in recently established clumps, or, by the clumps having existed for a very long time with a natural rate of mutation occurring (Adam *et al.* in press) or by propagules from other locations having established within these clumps.

Although several theories can be put forward to explain the variation seen within clumps, these levels are insignificant when compared to levels of variation within a (sub)population. For example only eight polymorphic bands were scored for isolates taken from field sample EP798 and most individuals were genetically identical, while 87 polymorphic bands scored within transect MVB and no two individual were genetically identical (cf. section 4.3). The shoots analysed can be presumed to be largely ramets (Harper 1977) of a single genetic individual because within-clump genetic differences represent only a small number of polymorphic bands. Therefore, the most likely scenario is that mutation rates (natural or high) cause the level of variation occurring within *P. heimii* clumps. Similar results were gained during independent experimental work on *P. heimii* and several other antarctic moss species including *Bryum argenteum, Campylopus pyriformis* and *Ceratodon purpureus* (Skotnicki *et al.* in press).

4.6.2 Variance within *Pottia heimii* transects

The small values at node A in Figures 4.2 and Figure 4.3 suggested that the Miers Valley isolates were genetically very similar (cf. section 4.3). The separation of the two Edmonson Point samples on a different branch showed these isolates were genetically more distant from the Miers Valley group of isolates. A significant level of genetic variation existed within transects MVB and MVE because no two isolates produced the same RAPD profiles. Both dendrograms indicated field samples collected ten metres or one metre apart were as genetically similar as those field samples collected ten centimetres or one centimetre apart, which suggested there was no genetic spatial structure to the transects and that the distribution of genotypes was random.

These results have recently been corroborated by similar studies on *P. heimii* in East Garwood Valley (Skotnicki, unpublished). Although sampling was less intensive, the dendrogram results showed that, within drainage channels, samples collected some distance apart were genetically as similar as samples collected in close proximity.

The lack of genetic spatial structure within the *P. heimii* transects in Miers Valley is in direct contrast to results from comparable studies on *B. argenteum* collected from East Garwood Valley (Skotnicki, unpublished). In that case transects showed definite genetic spatial structure because samples collected in close proximity e.g. at the top of a drainage channel were more genetically similar than samples collected further apart e.g. at the top and bottom of a drainage channel.

The exact mechanism of *P. heimii* reproduction and dispersal within Antarctica is unclear. Plant material has been collected from Granite Harbour with rhizoidal gemmae that perhaps act as vegetative propagules (Seppelt pers. comm.). Other plant fragments such as shoots, shoot pieces and leaf fragments are also likely to serve as vegetative propagules. Sporophytes have been recorded from Southern Victoria Land (Seppelt *et al.* 1992) however, these are generally frosted off early in development.

During the field work for this study, no evidence of sporophytes were seen. *Pottia heimii* samples collected were presumed to have dispersed locally by asexual means, probably by the regeneration from unspecialised fragments of the gametophyte (Smith 1984, Wyatt 1994).

The random distribution of genotypes within the transects MVB and MVE suggests that local dispersal of *P. heimii* must be a process that is less directional than that found for *B. argenteum.* Wind is the most likely dispersal agent. *Bryum argenteum*, which generally occupies the wetter sites, has deciduous shoot tips or axillary buds which are easily dislodged by water, particularly with the first flush of water over the plants in early summer (Adam *et al.* in press, Seppelt pers. comm.) and, as a result, they are easily dispersed downstream within the flush. However, *P. heimii* occupying the drier areas may not be subjected as frequently to this dispersal mechanism and is probably more dependent on wind for its dispersal of propagules.

4.6.3 Variance between *Pottia heimii* transects

Genetic subdivision is assumed to exist where genetic variation between (sub)populations is significantly greater than within (sub)populations (Adam *et al.* in press). This is clearly not the situation for the three *P. heimii* transects collected within Miers Valley because the dendrogram analysis did not assemble the isolates into their respective transects (Figure 4.5) and the percentage of molecular variance between the three transects was significantly lower than within each of the transects. Therefore, the Miers Valley transects could be considered to have been taken from a single population.

