Development of Microsatellite Markers for Antarctic *Bryum* Hedw. Species

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ABSTRACT

The objective of this thesis was to isolate microsatellite markers using the Glenn (2001) method from the species *Bryum argenteum* so as to be able to study these markers in Antarctic populations of *Bryum* species. Microsatellite regions have been found to be highly polymorphic and neutral markers, and usually genus specific, thus making them ideal for population genetic studies. The populations to be studied in the future have a large distribution over the Southern Victoria Land area, ranging from the Dry Valleys to Granite Harbour and Ross Island.

Mosses are the most abundant and widespread of the vascular plant groups within continental Antarctica. They inhabit locations that are some of the more extreme on earth and experience periods of desiccation and darkness that can last as long as four months. For these reasons the establishment of mosses in Antarctica is a subject that has attracted great debate. One hypothesis suggests that mosses first became established when the ice retreated from the land approximately 17000 years ago, at the end of the last glacial maximum. The alternative hypothesis is that mosses survived as relictual populations over this period and have recently increased their habitat range. The study of microsatellite length polymorphism in populations will allow these hypotheses to be tested.

The genus *Bryum* Hedw. (Bryaceae) is a highly polymorphic, cosmopolitan genus that is abundant in Antarctica over a wide range of locations. It is found from the Sub-Antarctic zone (Sub-Antarctic islands) to the continental zone (Continental Antarctica and Southern and Eastern Antarctic Peninsula. Thus covering a wide range of habitats from warm and wet (e.g. the Sub-Antarctic islands) to cold and very dry (e.g. the McMurdo Dry Valleys).

To study the population genetics of Antarctic *Bryum* species, development of microsatellite markers was necessary as it has been found that with less specific methods such as RAPD-PCR, the DNA used for the analyses had been contaminated by co-extracted DNA from fungi living on the mosses, thus confounding the results obtained. Microsatellites, once developed, are genus or family specific, thus there is little risk of amplifying a contaminant when using microsatellite markers.

This project failed to isolate any microsatellite markers from *Bryum argenteum*, due to experimental difficulties that occurred at three major stages; ligation, transformation and hybridisation screening of the genomic library. Future research should be focussed on completion of microsatellite isolation for this genus and on evaluation of the population relationships among Antarctic localities.

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Abbreviation/ Unit	Full version
3′	Three prime end of DNA (hydroxyl end)
5′	Five prime end of DNA (phosphate end)
β	Beta particle; emitted during radioactive decay.
γ ^{33}P-NTP	Nucleotide radio-labelled in the γ position with phosphorus-33
°, ′	Degrees, minutes of latitude
°C	Degrees Celsius
a	Mean number of alleles at a locus
BLAST	Basic local alignment search tool
bp, kb	Base pairs, kilo base pairs (1000 bp)
BA, BS	***Bryum argenteum*, *Bryum subrotundifolium***
C	Concentration (mol L^{-1})
DNA	Deoxyribose nucleic acid
G	Gravity (relative centrifugal force)
g, mg, µg, ng, pg	Gram, milligram, microgram, nanogram, picogram
gEW	Gram equivalent weight
GPS	Global positioning system
IAM	Infinite allele model
ITS	Internal transcribed spacer
KAM	*k*-allele model
km, m, cm, mm, nm	Kilometre, metre, centimetre, millimetre, nanometre
LGM	Last glacial maximum
M, mM, µM, nM, pM	Mole, millimole, micromole, nanomole, picomole
Ma	Million years before present
MAF	Ministry of Agriculture and Fisheries
MCS	Multiple cloning site
L, mL, µL	Litre, millilitre, microlitre
mJ	Millijoules
MP-PCR	Microsatellite primed-PCR
m_r	Molar mass
n	Number
N	Normal solution
n	Number of mole
N, E, S, W	North, East, South, West
nrDNA	Nuclear ribosomal DNA
OD	Optical Density
P	Percent of polymorphic loci
PC1, PC2	Physical containment level 1/2
PCR	Polymerase chain reaction
pfu	Plaque-forming units
PNK	Polynucleotide kinase
RAPD	Randomly amplified polymorphic DNA
RNA	Ribose nucleic acid
Rnase	Ribonuclease

rpm	Revolutions per minute
s	Second
SAP	Shrimp alkaline phosphatase
SMM	Stepwise mutation model
SPA	Specially protected area
ssDNA, dsDNA	Single stranded DNA, double stranded DNA
SSR	Simple sequence repeat
SSSI	Site of special scientific interest
STR	Simple tandem repeat
T_M	Melting temperature
U	Unit, measuring unit for enzymes
UV	Ultra-violet light
V	Volume
VNTR	Variable number tandem repeat
v/v, w/v,	Volume per volume, weight per volume,

LIST OF APPENDICES

AUTHOR'S NOTE

It should be noted that this thesis describes the development of procedures aimed at isolating microsatellite sequences from *Bryum* species and as such does not present the material and methods, results and discussion in a conventional manner. Thus, there is some discussion as to why particular steps were performed in the materials and methods chapter. Some theoretical background is given in the results sections and there is reference to various aspects of the materials and methods in the discussion chapter.

The materials and methods chapter also presents future development steps and alternative methods of development that may be of some use to the reader. Results presented are those that are relevant to the development of the microsatellite markers, including pictures of results at various stages to elucidate how and why various steps were performed. Much of the discussion is based around the method and the experimental difficulties experienced, with a large amount of background in an effort to explain the reasoning behind the use of a particular method.

R. T. Harfoot

5 April 2002.

CHAPTER 1

INTRODUCTION

OBJECTIVES

The focus of this research was to develop *Bryum* specific microsatellite primers for PCR (Polymerase Chain Reaction) based on flanking regions of the microsatellite sequences found in Antarctic *Bryum* species. This work was carried out as it is known that Antarctic mosses have low levels of genetic variability within Antarctica, and are not greatly different in slowly evolving genetic markers, such as the ITS region of nrDNA, from specimens collected in New Zealand and Australia. Future research will use these markers to estimate genetic variability within and among Antarctic *Bryum* species to reconstruct relationships and infer dispersal patterns of mosses on the Antarctic continent.

ANTARCTICA

Geological History

Antarctica consists of a large continent, approximately 14 million km^2 in area, of which 98% is covered in ice. Antarctica is the only polar land mass and the only significant landmass that is almost entirely ice covered. This setting means that Antarctica has a unique climate and hence biota. Antarctica has not always been geographically isolated or in a polar position, previously it was part of an aggregation of all the landmass on Earth, the super-continent called Pangaea (Valentine and Moores, 1970). Pangaea separated into three smaller super-continents (Gondwana, North America and Eurasia) during the late Phanerozoic (Gurnis, 1988) leaving Gondwana as the southern continent. Gondwana consisted of the present-day continents of South America, Africa, Antarctica, India and Australia (Du Toit, 1937, Figure 1.1). The break up of the Gondwanan super-continent over a period from the mid-Jurassic (180 million years before present (Ma)) to the early Eocene (55 Ma) left scattered portions of land progressing in different directions according to the motion of the tectonic plates. During this time, Antarctica moved south from a formerly semi-tropical position to its current polar position. It has been in this position for approximately 100 million years (Walton and Morris, 1990), but has been completely isolated for only 22 – 30 Ma (Craddock, 1977).

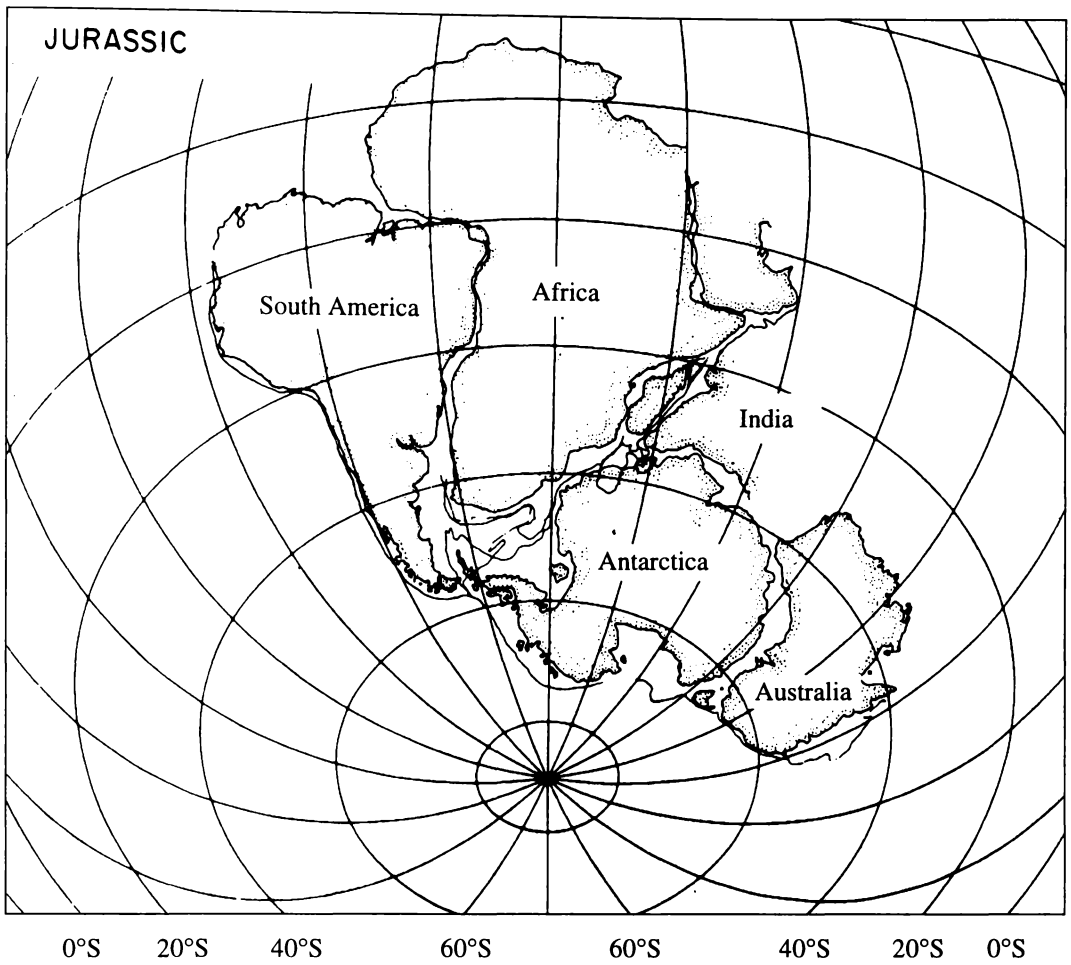

Figure 1.1 Gondwana showing hypothesised positions of continents in the Jurassic period (adapted from Walton, 1984). Reproduced with the permission of the publisher.

The opening of the Drake Passage between South America and Antarctica at approximately 22 - 30 Ma (Craddock, 1977) has isolated Antarctica from the rest of the world by expanses of ocean that are, at a minimum, 1100 km wide. The oceanic and atmospheric currents that flow within this channel maintain a close to constant temperature over the continent. These currents flow in a circular, clockwise motion around Antarctica, unimpeded by land, creating an isolating barrier that has stood since the opening of the Drake Passage between South America and the Antarctic Peninsula (Elliot, 1985). This region is called the Antarctic convergence or the Polar Frontal Zone (Hansom and Gordon, 1998) and is maintained by out-flow of cold water from the ice-sheets on the continent meeting the warm currents flowing south from the tropics. Air currents also play a role in this system through a large vortex of air, generated by the rotation of the Earth, over the Antarctic continent. The winds formed in the vortex drive the water currents in a clockwise (eastward) direction (Hansom and Gordon, 1998),

thereby maintaining the circumpolar current. The Antarctic convergence roughly coincides with the 0°C isotherm and is generally between latitudes 50° and 60° south. Areas south of the Antarctic convergence tend to have a cold climate (sea temperatures average 0°C or below) and are classified as Antarctic.

Glacial Development

Widespread glaciation on the Antarctic continent has probably existed for approximately the last 36 million years (Hambrey *et al.*, 1989). However, there has been some variation in the extent of glaciation over more recent history and up to the present-day. It is thought that the extent of glaciation seen today has existed for the past 14 million years (Shackelton and Kennett, 1975), but there have been substantial fluctuations over this time, as paleobotanical evidence suggests that there were trees (*Nothofagus* sp.) and other vegetation present on the continent as recently as 3 Ma (Barrett, 1991). It is also thought that the East and West Antarctic ice sheets developed at different times. The East Antarctic ice sheet (land based) probably developed first, reaching a size that caused direct deposition of glacial till into the ocean at a date of about 30 Ma (Robin, 1988). The West Antarctica ice sheet (sea based), on the other hand, did not develop until 7 Ma (Elliot, 1985), when the climate at sea level became cold enough to allow the development of ice shelves.

The Antarctic Environment

Antarctica can be divided into three climatic regions (Figure 1.2); sub-Antarctic (contains the sub-Antarctic islands, out to approximately the 0°C isotherm), which has a cool climate (mean temperature +2°C); the maritime-Antarctic (areas including the west side of the Antarctic Peninsula and surrounding islands, extending out into the Scotia Sea), which has a cold climate (mean monthly air temperatures over summer 0–2°C); and the continental zone (continental Antarctica, excluding the west side of the Antarctic peninsula), which has a frigid climate (mean monthly summer air temperatures below 0°C) (Longton, 1985). These classifications can also be applied to the vegetation, as the diversity and type vegetation is dependent on the climate. Sub-Antarctic regions have relatively lush vegetation with tussock grasses (e.g. *Poa foliosa*) and other macro-vegetation (e.g. *Stilbocarpa polaris*) dominating the ecosystem. Maritime-Antarctic regions have lower vegetation forms, mostly dominated by extensive moss (75 species)

and lichen (150 species) tundra, though two species of angiosperm (*Colobanthus quitensis* and *Deschampsia antarctica*) have been found in this area, as have liverworts (25 species) (e.g. *Cephaloziella varians*) (Lewis-Smith, 1984). Finally, continental Antarctica has very limited vegetation, restricted to the ice free areas, consisting almost entirely of localised moss (30 species) and lichen (125 species) communities (Lewis-Smith, 1984), though one species of liverwort (*Cephaloziella exiliflora*) has also been found on infrequent occasions (Longton, 1985).

All terrestrial ecosystems in the Antarctic are depauperate when compared to Arctic ecosystems of the same latitude. The main reason for this is that the Antarctic is several degrees cooler than the Arctic at equivalent latitudes, which affects water availability (Kennedy, 1993) and length of growth period, but may also be a function of geographical isolation (Vincent, 1997).

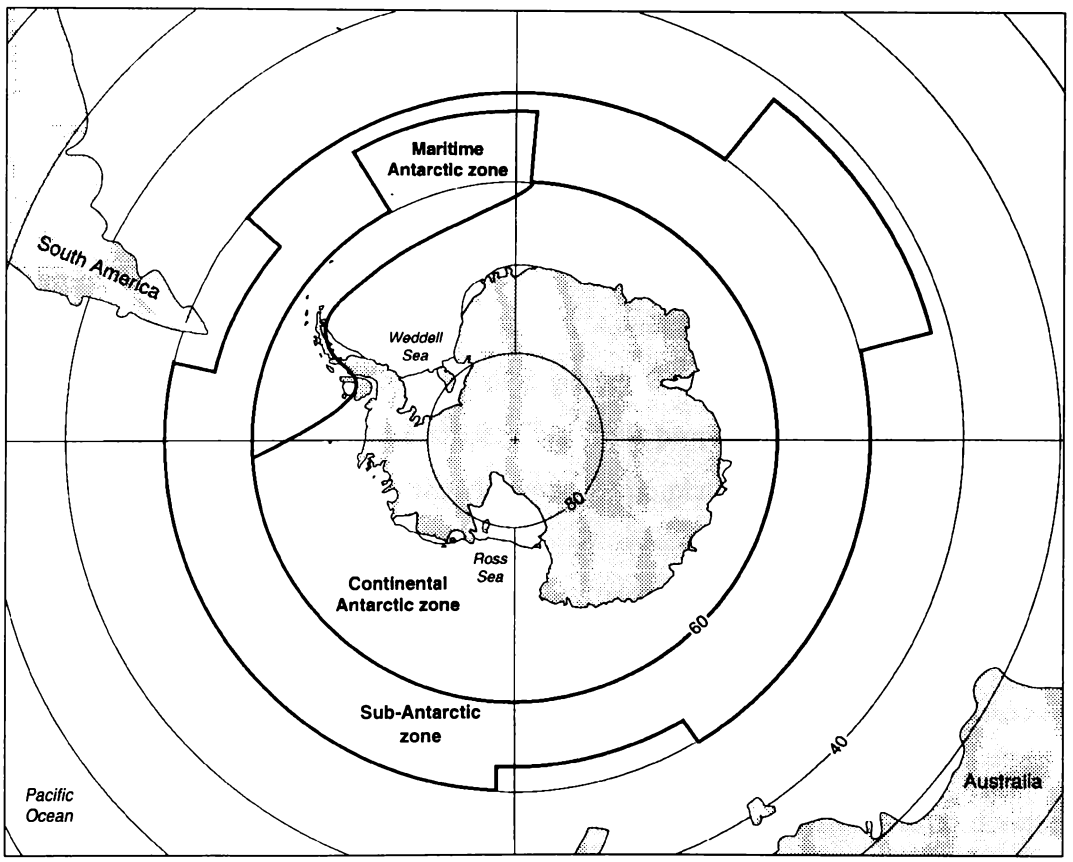

Figure 1.2 Climatic zones of the Antarctic region. Modified from Hansom and Gordon (1998).

Antarctica, as the coldest continent on earth, has little available water for the majority of the year and a limited period where growth of mosses and other floral components can occur. This period is over the Austral summer when there is

continuous sunlight in latitudes below the Antarctic Circle and air temperatures can reach as much as +12.5°C (Bull, 1966). In contrast to this period of relative warmth, winter temperatures on continental Antarctica can reach as low as -80°C inland and may be lower than –50°C in coastal areas (Bull, 1966). Mean annual temperatures vary around the coast but on average are approximately –12.5°C (Ugolini, 1970). These temperatures, combined with four months of continuous darkness and the resulting extreme dryness of the air make Antarctica a very difficult place for life to exist (Llano, 1965). Despite this, Antarctica has 130 species of bryophytes (Steere, 1961), which are restricted to the ice-free areas of the continent and the surrounding islands. The ice-free areas constitute approximately 2% of the total continental landmass (Melick and Seppelt, 1997) and apart from a few isolated nunataks, are all in coastal regions. The most southern vegetation (a moss, species unknown) has been found at 84° 35′ S, however lichens have been found at 86° 09′ S, in southern Victoria Land (Wise and Gressitt, 1965), these are presumed to be the southern limits for vegetative life. The probable constraints at these latitudes are related to water availability, lack of ice-free areas and are ultimately dependent on temperature.

Vegetation History

J. D. Hooker was the first to postulate that Antarctica has been vegetated since before the Pleistocene and was part of a greater continent, an idea that has been widely debated in the literature since it was first proposed in 1851. At the time Hooker was writing nothing was known about plate tectonics or continental movement, however since the theory and mechanisms of continental drift (Wegener 1924) were established (Holmes, 1965), an abundance of evidence has been compiled that Hooker's theory could be borne out if there were refugia for the present-day vegetation to have dispersed from after the end of the Last Glacial Maximum (LGM), between 17000 and 21200 years before the present (Elliot, 1985). It is known from fossil records, that Antarctica's vegetation was as diverse as any found at low latitudes, before the dispersal of Gondwana, and for much of the more recent history (Elliot, 1985). This vegetation was very similar to that found on other regions that had made up the Gondwanan super-continent, and was probably derived from the common origin: Gondwana. However, whether some of this vegetation could have survived the harsh environments and extensive glaciation of the LGM is not known and is unlikely to be deduced in the near

future, due to the paucity of sub-fossil evidence. It is known that there were *Nothofagus* trees (evidence supporting the presence of a complex ecosystem) along the Ross Sea coast up until relatively recent times (approximately 3 Ma) (Barrett, 1991). The conditions prevailing at that time probably did not reach temperatures much above 5°C, so it is possible that there were species within this ecosystem that could have survived such conditions as those found in the LGM (Barrett, 1991), provided there were some areas free of ice, such as beyond the range of glaciation, or near geothermally heated land. The alternative hypothesis is that the present-day vegetation consists entirely of newly introduced plants (from outside the Antarctic zone) dispersed to Antarctica by wind, water and seabirds such as skuas and gulls (Lewis-Smith, 1997). Spores from fungi and bacteria have been observed in the air streams over Antarctica (Marshall, 1996) therefore it is reasonable to believe that there could be dispersal of moss and lichen spores along the same pathways (Marshall and Convey, 1997). Much of the plant life is found in regions near penguin colonies or other inhabited areas, though this is not necessarily due to the dispersal by animal vectors, but probably more related to the abundance of nutrients in the form of guano (ornithogenic soils) and the fact that the colonies are near the coast and in relatively warm sites, and often have liquid water near by. The lack of evidence for animals being vectors is backed up by the many examples of bryophytes being found in areas which have never been the site of colonisation by birds, such as the Dry Valleys on continental Antarctica (see Ugolini, 1970).

ANTARCTIC MOSSES

Mosses (Plantae, Bryophyta) are a cosmopolitan group of simple, non-vascular plants that are present in many environments where there is an abundance of water for at least part of the year. Many species of moss are able to colonise extreme environments because they require little in the way of substrate or nutrients to grow. In the Antarctic continental zone, mosses [nine genera: *Bryoerythrophyllum*, *Bryum*, *Ceratodon*, *Campylopus*, *Didymon*, *Grimmia*, *Plagiothecium*, *Pottia* (= *Hennediella*) and *Sarconeurum* (Seppelt and Selkirk, 1984; Seppelt, 1986), within which there are thought to be 21 species (Lewis-Smith, 1997), however this number is under debate (Seppelt and Green, 1998)], along with liverworts are the highest forms of plant life to be found (Lewis-Smith, 1984). They create a very simple ecosystem that consists of no more than two or

three tropic layers (they are a habitat for mites and springtails) and are to be found in areas as small as a few centimetres across (e.g. Block, 1984). The most common species of mosses on continental Antarctica belong to the "silver" (informal grouping, *sensu* Seppelt) *Bryum* species such as *Bryum psuedotriquetrum* (Hedw.) Gaertn., Meyer et Scherb., and *B. subrotundifolium* Jaeg., which inhabit areas moistened by melt-water from glaciers and snow.

The genus *Bryum* Hedw. (Bryaceae) consists of a large group (194 species, Ochi, 1992) of cosmopolitan mosses with many species. All species exhibiting very similar morphological characters, and species are often only distinguished by close examination of the cellular structures of the leaves and capsules (see Cox, 1998, and Figure 1.3). Therefore, *Bryum* is a taxonomically problematic genus and has undergone much taxonomic revision (Ochi, 1979, 1992; Catcheside, 1980; Ochi and Ochyra, 1985; Cox, 1998), with debate over whether or not some of the present species assignments are valid, on both genetic and morphological bases (e.g. Adam *et al.*, 1997; Seppelt and Green, 1998). The major difficulty with continental Antarctic *Bryum* taxa [17 species (Ochi, 1979; Ochi and Ochyra, 1985; Ochyra, and Ochi, 1986), more recently revised to two species (*B. pseudotriquetrum* and *B. subrotundifolium*), (Ochi, 1979; Kanda, 1981; Ochi and Kanda, 1991; Seppelt and Green, 1998)] is the lack of field evidence for sporophytes on the plants collected (Horikawa and Ando, 1960; Steere, 1965; Ochi, 1979; Ochi and Ochyra, 1985; Ochyra, and Ochi, 1986). Sporophytes are essential in characterising moss species, as many different species or even genera can resemble each other on a purely vegetative basis (Greene, 1962). Ochi (1979) found, in particular, that it was not possible to distinguish Antarctic *Bryum* species purely on vegetative characters; sexual organs were needed for identification.

Compounding the taxonomic problem is that many of the species are highly polymorphic in their characters, for example, *B. pseudotriquetrum* has two forms; dioicous and synoicous, which exhibit costa (Figure 1.3) that are slightly excurrent and long-excurrent respectively (Ochi, 1979) as well as variable leaf and stem structures. Additionally, characters can vary according to the age of the plant, and can be even more variable under extreme conditions such as those found in Antarctica (Ochi, 1979; Seppelt and Selkirk, 1984). For these reasons Ochi (1979) recommends that classifications of Antarctic mosses be only made on

specimens that have well developed stems and bear sexual organs, though this does not solve the problem of how to classify samples that exhibit many characters of a certain species, but can not be fully identified as no sporophytes are present.

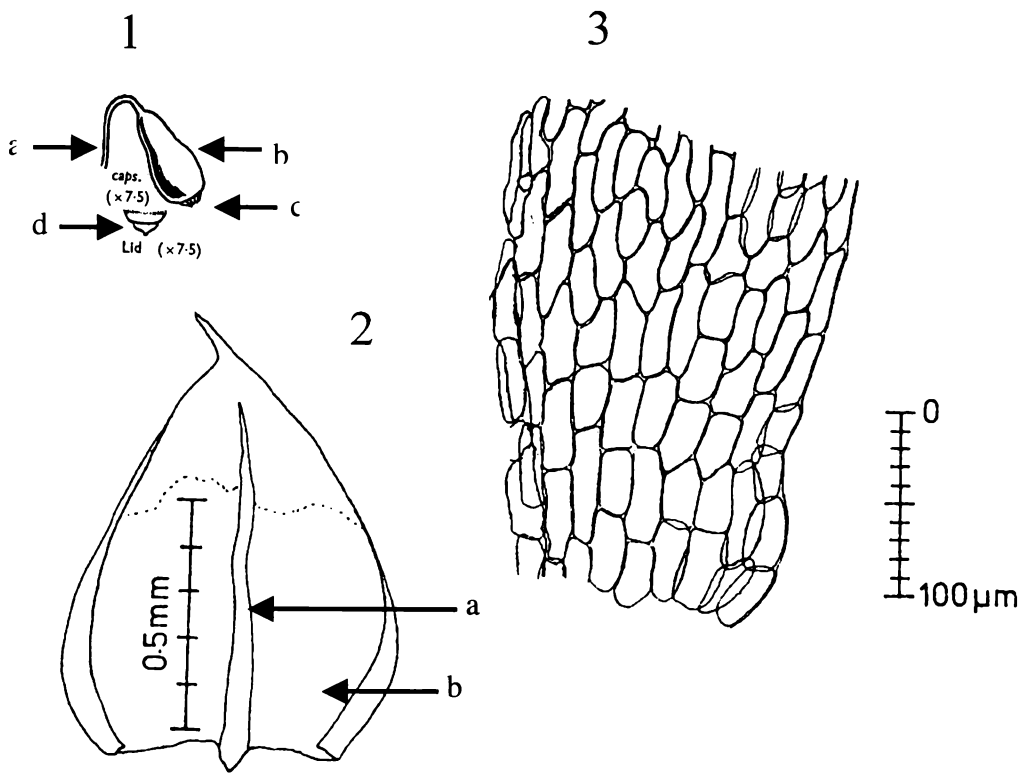

Figure 1.3 Taxonomic features of mosses. 1. Sporophyte from *B. pallescens* (a) seta (b) capsule (c) peristome teeth (d) operculum, 2. Leaf from *B. subrotundifolium* (a) nerve (costa) (b) lamina, 3. Lower lamina cells from *B. psuedotriquetrum*. (Adapted from Watson, 1968 and Seppelt and Green, 1998).

Species assignments within and around Antarctica have been debated almost since the first expeditions to collect mosses returned to lower latitudes. For example, Cardot (1908) questioned the number and assignments of taxa collected by Müller in South Georgia (Greene, 1962). This position was exacerbated by the opinion then held by many bryologists, that each island must have its own individual species (Steere, 1965). More recent taxonomic investigations have found that this is not the case and have collapsed many of the taxa described by the original botanists into the several presently known species (Ochi, 1992). There is still, however, debate on which species are actually present, with taxonomic revisions continually taking place (e.g. Ochi, 1979, 1992; Ochyra and Ochi, 1986). For example recent research has found that two morphologically similar species

(*B. subrotundifolium* and *B. argenteum* L.) that were both thought to inhabit Antarctica have been mis-identified and are likely to be only one species, most closely related *B. argenteum* from New Zealand (Hunger, 2000), thus there has been little clarification of the status of the Antarctic *B. argenteum/ subrotundifolium* complex as no formal nomenclature changes have been put forward. This conclusion was reached on the basis of comparison of sequence data of the ITS (Internal Transcribed Spacer) region of nuclear ribosomal DNA (nrDNA) for specimens that had been classified as either species from Antarctica and comparing these with sequence data obtained from isotype specimens of *B. subrotundifolium* and *B. argenteum.*

Dispersal and Colonisation

The absence of sporophytic structures on *Bryum* within Antarctica, has led to debate over the means of dispersal and colonisation events on the continent (e.g. Linskens *et al.*, 1993; Marshall, 1996). It is presently thought that asexual structures such as gemmae have been the main means of dispersal within Antarctica (e.g. Steere, 1965), but nothing is known about the number of colonisation events from outside Antarctica, or indeed, how the first mosses are likely to have arrived (as spores or as asexual structures), a problem, the answers to which may help elucidate the relationships among present-day populations.

Lewis-Smith (1984) states four reasons why sporophytes are unlikely to be found in any given region on continental Antarctica. Firstly, there is often wide spatial separation of unisexual plants in dioicous species (Longton and Greene, 1967). Secondly, there is an imbalance of male and female plants found within populations, frequently with either sex being entirely absent (Horikawa and Ando, 1967). Thirdly, the lack of liquid water at higher latitudes in Antarctica creates a physical barrier over which the male gametes cannot pass, thus the eggs within the archegonia are not fertilised and no sporophytes can develop (Steere, 1965). Fourthly, the Antarctic has a short duration growing season with cool temperatures, freeze-thaw activity and short photoperiod, causing inhibition of maturation of both sporophytes and gametangia (Clarke and Greene, 1970a, b). A fifth problem may be that the relatively cool air temperatures over the growing season mean that it is infrequent for the temperature to rise over freezing point for more than 24 hours, a critical temperature for growth of plants. Soil temperatures,

however, can stay close to or above 0°C for up to three months (Campbell and Claridge, 2000), which may in some cases aid the growth of mosses. Sporophytes have been observed on some of the sub-Antarctic islands and on the northern parts of the Antarctic Peninsula, where the growing season is longer and where liquid water is present for much of the summer months; however these sporophytes have not been on *Bryum* species.

The lack of evidence for sexual reproduction does not, however, indicate that there is no gene flow between populations on the continent as it has been observed that there are abundant propagules [gemmiferous axillary bulbils and stem apices (Seppelt and Green, 1998)] transported on wind currents (Lewis-Smith, 1997; M. I. Stevens, *pers. comm.*). The distances over which propagules can travel has not yet been investigated, so the extent of gene flow may be quite limited in some areas and extensive in others, depending on the prevailing wind and fresh water currents and the strength of the above. Thus, it can be hypothesised that the genetic similarity between moss populations will be correlated with geographical distance between populations, and other factors such as terrain, and prevailing wind and water currents. Lewis-Smith (1997) found such a pattern for several species (*B. argenteum*, *Ceratodon purpureus*, *Encalypta patagonica*, *Tortula princeps*) of moss dispersing from a central point (seal carcass) on James Ross Island in relation to wind direction and melt-water streams (slope), but this patterns was also dependent on the type of structure being used for dispersal; *E patagonica* and *T. princeps* were dispersing by spore production and the pattern produced reflected wind as the major component, while *B. argenteum* and *C. purpureus* were dispersing using propagules, and the pattern produced reflected water dispersal. These experimental results are probably reflected by the size of the dispersing bodies, spores, being light are able to be dispersed by wind, and as the sporophytes are elevated in most cases, wind is likely to be the major vector for spores. Propagules on the other hand are larger and heavier, and thus less likely to be dispersed by wind, but are a suitable size to be carried by water currents. Skotnicki *et al.* (1997) also came to the conclusion that this was the case, at least for local dispersal, on the basis of higher levels of genetic similarity between mosses from individual drainage channels, when compared to larger areas and other drainage channels. Salt-water dispersal is an unlikely prospect, as

the salt content would kill the propagules, although rafting on sea ice is a possibility.

In all species present in Antarctica, there have been observations of male and female structures on mosses, implying that the lack of sporophytes is not due to the lack of one sex or other, although this may be the case locally (Longton and Greene, 1967), but is rather due to the lack of fertilisation and due to the slow growth rate of the plants in Antarctica. It may also be that in dioicous species, such as *B. subrotundifolium,* spatial separation of individual clumps, which commonly show no genetic variation (Skotnicki *et al.*, 1998b) and as such may be one individual genetically, means that the male gametes cannot fertilise the eggs of the female plants due to the spatial separation of the individual plants (clump to clump distance). If this is the case, the absence of sporophytes is due to the spatial separation of the male and female plants, not necessarily the habitat conditions of the mosses (Longton and Greene, 1967), although a situation like that would not be expected to be seen in areas where the ground is covered in a continuous turf, such as on Beaufort Island. van der Velde *et al.* (2001a) found that dispersal of male gametes in *Polytrichum* was possible upwards of 1.5 metres, although the conditions in which this species was studied were far less severe than those found in Antarctica, thus there was a higher chance of (relatively) long distance dispersal of the gametes in the area studied than in Antarctica. Longton and Greene (1967) found that fertilisation of female *Polytrichum alpestre* could take place over distances of up to 75 cm on South Georgia, but again these conditions are much less severe than those found on continental Antarctica. An interesting point of note is that mites and springtails along with other invertebrates have been observed carrying sperm of *Polytrichum commune* in temperate-climate populations (Wyatt and Derda, 1997), thus it is possible that this could also occur in the Antarctic where mites and springtails are commonly found among mosses.

Genetic Variation in Mosses

Mosses form small patches or turfs of dense shoots that can usually be assumed to be from one plant, but occasionally will have more than one species in the same turf (*pers. obs.*). Previous studies of population genetics on Antarctic mosses have detected variation based on geographic location, though the levels of variation were low (25%) when compared to the within population variation,

which accounted for 75% of the variation seen (Skotnicki *et al.*, 1998a). However, these figures depend on the size of the region defined as containing a population (Skotnicki *et al.*, 1998a). Skotnicki *et al.* (1998a, b, c) performed Random Amplified Polymorphic DNA (RAPD) analyses on adjacent shoots from a single turf and found that there was resolvable genetic variation between shoots, a discovery that implies very slow growth rates and long establishment of the individual moss turfs. However, Hunger *et al.*, (In Prep.) found that this observed variation may have been due to fungal contamination of some shoots and not others. Fungal hyphae are essentially inseparable from the moss shoots found in Antarctica, due to the small size and high abundance of the fungi. It has been observed that a large proportion of moss samples from Antarctica, when taken back to room temperature environments, will rapidly develop obvious signs of fungal growth (*pers. obs.*), and signs of fungal growth on mosses have been observed in the field (Longton, 1973; T. G. A. Green, *pers. comm.*, Figure 1.4). This abundance of fungi has made it very difficult to extract DNA from Antarctic moss samples without also extracting fungal DNA contaminants simultaneously, creating the need for moss-specific probes such as microsatellite markers as less specific methods (e.g. RAPDs) are prone to amplifying contaminant DNA as well as the DNA from the organism being studied, creating misleading results.

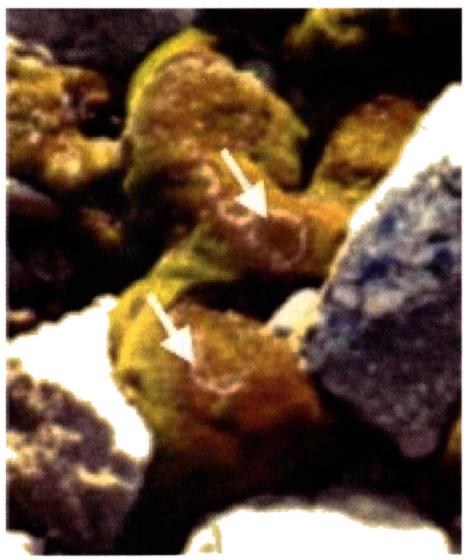

Figure 1.4 Fungal growth rings on Antarctic *Ceratodon purpureus* (Photograph: T. G. A. Green)

Temperate-boreal populations of the moss *Messia triquetra* have been found to have higher levels of genetic diversity than those from sub-Arctic and high Arctic populations, this was attributed to the higher frequency of sexual reproduction

found in boreal regions (Montagnes *et al.*, 1993). A similar situation may exist for Antarctic species, where the frequency of sexual reproduction is low (Steere, 1965; Horikawa and Ando, 1967; Ochi, 1979; Ochi and Ochyra, 1985; Ochyra, and Ochi, 1986; Seppelt, 1986) compared to that found in temperate regions, such as New Zealand or Australia, and the levels of genetic variation in Antarctica have been found to be low compared to Australia and New Zealand (Skotnicki *et al.*, 1997).

Genetic variation in bryophytes, has until recently, been studied solely by the use of isozyme electrophoresis to determine the genetic structure of populations and the geographical variation. In general the findings have been that bryophytes are not genetically depauperate, instead they show levels of isozyme variability comparable to those found in vascular plants (Stoneburner *et al.*, 1991 Daniels, 1993), however van der Velde and Bijlsma (2000) found significantly lower levels of genetic variation in *Polytrichum*, using isozyme analysis. This disparity in the figures seen over different studies could be due to a number of different causes, but is probably due to the variety of modes of reproduction and the haploid life style. Life history and habitat probably play a large part in this, in that some types of habitat are more conducive to clonal reproduction, while others will encourage sexual reproduction (van der Velde and Bijlsma, 2000). The mode of reproduction affects the genetic variability in that if the population is reproducing asexually, then it is essentially cloning itself, thus introducing no variation into the genome. Sexual reproduction on the other hand, results in genetic variation being introduced at the meiotic stage of gamete formation, which is then dispersed by the diploid parent (sporophyte) through the production of spores.

Genetic variation within *B. argenteum* (*sensu lato*, referred to *B. subrotundifolium* after Seppelt and Green, 1998, for Antarctic material) from Antarctica, New Zealand and Australia, has been investigated through the used of RAPD markers by Skotnicki *et al.*, (1998a), they found that 19% of the variation seen occurred between the Antarctica group and Australia and New Zealand as a single group, 7% of the variation occurred between the Australian and New Zealand populations and 75% of the total variation occurred within the populations. Within-clump variation has been observed, in the form of varying RAPD banding patterns produced by samples of Antarctic *B. pseudotriquetrum* (Skotnicki,

1998b). However, the levels of genetic variability were such that some samples from distant populations (over 40 km apart) were found to produce identical RAPD profiles, a situation which either indicates that there is long distance dispersal, or that the high level of genetic variation within clumps, seen in these samples are an artifact due to some unknown cause.

VEGETATION ORIGINS

There are two theories about the present-day vegetation of continental Antarctica. The first postulates that bryophytes could have survived the LGM in remote refugia (i.e. that the present-day populations are relict from a more extensive vegetations) such as nunataks, coastal areas (Llano, 1965) and areas warmed by volcanic activity (Broady *et al.*, 1987). Many species of bryophytes have been found in geothermally heated areas within Antarctica including species that are known to be cold-intolerant (e.g. *Campylopus pyriformis* (Schultz) Brid., see Broady *et al.*, 1987), thus there is some evidence that there are likely to have been refugia at the LGM.

The second theory postulates that the present-day vegetation is entirely due to recent invasion from sites that were outside the area influenced by the LGM (i.e. from outside continental Antarctica, including New Zealand, Australia and South America). This theory is supported by evidence that shows there was little, if any, land that was not covered by glaciers, on the continent, even some islands in the vicinity of the continent have been shown to have been completely covered by ice at this time (Holdgate, 1967). In debate of this, Llano (1965), referring to lichens, was of the opinion that it was not possible for some of the slower breeding endemic species to have evolved in such a short space of time. Castello and Nimis (1997) also state "The lichen flora of Antarctica, and especially of continental Antarctica, is a young one, which mainly originated during the quaternary period by long distance dispersal". This recent statement suggests that other components of the Antarctic flora are also likely to have arrived by similar mechanisms. Another piece of evidence supporting the recent arrival of the flora, is that studies on bryophytes have shown that there is little, if any, difference between Antarctic mosses and temperate species in terms of the relationship between net photosynthesis and temperature, whereas Arctic mosses have lower temperature optima (Convey, 1997) for both respiration and photosynthesis,

supposedly due to the longer adaptation time the Arctic species have had. It has been pointed out, however that even with complete glaciation, large peaks near the coast would have been exposed, if the plasticity of the ice is such that the gradient on the ice sheet is 1:100 or less, making this a potential site for refugia (Dahl, 1946). It is likely, however, that neither hypothesis represents the complete story, rather that the present-day vegetation is likely to be a combination of all of the above (see Lamb, 1970).

MICROSATELLITE MARKERS

An Introduction

Microsatellites, sometimes known as simple sequence repeats (SSR) or short tandem repeats (STR) are a class of repeat sequences known as variable number tandem repeats (VNTR), which also includes minisatellites (Nakamura *et al.*, 1987). These markers consist of tandem repeats in the DNA sequence, such as CACACACA (denoted $(CA)_n$, where n is the number of repeats), which are highly variable in length and are distributed randomly about the genome (Bruford and Wayne, 1993; Lagercrantz *et al.*, 1993; Morgante and Olivieri, 1993; Armour *et al.*, 1994). This means they can be used effectively as genetic markers for DNA profiling (Litt and Luty, 1989; Tautz, 1989; Weber and May, 1989). The composition of microsatellites ranges from mono- to hexanucleotide motifs that can be repeated many times (Schlötterer and Pemberton, 1998), usually with $n \leq 60$ (Akkaya *et al.*, 1992). Microsatellites can be classed into three different types of repeat sequence; 1) pure, where the sequence consists of only the repeat unit e.g. CAGCAGCAG; 2) compound, where there are two repeat sequences joined end to end e.g. CAGCAGCAGTGTGTGTG; 3) imperfect, where the repeat sequence is interrupted by a non-repetitive unit such as CAGCAGCAGCTTAGCAGCAGCAG (Weber, 1990; Rosenbaum and Deinard, 1998).

Each microsatellite marker can, depending on the repeat length, have up to 20 alleles and be up to approximately 150 base pairs (bp) in length (Schlötterer and Pemberton, 1994), however Primmer *et al.* 1996 reported examples of large, polymorphic microsatellites in swallows and Nybom *et al.* (1992) even larger alleles in box elder. As microsatellites are codominantly inherited and usually selectively neutral, they are ideally suited to population genetic analyses (Ashley

and Dow, 1994; Schlötterer and Pemberton, 1994) if suitable primers can be developed.

The abundance of microsatellite markers in the genome is variable according to the type of organism being studied. For example, they are almost non-existent in prokaryotes, but are found at an abundance of one every 10-15 kb (kilobase pairs) in higher plants (Barrier *et al.*, 2000) and even more abundantly in mammals and insects (Lagercrantz *et al.*, 1993; Van Treuren *et al.*, 1997). It has been found that the most frequently typed microsatellites in plants are of the $(AT)_n$ type (Morgante and Olivieri, 1993; Gupta *et al.*, 1996; McCouch *et al.*, 1997). Other forms of microsatellite repeat (both sequence difference and length) are also found in plants, but at a lesser abundance (Lagercrantz *et al.*, 1993). There is also variation in the frequency of microsatellite sequences among groups of plants, for example Wang *et al.* (1994) and Gupta *et al.* (1996) found that there was a higher frequency of microsatellites in monocotyledons than in eudicotyledons. Levels of polymorphism also vary among both genus and type of microsatellite, for example Condit and Hubbell (1991) found that *Piper* and *Zea* had five to ten fold fewer $(AC)_n$ and $(AG)_n$ sites than other genera (*Malmea*, *Virola*, *Trophis*, *Poulsenia*), while Bell and Ecker (1994) found that $(CA)_n$ microsatellite loci were mostly uninformative (non-polymorphic) in *Arabidopsis thaliana*, and that $(GA)_n$ loci were much more informative (polymorphic). However, these data are restricted to a few plant groups and as such may not be applicable to other plant groups.