This result has recently been substantiated by RAPD analysis of *P. heimii* transects corresponding to five drainage channels within East Garwood Valley (Skotnicki, unpublished). Dendrogram analysis showed there was no genetic subdivision between the transects, inferring a random distribution of genotypes within East Garwood Valley. Molecular variance analysis within and between the transects (drainage channels) were very similar to those found within and between transects in Miers Valley. The random distribution of genotypes throughout Miers Valley was similar to that seen within the transects MVB and MVE (section 4.6.2). Wind dispersal could be a valid explanation for this pattern.

The lack of genetic subdivision and random distribution of genotypes within Miers Valley is in direct contrast to comparable studies on five transects of *B. argenteum*, where isolates assembled into their respective drainage channels within East Garwood Valley and molecular variance analysis showed genetic differentiation to be substantially larger between transects than was obtained for *P. heimii* isolates (Skotnicki, unpublished). This suggests that *B. argenteum* has an initial colonisation followed by dispersal via meltwater channels, as opposed to repeated colonisations for *P. heimii*.

Because no two isolates produced the same RAPD profile a significant level of genetic variation is suggested within the Miers Valley population. This could be explained by several hypotheses; (1) the Miers Valley population has been present for a long period of time which has allowed natural rates of mutation to occur over the millennia despite mainly or purely vegetative reproduction; (2) colonisation of *P. heimii* in Miers Valley has occurred via a number of propagule sources; (3) recent establishment of *P. heimii* in Miers Valley has been followed by high rates of mutation, perhaps facilitated by historically high levels of ultra violet (UV) light during its growing season (cf. section 3.7). However, from the results of this study it is difficult to more than speculate on possible colonisation events.

4.6.4 Comparison between isolated *Pottia heimii* populations in East Antarctica

The geographic distribution of *P. heimii* genotypes can be used as a basis for speculation on the possible colonisation history of East Antarctica. Geological records show that the continent of Antarctica, as part of Gondwana Land, was richly vegetated in the Late Palaeozoic, Late Cretaceous and Tertiary but flora disappeared as the conditions for plants deteriorated with the Pleistocene Glaciation producing a continuous ice cover over the continent, except for isolated nunatuks (Smith 1984). Recolonisation or initial colonisation of flora into these areas occurred as the ice retreated on what was now an isolated continent (Longton 1985). There is much speculation by a large number of authors explaining the origin and distribution of the past and present south polar flora. Smith (1984) gives a comprehensive summary of these works.

From the dendrogram and molecular variance analyses (cf. section 4.5) it is apparent that the Edmonson Point population is genetically distinct from the Miers Valley, East Garwood Valley and Bratina Island populations. Because of this the assumption can be made that the population was either originally established from a different source or the original population (which may have been similar to the other populations sampled) became extinct and the site re-colonised by a different source. If the establishment of *P. heimii* is a recent event then time might not have permitted gene flow to and from the more southerly populations. Alternatively, *P. heimii* might be part of the relictual antarctic flora where genetic differentiation has arisen by adaptation to the local environment and/or a drift-like effect of random elimination of genotypes during periods of population decline (Adam *et al.* in press).

If recent colonisation is indeed true for the Edmonson Point population or any other *P. heimii* population within East Antarctica a further question arises; how did *P. heimii* propagules arrive in East Antarctica? Indirect evidence by Van Zanten (1978) established that spores may be wind-borne over great distances. Schuster (1979) and Smith (1984) suggest that the most likely means of population establishment within the southern polar region, since the Pleistocene, has been by various forms of trans-oceanic migration from temperate regions. This may well be the situation for *P. heimii* within East Antarctica, where southern South America is a possible propagule source (Greene 1986).

The small value at node A in Figure 4.7 and the low molecular variance percentage between Miers Valley, East Garwood and Bratina Island groups suggested these populations could be considered as a single larger population. This finding is plausible as it could be presumed that propagules that have established in the Miers Valley and the East Garwood Valley are from a similar source or propagules have migrated between the two populations as the two valleys are approximately only 10 km apart (Figure 1.2). Periodic winds down the Dry Valleys in an west to east direction (Selkirk and Green pers. comm.) would facilitate the transport of propagules in the direction of Bratina Island, which is situated approximately 25 km east of East Garwood Valley and approximately 30 km north east of the Miers Valley (Figure 1.2).