Microsatellite DNA markers are largely species specific but can often be used in closely related taxa (Strassmann *et al.*, 1996) though not normally above the genus level. Despite this, in the literature, there are examples of amplification at the family level (in Leguminosae and Myrtaceae) using the same primers, (Dayanandan *et al.*, 1997; Rossetto *et al.*, 2000). The specificity of the primers used in microsatellite DNA analysis reduces the chance of amplifying contaminant DNA from unrelated organisms. For example, previous RAPD analyses of Antarctic mosses have shown that they have extremely high levels of genetic variation (Skotnicki *et al.*, 1998a, b, c), however recent studies have shown that the variation is likely to have been over-estimated due to fungal contamination (Hunger, 2000). This gives microsatellite markers an advantage over other methods of analysis such as RAPDs that are prone to amplifying target

and contaminant DNA alike, but is disadvantaged by the time and expense spent in developing these markers.

Microsatellite Polymorphism

Variations in microsatellite sequence (length polymorphism), causing new alleles to be generated for a particular marker, are thought to occur through slippage mechanisms (Levinson and Gutman, 1987; Gaggiotti *et al.*, 1999), such as slipped strand mis-pairing of complementary bases in a repeat sequence during DNA replication, resulting in the insertion or deletion of whole repeat units each time this occurs (Levinson and Gutman, 1987). However, this scenario does not seem to occur all the time, and other mechanisms of change have been recently proposed (Ellegren, 2000; Sibly *et al.*, 2001). The mutation rate of microsatellite loci has been estimated to be in the order of 10^{-3} per locus per generation (Weber and Wong, 1993; Jarne and Lagoda, 1996), which is one of the higher mutation rates observed at molecular loci (Goldstein and Pollock, 1998), however these rates were estimated from sequences found in humans and *Drosophila melanogaster*, and as such may not be transferable to plants. It has been found that one of the most important factors in the rate of microsatellite mutation is the length of the locus, indeed a directly proportional relationship has been shown to exist between the average repeat number and degree of length polymorphism (Weber, 1990). This relationship indicates that longer alleles mutate more rapidly than short alleles, a fact that bears up the slippage-mechanism model of microsatellite allele mutation, through the fact that a longer sequence with more repeat units is more likely to have a slippage occur at any one of the repeats (Ellegren, 2000). It has been found that the degree of polymorphism is related to the length of the microsatellite with variability being very low in microsatellites of less than 10 repeats in length (Beckmann and Weber, 1992; Ashley and Dow, 1994). A microsatellite that is highly polymorphic in one species may be monomorphic or even entirely absent from a closely related species, as the levels of polymorphism and frequency of microsatellites have been found to vary widely among different genera (Condit and Hubbell, 1991; Bell and Ecker, 1994 Wang *et al.*, 1994; Gupta *et al.*, 1996).

Evolution of microsatellites is thought to follow either of two main models: the infinite allele model (IAM), or the stepwise mutation model (SMM) (see

Rosenbaum and Deinard, 1998). Both models are based around the strand slippage model of DNA mutation (Schlötterer and Tautz, 1992). The IAM postulates that any allele generated through mutation will be completely different from any other allele that has been discovered. This model, along with a closely related model, the *k*-allele model (KAM), in which the new alleles can occupy *k* pre-existing states, has been found to be problematic as the assumptions implicit in the model are violated by the high rate of mutation found in microsatellite loci and by the assumption of the KAM that prior allelic states are non-existent, a situation that is not always true in microsatellite studies (Slatkin, 1995). The SMM states that the there are no constraints on allele size and that the mutation process does not depend on allele size, with an equal probability of addition and deletion occurring. These assumptions have been shown to be violated (Takezaki and Nei, 1996) in that the longer the sequence, the more likely it is to mutate via slippage mechanisms (Weber, 1990), and that alleles seem to have a maximum size of approximately 100 repeats (Tautz, 1993), with some notable exceptions such as Huntington's disease. Finally, there appears to be some bias towards additions rather than deletions in some loci and *vice versa* at other loci (Primmer *et al.*, 1996). None of these models completely describe the observed mutation patterns found in microsatellite sequences, in particular that they do not follow a simple stepwise model. New models are being postulated, most of which are based around Markov chain models (Kruglyak *et al.*, 1998). Sibly *et al.* (2001) used maximum likelihood methods to calculate parameters for models based on Markov chain methods and some earlier models, such as the slippage models, and found that a "full" model, where parameters are fitted for each microsatellite length, described the rate and evolution of microsatellites better than the older methods, which tended to have limited ability to predict events in the evolution of very small and very large microsatellite alleles.

Microsatellites and Mosses

The evidence for microsatellites in mosses is sparse, however in recent years there have been several papers describing microsatellite studies in mosses, especially for the genus *Polytrichum* (van der Velde *et al.*, 2000). Subsequent work by the same authors has produced studies on the genetic structure, reproductive biology and mating systems of moss species within this genus. Their findings are that there is some genetic variation between populations over both large- (200 km +)

and small- (1-2 m) scale population studies. In particular, van der Velde *et al.* (2001b), found that on a large geographical scale, sexual reproduction is the more important factor in the genetic structure of *P. formosum*. These findings have been in mosses from in temperate regions, where sexual reproduction is common, thus they may not apply to the situation in the continental Antarctic, where sexual reproduction is the exception rather than the rule (Longton, 1985). It is possible however that most of the populations of mosses in Antarctica are the result of long distance dispersal from areas outside Antarctica (South America, Australia, New Zealand, South Africa), and that they are genetically heterogeneous due to many colonisation events from some or all of these localities.

The abundance of microsatellites in mosses has never been fully investigated. The majority of investigations of microsatellites in mosses have been in the genus *Polytrichum*, where it was found that the percent of polymorphic loci (*P*) were amongst the lowest (48.8%) found for any plant group (usual range 80 – 100%), and that the mean number of alleles at a locus (*a*) was also substantially lower (2.8) than found in other plant groups (range: 4.7 – 16.2) (van der Velde *et al.*, 2001b). When the monomorphic loci were removed from these data, the levels of microsatellite variability were still low (P = 90.6%, a = 4.3), but much closer to the range of data found amongst other plant groups (van der Velde *et al.*, 2001b). How applicable these data are to other plants groups or indeed other mosses is debatable, as it has been found that there are often large differences in microsatellite frequency and polymorphism between different groups of plants (Condit and Hubbell, 1991; Wang *et al.*, 1994; Gupta *et al.*, 1996).

CHAPTER 2

MATERIALS AND METHODS

INTRODUCTION

As this project is an experimental development of microsatellite markers in mosses for use in clarifying the population genetic structure of Antarctic moss populations, the materials and methods chapter will also include some discussion on why various methods were attempted.

FIELD COLLECTIONS

Population-level samples of mosses, representing *Bryum* and *Hennediella* (= *Pottia*) were collected from the Ross Sea Region of Antarctica and New Zealand (Appendix 1, Figure 2.1) over the 2000 and 2001 Antarctic summer seasons at six different localities, these were; Beaufort Island, Cape Bird and Miers Valley in 2000 and Cape Crozier, Granite Harbour and Marble Point in 2001. The samples collected included *Bryum subrotundifolium* Jaeg., *Bryum psuedotriquetrum* (Hedw.) Gaertn., Meyer et Scherb., and *Hennediella heimii* (Hedw.) [=*Pottia heimii* (Hedw.) Hampe]. In total 765 samples (Appendix 1) were collected from Antarctica, consisting of 192 populations of three different species (Table 2.1). Antarctic samples that were otherwise comparable to *B. argenteum* were classified as *B. subrotundifolium* following Seppelt and Green (1998) in their reduction of the *Bryum* species within continental Antarctica to two: *Bryum subrotundifolium* and *Bryum psuedotriquetrum*. These two species are quite distinct when found in the field based on leaf apex shape, size and colour of the leaves, and nerve characteristics; *B. subrotundifolium* has small silvery-yellow leaves, a rotund to subrotund leaf apex and a nerve failing before the leaf point (Seppelt and Selkirk, 1984; Seppelt and Green, 1998). *B. pseudotriquetrum*, on the other hand has larger leaves, with a darker green colour and long tapering, slightly toothed leaf apices and a nerve that reaches or fails just below the apex (Watson, 1968). Of these, both species can be confused with others; *B. pseudotriquetrum* is very similar to *Hennediella heimii* (= *Pottia heimii*), another common species in Antarctica, while *B. subrotundifolium* closely resembles *B. argenteum*, with small silvery-green leaves, rotund to acuminate leaf apices and a nerve that fails in or before the apex. *B. argenteum* is a cosmopolitan species which was previously

thought to be present in Antarctica, though the work of some authors recently has classified all Antarctic specimens of this type as *B. subrotundifolium* (Seppelt and Green, 1998), despite the two distinct morphotypes, one of which is very similar to *B. argenteum*, found at various localities (Hunger, 2000).

Specimens of *H. heimii* were also collected from Antarctica, this species is relatively easy to identify in the field as it has a darker greenish-brown, shading to reddish-brown coloration and finely denticulate apices on the leaves, the nerve fails in or shortly below the apex (Seppelt and Green, 1998).

A total of 70 populations and 393 samples were collected for *Bryum* (Table 2.1). Collections followed the classification of Seppelt and Green (1998), in reducing the *Bryum* species within Antarctica to two.

Seven samples from one population were also collected from New Zealand these were of the species *B. argenteum* (Hedw.) and were collected for purposes of development of the microsatellite markers.

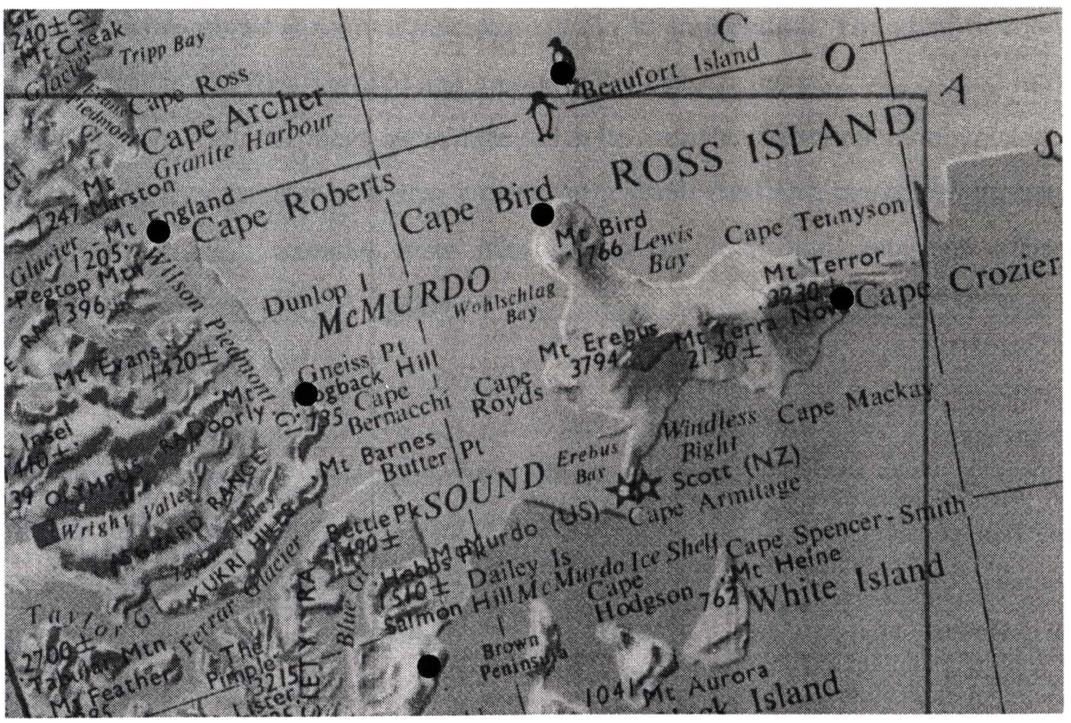

Figure 2.1 Map of the Ross Is., South Victoria Land region showing locations of collection sites, marked by dots. Left to right: Granite Harbour, Marble Point, Miers Valley, Cape Bird, Beaufort Is., Cape Crozier. Reproduced from NZMS 135 Ross Sea Regions 2nd Edition, Department of Lands and Survey New Zealand.

The size of each sample collected was dependent on the abundance of moss in the locality; generally samples were approximately two centimetres in diameter, but frequently smaller. Samples were obtained using a cork borer or a pocketknife. All sampling sites were refilled with gravel or sand to prevent the exposed surfaces drying out and the rest of the moss patch dying. Individual samples were identified by location, species and sample number (Appendix 1), for example; sample 'MV 10/1 BS' would be from Miers Valley (Dry Valleys, continental Antarctica), population ten, sample one, and is the species *Bryum subrotundifolium*. Samples were collected into labelled paper bags and left to dry at room temperature. The number of samples taken from a population also depended on the size of the population, and ranged from 1 to 52 (see Appendix 1). Field data recorded included preliminary species identification, site locations, descriptions of the sites, as well as other details such as abundance and condition of mosses in the area, presence of algae, and other species in the same area. When possible each population was also given a GPS (global positioning system) location, however for some localities in 2000 no GPS signal was available and in the 2001 season this was not possible as no GPS unit was available.

Samples were stored at room temperature to dry to completion. The identification of the sample was then verified and a portion for genetic analyses was placed into a plastic bag. Plastic bags were labelled with sample accession numbers (see below) and this number was also written on a small card and placed in the bag with the sample. Samples were then stored at –76°C until required. The remaining portion of each specimen was lodged at the University of Waikato herbarium (WAIK).

Permits to collect in Antarctica were obtained from Antarctica New Zealand (under permit numbers 99/053 and 00/008) under the Antarctica (Environmental Protection) Act, 1994. Approval was obtained for entry to SSSI (Site of Special Scientific Interest) No. 10 (Caughley Beach, Cape Bird, Ross Island), SSSI No. 4 (Cape Crozier) and SSSI No. 37 (Granite Harbour, Victoria Land) and to SPA (Specially Protected Area) No. 20 (New College Valley, Cape Bird, Ross Island) and SPA No. 5 (Beaufort Island), Conditions made upon granting approval were followed. All samples were imported to New Zealand under MAF permit numbers 1998004318, 1999007502 and 200010764 and stored in a transitional containment

facility.

Table 2.1 Moss collections sorted by population and number of samples within a locality.

Locality	Species	# of populations	# of Samples
Beaufort Island	*B. subrotundifolium*	5	99
	H. heimii	1	1
Cape Bird	*B. subrotundifolium*	19	110
	B. pseudotriquetrum	3	66
	H. heimii	9	46
Cape Crozier	*B. subrotundifolium*	9	23
Granite Harbour	*B. subrotundifolium*	7	23
	B. pseudotriquetrum	2	2
Marble Point	*B. subrotundifolium*	5	21
	H. heimii	7	38
Miers Valley	*B. subrotundifolium*	24	110
	B. pseudotriquetrum	6	24
	H. heimii	95	202
Hamilton, NZ	*B. argenteum*	1	7
	Totals	193	772

Samples were collected in 2000 by S. A. Hunger, R. D. Seppelt and the author. 2001 collections were by C. E. C. Gemmill, C. Beard and the author.

PROTOCOLS AND REAGENTS

Protocols for making solutions, including ratios and concentrations of solutions, and other materials used in the laboratory work are found in Appendix 2, along with equations useful for making the above. Lists of chemicals and supplies used are also included under this appendix.

GENOMIC DNA EXTRACTION

The protocol of Rogers and Bendich (1985) was chosen as the extraction protocol for this project because of its simplicity and high yield, also the small preparation size enables a relatively high number of samples to be extracted at one time (upwards of 30). In many respects it is similar to many other extraction procedures, in that it uses CTAB buffers to lyse cells and disrupt the cell membranes and C:I extractions to denature and remove proteins and other material before the DNA is precipitated and purified (Murray and Thompson, 1980). However, this procedure has been designed and proven to work on small amounts of tissue and provides instructions for the optimisation of the yield. The precipitation of the DNA is performed in ethanol at -20°C, this has been shown to

precipitate DNA at concentrations as low as 20 ng mL^{-1}, such that it can be recovered quantitatively by centrifugation (Sambrook *et al.*, 1989).

The main difficulty with extraction of DNA from plants is the high polysaccharide content of the cells. The polysaccharides make up the majority of the cell wall and as sugars, are often co-extracted with the DNA (Porebski *et al.*, 1997). This problem, along with the tendency of secondary compounds, such as polyphenolics, to co-precipitate with the DNA is often a major problem in DNA extraction and further use of the DNA, such as PCR, which can be inhibited by these compounds (Li *et al.*, 1994). Often it is necessary to remove these compounds by further extraction of the stock DNA. Mosses are simple plants, producing little in the way of secondary compounds, and having a simple cell wall structure. For this reason the extraction of DNA is relatively simple from these plants, and the DNA obtained is usually "clean" in that it contains little in the way of polysaccharides or secondary compounds.

Extraction of total DNA from all moss samples followed a modified Rogers and Bendich protocol, (1985). Approximately 100 mg of shoot tissue was mixed with liquid nitrogen in a mortar to freeze the tissue. The frozen tissue was then ground with a chilled pestle and transferred to a 1.5 mL Eppendorf tube using a spatula chilled in liquid nitrogen. After the tissue had thawed, approximately one microlitre of 65°C 1× CTAB extraction buffer per milligram of tissue was added. If not all the tissue was moistened, more extraction buffer was added. This mixture was then incubated at 65°C for three minutes. Removal of cellular debris and proteins was performed by the addition of 1.5 volumes of chloroform: *iso*-amyl alcohol (C:I) and the solution mixed by shaking thoroughly for one minute. Separation of the aqueous and organic phases was carried out by centrifugation at 11000 times gravity (G) for 30 seconds (s) on an Eppendorf 5415 D bench-top centrifuge. The aqueous phase was removed and placed in a new tube, taking care not to disturb the layer of tissue and protein between the two liquid phases. One tenth of the aqueous volume of 65°C 10% CTAB buffer was added and the C:I extraction repeated once. One volume (~100 µl) of CTAB precipitation buffer was added and mixed gently by pipetting, followed by centrifugation for five minutes at 11000 G, after which the pellet was re-hydrated in 1× STE buffer.

After re-hydration, the DNA was precipitated with two volumes (~200 µl) of -20°C, 95% ethanol and pelleted by ten minutes of centrifugation at 11000 G. The supernatant was then aspirated off and the pellet washed in 80% ethanol at room temperature, after which the ethanol was poured off and the pellet was completely dried in a DNA 120, DNA-speedvac (Savant) using the medium heat setting. Once dry, the pellet was re-suspended in Milli-Q water (20 - 50 µL, depending on the size of the pellet) and RNA digested with one hundredth the volume of 10 mg mL^{-1} Ribonuclease A (Sigma) by incubation at 37°C for one hour. Ribonuclease A was deactivated by heating the tubes to 65°C for 5 minutes. Two to five microlitres of DNA was electrophoresed on a 1.5% agarose gel in 1× TBE buffer, containing 0.1 ng mL^{-1} ethidium bromide (Appendix 3), to determine the quality of each DNA sample. Gels were visualized on an EagleEye II gel documentation system running EagleSight® software, version 3.2 (Stratagene) using ultra-violet light as a fluorescent source. DNA concentrations in each sample were determined using 100, 50, 25 and 10 ng uncut λ virus DNA (Life Technologies) as concentration standards, allowing a fluorimetric approximation of DNA concentration following the Saran™ wrap method of Sambrook *et al.* (1989).

The use of a Hoefer DNA fluorometer (Hoefer Scientific Instruments) and GeneQuant II (Pharmacia Biotech) with a 5 µL cuvette, were also investigated for estimating DNA concentrations.

FUNGAL DETECTION

Internal Transcribed Spacer Regions (ITS)

For the development of *Bryum* specific microsatellite markers it is essential that sample DNA is free of contaminating DNA from other organisms, so as to be absolutely certain that the markers developed are found in the taxon being investigated. If the DNA is contaminated, the markers developed may give false results, in that the patterns established may not be those for the group being investigated, instead they may be microsatellite sequences of the contaminant organism. Previous work by Hunger (2000) established that DNA extracted from Antarctic moss samples were often contaminated by fungal DNA, thus it was necessary to develop methods by which sample DNA could be screened for the

contaminants. The presence or absence of fungal contaminants in extracted DNA was established by two methods: The first method used was PCR amplification of the ITS (Internal Transcribed Spacer) region of nuclear ribosomal DNA from the extracted moss samples. An indication of the presence of fungi contamination was taken as the presence of a second band of DNA of approximately 550 bp in length when the products were visualised on the gels after electrophoresis (Figure 2.2). That this was likely to be a band from fungal contaminants was established by Hunger (2000) through extraction and sequencing of this band and then performing a BLAST (basic local alignment search tool, www.ncbi.nlm.nih.gov/BLAST/) search for sequence similarity. Through cultivation of fungi from moss samples and amplifying the ITS region from these samples, and comparing sequence information between the extracted band and the cultivated samples it was possible to determine the origin of the contaminant.

ITS regions are non-coding parts of the nuclear ribosomal DNA, found between the exons of the ribosomal genes. The ITS regions are situated between the 18S and the 5.8S ribosomal genes (ITS1) and the 5.8S and the 26S genes (ITS2) (Baldwin, 1992). ITS 1 and 2 are mutated at a defined rate, but are surrounded by highly conserved genes (ribosomal genes), making them ideal markers for genetic studies in plants, as primers anchored in the ribosomal genes will allow amplification of the ITS regions in almost any organism (Hamby and Zimmer, 1992). The size of the ITS amplification product depends on the length of the spacer regions and is consistent within species. As the ITS sequence amplified by the "ITS4" and "ITS5HP" primers (White *et al.*, 1990) overlaps the 5.8S ribosomal gene, this conserved region within the marker can be used as an alignment tool to arrange sequences before analysis (Baldwin *et al.*, 1995)

The contaminant was first seen as two bands (Figure 2.2) on a gel of an ITS-PCR product, isolation and sequencing of some of these bands led to the identification of this as a product of the ITS region of any one of three fungal species: *Phoma glomerata*, *Ampelomyces humuli* or *A. quercinus* (97% sequence homology), though subsequent morphological identification of fungal cultures isolated from Antarctic moss samples led to the characterisation of these specimens as *Phoma* sp., thought to be *Phoma herbarum* Westend. (Hunger 2000).

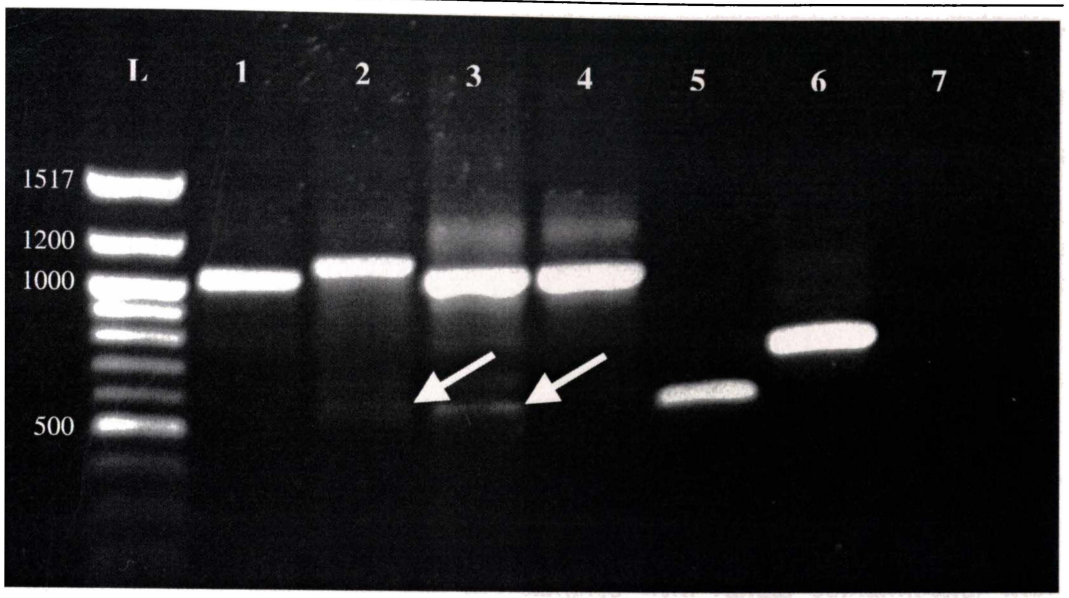

Figure 2.2 ITS products showing the multiple bands characteristic of contamination of the DNA. Lane marked "L" contains 0.3 µg 100 bp ladder (New England Biolabs), band sizes in base pairs are marked next to the ladder. Lane 1 contains an un-contaminated Antarctic moss sample (SH 16 BA), lanes 2 and 3 contain contaminated Antarctic moss samples (SH 21 BA, SH34 BS), note bands marked by arrows, lane 4 contains a New Zealand moss sample (HR 1/2 BA) with no fungal contaminant, and lane 5 contains amplified fungal DNA. Lanes 6 and 7 contain positive (*Pittosporum cornifolium*) and negative controls respectively.

PCR conditions for the ITS analyses were as follows for a 50µL reaction: 2.5 mM $MgCl_2$, 1.0 µM each of ITS4 and ITS5HP primer (Life Technologies), 0.15 mM each dNTP (Boehringer Mannheim), 1.0 U *Taq* DNA Polymerase (Boehringer Mannheim and Roche), with five microlitres of DNA at between 4 and 36 ng μL^{-1}. Cycling parameters were as follows for an Eppendorf MasterCycler Gradient thermocycler; 96°C for 5 minutes, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, final extension was performed at 72°C for 10 minutes. Reactions were then held at 4°C. PCR products were visualised on 1.5% agarose gels (Appendix 3) in 1× TBE buffer, on an EagleEye DNA visualisation system (Stratagene) as in DNA extraction.

Random Amplified Polymorphic DNA (RAPD)

The second method used to screen mosses for contamination was using RAPD (Randomly Amplified Polymorphic DNA) primers in a RAPD-PCR (Figure 2.3). This technique involves the use of arbitrary primers, ten bases in length, composed of 60-70% G or C, to amplify random regions of the genome. The fragments (bands) produced are visualised using electrophoresis on either a

polyacrylamide or an agarose gel. It has been proven that the majority of polymorphic RAPD bands are dominantly inherited (Clark and Lanigan, 1993) and can be assumed to follow Mendelian segregation (Williams *et al.*, 1990), although it would appear that some alleles do not (Grosberg *et al.*, 1996). The mode of inheritance and Mendelian segregation makes RAPD analysis a highly useful tool for studies of population genetics.

Observed banding patterns were compared with banding patterns from known fungal contaminants and with moss samples of the same species that are known to be free of fungal DNA. The observed banding patterns were not completely what was expected, it was found that the samples with fungal contaminants were without some bands that were present in the un-contaminates samples. It had been expected that the two banding patterns (fungal and moss) would show the total bands from each of these species.

PCR conditions were: 2.0 mM $MgCl_2$, 0.1 µM each of dATP, dTTP, dCTP, dGTP (Boehringer Mannheim), 1.5 µM primer (Operon Technologies Inc., Kit A) and 0.5 U *Taq* DNA Polymerase (Boehringer Mannheim and Roche) in a total reaction volume of 25 µL. Two and a half microlitres of DNA (concentrations ranging between 4.0 and 36.0 ng μL^{-1}) were added per reaction. Amplification was carried out on an Eppendorf MasterCycler Gradient thermocycler, using the following cycling parameters; 94°C for 3 minutes followed by 35 cycles of 94°C for 1 minute, 45°C for 1 minute and 72°C for 2 minutes, and a final extension of 72°C for ten minutes after which the reactions were held at 4°C until they were loaded onto a gel. PCR products were visualised on a 1.5% agarose gel (Appendix 3) in 1× TBE buffer. Using the EagleEye DNA visualisation system above.

Amplification of these samples was problematic in that the samples had different optimal annealing temperatures with the primers used, thus experimentation to determine the best temperature at which to perform the PCR was necessary. This was carried out using the gradient function on an Eppendorf MasterCycler Gradient thermocycler, with the gradient set to 36 ± 10°C. It was found that this temperature was 36°C, as per Williams *et al.* (1990). However, note the faint

banding pattern in lane 1 of Figure 2.3, which is probably the result of degraded DNA.

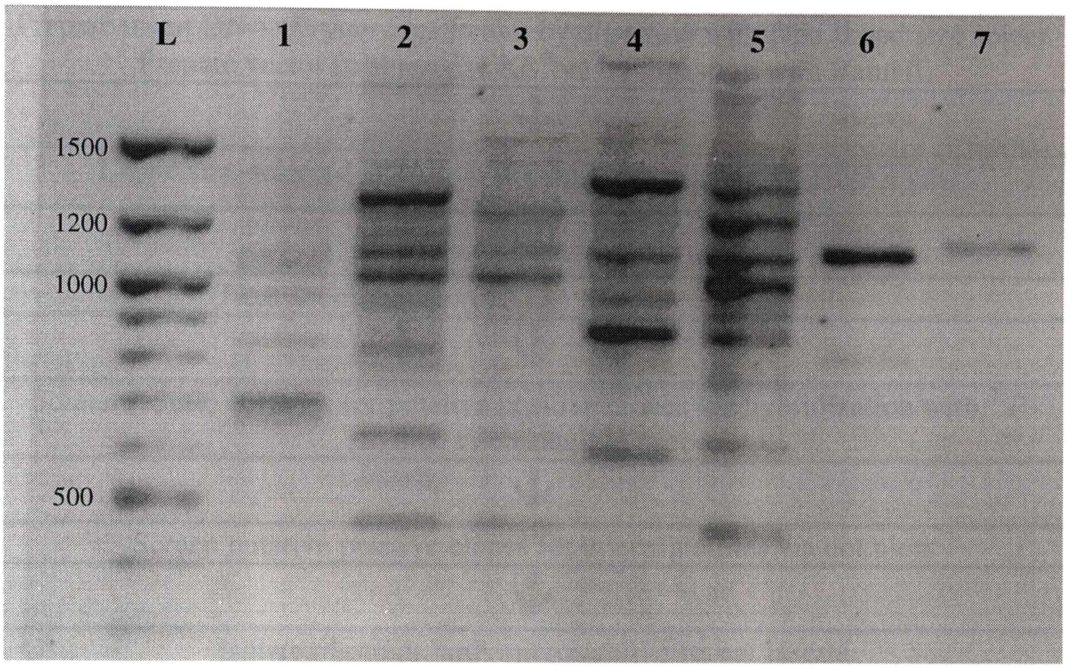

Figure 2.3 RAPD banding pattern produced by three Antarctic samples, a fungal sample and a combined moss and fungal sample. Note band in negative control (lane 7). Lanes 1 – 6 contain samples SH 16 BA, SH 27-2 BS, SH 23-1 BS, Fungal extract, Spiked, and positive control (*Pittosporum cornifolium*). Lane marked L contains 0.05 μg 100 bp ladder (New England Biolabs).

MICROSATELLITE DEVELOPMENT

Experimental protocols followed those outlined in Glenn (2001), as closely as possible, with exceptions made for DNA extraction as the original protocol gives instructions for extraction of animal DNA as opposed to plant DNA. Variations were also made for competent cell preparation and for probing.

In general, the procedure for development of the microsatellites followed Figure 2.4. A more detailed description of the protocol is given below as the main body of the text. This also includes work that was not completed during the study.

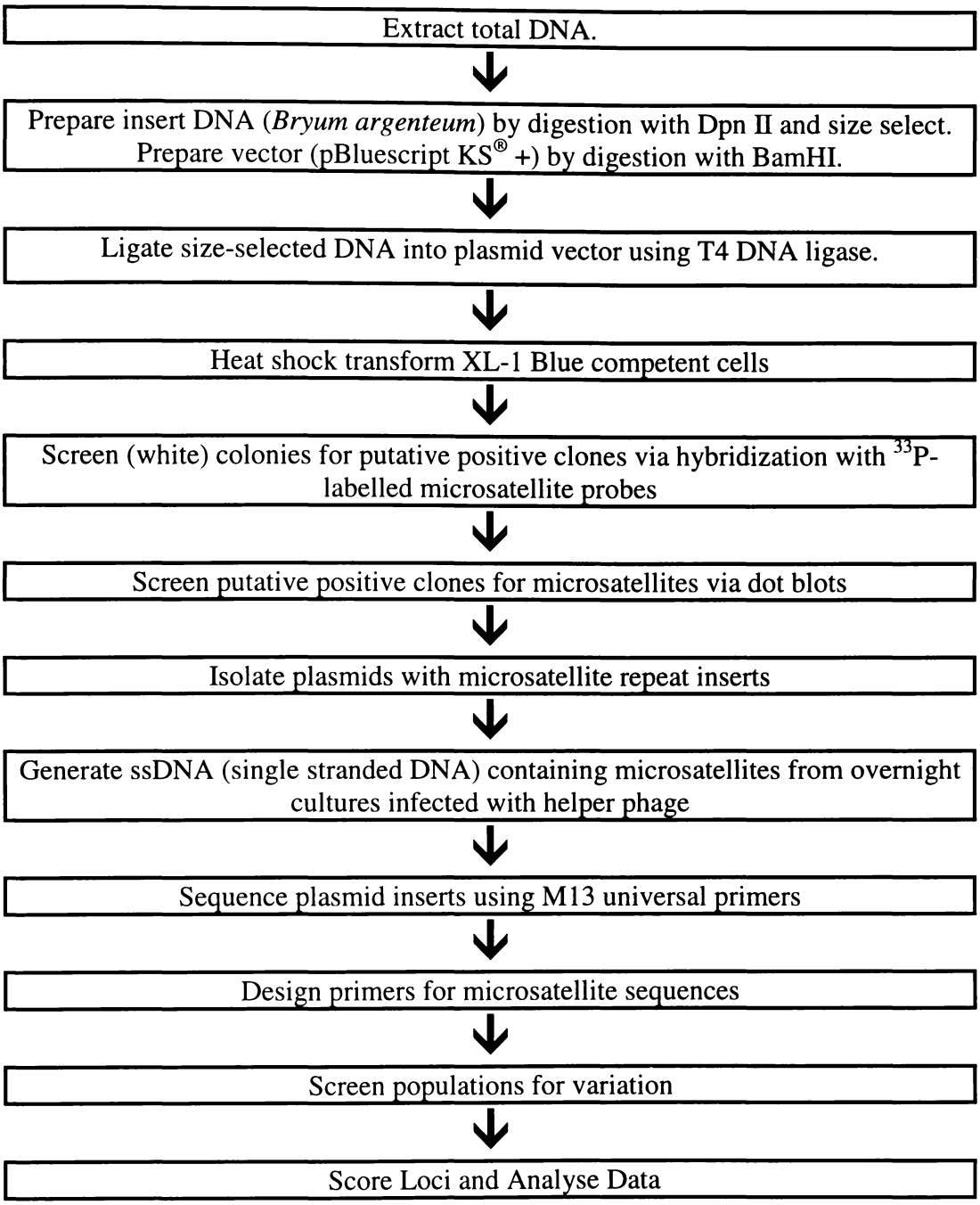

Figure 2.4 Flowchart of microsatellite development steps.

Genomic DNA Preparation and Size Selection

DNA was size selected for microsatellite development so as to be able to increase the efficiency of the protocol, this involved the extraction of the 300 – 700 bp region of the genomic DNA after digestion with a restriction enzyme.

For microsatellite development, specimens of New Zealand *Bryum argenteum* were used, as these were found to have no fungal contamination. DNA was

extracted as above from six New Zealand moss samples (HR 1/1 BA, HR 1/2 BA, HR 2/1 BA, HR 2/2 BA, HR 3/1 BA, HR 3/2 BA) and quantified on a Hoefer DNA Fluorometer (Hoefer Scientific Instruments). Extractions were then tested for the presence of fungal contaminants using PCR amplification of the ITS region.

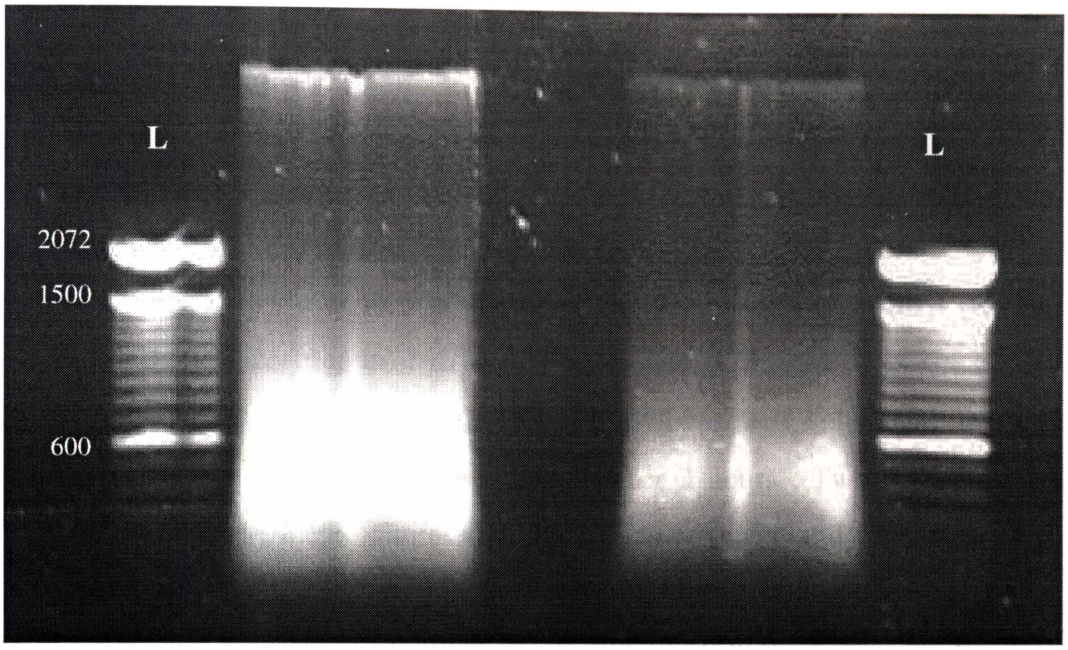

Figure 2.5 Digested *Bryum argenteum* DNA from samples HR 2/1 BA and HR 3/2 BA. Lanes marked "L" contain 0.3 μg 100 bp ladder (Life Technologies), band increments around the 600 bp band are in 100 bp sizes. The region 300-700 bp in length of the digested DNA was excised and extracted from the gel for use in the ligation step.

Those extractions with high (20-70 ng μL^{-1}) DNA concentrations (HR 1/2 BA, HR 2/1 BA, HR 2/2 BA, HR 3/2 BA) were digested using Dpn II restriction enzyme (New England Biolabs) to completion (approximately one hour), using a 50 μL reaction volume containing 1× Dpn II restriction buffer (10 mM $MgCl_2$, 1 mM dithiothreitol, final concentrations), 20 U Dpn II, and 2 μg DNA. Digested DNA was electrophoresed on a 1.5% agarose gel with 0.3 μg of a 100 bp (base pair) ladder (Life Technologies) as a size standard in a separate lane, until separation of the DNA was seen (Figure 2.5). The 300-700 bp region was excised from the gel, keeping UV exposure to an absolute minimum. Extraction of the DNA from the gel slices was performed using a gel extraction kit (Life Technologies, Concert™ Gel Extraction Systems) and the DNA re-suspended in 25 μL 1× TE buffer (pH 8.0), quantified and diluted to make a concentration of 25 ng L^{-1} in 1× TE buffer (pH 8.0).

Vector Preparation

Vector preparation was performed so as to enable the vector to take up the insert DNA though a ligation reaction. The vector preparation step cuts the vector in one place, so that there are overhanging ends that are complementary to those produced by the restriction of the genomic DNA, this allows the vector and the genomic DNA to bind together, re-circularising the vector and enabling it to be taken up by a bacterium.

The vector used was a pBluescript KS® + (Stratagene) phagemid vector. Ten micrograms of vector were prepared as follows:

Ten micrograms of vector were restricted with 100 units (U) of BamHI (Roche) with 1× BamHI restriction buffer (10 mM NaCl, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, final concentrations) in a total reaction volume of 100 µL. The digestion reaction was incubated at 37°C for 3 hours. Two microlitres of digested vector were electrophoresed on a 1.0% agarose gel, and visualised using UV light at 312 nm. Because no-uncut vector was observed, dephosphorylation of the vector was carried out. Five microlitres of shrimp alkaline phosphatase (SAP, Roche) was used to dephosphorylate the remaining (95 µL) vector by incubation at 37°C for 1 hour. One hundred microlitres of 1× TE buffer (pH 8.0) was then added and the solution was extracted once with P:C:I (25:24:1, Sigma) [by adding one volume of P:C:I, mixing, centrifuging at G_{max} (13000 G) for 1 minute, then removing the aqueous layer to a new tube., and twice with C:I. Twenty microlitres of 3.0 M sodium acetate (NaOAc) and 450 µL of –20°C, 95% ethanol were then added to the aqueous solution. This was then mixed gently by inversion and incubated at -20°C for 30 minutes. To pellet the DNA the tubes were centrifuged at G_{max} for 15 minutes. The supernatant was then poured off and the pellet washed by adding 500 µL of 70% room temperature ethanol without mixing, and the tubes centrifuged again at G_{max} for five minutes. Finally, the ethanol was poured off, and the pellet dried in a DNA 120, DNA-speedvac (Savant) using a medium heat setting. The pellet was re-suspended in 1× TE buffer (pH 8.0), quantitated on the EagleEye II, as for DNA extractions, and diluted in 1× TE buffer (pH 8.0) to make a concentration of 200 ng μL^{-1}.

Ligation of Genomic DNA into the Vector

Ligations were performed to join the digested genomic DNA to the vector, forming a circular piece of DNA that is able to be taken up by a bacterium, thus enabling fast and efficient screening of the genomic DNA for microsatellite sequences.

Ligation of the digested, size-selected genomic DNA into the prepared vector was performed using T4 DNA Ligase (Boehringer Mannheim). Ratios of 1:1 and 3:1 (insert: vector) were trialed in an effort to optimise the ligation efficiency. The reaction consisted of: 1× ligation buffer (5.0 mM $MgCl_2$, 1.0 mM dithiothreitol, final concentrations), 2.0 U of T4 DNA ligase, 400 ng digested vector and 225 ng insert DNA in a total reaction volume of 20 µL. Transformations were incubated at 16°C for 16 hours on an Eppendorf MasterCycler Gradient thermocycler with the lid set to 16°C. Two volumes of 1× TE buffer (pH 8.0) were added to each reaction and then the reactions were heated to 65°C for 15 minutes. Ligation reactions were stored at -20°C.

Table 2.2 Control ligation reactions.

Reagent volume (µL)	**Control**		
	One	**Two**	**Three**
10× Ligation buffer	1	1	1
T4 DNA Ligase (1.0 U μL^{-1})	0	1	1
Vector (200 ng μL^{-1})	1	1	0
Insert DNA (20 ng μL^{-1})	0	0	0
Uncut Plasmid (10 ng μL^{-1})	0	0	1
Milli-Q Water	8	7	7
Total reaction volume (µL)	10	10	10

Three controls were also set up at the same time and under the same reaction conditions in a 10 µL volume. These are shown in Table 2.2. Control 1 is a negative control that tests the amount of uncut vector in the preparation. Control 2 tests the ability of the vector to ligate to itself (re-circularise). Control 3 tests the ability of the vector without insert to be taken up by the competent cells. A fourth control, called the background control, was also used; this consisted of transforming 100 µL of competent cells (see below) with 50 ng of uncut plasmid to test the level of vector uptake under non-ligated conditions.

Competent Cell Preparation

Competent cells are the medium by which it is possible to obtain large amounts of DNA containing an insert in a short amount of time. To enable this the bacterial cells must be prepared to make them receptive ("competent") to the uptake of DNA from outside the cell.

Initially, a modified Chung *et al.* (1989) method was used for preparation and transformation of competent cells. It was recommended that fresh competent cells be prepared before each set of transformations (R. Cursons, *pers. comm.*), for this reason the Chung *et al.* (1989) method was selected, based on the speed, simplicity of preparation and the high levels of transformants (~1 $\times 10^8$ per microgram of insert DNA) produced. The second method trialed followed Nishimura *et al.* (1990). This method was used as it gave extremely high transformation efficiency (~1 $\times 10^8$ per μg insert DNA, Nishimura *et al.*, 1990), was relatively simple in preparation and allowed storage of the cells in the transformation medium for several months with little loss of competency, which is in contrast to the Chung *et al.*, (1989) method which was reported to be able to be stored, however it was found that this decreases the competency of the cells.

Competent cell preparation and transformations were performed by following either the protocol based on the protocol of Chung *et al.* (1989) modified by G. Jacobsen (*pers. comm.*) to make fresh competent cells each experiment, or by the method of Nishimura *et al.* (1990) for storage of pre-pared competent cells. All work was performed under sterile conditions either in a laminar flow cabinet or under PC2 conditions in a biological safety cabinet.