The scenarios put forward in section 4.6.3 to explain the significant levels of genetic variation seen in the Miers Valley population are also possible explanations for the level of genetic variation seen within the East Garwood Valley, Bratina Island and Edmonson Point populations. However, again it is difficult to more than speculate on possibilities for *P. heimii* colonisation within East Antarctica.

4.6.5 Genetic variation in *Pottia heimii*

Paleobotanical research supports the hypothesis that bryophytes evolved early and have remained morphologically stable through geologic time, therefore slow evolutionary rates and genetic uniformity within species have been presumed in the past (Cummins and Wyatt 1981). The present study of *P. heimii* populations within East Antarctica adds to the increasing evidence that levels of genetic variability within the Bryophyta are comparable to those found for other plants (Newton and Mishler 1994). To date there is no clear explanation for these findings, as levels are still higher than expected for haploid-dominant organisms where asexual reproduction is predominant.

Although most studies are based on isozyme analysis, comparable studies using RAPDs are shown to agree with isozyme data (Boisselier-Dubayle *et al.* 1995). However, several studies suggest that isozyme markers may indeed underestimate levels of genetic variation in comparison to RAPD markers (Liu and Furnier 1993, Bachmann 1994, Adam *et al.* in press).

The level of genetic variation for *P. heimii* populations in East Antarctica are similar to results for RAPD analysis of antarctic *B. argenteum* and *S. glaciale* populations (Adam *et al.* in press, Selkirk *et al.* in press, Skotnicki *et al.* in press). This proposes that these mosses might form a community that has established in a similar time frame. It is interesting that levels of genetic variation within individual populations are similar for *P. heimii* and *B. argenteum* but the pattern of genotypic dispersal/structure within populations are opposite, implying a different mode of local propagule dispersal. This suggests that if mutation is responsible for the level of variation seen within populations, then the mutation rates are not high enough to obscure the different genotypic patterns (cf. section 4.6.3 and 4.6.4) imposed by other factors e.g. dispersal.

RAPD analysis has not be performed on temperate *P. heimii* populations. However, RAPDs have been used to analyse *B. argenteum* samples from Antarctica, Australia and New Zealand (Skotnicki, in press). Dendrogram analysis of RAPD banding patterns showed that isolates from Australia and New Zealand clustered together and the isolates from Antarctica were less closely related. Antarctic *B. argenteum* isolates showed less genetic variation (as judged by the number of polymorphic bands) when compared to the groups from Australia and New Zealand. Because the level of genetic variation within antarctic *P. heimii* and *B. argenteum* populations are similar, patterns resembling the results of Skotnicki's (in press) work might be expected if comparable studies were performed on antarctic and temperate populations of *P. heimii* .

4.7 Management of the antarctic ecosystem

One aim of conservation is to preserve genetic diversity and thus the evolutionary potential of species. Knowledge of genetic structure is therefore important in conservation. An important issue revealed by this study is the accidental or deliberate transfer of plant material by humans between populations. The genetic differences between the Edmonson Point and the Southern Victoria Land populations suggests that movement of plant material between these populations could potentially change the genetic make-up of local (sub)populations. If these populations did indeed originate from different sources it is imperative that any immigration of plant material other than by natural means should be avoided.

Reducing human disturbance is of particular importance as the antarctic environment is unique and the vegetation as a whole has few direct counterparts in arctic or alpine areas (Longton 1988). It is essential that significant major terrestrial vegetation types should be shielded from human induced modification. Studies were conducted by Schwarz (1990) at several sites within the Canada Glacier flush where *P. heimii* turfs had been removed ten years earlier. The results of this study showed that *P. heimii* recovery was slow and patchy. Therefore, removal of plant material and accidental trampling of vegetation for scientific research purposes should be minimised. The knowledge gained from this study potentially allows the development of efficient sampling strategies for future collections, permitting minimal impact on antarctic *P. heimii* populations. Analysis of Miers Valley samples has shown that a small number of individuals provide genetic information representative of a population, therefore, future sampling of other *P. heimii* populations can potentially to be kept to a minimum.