Method 1, Chung *et al.* (1989): LB-agar plates containing 50 μg mL^{-1} tetracycline (Sigma) were streaked with stock cultures of *Escherichia coli*, strain XL1 - Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F´ *proAB lacI*^{q}ZΔ*M15* Tn*10* (Tet^r)]) (Stratagene). Plates were incubated inverted at 37 °C overnight. From the streak plates, individual colonies were picked using a sterile toothpick and placed in 3 mL of LB-broth containing 10 mM $MgSO_4$, 0.2% glucose (supplemented LB-broth), and 50 μg mL^{-1} tetracycline to make over-night cultures, which were incubated at 37°C in a shaking incubator at approximately 250 revolutions per

minute (rpm). A 1.0% inoculum of over-night culture was added to supplemented LB-broth (i.e., for a 50 mL culture, 0.5 mL of overnight culture was added to 50 mL of supplemented LB-broth) and incubated at 37°C and 225 rpm in a shaking incubator. The number of cultures was determined by the number of ligations, including controls, and one or two extra (to account for error). When the OD_{600} of the cell suspension was between 0.4-0.5 as measured on a Shimadzu UV-160 spectrophotometer, 1 mL aliquots of the cells were transferred into sterile 1.5 mL Eppendorf tubes and centrifuged for 15 s at G_{max} on a bench-top centrifuge (Eppendorf 5415D). The pelleted cells were re-suspended in 100 µL of ice-cold 1× TSS by gently flicking the tube and iced for 5 – 10 minutes.

Method 2, Nishimura *et al.* (1990) The second method to prepare competent cells followed the methods of Nishimura *et al.* (1990) exactly. Cells were grown in pre-warmed supplemented LB-broth (see above), to which a 1.0% inoculum of over night culture (as above) had been added. Cultures were incubated in an orbital shaker at 37°C until the optical density (OD_{600}) of the cells suspension was between 0.4 and 0.6. When this point was reached (approximately 4 – 6 hours), the cell suspension was iced for 10 minutes. The suspension was then aliquoted into chilled centrifuge tubes (for a 50 mL solution, 12.5 mL per tube for 4 tubes) and centrifuged at 1500 G on an Eppendorf 5810R centrifuge for 10 minutes at 4°C and the supernatant poured off. The cells were then re-suspended by gently flicking the tube, in a total volume of 500 µL of ice-cold supplemented LB-broth (see above) while on ice. Once the cells were re-suspended 2.5 mL of ice cold LB-broth containing 36% glycerol, 12% PEG 8000, and 12 mM $MgSO_4$ was added and mixed gently. The resulting cell suspension was then aliquoted (100 µL) into chilled 1.5 mL Eppendorf tubes and stored at –76°C until use.

For use, cells from the above method, were taken out of the freezer and thawed on ice. Immediately the cells were thawed, 5.0 µL of ligated DNA or control was added and the cells transformed as in the transformation section, below.

Heat Shock Transformations

Transformations are the procedure by which the cells take up the vector, cells without a vector will not grow on the antibiotics in the plates, and those with a

vector can be selected by blue/white phenotype expressed by those with or without an insert respectively.

Transformations were conducted under New Zealand Environmental Risk Management Authority (ERMA) permit number GMO99/UOW005, obtained for genetic modification of *E. coli* in the laboratory. Transformations were conducted in an approved PC2 facility.

Five microlitres of ligated vector and insert, or control, were transformed into 100 µL of competent cells (XL-1-Blue, Stratagene) prepared as above, using heat shock following Nishimura *et al.* (1990). Two to five microlitres of ligation product or control was added to 100 µL of thawed or fresh competent cells on ice, in a 1.5 mL Eppendorf tube using a chilled pipette tip, the tubes were then incubated at 4°C for 20 – 30 minutes. Tubes were incubated on ice for 1 minute, heat shocked at 42°C in a water bath for 1.5 minutes and immediately placed back on ice for 2 minutes. The cells were allowed to recover by adding 895 µL, 37°C, LB-broth (un-supplemented) and incubating for 1 hour at 37°C in the shaking incubator. Finally, the cells were centrifuged to form a pellet, and the pellet re-suspended in 100 µL of room temperature, LB-broth (un-supplemented), by gently flicking the tube. Once cells had been re-suspended, 25 – 50 µL of the resulting cell suspension was spread onto 90 mm diameter LB-agar plates containing 50 µg mL^{-1} tetracycline and ampicillin (Sigma) and spread with X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, Sigma) and IPTG (isopropyl β-D-thiogalactopyranoside, Sigma) to make final concentrations of 50 mM and 25 mM respectively (25 µL and 5 µL per 90 mm diameter plate, respectively). The X-gal and IPTG allow the cells to exhibit blue/white phenotypes according to the presence or absence of an insert into the pBluescript KS® + phagemid vector. Plates were inverted and grown overnight (or until colonies were pinhead-sized) at 37°C and then incubated at 4°C to enhance expression of the blue/white colour (Sambrook *et* al., 1989).

Insert Size Checks

To check that white colonies contained inserted sequences, white colonies from libraries of transformed cells were selected for PCR-based insert checks.

pBluescript KS® + phagemid vector has a multiple cloning site (MCS) of 223 bp when amplified using M13 primers. Thus, if the amplified plasmid has no insert, the size of the PCR product will be 233 bp; any product with a larger size will have an insert. The size of the inserts was determined by subtracting 220 from any product larger than 220 bp in length, an average of the results gave the average size of the inserts.

Twenty PCR tubes (0.2 mL) were labelled for each library being tested. Twenty-five microlitres of Milli-Q water was then added to each tube. A sterile toothpick was touched to a white colony and then twirled in the water for two seconds; this was repeated using a fresh toothpick for each colony selected until all twenty tubes had been inoculated.

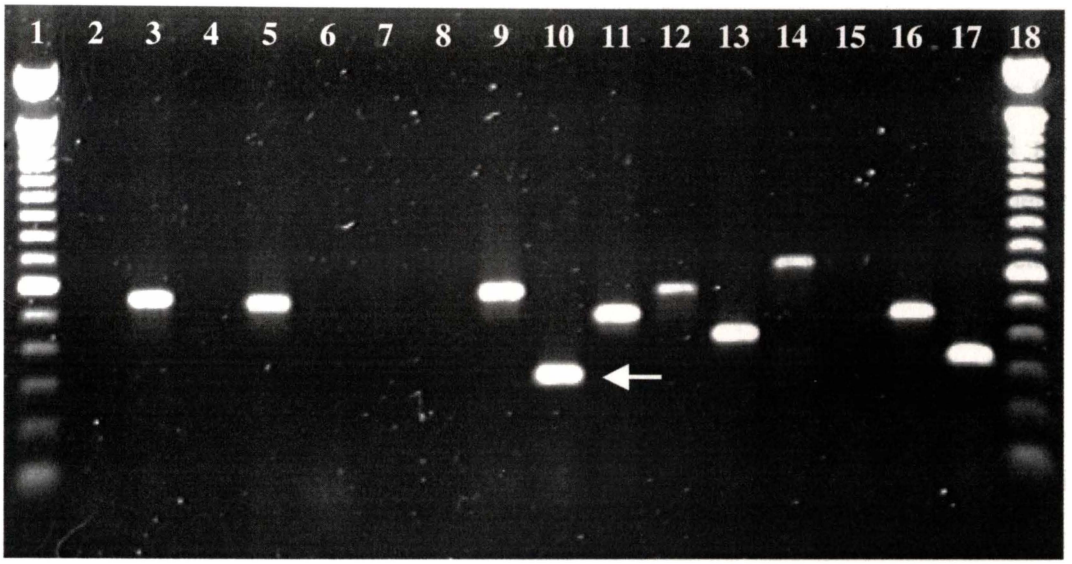

Figure 2.6 Photo of an agarose gel of DNA bands from the M13 multiple cloning site of pBluescript KS® +. Lanes 2 – 17 contain the PCR products of the amplification reaction above. Lanes 1 and 18 contain 0.3 µg 100 bp ladder. The top bright band of the ladder is at 2072 bp, the bright band halfway down the ladder is 600 bp in size, other bands are at 100 bp intervals. Positive and negative controls were run on another gel with other picked clones. Arrowed band contained no insert

Twenty-two new PCR tubes were labelled (twenty as for the first set, above, and a positive and negative) and a PCR master mix set up according to the following protocol: 1.5 mM $MgCl_2$, 0.5 µM M13 Universal forward primer, 0.5 µM M13 Universal reverse primer (Life Technologies), 0.15 mM each dNTP (Roche), 0.5 U *Taq* DNA polymerase (Roche), in a total reaction volume of 25 µL. Five

microlitres of the water/bacteria mix above was added per reaction. The reactions were amplified on an Eppendorf MasterCycler Gradient thermocycler using the following parameters: 25 cycles of 94°C for 1 minute, 50°C for 30 s, 72°C for 90 s followed by 72°C for 7 minutes and then held at 4°C until run on a gel. Ten microlitres of PCR product was electrophoresed on a 1.5% agarose gel in 1× TBE buffer with 0.3 μg of 100 bp ladder as reference and visualised on an EagleEye DNA visualisation system (Stratagene) using ultra-violet light (Figure 2.6).

Lifting of White Colonies onto Filters

Lifts are performed to enable the hybridisation step to take place. Hybridisation is not possible *in situ* for bacterial colonies, thus it is necessary to remove the colonies onto a solid substrate that will allow extraction of the DNA from the cells and then bind to the DNA, and allow it to be screened by hybridisation.

Total colony counts were taken, as well as numbers of blue and white colonies for calculation of transformation efficiency. White colonies were picked and re-plated (streaks about 0.5 cm long in a spiral design) onto replica 150 mm diameter LB-agar plates containing 50 μg mL^{-1} ampicillin at a density of about 200 streaks per 150 mm diameter plate.

These colonies were then lifted onto positively charged nylon filters (N^+, Boehringer Mannheim) by 15 minutes incubation at 4°C, the filter must be layed carefully on the plate so that all of the filter becomes wet, while excluding air bubbles. Filters were labelled with direction-orienting arrows, and an identification number to match the plate to the filter. The filters were then punctured with a needle in three places and these places marked on the plate, as further identification. The bacteria are then lysed by placing the filter colony side up on a mild detergent (10% SDS); at this stage, the surface of the filter becomes yellow with the digested cells. After this, the DNA is denatured to allow binding of the DNA to the positively charged membrane, performed on a saline base solution. The filter is then placed on a neutralising solution that allows the DNA to bind to the filter and finally washed to remove excess bacterial proteins. After this, the filter is dried and the DNA more firmly bound to the filter by cross-linking with short-wave ultra-violet light (UV) in a BLX-254 UV crosslinker

breaks some of the DNA: DNA bonds that have formed during the extraction process, creating sites with a negative charge that can bind to the positively charged filter by covalent bonds (Brown *et al.*, 1991). The crosslinked filters were stored wrapped in aluminium foil under a dry vacuum (Sambrook *et al.*, 1989). A schematic of this process is shown in Figure 2.7. Six filters were lifted with a total of 1301 white colonies divided between them.

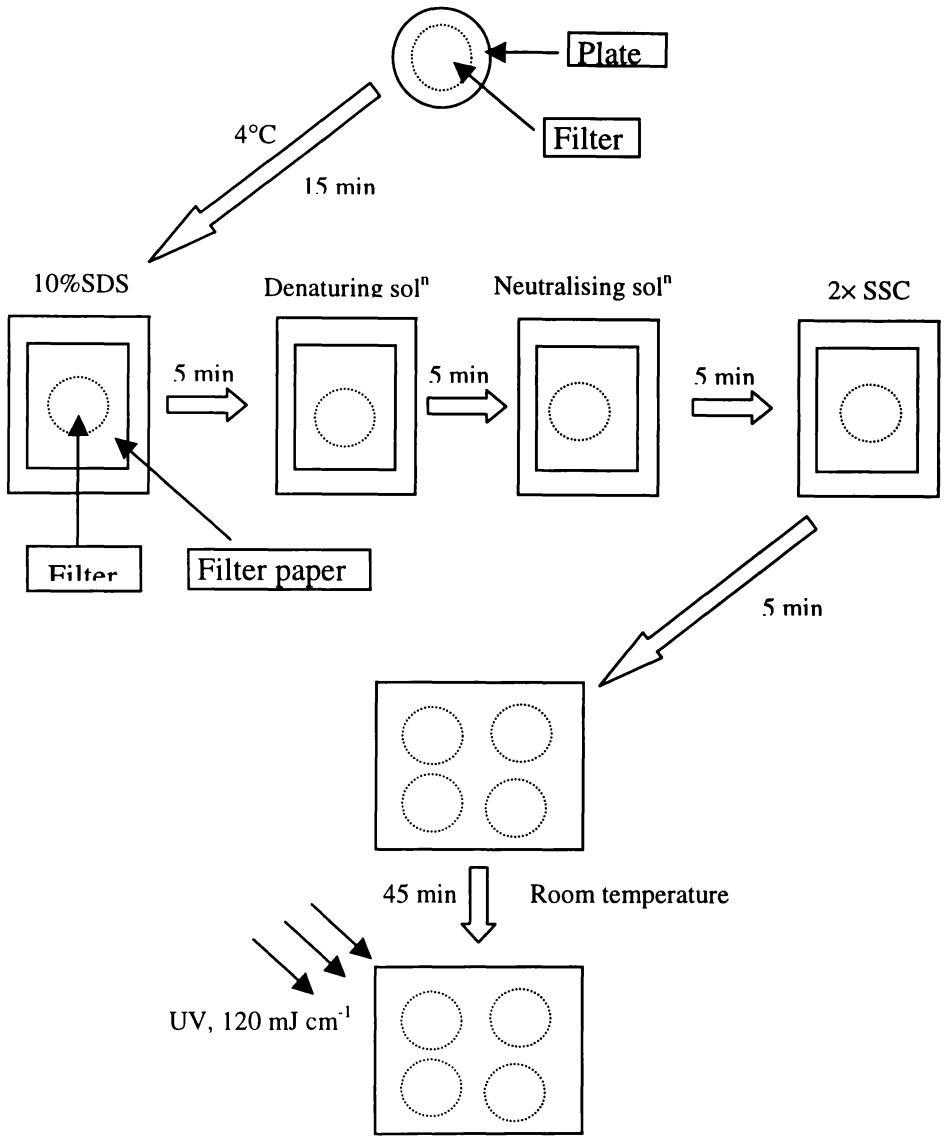

Figure 2.7 Schematic of the lifting procedure, the first step it to lay the filter on a plate, then incubate for 15 minutes at 4°C, followed by 5 minutes, colony side up on filter paper wet with 10% SDS, then 5 minutes on each of the other three solutions, finally the filter is dried and crosslinked.

Probing of Filters

Probing is the step at which the genomic DNA is screened for microsatellite sequences, this is carried out through the use of radio-labelled probes

complementary to the sequence being screened for (in this case microsatellite sequences). These probes bind to the DNA on the bacterial filters from the lifting step above and as the probes are radioactively labelled, the filters with bound probes can be exposed to a film and areas that have probe bound to them can be seen as dark patches on the film.

The filters were probed with ^{33}P-labelled microsatellite sequences $(GT)_{15}$, $(GA)_{15}$, $(CAA)_{10}$, $(AT)_{15}$, $(ATT)_{10}$, $(TCC)_{10}$ (Life Technologies). All work with radioisotopes was performed in a registered isotope facility, following standard procedures for health and safety.

DNA oligonucleotide probes were labelled using Polynucleotide Kinase (PNK, Roche) in a 50 µL reaction with the following conditions: 1× PNK direct buffer, 50 µCi γ^{33}P-ATP (Amersham), 20 U PNK, 50 pM oligonucleotide (or combination of nucleotides, see Table 2.2). Tailing reactions were incubated at 37°C for 2 – 4 hours and the probes stored at -20°C until use.

The radio-labelled probes were then hybridised to the nylon filters (see below) and the filters washed at appropriate temperatures (see Table 2.3) following a modified Sambrook *et al.* (1989). Melting temperatures for the oligonucleotides were calculated on the basis of the following equation:

$$T_M = 81.5 + 41(\%GC) - (675/\text{primer length}) \qquad \text{(Equation 1)}$$

where the %GC is the decimal value (e.g. 60% GC = 0.6), and the primer length is in base pairs. This value had approximately 20 - 25°C subtracted and rounded to a convenient number. The $(GT)_{15}$, $(GA)_{15}$, $(CAA)_{10}$ probes were hybridised at 55°C on the advice of R. J. Wilkins (*pers. comm.*).

A shaking incubator (Hybaid Midi Duel 14) was turned on and set to the appropriate temperature, then approximately 125 mL of pre-hybridisation solution (6× SSC, 0.1% SDS, 1× Denhardt's solution) was poured into a plastic container, and another 125 mL of the same solution measured, placed in a glass bottle and both the solutions were placed in the incubator. After the incubator and solutions

had reached the set temperature, the filters were placed (one by one) into approximately 200 mL of room temperature 2× SSC in another container to pre-wet the filters. The container was agitated gently by hand as the filters were being added so that the solution covered each filter before the next was added.

Table 2.3 Probe hybridisation and washing temperatures.

Probe	Hybridisation Temp. (°C)	Washing Temp. (°C)
$(GT)_{15}$, $(GA)_{15}$, $(CAA)_{10}$	55	48
$(AT)_{15}$, $(ATT)_{10}$	40	30
$(TCC)_{10}$	68	55

After the filters were wet (approximately 10 minutes) the warmed container with pre-hybridisation solution was taken out of the incubator and the filters transferred to it one by one as for the 2× SSC. The container with 2× SSC was emptied and rinsed in dH_2O. Ten millilitres per filter of pre-hybridisation solution (6× SSC, 0.1% SDS, 1× Denhardt's solution) was measured into the appropriate number of hybridisation tubes (one per filter, as close as possible to filter size, so as to allow the filter to fit in the tube with minimal overlapping of the edges of the filter) and these were placed in the incubator and allowed to warm to the hybridisation temperature. The bacterial side of the filters was rubbed with a Kim-wipe while under the warm pre-hybridisation solution in the plastic container. The filters were then rolled into a cylinder without touching the colony side of the filter, and transferred into the warm hybridisation tubes (one filter per tube, colony side innermost) and the solution swirled to wet the filter. They were then placed in the incubator and pre-hybridised for 15 – 20 minutes with rotation to ensure the surfaces of the filter were coated in a film of solution. During the pre-hybridisation the probe was removed from the freezer and placed behind a perspex shield. After the pre-hybridisation was completed, the pre-hybridisation solution was poured out of the tubes and 10 mL of fresh, pre-warmed hybridisation solution was added. The defrosted probe was then divided equally among the tubes and placed directly into the buffer in the tubes, the lids were put back onto the tubes and the filters incubated at the hybridisation temperature for 1 – 2 hours.

After the hybridisation time had elapsed, the hybridisation tubes were taken out of

the incubator and the incubator reset to washing temperature, leaving the door open. Probe solution was poured into labelled 50 mL tubes, and stored at 4°C [ssDNA (single stranded-DNA) probes can be used for 1 – 2 weeks with minimal loss of signal]. The filters were removed from the tubes and immediately placed in approximately 120 mL of 2× SSC, 0.5% SDS at room temperature, after 5 minutes the filters were removed from this solution and placed in approximately 120 mL of 2× SSC, 0.1% SDS also at room temperature. These were incubated for 15 minutes with occasional agitation. When the 15 minute incubation had elapsed, the filters were placed in 120 ml of 0.1× SSC, 0.5% SDS at wash temperature and incubated with agitation for 15 minutes. Used wash-solutions was poured directly into a sink drain without splashing, with water run during and after for 15-20 minutes to dilute the residual isotope a much as possible.

The filters were removed from the final wash-solution and placed colony side up on filter paper to dry (45 minutes at 45°C in an incubator). A Geiger counter was passed over the filters to check that there was some radioactivity present on the filters (an indication that the hybridisation had worked). Probed filters were then exposed to X-ray film (Kodak, X-Omat AR) (see Exposing and Developing Films section below).

Filters were stripped between hybridisations with different combinations of probes (see Table 2.3) by pre-wetted in room temperature 2× SSC, removing these to a fresh container and pouring boiling 0.5% SDS over the filters, allowing them to cool to room temperature and then drying the filters again (Amersham International plc, Hybond™-N^+ product information sheet). A Geiger counter was passed over the dry filters to check for residual radiation.

After the filters had been hybridised for the first time, there was no need for the Kim-wipe step in subsequent hybridisations, so this step was omitted and the filters were simply pre-wetted as above, and then placed directly into the warm pre-hybridisation buffer. Hybridisation procedures were followed from this point. In the optimisation stages for this procedure several different methods were used for hybridisation, these were: Variations in the hybridisation time from one hour to overnight (16 hours); variations in washing solutions, the Glenn (2001)

protocol recommends washing twice in 6× SSC, 0.1% SDS at the washing temperature, however this was found not to be effective in eliminating non-specific hybridisation, so the recommendations of Sambrook *et al.* (1989), were followed to create the above procedure.

Exposing and Developing Film

Exposure to the hybridised filters to a film allows the colonies that had a microsatellite-containing insert to be selected. This is done by examining the developed film for dark patches, which indicate the presence of a microsatellite-containing sequence in the colony.

Once dry, the top left corner of the filter paper was marked, the filters taped in place on the filter paper and this assemblage placed in a film cassette and taken to the dark room. In the dark room, under the safe light, a piece of X-ray film was taken out of the packet and the top left corner folded over. This was then placed in the cassette so that the top left corner of the film aligned with the marked left corner of the filter paper. The cassette was closed and the sides taped. Films were exposed for 6 – 24 hours at room temperature.

Once exposure was complete, films were developed manually by the following method: Developer and fixer trays were set up with the respective solutions, using a minimal amount of developer (Kodak) in the developer tray and 1 – 2 centimetres depth of fixer (Kodak) in the fixer tray. The safe light was turned on and the normal lights turned off. The film cassette was opened and the film lifted off the filters, and lowered into the developer with gentle agitation so that the solution covered the film on all sides. The cassette was closed and agitation of the developer was continued for 1 – 2 minutes. Images started to appear towards the end of this time. Once the images started to form, the film was lifted out of the developer and allowed to drip-dry for a few seconds. Then the film was placed in the fixer so that the film was totally covered. The normal lights were turned on and the film fixed with agitation for twice the time it was in the developing solution (2 – 4 minutes). The film was then rinsed under cold flowing water for 5 minutes and allowed to dry completely before touching (2 – 3 hours).