Historically high ultra violet radiation levels above Antarctica have caused an increased interest in its effect on terrestrial communities. The relative increase in UV is facilitated by the rapid breakdown of ozone over Antarctica. The loss of ozone in the Antarctic each year removes about 60% over the continent of Antarctica in September and October, compared with measurements over the twenty years from 1957 (Wood 1996). During the growing season UV radiation has the potential to impact on moss species that cannot adapt to these changes, alongside increasing mutation rates above natural levels (Weaver and Hedrick 1992). The present study has documented the level of genetic variation within the Miers Valley *P. heimii* population in 1995. This information has the potential to be used as a reference to compare levels of genetic variation from similar areas in the future, allowing speculation on the effect of increased levels of UV radiation on *P. heimii* populations at a DNA level.

4.8 Future research

Proposals are currently in progress to use moss chloroplast DNA (cpDNA) and restriction endonucleases as an alternative molecular tool to analyse not only *P. heimii* but also other antarctic moss species (Skotnicki pers. comm.). Chloroplasts contain their own autonomously replicating DNA genome and ribosomes (70S) which are active in protein synthesis. About one-third of the ribosomal proteins are encoded by the chloroplast genome and the remainder by the nuclear genome (Dorne *et al.* 1984). Genes encoding ribosomal proteins have been identified for the liverwort *Marchantia polymorpha* (Ohyama *et al.* 1986). Using standard polymerase chain reactions experiments involving specific cpDNA oligonucleotides, the amplification products can be cut by restriction endonucleases to produce diagnostic restriction site maps that can be compared between individuals (Arnold *et al.* 1991). This technique potentially could provide information about the time of colonisation of the mosses on the continent of Antarctica.

Continued research using RAPDs is necessary, with the analysis of further populations throughout East Antarctica needed to weave the bigger picture of *P. heimii* colonisation within the area. These antarctic populations should be compared with temperate *P. heimii* populations e.g. southern South America, as done in the studies on *B. argenteum* (Skotnicki, unpublished) (cf. section 4.6.5).

A combination of RAPD analysis and the production of endonuclease restriction maps from cpDNA provides a powerful tool on which to base future research, where the questions pertaining to antarctic moss are limitless and the answers to date are few.

4.9 Conclusions

From this study a number of conclusions have be made about antarctic *P. heimii*:

(1) A method for application of RAPDs to *P. heimii* has been developed by establishing:

(a) RAPD profiles can be produced for antarctic *P. heimii* DNA;

(b) RAPDs profiles for *P. heimii* are reproducible;

(c) RAPDs give identical profiles for genetically identical *P. heimii* tissue;

(d) RAPDs are able to detect genetic differences between individual *P. heimii* shoots.

(2) Genetic variation can occur within a single clump of *P. heimii.*

(3) A significant level of genetic variation exists within *P. heimii* transects MVB and MVE in Miers Valley.

(4) No genetic subdivision exists between the *P. heimii* transects MVB, MVC and MVE in Miers Valley. Therefore, individuals can be considered to be taken from a single population.

(5) Genetic variation exists between geographically isolated *P. heimii* populations in East Antarctica. Individuals from the Miers Valley, East Garwood Valley and Bratina Island can be considered to be taken from a single larger population which is separate from the more northerly population at Edmonson Point.

(6) The genetic spatial structure within *P. heimii* transects MVB and MVE is random indicated by the distribution of genotypes.

(7) The random pattern of genotypic distribution within transects MVB and MVE suggests that the most likely agent for *P. heimii* propagule dispersal is wind

The RAPD protocol developed for *P. heimii* during this study is presently being used for continuing research on this species. Initial studies have concluded RAPDs are a valuable tool for studying genetic variation of antarctic *P. heimii* at a population level. This information has allowed an insight into local population structure and speculation on processes of local dispersal/colonisation of *P. heimii* within East Antarctica. These studies and more advanced genetic analysis will allow insight into the origins of the antarctic flora as well as providing a solid basis for management to protect the existing vegetation. The simplicity of the antarctic flora, in diversity and abundance, is particularly helpful and should allow excellent genetic investigation unhindered by the complexity of the temperate vegetation.

REFERENCES

Adam KD, Selkirk PM, Connet MB, Walsh SM (in press) Genetic variation in populations of the moss *Bryum argentum* in East Antarctica. In: Battaglia B, Valencia J, Walton DWH (Eds) Antarctic Communities. Cambridge University Press, Cambridge.

Anon.(a) (in preparation) *Joyce Glacier draft map*.USGS .

Anon.(b) (1995) *PCR Applications Manual.* Boehringer Mannheim GmbH, Biochemica.

Anon.(c) *FTS-4000 Capillary Thermal Sequencer Operators Manual*, Corbett Research

Armstrong JS, Gibbs AJ, Peakall R, Weiller G (1995) The RAPDistance Package. WWW: http://life.anu.edu.au/molecular/software/rapd.html.

Arnau I, Cooper S (1991) Polymerase chain reaction. In: Berry RJ, Crawford TJ, Hewitt GM (Eds.) *Genes in Ecology.* The 33rd Symposium of the British Scientific Society, Blackwell Scientific Publications. pp 457-459.

Arnold ML, Buckner CM, Robinson JJ (1991) Pollen-mediated intogression and hybrid speciation in Lousisana irises. *Proceedings of the National Academy of Science USA* 88: 1398-1402

Bachmann K (1994) Tansley review No. 63: Molecular markers in plant ecology. *New Phytologist* 126: 403-418.

Baker A (1967) Algae from Lake Miers, a solar-heated Antarctic lake. *New Zealand Journal of Botany* 5: 453-468.

Beever J, Allison KW, Child J (1992) *The mosses of New Zealand* (2nd edition). University of Otago Press.

Boisselier-Dubayle MC, Jubier MF, Lejeune B, Bischler H (1995) Genetic variability in the three subspecies of *Marchantia polymorpha* (*Hepaticae*): isozymes, RFLP and RAPD markers. *Taxon* 44: 363-376.

Burke T, Rainey W, White T (1992) Molecular variation and ecological problems. In: Berry R, Crawford T, Hewitt G (Eds.) *Genes in Ecology*. The 33rd Symposium of the British Ecological Society. Blackwell Scientific Publications. pp 229-254.

Campbell IB, Claridge GC (1987) Antarctica: soils, weathering processes and environment. *Developments in Soil Science* 16. Elsevier Science Publishers.

Campbell IB, Claridge GGC, Balks MR (1992) The properties and genesis of cryosols at Marble Point McMurdo Sound Region, Antarctica. In: Proceedings, 1st International Conference on Cryopedology. Russian Academy of Science. pp 59-66.

Campbell IB, Claridge GGC, Campbell DI, Balks MR (in press) The soil environment. In: Priscu JC (Ed.) *The McMurdo Dry Vallys, Antarctica: A Cold Desert Ecosystem.* Antarctic Series. American Geophysical Union.

Clayton-Greene JM, Hendy CH, Hogg AG (1988) Chronology of a Wisconsin age preglacial lake in the Miers Valley, Antarctica. *New Zealand Journal of Geology and Geophysics* 31: 353-361.

Cummins H, Wyatt R (1981) Genetic variability in natural populations of the moss *Artichum angustatum*. *Bryologist* 84: 30-38.

Dorne AM, Lescure AM, Mache R (1984) *Plant Molecular Biology* 3: 83-90.

Doyle J, Doyle J (1990) Isolation of plant DNA from fresh tissue. *Focus* 12: 13-15.

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Research* 19: 1349.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitocondrial DNA restriction sites. *Genetics* 131:479-491.

Faith DP, Cranston PS (1991) Could a cladogram this short have arisen by chance alone? On permutation tests for cladistic structure. *Cladistics* 7: 1-28.

Ferguson JWH (1990) TDRAW computer program. Centre for Demographic and Population Genetics, University of Texas, Houston, USA.