Screening Putative Positive Clones

Putative positive clones are those colonies that had a positive first hybridisation. In this step these colonies are picked off the plates and the insert amplified and re-screened for microsatellites. This step is similar to the Insert Size Checks step above in the methods used, but this step is carried out to eliminate sequencing of false positives from the probing step above. The amplification can be done straight from bacteria ruptured in distilled water, or from plasmid extracts (see dsDNA extraction).

Once developed and dried, the 12 o'clock arrows, plate identification numbers and the needle hole positions were marked on the film and the films aligned with the appropriate plate. Colonies on the blots that showed up as dark images, potentially containing microsatellite sequences, were picked from the re-plated colonies using sterile pipette tips. For each colony picked, the tip used for picking was pipetted up and down in a PCR tube containing 25 µL Milli-Q water, and then the tip ejected into a culture tube containing 1.5 mL of LB-broth with 50 µg mL^{-1} ampicillin, these were then grown overnight at 37°C in a shaking incubator (225 rpm), for manufacture of glycerol stocks and plasmid extraction the next day.

The PCR tubes containing water with bacterial isolates were PCR amplified using M13 primers (see Insert Size Checks section above) and visualised on a 1.5% agarose gel in TBE buffer. Five microlitres of M13-PCR products of the appropriate size (>220 bp) were mixed with 5 µL of sequencing stop dye and heated to 90°C for 1 minute. Small square nylon filters (N^+, Boehringer Mannheim), were marked in an approximately 1.5 cm^2 grid pattern, and 5 µL of the M13-MCS PCR product spotted (dot-blot) onto the grid pattern, which were allowed to dry and then cross-linked by the same methods used for other filters. Dot-blots were probed and visualised using the same methods as in the probing section above, without the Kim-wipe step.

Double-Stranded DNA Extraction

Plasmids are the insert-containing circular DNA that was screened in the previous few steps. In this step the plasmids are isolated and the DNA used for amplification of the insert, or for sequencing of the insert.

Glycerol stocks of the picked cultures were made using 100 µL of the overnight culture of bacterial cells grown in the step above in LB-broth with ampicillin, tetracycline (both at 50 µg mL^{-1}) and 30% glycerol, and then the glycerol stocks were stored at –76°C.

Plasmid DNA was extracted from the 1.4 mL of overnight culture remaining from the screening of putative positive clones, above. This was performed using a modified X-Gen protocol (modified alkaline lysis) leaving out the guanidine hydrochloride resin step and simply precipitating the DNA with ethanol (Appendix 3). Pellets were re-suspended in 80 µL 1× TE buffer (pH 8.0)

FURTHER MICROSATELLITE DEVELOPMENT STEPS

For complete development of microsatellites further steps in the protocol need to be performed. As the development was not completed, these are outlined below, followed by a second microsatellite development protocol that was attempted.

Single-Stranded DNA Extraction

Isolation of the single-stranded plasmid DNA (ssDNA) is performed using helper phage. Two millilitres of 2× YT-broth containing tetracycline (50 µg mL^{-1}) and ampicillin (50 µg mL^{-1}) are inoculated with Helper Phage (10 pfu) and then with 100 µL of cells from the screening putative positive clones step, above. The infected cultures are then grown at 37°C in a shaking incubator for one hour, after which 1.0 mL of 2× YT-broth with 50 µg mL^{-1} ampicillin, tetracycline and kanamycin (Sigma) is added and the incubation continued.

After the infected cells have grown to saturation (approximately 24 hours), 1.5 mL aliquots are placed in 1.5 mL Eppendorf tubes (two per culture tube) and centrifuged at G_{max} on a benchtop centrifuge for 10 minutes. One and a half millilitres of the supernatant is then transferred to a new tube containing 200 µL PEG, mixed by inversion and incubated at room temperature for 15 minutes. DNA is removed from suspension by centrifuging the tubes at G_{max} on a bench-top centrifuge for 15 minutes, the supernatant aspirated off and the tubes centrifuged again at G_{max} for 2 minutes, the supernatant is again aspirated off. The ssDNA

pellets are re-suspended in 50 µL of 1× TE buffer (pH 8.0) and the pellets combined. The tubes are then placed in boiling water for 2 minutes before storage at -20°C.

Insert Sequencing and Primer Design

Sequencing of inserts is to be performed on an ABI prism 377 automated DNA sequencer (Perkin Elmer Applied Biosystems), using the facilities available at the University of Waikato DNA Sequencing Unit. Reactions are performed using the dideoxy chain termination method (Sanger *et al.*, 1977) and BigDye Terminator Chemistry®.

Primers are designed using a program such as Oligo (Piotr Rychlik and Wojciech Rychlik

Optimisation of PCR conditions is performed on an Eppendorf Mastercycler Gradient thermocycler, using the gradient function to determine the optimal annealing temperature. Initial T_M was either calculated on the basis of the G/C content of the primers developed (Equation 2), or taken from the annealing temperature calculated by the manufacturer of the primers (Life Technologies). Annealing temperature optimisation is performed at 10°C on either side of this temperature (i.e. $T_M \pm 10°C$). The optimal annealing temperature is taken to be the temperature that produced the least bands on a gel, or produced a band that had significantly stronger signal strength than any other in the same lane on the gel.

For primers greater than 10bp in length in 50 mM salt solution (PCR conditions):

$$T_M = 59.9 + 41(\%GC) - (675/\text{ primer length}) \qquad \text{(Equation 2)}$$

Where percent G/C values are the decimal value (e.g. 46% G/C = 0.46) and the primer length is in base pairs.

Screening for Variation

Screening for polymorphisms is performed on DNA extracted from the population level Antarctic moss samples. Sequences that are found to be polymorphic are

developed for full-scale length-polymorphism analysis of the populations.

Screening is performed by labelling one primer with ^{33}P in a 50μL reaction: 1× PNK direct buffer (50 mM Tris-HCL, 10 mM $MgCl_2$, 5 MM dithiothreitol, 0.1 mM sepermidine, final concentrations), 16 U Polynucleotide Kinase (Boehringer Mannheim), 12.0 μL $\gamma^{33}P$-ATP, added to 310 pMol of primer. The reactions are then incubated at 37°C for 30 minutes in an incubator.

Fifty nanograms of DNA from each individual is placed in an appropriately labelled PCR tube and 10.5 μL of a master mix containing 1.6 mM $MgCl_2$, 0.52 μM un-labelled primer, 0.83 μM ^{33}P-labelled primer, 0.16 mM dNTPs (Roche) and 0.5 U *Taq* DNA polymerase (Boehringer Mannheim or Roche). The reactions are then amplified according to the appropriate parameters on an Eppendorf MasterCycler Gradient thermocycler: 94°C for 2 minutes, followed by 10 cycles of 94°C for 1 minute, optimal annealing temperature for 30 s, 72°C for 30 s. Once the amplification is finished, 13 μL of sequencing stop solution is added per reaction, and reactions are heated to 90°C for 1 minute and then iced until loaded on a pre-warmed 6.0% polyacrylamide gel (Appendix 3). The gel is run for different lengths of time according to the expected size of the product (Table 2.3). When the gel run is complete, it is dried and exposed to X-ray film (see Exposing and Developing Films above).

Table 2.4 Polyacrylamide gel electrophoresis times for different PCR product sizes.

Expected product size	Run time (hours)
100 bp	1.5
200 bp	2.0
300 bp	3.0

Microsatellites that exhibit variation will show as different length bands on the gels, these can be assumed to be variants in the microsatellite length. Those sequences that exhibit such a pattern can be used for population analysis.

Scoring Microsatellite Loci

Once polymorphic loci had been determined, the primers designed for each are used to amplify DNA from individuals within each population. This is performed

on polyacrylamide sequencing style gels (Appendix 3), like the ones used in screening for variation. The resulting data is scored by eye or by GeneScan software (Version 2.5) and analysed using Arlequin software, Version 2.0 (Schnieder *et al.*, 2000), Phylip Version 3.5 (Felsenstein, 1993) or PAUP* (phylogenetic analysis using parsimony *and other methods) (Swofford, 1998).

MP-PCR DEVELOPMENT

Based on the protocols of Weising *et al.* (1995), microsatellites were developed using RAPD-PCR protocols to amplify DNA from the moss samples and extract those sequences that contained microsatellite sequences.

This was performed using a RAPD reaction, primed with a microsatellite primer as a second primer in a reaction known as MP-PCR or microsatellite-primed PCR (Ramser *et al.*, 1997a). At the same time a RAPD reaction was set up with a RAPD primer. MP-PCR and RAPD reactions were performed following Balakrishna (1995) with the following conditions: 1× PCR buffer (1.5 mM $MgCl_2$, 50 mM KCl), 0.1 mM each dNTP, 0.5 µM Primer [Operon Technologies (RAPD) or Gibco BRL (MP-PCR)], 1.5 U *Taq* DNA polymerase (Roche) in a 25 µL reaction volume. Cycling conditions on an Eppendorf MasterCycler Gradient thermocycler were as follows: 94°C for 2 minutes, followed by 41 cycles of 94°C for 1 minute, 37°C for 1 minute and 72°C for 2 minutes, with a final extension of 72°C for 4 minutes. Samples were held at 4°C before loading onto a gel. Small amounts (2.0 µL) of these reactions were electrophoresed on small 1.5% TBE-agarose gels containing 0.1 ng mL^{-1} ethidium bromide (Appendix 3) for three hours so that the banding patterns could be fully seen. Each lane that had a MP-PCR electrophoresed in it had a normal RAPD reaction run beside it so that the difference in banding pattern could be determined. Gels were visualised using an EagleEye DNA visualisation system (Stratagene).

MP-PCR development was unable to proceed past the visualisation on gels due to time constraints, however, further steps are given below:

When it has been determined which MP-PCR amplified samples contained bands that are different to those found in the standard RAPD reaction, the MP-PCR samples are again electrophoresed on a large polyacrylamide gel (Appendix 3),

the banding pattern is Southern blotted onto a nylon membrane (N^+, Boehringer Mannheim) and the gel dried to preserve the banding pattern for a later step. The filter is probed with a repeat oligonucleotide labelled with γ^{33}P-ATP (see Probing section above). The resulting bands seen on an autoradiograph are those that contain a microsatellite sequence. The autoradiograph is then aligned with the original gel and those bands that had show a positive result are excised and the DNA extracted using a Concert™ Gel Extraction System (Life Technologies). Form here, the extracted DNA is cloned into a vector that will ligate PCR products such as pGEM-T easy vector systems (Promega), or via TA cloning. The ligated MP-PCR-product and vector are then transformed into a bacterial host, such as XL1-Blue (Stratagene) and the resulting clones are grown up as in the main microsatellite development protocol above. After this the plasmids are extracted, M13 MCS amplified and dot-blotted to confirm the presence of a microsatellite containing insert. The extracted DNA is to be sequenced using BigDye Terminator Chemistry® (ABI Prism, Perkin Elmer Applied Biosystems) at the University of Waikato DNA Sequencing Facility.

Microsatellite containing sequences obtained from this are used to design primers for the amplification of the microsatellite regions. These primers are then used to screen populations for variation as in the main microsatellite protocol.

CHAPTER 3

RESULTS

GENOMIC DNA EXTRACTION

DNA extractions generally resulted in large quantities of high molecular weight DNA being produced from the samples extracted (Figure 3.1). The DNA concentrations of extracted stock solutions ranged from 4 to 90 ng μL^{-1} as determined by a modified Saran™ wrap method (Sambrook *et al.*, 1989), using an EagleEye DNA still visualisation system (Stratagene) to estimate the concentrations, based on the strength of fluorescence of samples relative to standards.

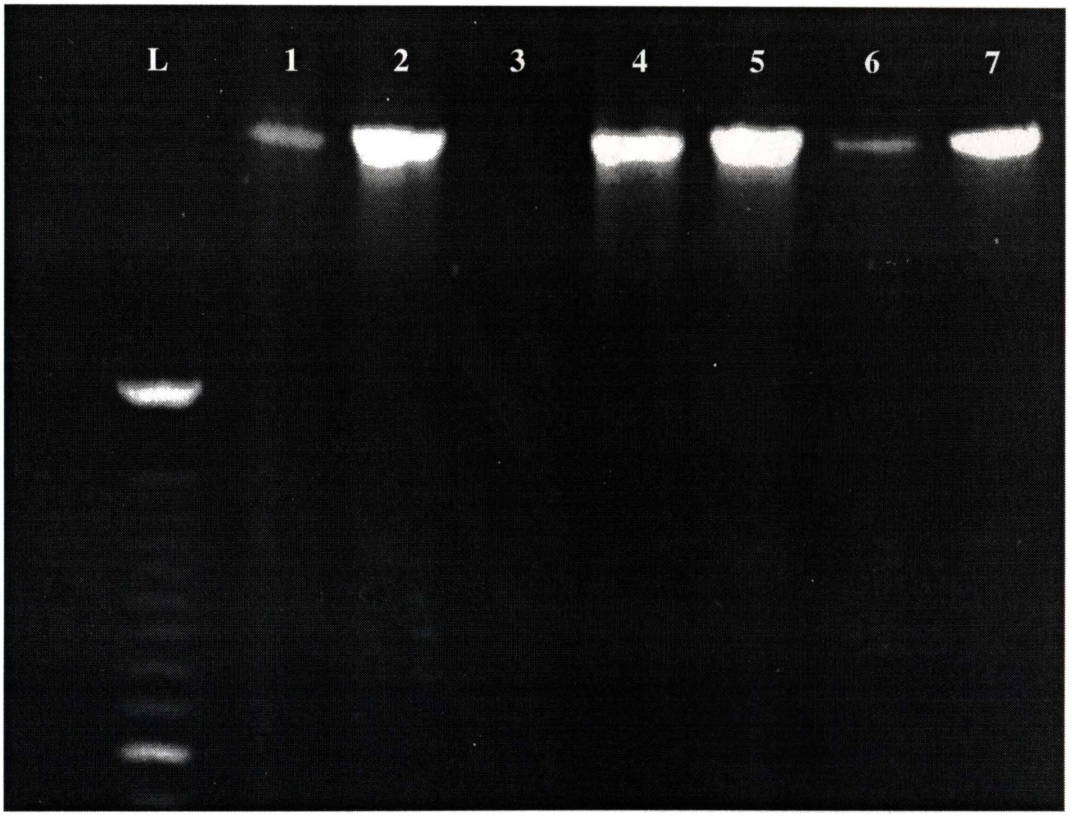

Figure 3.1 Extracted DNA from New Zealand *Bryum argenteum*, lane numbers 1-7 are samples HR1/1 BA, HR 1/2 BA, blank, HR 2/1 BA, HR 2/2 BA, HR 3/1 BA, HR 3/2 BA respectively. Lane marked (L) contains 0.3 µg 100 bp ladder (Life Technologies). Lane 3 was left as a blank as the well was damaged. Concentrations of the samples in ng μL^{-1} are, left to right: 10, 36, 18, 34, 4, and 28 as determined by a Hoefer DNA fluorometer (Hoefer Scientific Instruments).

It was found that the GeneQuant II (Pharmacia Biotech) gave inaccurate readings of DNA concentrations, commonly producing zero readings for the DNA

concentration, such as for the samples above. However, this instrument was useful for determining the purity of the DNA. This is performed by calculating the ratio of absorbance readings at 260 and 280 nm. Pure DNA has a reading of 1.8 (Sambrook *et al.*, 1989). 260:280 nm ratios for the above samples ranged from 1.917 to 1.300 with an average of 1.714, indicating that the DNA was reasonably pure.

FUNGAL DETECTION

Internal Transcribed Spacer Regions (ITS)

Polymerase chain reaction (PCR) amplification of the ITS (internal transcribed spacer) region of nuclear ribosomal DNA involves the use of specific primers to amplify between the 18S and the 26S genes, encompassing ITS1 and ITS2 as well as the 5.8S gene (Baldwin, 1992) (Figure 3.2).

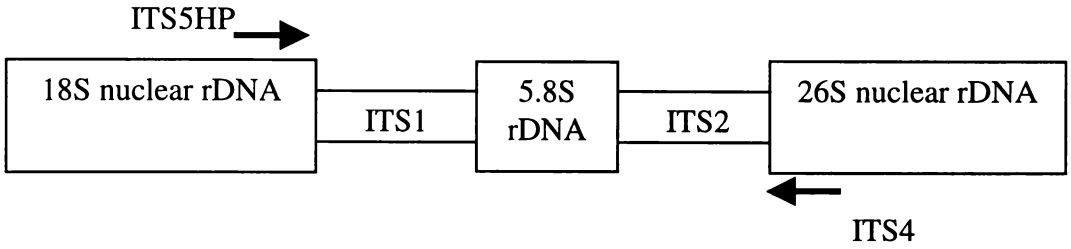

Figure 3.2 A schematic of the ITS regions of nrDNA showing the positions of the 18S, 5.8S and 26S ribosomal genes with ITS1 and ITS2 between them (not to scale). Primer binding sites are shown as arrows marked as "ITS4" and "ITS5HP".

Amplification of the ITS regions for the purposes of this project, was to detect fungal contamination of the DNA extracted from Antarctic mosses. Hunger (2000) established that contamination of mosses by fungi could result in DNA extracted from the moss being contaminated by fungal DNA extracted simultaneously. Amplification of the ITS region of nuclear ribosomal DNA from Antarctic moss samples indicated the presence of a contaminant in the moss. Un-contaminated samples produced a single band of approximately 1100 bp in length (Figure 3.3). Contaminated samples produced a band approximately 550 bp in length (Figure 3.4). This test was performed on all samples used for microsatellite development to determine if there was likely to be a fungal contaminant in the extracted DNA from these samples (Figure 3.3).

The distinctive second PCR-amplified band of the ITS region in some Antarctic samples allowed subsequent easy identification of contaminants in moss DNA

extracts. However, not all contaminated samples were able to be identified in this manner, thus the RAPD-PCR analysis was also performed.

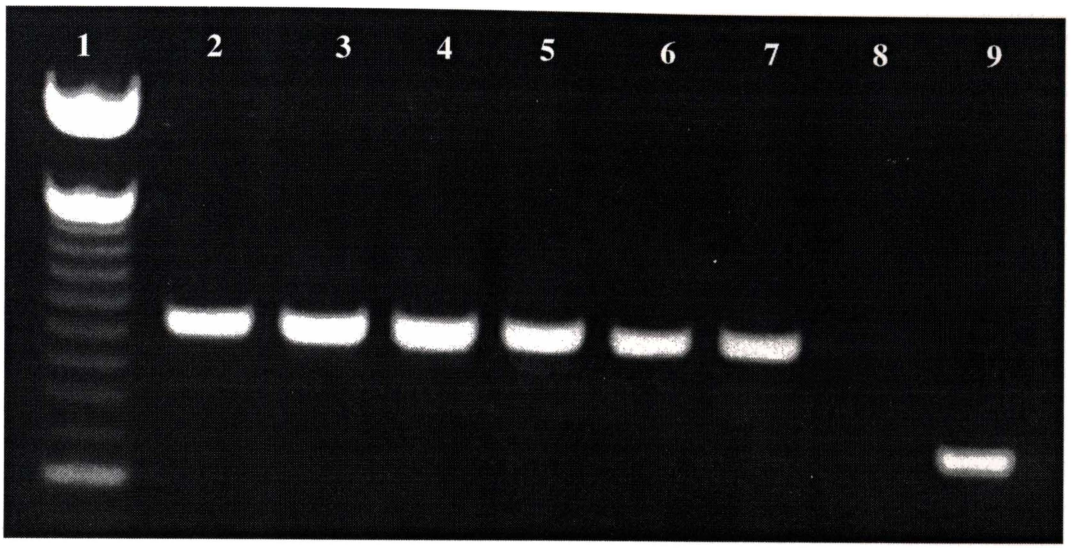

Figure 3.3 ITS bands from un-contaminated samples. Lane 1 contains 0.3 µg 100 bp ladder (Life Technologies), the three brightest bands on the ladder are; top to bottom, 2072, 1500 and 600 bp, other bands are at 100 bp intervals. Lanes 2 - 7 are bands from the New Zealand moss samples (HR series) used in microsatellite development. Lanes 8 and 9 contain negative and positive controls (*Pittosporum cornifolium*) respectively.

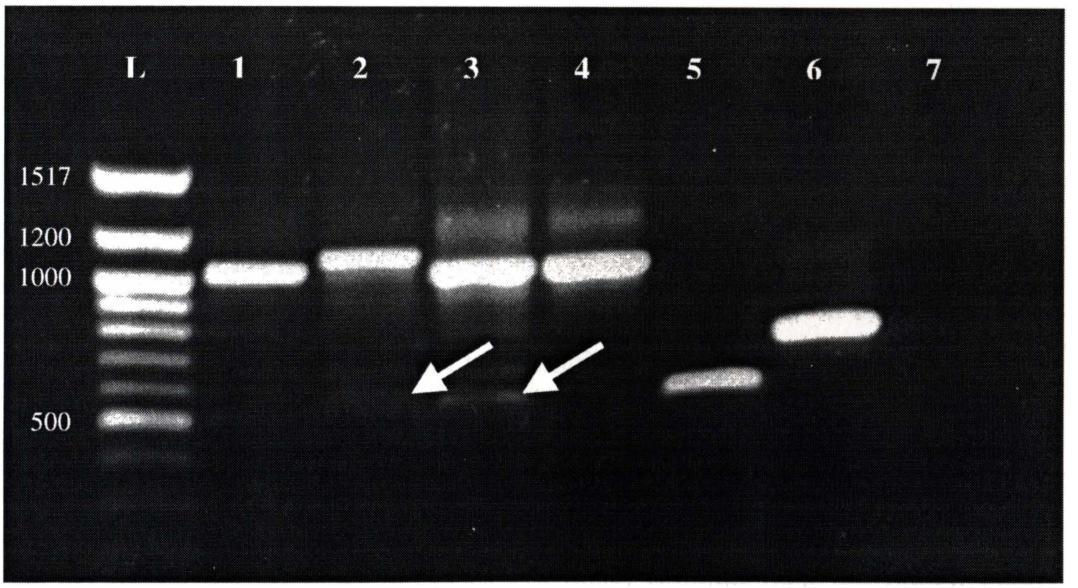

Figure 3.4 ITS products showing the multiple bands characteristic of contamination of the DNA. Lane marked "L" contains 0.3 µg 100 bp ladder (New England Biolabs), band sizes in base pairs are marked next to the ladder. Lane 1 contains an un-contaminated Antarctic moss sample (SH 16 BA), lanes 2 and 3 contain contaminated Antarctic moss samples (SH 27-2 BS, SH 23-1 BP), note bands marked by arrows, lane 4 contains a New Zealand moss sample (HR 1/2 BA) with no fungal contaminant, and lane 5 contains amplified fungal DNA. Lanes 6 and 7 contain positive (*Pittosporum cornifolium*) and negative controls respectively.

Random Amplified Polymorphic DNA (RAPD)

RAPD-PCR was used to determine the presence of fungal DNA in the DNA extracted from the moss samples that had been extracted. It was determined that this system was possible by Hunger (2000) after the discovery that some Antarctic moss samples had fungal contaminants in them. The work of Skotnicki *et al.* (1998a, b, c) using RAPD analysis to determine the relatedness of populations of Antarctic mosses had found that there was hyper-variability among the populations studied (mostly in Victoria Land), and it was thus brought into debate as to whether it was possible that the extreme variability seen was due to fungal contamination of the samples that were used in this analysis. Figure 3.5 presents a typical RAPD banding pattern produced by a sample of Antarctic *Bryum subrotundifolium* free of fungi (SH 16 BA), a sample of Antarctic *B. argenteum* known to have fungal contaminants, (SH 21 BA), an Antarctic *B. subrotundifolium* known to have fungal contaminants (SH 34 BS), one New Zealand specimen of *Bryum argenteum* (HR 2/1 BA), a fungus (*Phoma* sp.) and lastly a reaction in which fungal DNA and moss DNA (SH 16 BA) were combined ("spiked").

A comparison of the of the banding patterns from the amplified DNA samples indicated that fungal contaminants were present in many Antarctic moss samples, and that this could often be verified on whole voucher specimens under magnification through a stereo-microscope (20×), although fungal hyphae were not observed on all samples that the RAPD analysis indicated had fungal contaminants. It can be seen in Figure 3.5 that there are common banding patterns among the moss samples and with the combined fungal and moss DNA samples. As can be seen there is a significant difference between the moss samples and the fungal sample, although there are some similarities between the contaminated samples and the fungal sample, some of the bands obtained from the "spiked" sample match the moss bands, these are much brighter than the fungal bands, however these are still present, but the pattern obtained is not the same as from those samples that were contaminated at extraction. This pattern for the "spiked" sample may be due to the different levels of fungal DNA in each, with this sample having a much higher level of fungal component than the contaminated samples.

RAPD amplification of fungal DNA alone produced a banding pattern distinct from that produced using non-contaminated *Bryum* DNA, however the banding pattern produced by samples that were contaminated was a combination of both of the banding patterns above, with some bands missing from either of the first two patterns. It is not known why there should be some bands absent from patterns produced by the contaminated samples, when compared to those produced by the uncontaminated moss and fungal extractions, but it is thought that there could be preferential binding of primers to one DNA over the other for some binding sites in the PCR (Black IV, 1993). It should be noted that potentially, not all fungi present on the moss samples have been identified, and other species may be present that would produce different banding patterns in a RAPD reaction, thus this method is not an absolute for detection of potential contaminants in the moss DNA.

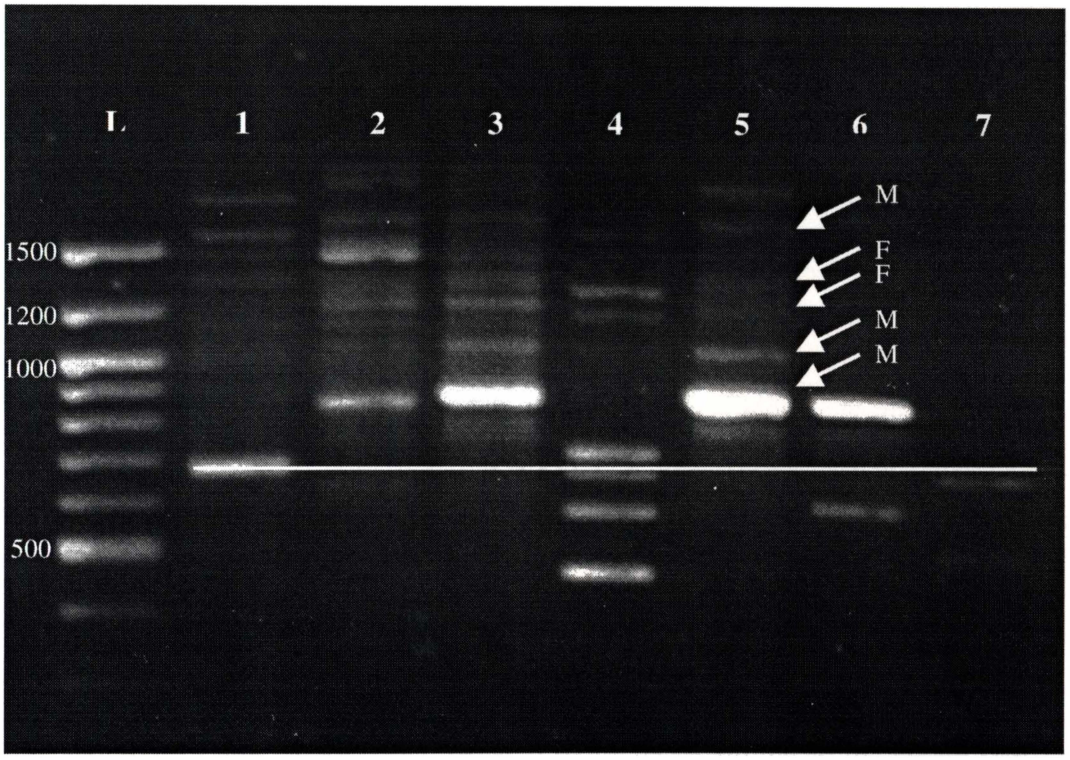

Figure 3.5 Banding patterns produced by RAPD-PCR of three moss samples (Lanes 1 – 3, SH 16 BA, SH 21 BA, SH 34 BP), a fungal sample isolated from an Antarctic moss (lane 4), and a combined moss (SH 16 BA) and fungal DNA sample (Lane 5). Lanes 6 and 7 contain positive (*Pittosporum cornifolium*) and negative controls respectively. Lane marked L contains 0.3 μg 100 bp ladder (Life Technologies). Note the band (line) in the negative control, this should be ignored in any other lane in which it is found. Shared bands are marked by arrows in the spiked sample lane, M indicates moss, F indicates fungal.

The faint band seen in lane 1 of the above figure is probably the result of degraded DNA. Incorrect annealing temperature is seen in other lanes (2, 3, 5) where a

higher level of background amplification was observed, making it harder to distinguish the banding pattern (not shown). Note the inconsistency in the banding pattern in lanes 1 and 5, both of which contain the same moss DNA sample.

MICROSATELLITE DEVELOPMENT

Microsatellite development followed a modified Glenn (2001) protocol as extracted from the website (http://www.uga.edu/srel/DNA_Lab), January 2001. The modifications made to this protocol were to suit the facilities available at the University of Waikato and as suited the results obtained at each step. Changes to the protocol were also made in areas where there were incomplete or incorrect instructions for a particular step. The results for each step will be discussed under the same titles used in the materials and methods chapter.

Genomic DNA Preparation and Size Selection

Genomic DNA was digested using DpnII restriction enzyme, this enzyme produces ends on the DNA, after restriction that are complementary to those produce by Bam HI, the enzyme used in vector preparation. The restriction sequence for Dpn II is:

5′- ↓ GATC -3′

3′- CTAG ↑ -5′

An example of digested genomic DNA is seen in Figure 3.6. For use in later steps, the region between 300 and 700 bp in length was excised from the gel and the DNA extracted. This was the size selection step, which is purported to increase the number of informative microsatellites [those over approximately 10 repeats (Ashley and Dow, 1994)] isolated by methods similar to the Glenn (2001) protocol.

Vector Preparation

Vector preparation involved digestion of the pBluescript KS® + phagemid vector with Bam HI, which has a recognition and restriction sequence:

5′-G ↓ GATC -3′

3′- CTAG ↑ G -5′

The ends produced by BamHI, as can be seen, are complementary to those produced by Dpn II and as such will allow the complementary binding of the two DNA strands together in a ligation reaction using T4-DNA ligase, if the ends of the vector are dephosphorylated, otherwise the DNA ligase cannot join the DNA strands correctly.

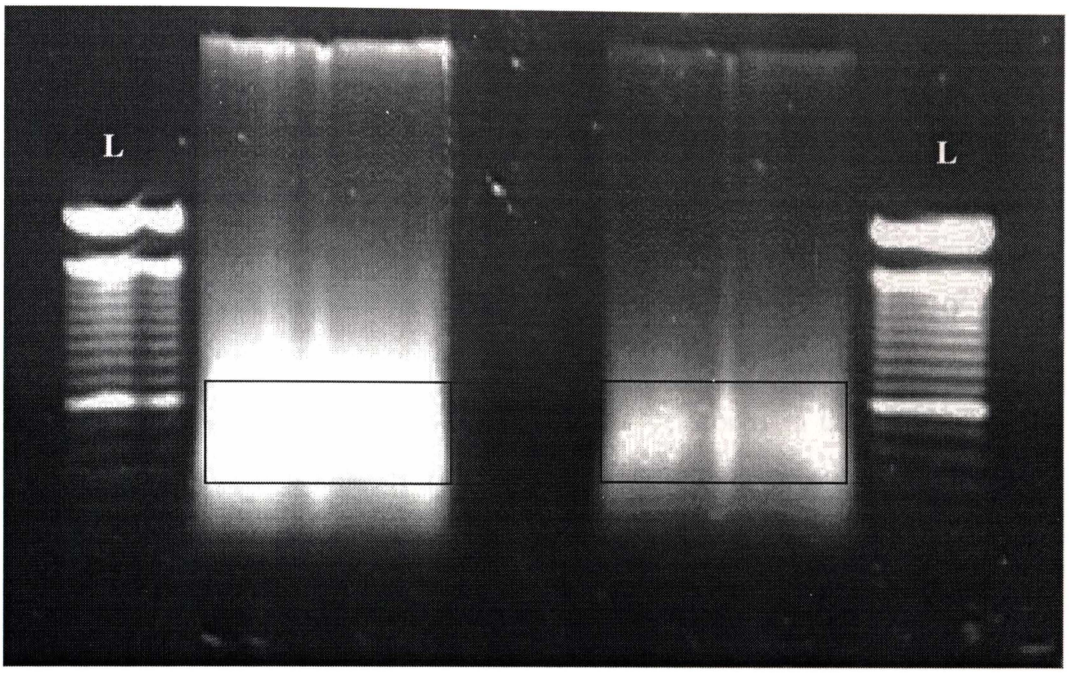

Figure 3.6 Digested genomic DNA from samples HR 2/2 BA and HR 3/2 BA. Lanes marked L contain 0.3 μg 100 bp ladder. The area marked with a box contains the regions extracted from the gel at the size selection step. The bright band half way down the ladder is 600 bp in size, bands above and below this are in 100 bp increments. The regions excised for DNA extraction are marked by blue boxes.

Ligation of Genomic DNA into the Vector

Ligations were performed between the digested genomic DNA and the digested, dephosphorylated vector using T4-DNA ligase. A 1:1 ratio of vector to insert (genomic DNA) was initially trialed, however it was found that this did not give sufficient numbers of transformants at the transformation step (more blue colonies that white in a ratio of roughly 3:1). To improve the ligation efficiency, a ratio of 3:1 (insert to vector) was used. This gave an average of 63% white colonies per library plate. An empirical method of determining the efficiency of the ligation has not been established, although Sambrook *et al.* (1989) recommend electrophoresis of small portions of this to see the relative brightness of the bands produced as re-circularised vector and vector with an insert in it should be

different sizes. This method, however, will not give an exact measure of the relative amounts of ligated vector and insert and non-ligated vector.

Competent Cell Preparation

Completion of the competent cell preparation required optimisation to obtain high transformation efficiencies. Attempts at making large quantities of competent cells were unsuccessful initially by a scaled-up Nishimura *et al.* (1990) method, possibly due to the cells warming too much during the re-suspension step thus losing their competency. This resulted in the cells having low competency and hence the transformation efficiency was low. When the preparation of competent cells had been optimised, for both methods, it was found that fresh preparations of competent cells were needed for a high transformation efficiency (~1 $\times 10^7$ per µg insert DNA) using the modified Chung *et al.* (1989) method, despite the recommendation of the paper that the cells can be stored in the 1× TSS buffer (~5 $\times 10^6$ per µg insert DNA after one week storage). In contrast, the Nishimura *et al* (1990) method produced cells of high transformation efficiency both with fresh preparations (~8 × 10^7 per µg insert DNA) and with cells stored at -76°C (~3 $\times 10^7$ per µg insert DNA after two weeks storage), these values are taken from the control three plates.

Heat Shock Transformations

Transformation efficiencies varied according to the volume of ligation reaction added to the cells and by the competent cell preparation method used. It was found that the optimal volume of ligation reaction to add to a 100 µL aliquot of competent cells was 5.0 µL (data not shown). On average library transformation efficiencies were approximately 5.5× 10^6, with a range from 9.1× 10^5 to 1.5× 10^7. Upwards of 300 colonies could be found on many of the 90 mm diameter plates used for this step. Colony size was generally small, commonly being 1-2 mm in diameter, compared to the suggested colony size of 2-3 mm for these results (Glenn, 2001). This was attributed to the plates being too dry for proper growth of the cells. Small satellite colonies were also often observed on the plates, especially surrounding blue colonies. These may have been due to old ampicillin, however no change was noted when fresh stocks of ampicillin were used.

Insert Size Checks

Insert checks were performed by PCR amplification of the M13 multiple cloning site (M13 MCS) on the vector. This was performed to determine if the ligations had succeeded. Amplification was only performed on colonies that exhibited a white phenotype, indicating the presence of an insert in the vector. A typical gel from the insert checks is shown in Figure 3.7. Amplification of this region was not always successful, as can be seen from the absence of a band in some lanes.

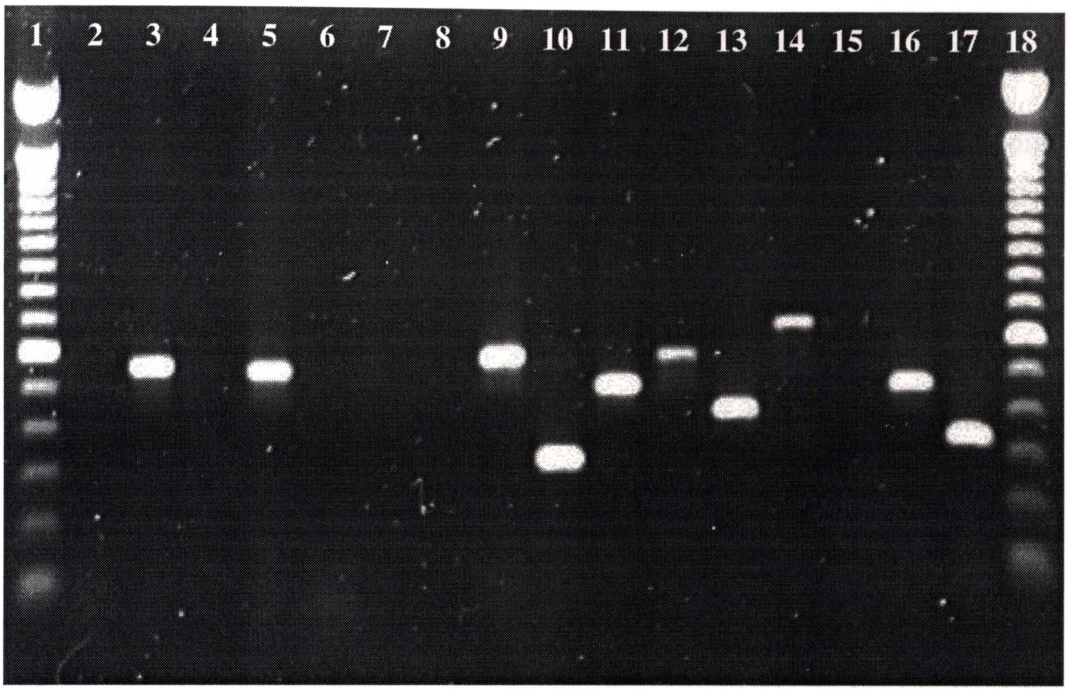

Figure 3.7 A typical gel from the insert checks, lanes 1 and 18 contain 0.3 µg 100 bp ladder (Life Technologies), the bright band at the top of the gel is 2072 bp, the band half way down the ladder is 600 bp. Lanes 2 - 17 contain the PCR products of the amplification of the M13 MCS in pBluescript KS® +. Negative and positive (amplified 10 ng of pUC 18 control plasmid) controls are not shown.

Early attempts at ligation and transformation produced average insert sizes of approximately 220 bp, although the size of the inserts ranged from 0 (no insert) to approximately 425 bp (650 - 223 bp). Only one out of the 60 white colonies picked contained no insert, although some appeared to have very small insert sizes (~100bp). This result is unexpected, as the average size of the insert should be between 300 and 700 bp as a result of the size selection step. These results suggest that shorter insert lengths may have been preferentially incorporated into the vector. This is because the shorter lengths of DNA produced by the restriction reaction have a greater molar concentration of ends in solution, relative to the larger fragments, thus there is preferential incorporation of the smaller fragments

into the vector as the rate of reaction is controlled by the molar concentration of ends in solution (Sambrook *et al.*, 1989; Beckler *et al.*, 1996).

Subsequent attempts at amplifying this region generated plasmids that contained larger inserts. For example, amplification of the M13 MCS for the screening of putative positive clones gave an average insert size of approximately 330 bp with a range of 100-700, which is significantly higher than that seen for the first data set.

Lifting of White Colonies onto Filters

Lifting of colonies onto filters is a routine step in most genomic library screening procedures. To transfer the library onto a filter in such a manner that the DNA can later be bound to the filter involves careful handling of the plates and sensitive membranes. First of all the membrane must be placed carefully on the plate so that all of the filter becomes wet, while excluding air bubbles, once this is performed the plate and filter can be left to incubate at 4°C for several minutes. The bacteria are then lysed by placing the filter colony side up on a mild detergent (10% SDS); at this stage, the surface of the filter becomes yellow with the digested cells. After this, the DNA is denatured to allow binding of the DNA to the positively charged membrane, performed on a saline base solution. The filter is then placed on a neutralising solution that allows the DNA to bind to the filter and finally washed to remove excess bacterial proteins. After this, the filter is dried and the DNA more firmly bound to the filter by cross-linking with short-wave ultra-violet light (UV). The UV light breaks some of the DNA: DNA bonds that have formed during the extraction process, creating sites with a negative charge that can bind to the positively charged filter by covalent bonds (Brown *et al.*, 1991). Six filters were lifted with a total of 1301 white colonies divided between them.

Probing of Filters

There are two main steps to probing of the filters. The first step involves end-labelling of oligonucleotides that are complementary to the sequences being screened for; in this case, this involved the use of γ^{33}P-ATP, a weak β emitter. The second step involves the hybridisation of the labelled probes to the filters.

End-labelling of oligonucleotides is carried out by Polynucleotide Kinase (PNK) and enzyme that detaches the α and β phosphates from the nucleotide tri-phosphate and uses the energy released by this process to catalyse the addition of the remaining phosphate onto the 5′ end of the oligonucleotide. For this reason a γ-labelled nucleotide-triphosphate is used in this reaction. There is no empirical method of determining the effectiveness of this reaction until after the hybridisation and film development steps.

The effectiveness of a hybridisation is measured in the degree of background labelling of the filter, if none is detected, then the hybridisation can be termed a success. The hybridisations performed in this project showed little background hybridisation, but exhibited a high degree of non-specific hybridisation, with all or most of the colonies showing hybridisation, making it difficult to determine the presence or absence of positive clones. Some colonies however, showed as significantly darker patches on the developed film (Figure 3.8), these were taken as putative positive clones.

The first attempts at hybridisation were performed using $(GA)_7$ and $(GT)_7$ oligonucleotide repeats using a 12 hour hybridisation and two washes as in 6× SSC, 0.1% SDS (R. J. Wilkins, *pers. comm.*). These probes failed to hybridise at all, even to definite $(CA)_n$ and $(CT)_n$ sequences amplified from mouse DNA, that had been spotted on to a small piece of filter to test the hybridisation reaction.

Initial attempts at hybridisation with the 30-mer probes were performed using a twelve hour hybridisation and two washes as above. It was observed that the levels of non-specific hybridisation were high, thus making resolving of positive clones difficult. To avoid this problem, the hybridisation time was shortened to one hour, with the same washes performed afterwards as recommended by Glenn (2001). The levels of non-specific hybridisation remained high. Thus for the final hybridisation protocol, more-stringent washes were performed after the one hour hybridisation. In all subsequent hybridisations the filters were washed stringently in three washes firstly in 2× SSC, 0.5% SDS, secondly in 2× SSC, 0.1% SDS and finally in 0.1× SSC, 0.5% SDS, the first two at room temperature and the final wash at the wash temperature (Table 2.2). This resulted in a lower level of non-specific hybridisation, and showed 14 putative positive clones

containing a $(CT)_n$, $(CA)_n$ or $(GTT)_n$ repeat microsatellite sequences being probed for using $(GA)_{15}$ and $(GT)_{15}$ $(CAA)_{10}$, γ^{33}P-dATP labelled oligonucleotides. Other oligonucleotide sequences were attempted in an effort to isolate microsatellite sequences from the clones. These sequences were $(TA)_{15}$, $(TG)_{15}$ and $(AGG)_{10}$, hybridisation with these sequences produced no more putative positive clones. The reason for the absence of positive clones with these sequences is not known as they are reported to be more frequent in plants than the oligonucleotide probes initially used for these steps (Lagercrantz *et al.*, 1993; Morgante and Oliveri, 1993; Gupta *et al.*, 1996).

Attempts were made to remove bound probe from the filters before a new probe (or combination of probes) was hybridised to the filters, initially this was a success, with little radioactivity exhibited by the filters when they were scanned with a Geiger counter (<100 counts per minute, data not shown). Failure to remove much of the probe at later washes was probably due to accumulation of the probe over several hybridisations, through the probe binding to the filter during the drying step. The drying step was necessary as the filter would otherwise stick to the film while being exposed, if the filter was wrapped in plastic wrap (as filters with ^{32}P are), the weak β-emission from the ^{33}P would be unable to penetrate the plastic, thus giving no result on the film.