GeneQuant II RNA/DNA Calculator User Manual (1996) Pharmacia Biotech.

Green TGA (1985) Dry valley terrestrial plant communities the problem of production and growth. *New Zealand Antarctic Record* Vol. 6, Special Supplement: 40-43.

Greene DM (1986) *A conspectus of the mosses of Antarctica, South Georgia, the Falkland Islands and Southern South America.* British Antarctic Survey, Natural Environment Research Council, Cambridge. p 215.

Greene SW (1967) Bryophyte distribution. In: Bushnell V (Ed) *Terrestrial life in Antarctica.,* Antarctic Map Folio Series, Folio 5: pp 11-13.

Harper JL (1977) *Population Biology of Plants.* Academic Press, London.

Helm AS (1958) *Provisional gazetter of the Ross Dependency.* Government Printer, Wellington, New Zealand.

Holdgate MW (1970) *Antarctic Ecology* Volume 2, Academic Press.

Howland D, Arnau J (1991) RAPDs: Random Amplified Polymorphic DNAs. In: Berry RJ, Crawford TJ, Hewitt GM (Eds) *Genes in Ecology*, The 33rd Symposium of the British Ecological Society, University of East Anglia. Blackwell Scientific Publications, Oxford. pp 466-468.

Huff DR, Peakall R, Smouse PE (1993) RAPD variation within and among natural populations of outcrossing buffalograss [*Buchloe dactyloides* (Nutt.) Engelm.]. *Theoretical and Applied Genetics* 86: 927-934.

Jin L (1988) NJTREE computer programme. Centre for Demographic and Population Genetics, University of Texas, Houston, USA.

Longton RE (1985) Terrestrial habitats - Vegetation. In: Bonner WN, Walton DWH (Eds) *Antarctica*, Pergamon Press.

Longton RE (1988) Biology of polar bryophytes and lichens. Cambridge *University Press.*

Longton RE (1988) Adaptations and strategies of polar bryophytes. *Botanical Journal of the Linnean Society* 98: 253-268.

Longton RE (1994) Reproductive biology in bryophytes the challenge and the opportunities. *The Journal of the Hattori Botanical Laboratory* 76: 159-172.

Liu Z, Furnier GR (1993) Comparison of allozyme, RFLP, and RAPD markers revealing genetic variation within and between trembling aspen and bigtooth aspen. *Theoretical and Applied Genetics* 87: 97-105.

Kanda H (1981) Two mosses of the genus *Pottia* collected from the vicinity of Syowa Station, East Antarctica. *Antarctic Record* 71: 96-108.

Kappen L, Straka H (1988) Pollen and spores transport into the Antarctic. *Polar Biology* 8:173-180.

Magdefrau K (1982) Life-forms of bryophytes. In: Smith AJE (Ed.) *Bryophyte Ecology.* Chapman and Hall. pp 45 - 57.

Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings from the National Academy of Science USA* 76: 5269-5273

New Zealand Antarctic Programme (NZAP) (1994) Operations Manual.

Newton A, Mishler B (1994) The evolutionary significance of asexual reproduction on mosses. *The Journal of the Hattori Botanical Laboratory* 76: 127-145.

Ohyama K, Fukuzawa H, Kohchi T, Shirai H, Sano T, Sano S, Umesono, Shiki Y, Takeuchi M, Chang Z, Aota S, Inokuchi H, Ozeki H (1986) Chloroplast gene organisation deduced from complete sequence of liverwort *Marchantia polymorpha* chloroplast DNA. *Nature* 322: 572-574.

Pickard J (1986) *Antarctic oasis terrestrial environments and history of the Vestfold Hills*. Academic Press.

Rafalski JA, Tingey SV (1993) Genetic diagnostic in plant breeding: RAPDs, microsatellites and machines. Trends *in Genetics* 98: 275-280.

Saitou N, Nei M (1987) The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406-425

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning - A Laboratory Manual* Vol. 3 (2nd ed.). Cold Spring Harbour Laboratory Press, Plainview, New York.

Schuster RM (1979) On the persistance and dispersal of transartic Hepaticae. *Canadian Journal of Botany* 57: 2179-2225.

Schwarz AMJ, Green TGA, Seppelt RD (1992) Terrestrial vegetation at Canada Glacier, Southern Victoria Land, Antarctica. *Polar Biology* 12: 397-404.

Selkirk PM, Skotnicki ML, Adam KD, Connet MB, Dale TM, Joe TW, Armstrong J (in press) Genetic Variation in Antarctic populations of the moss *Sarconeurum glaciale. Polar Biology* .

Seppelt RD, Selkirk PM (1984) Effects of submersion on morphology taxonomic interpretation of selected Antarctic moss species. *Journal of the Hattori Botanical Laboratory* 55:273-279.

Seppelt RD, Green TGA, Schwarz AMJ, Frost A (1992) Extreme southern locations for moss sporophytes in Antarctica. *Antarctic Science* 4: 37-39.

Seppelt RD, Green TGA, Schroeter B (1995) Lichens and mosses from the Kar Plateau, Southern Victoria Land, Antarctica. *New Zealand Journal of Botany* 33: 203-220.

Skotnicki MJ, Selkirk PM, Dale TM (in press) RAPD profiling of genetic variation in Antarctic mosses. In: Howard-Williams C, Lyons B (Eds) *Polar Desert Ecosystems. Balkema*, Rotterdam.

Smith RIL (1984) Terrestrial plant biology of the sub-Antarctic and Antarctic. In: Laws RM (ed) *Antarctic Ecology* Vol 1, London Academic Press: pp 61-162.

Smith RIL (in press) Oases as centre of plant diversity and dispersal in Antarctica. In: Howard-Williams C, Lyons B (Eds) *Polar Desert Ecosystems. Balkema*, Rotterdam.

Soderstrom L (1994) Scope and significance of studies on reproductive biology of bryophytes. *The Journal of the Hattori Botanical Laboratory* 76: 97-104.

Studier J, Keppler K (1988) A note on the neighbour-joining metric of Saitou and Nei. *Molecular Biology and Evolution* 5:729-731.

Tingey S, Del Tufo JP (1993) Genetic analysis with random amplified polymorphic DNA markers. *Plant Physiology* 101: 349-352.

Van Zanten BO (1978) Experimental studies on trans-oceanic long-range dispersal of moss species in the Southern Hemispere. *Journal of the Hattori Botanical Laboratory* 44: 455-482.

Walton DWH (1990) Colonisation of terrestrial habitats-organisms, opportunities and occurrence. In: Kerry K, Hempel G (Eds) *Antarctic Ecosystems, Ecological Change and Conservation.* Springer-Verlag Berlin Heidelberg. pp 51-60.

Wang H, QIM, Cutler AJ (1993) A simple method of preparing plant samples for PCR. *Nucleic Acids Research* 21: 4153-4154.

Weaver RF, Hedrick PW (1992) *Genetics*, 2nd edition.WCB Publishers.

Wharton R (1994) McMurdo Dry Valleys : A cold desert ecosystem. *Antarctic Journal*: 11-12

Williams JG, Hanafey MK, Rafalski JA, Tingey SV (1993) Genetic analysis using Random Amplified Polymorphic DNA markers. In: Wu R (Ed) *Methods in Enzymology Volume 218, Recombinant DNA, Part I.* Academic Press, Inc. pp 704-740.

Wood S (1996) "Is ozone depletion really a scam". *Antarctic.* Bulletin 14 No. 2. 63-64.

Wyatt R (1982) Population ecology of bryophytes. *The Journal of the Hattori Botanical Laboratory* 52: 179-198.

Wyatt R, Stoneburner A, Ordrzykoski (1989b) High levels of genetic variability in the haploid moss *Plagiomnium ciliare. Evolution* 43: 1085-1096.

Wyatt R (1994) Population genetics of bryophytes in relation to their reproductive biology. *The Journal of the Hattori Botanical Laboratory* 76: 147-157.

Zander RH (1993) Genera of the Pottiaceae: mosses of harsh environments. *Bulletin of the Buffalo Society of Natural Sciences,* Buffalo, N.Y. Vol 32.