Exposing and Developing Film

Film exposure length was dependant on the amount of radioactivity exhibited by the hybridised filters; generally, the signal strength was weak enough that the exposure times could take place over 16+ hours at room temperature. A typical autoradiograph is shown in Figure 3.8.

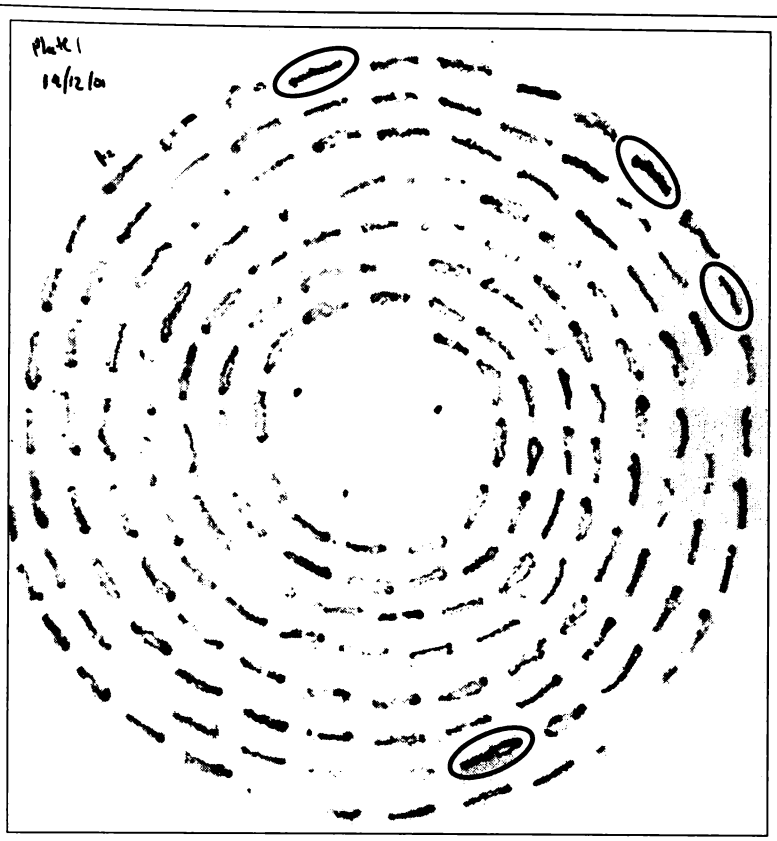

Figure 3.8 Typical autoradigraph of a filter after hybridisation with a combination of $(GA)_{15}$, $(GT)_{15}$ and $(CAA)_{10}$ radiolabelled oligonucleotides. Dark streaks represent colonies with non-specific hybridisation, "brighter bands" represent the putative positive clones. Clones picked for screening of putative positives are marked with circles, from bottom, anticlockwise around the filter, clones were numbered 1 – 4. Note the "12 o'clock" mark in the upper left and the sites of the needle holes (three spots) in the centre of the autoradiograph.

Screening Putative Positive Clones

The presence of putative microsatellite sequences in the cloned DNA, were indicated by the presence of very dark patches on the X-ray film. The corresponding clones, along with some that were not obviously positive (to act as a negative control for the next stage), were re-picked and grown overnight to enable extraction of plasmid DNA and to make a glycerol stock of these clones.

Plasmids containing inserts were extracted from the putative positive clones (Figure 3.10) and the M13 multiple cloning site (M13 MCS) amplified with universal primers to estimate the size of the insert and to establish if it was possible to sequence the plasmid and obtain the insert. This step was necessary as occasionally the plasmid will incorporate an insert into regions outside the M13 MCS due to non-specific cutting of the plasmid and as such is not easily

sequenced as the total length of the plasmid is over 2000 bp, which is too long for conventional dideoxy-sequencing methods.

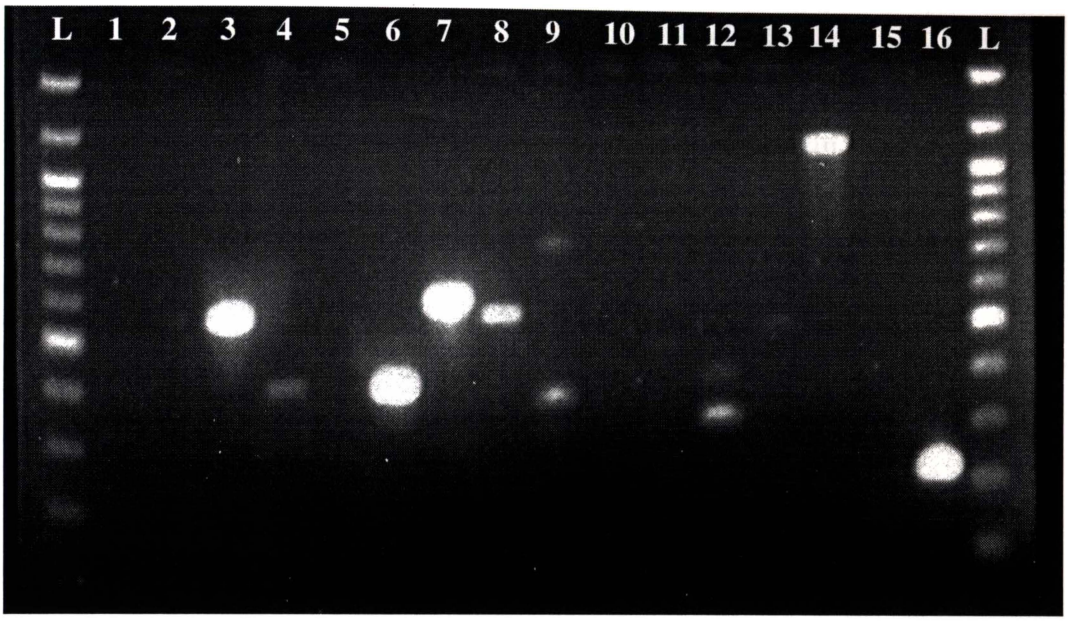

Figure 3.9 M13 MCS PCR-products from picked putative positive clones. Lanes 1 – 14 represent picked clones 1- 14 from filters screened with $(GA)_{15}$, $(GT)_{15}$ and $(CAA)_{10}$. Lane 15 is a negative control and lane 16 contains a positive control (amplification of 10 ng uncut pUC18). Lanes marked L contain 0.05 µg 100 bp ladder, the top band is 1517 bp in length, below this is a band at 1200 bp, followed by 1000 bp, all other bands are at 100 bp intervals. Note the double bands in lanes 9 and 12 as well as the very large band in lane 14.

Second, dot-blots of the M13 PCR products (from both dsDNA and direct from the bacteria) were hybridised to nylon filters and probed using the same labelled oligonucleotides as were used to probe the filters from which the clones were isolated. Dot-blots of the positive amplification products produced no result when hybridised with the same probes used to identify the original positive clones. It can be assumed from this that there were no microsatellite sequences present in the clones isolated as positives. The number of false positives seen in the probing step may be a result of the small difference between a signal and non-specific binding of the radio-labelled probes used to screen the colonies.

Double-Stranded DNA Extraction

dsDNA was extracted using a modified X-Gen method (Appendix 3), in which the bacterial cells are pelleted and then re-suspended in a pre-lysis buffer. After this, the cells are lysed in alkali, the solution neutralised and the protein debris

removed. DNA is precipitated with cold ethanol, pelleted and re-suspended in water or TE buffer.

During this process, several steps have obvious products; the lysis step results in the clearing of the cell suspension, due to the lysis of the cells, the solution also becomes very viscous from the high levels of denatured protein in solution. The neutralisation step results in the proteins "salting out" of solution forming a visible white precipitate, which is removed by centrifugation. Precipitation of the DNA produces and instant gelatinous layer in the solution, this is the DNA coming out of solution, incubation at -20°C aids this process, resulting in a higher yield of DNA. Re-suspension of the plasmid DNA from the bacteria requires only a short re-suspension period, otherwise co-precipitated genomic DNA will re-suspend as well, causing the plasmid preparation to be impure, making subsequent amplification and sequencing of the plasmid difficult (Figure 3.10).

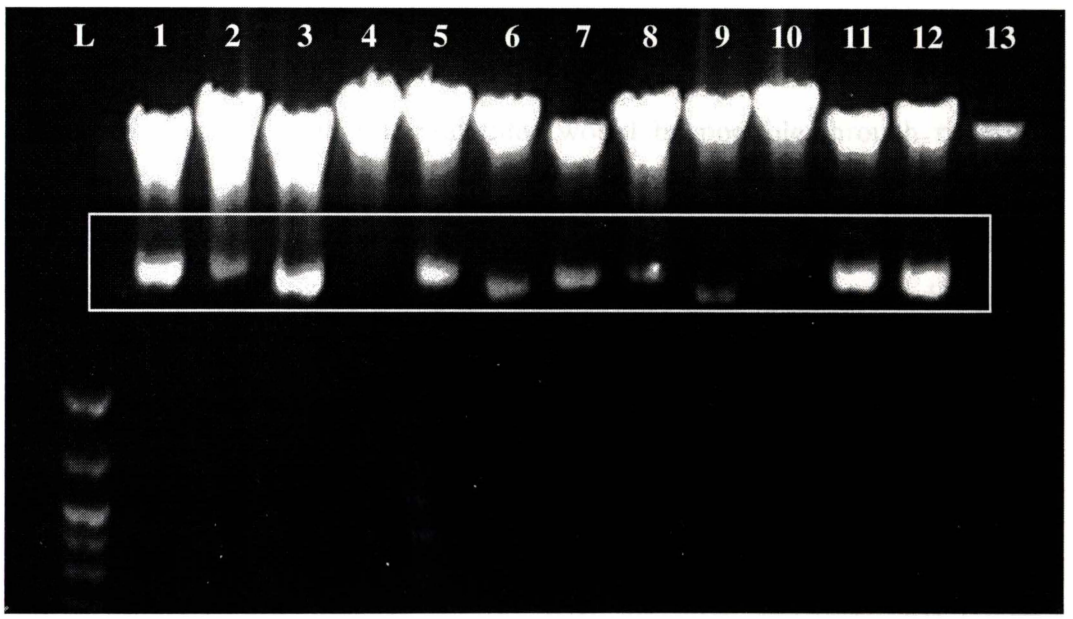

Figure 3.10 Double stranded plasmid DNA isolates from bacteria containing putative positive microsatellite clones. The large bright mass is genomic DNA from the bacteria; the smaller band below this is the plasmid (enclosed in the box). Variation in the degree of migration of this band is due to size differences of the insert. Note single band in lane 13.

As can be seen from the plasmid extracts below (Figure 3.10), there is a range of sizes of insert in the vectors, giving the variation in the migration of the smaller bands with electrophoresis. Note lane 13, which should be compared to lane 14 in Figure 3.9, as one plasmid extract failed to work. Lane 13 has no large bright

genomic DNA mass, but shows a band of comparable size to the genomic DNA. The amplification product of the M13 MCS for this band resulted in the large band seen in land 14 of Figure 3.9. Thus, it can be assumed that the band seen in lane 13 of the plasmid extracts, is the plasmid.

MP-PCR DEVELOPMENT

RAPD-based microsatellite development followed a modified Weising *et al.* (1995) protocol, in this the microsatellite regions were amplified using a microsatellite oligonucleotide as a primer in what is similar to a RAPD reaction, but is known as MP-PCR (Ramser *et al.*, 1997a), using the PCR conditions of Balakrishna (1995). As the system of Weising *et al.* (1995) required only the use microsatellite oligonucleotide, and involved Southern blotting of the resulting gel to enable the isolation of microsatellite sequences, it was decided to attempt to isolate microsatellites through the use of a RAPD reaction that used a RAPD primer, along with a microsatellite oligonucleotide as primers in a single reaction. It was reasoned that this system should produce banding patterns that were different from those that would be seen in a standard RAPD reaction. Isolation of those bands that contain microsatellites would be possible through running a standard RAPD reaction (primed using the same RAPD primer as was used in the MP-PCR) concurrently on the gel next to the reaction primed with the microsatellite oligonucleotide. This system should show those bands that are different between the two reactions, allowing isolation of the different bands for cleaning and sequencing.

Initially to establish whether the standard RAPD protocols could amplify the genomic DNA when a microsatellite sequence was used as a primer, RAPD reactions were set up with the microsatellite sequence as a sole primer. The products from this reaction were seen as high molecular weight smears (>2000 bp) on the gel with some faint and indistinct bands between 1500 and 500 bp (Figure 3.11). Bornet and Branchard (2001) found in a similar situation, that the smear was not due to non-specific primer binding, but rather due to high levels of PCR-product, for this they performed annealing temperature optimisation.. However, I suspect that the smear in this case is due to non-specific binding as the annealing temperature in the reaction was 37°C, this is 11°C below the Wallace temperature (Wallace *et al.*, 1979), potentially resulting in a high level of non-

specific primer binding, also too the smears did not disappear when a lower volume of PCR-product was loaded on the gel (Bornet and Branchard, 2001).

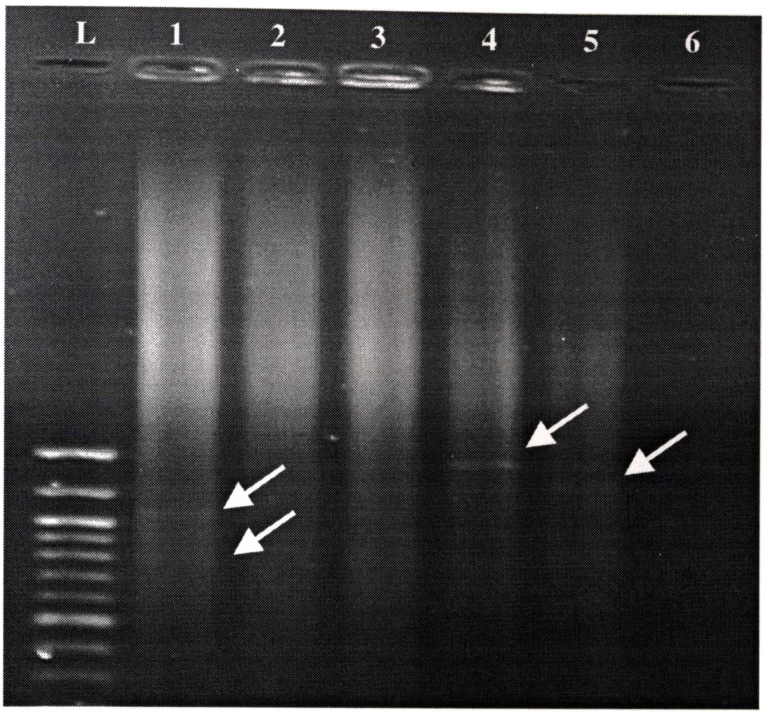

Figure 3.11 PCR-products of RAPD reactions primed with a $(CAA)_6$ microsatellite oligonucleotide. Lanes 1 – 4 contain amplified DNA from samples HR 1/2 BA, HR 2/1 BA, HR 2/2 BA, HR 3/2 BA. Lanes 5 and 6 contain positive (*Pittosporum cornifolium*) and negative controls respectively. Lane marked L contains 0.05 µL 100 bp ladder, top ladder band is 1517 bp in length followed by 1200, and then 1000, all other bands in the ladder are at 100 bp intervals. Note faint bands in the sample lanes marked with arrows.

Unfortunately, time constraints did not allow this avenue to be researched further. It would be interesting to see what a Southern blot of the smears would look like compared to the gel product.

It is not known why the MP-PCR reactions failed to work, but it is suggested that the primer lengths were too long to act as non-specific primers at the temperatures used for annealing. Other potential reasons include there being a very low incidence of microsatellite sequences in the genome of the samples used for this experiment. Other protocols performing similar methods with A/T rich primers and dinucleotide primers found that some samples produced smears similar to those seen above, and that it was not possible to optimise these reactions further (Weising *et al.*, 1995).

CHAPTER 4

DISCUSSION

GENOMIC DNA EXTRACTION

A protocol for DNA extraction, such as that produced by Rogers and Bendich (1985), is essential for situations where the sample size is small and the samples valuable in some manner. The ability to extract high yields of DNA from small samples reduces the risk of losing all or most of a sample from an error during the extraction process. The yields obtained using this protocol were variable (see Figure 3.1) according to the amount of tissue used for the extraction. This may have been due to the transfer of the ground sample from the mortar and pestle to a 1.5 mL tube as the amount of tissue transferred could have varied as some may have stuck to the mortar while the tissue was defrosting. This was probably mostly due to the low temperature of the mortar and pestle from being immersed in liquid nitrogen, condensing water out of the atmosphere and wetting the tissue thus making it adhere to the mortar. At the first chloroform: *iso*-amyl alcohol (C:I) extraction step some DNA may also have been lost due to particles of the ground tissue being less dense than the C:I mixture, and thus forming a dense mat of tissue in the aqueous layer. This lowered the amount of aqueous layer, containing dissolved DNA that could be transferred to the next step, as minimal amounts of tissue should be transferred to the later steps of the extraction.

The main difficulty with extraction of DNA from plants is the high polysaccharide content of the cells. The polysaccharides make up the majority of the cell wall and as sugars, are often co-extracted with the DNA (Murray and Thompson, 1980; Porebski *et al.*, 1997). This problem, along with the tendency of secondary compounds, such as polyphenolics, to co-precipitate with the DNA is often a major problem in DNA extraction and further use of the DNA, such as PCR, which can be inhibited by these compounds (Li *et al.*, 1994). Often it is necessary to remove these compounds by further purification of the stock DNA by further phenol: chloroform: *iso*-amy alcohol extractions (Dellaporta *et al.*, 1983). Mosses are simple plants, producing little in the way of secondary compounds, and having a simple cell wall structure. For this reason the extraction of DNA from these plants is relatively easy, and the DNA obtained is usually "clean" in that it contains little

in the way of polysaccharides or secondary compounds. The purity of a sample can be determined by the ratio of the absorbance at 260 and 280 nm, with a ratio of 1.8 indicating pure DNA, higher values indicate RNA contamination and lower values indicate salt or phenol contamination. For example, the DNA extracts used for microsatellite development had an average ratio of 1.714, indicating that the DNA extracted was reasonably pure. As phenol was not used in the extraction, the values obtained may indicate that polyphenolic-secondary compounds were present (Sambrook *et al.*, 1989).

FUNGAL DETECTION

Internal Transcribed Spacer Regions (ITS)

Hunger (2000) performed a study on the molecular phylogentics of Antarctic and New Zealand *Bryum* species, using the ITS region as a marker to determine the relationships between Antarctic (Victoria Land) *Bryum* species and those from Australia and New Zealand. The findings were that there was a high degree of similarity between *B. argenteum* from New Zealand, *B. subrotundifolium* from Australia, and the *B. subrotundifolium/argenteum* complex from Antarctica. Seppelt and Green (1998) classified Victoria Land specimens of this complex as *B. subrotundifolium*, a classification based on the morphological characters of the specimens collected by the authors, and through careful comparison of the features of the plants in culture. It was found that many of the characteristics of *B. argenteum*, such as the silvery colour of the leaves and colourless hyaline cells in the upper part of the leaf disappeared when grown under culture conditions, leaving plants that closely resembled *B. subrotundifolium* (Seppelt and Green, 1998). Hunger (2000) also noted the presence of two distinct morphotypes of *B. subrotundifolium* at Cape Hallett. One is similar to *B. subrotundifolium*, with silver-yellow appearance and the other is also silvery, but of a much darker green, more similar to *B. argenteum* in superficial appearance, but each had identical ITS sequences, implying that these were morphotypes of the same species. Skotnicki *et al.*, (1997) present some molecular data that *B. argenteum* may also be present in the Ross Sea region.

As a tool for analysing the contamination of plant DNA with fungal DNA, the ITS regions are ideal markers as the different products of the amplification for the ITS regions are very different sizes in the plant and in the fungus (Hunger, 2000). This

can be seen in the amplification of a contaminating DNA in the Antarctic moss analysis performed as part of this study (Figure 3.4). PCR Amplification of the ITS region in *Bryum argenteum*, results in a product that is approximately 1100 bp, whereas the fungal contaminant (*Phoma* sp., Hunger, 2000) an amplification product of approximately 550 bp. This size disparity of the two PCR products allowed the determination of the presence of fungal contaminants in the moss DNA extracted from Antarctic samples. Samples extracted from sites outside Antarctica were also subjected to the same examination as the Antarctic samples, and it was found that the incidence of fungal contaminant DNA being present in samples from outside Antarctica was very low. The reasons for this are not known. It can be speculated that the high incidence of fungal contaminants observed in continental Antarctica is because, as mosses are one of the few potential habitats for the fungus; as they provide a food source and are commonly found in damp areas, which is ideal fungal habitat (Hunger, 2000). This would explain why the majority of the fungi observed have been saprophytic in nature (Block, 1984). As to why the incidence of fungi seen growing on moss outside Antarctica is so low; the growth and survival rates of mosses is much higher in temperate climates, thus it may be that the fungi are still present, but at lower levels as there is less dead moss tissue for the fungus to inhabit. It could also be that sampling has an affect on this, in that samples taken in temperate climates are normally taken from the healthy growing shoots, where there is likely to be little dead tissue for the fungi, thus fungi are poorly represented in the samples taken.

ITS markers were initially developed in fungi (White *et al.*, 1990) making this marker ideal for determination of the presence of fungal DNA contaminants in the extracted moss DNA. However, the amplification conditions and binding sites of the primers of this region are such that not all potential contaminants will be amplified by the ITS primers used. For example, *Nostoc* sp. (Cyanobacteria) and *Prasiola crispa* (Chlorophyta, *incertae sedis*) are commonly found to be growing in and around moss patches on continental Antarctica (pers. obs., Figure 4.1). Thus, while it is generally possible to remove obvious patches of these organisms from the mosses before DNA extraction, there are still likely to be cells from them on the moss as it is being extracted. Why they are not amplified concurrently, as the fungi are, is unknown, but may be due to low levels being present in the samples extracted.

Figure 4.1 *Bryum subrotundifolium* (silvery yellow) overgrown with *Nostoc* (black patches in foreground) and *Prasiola crispa* (bright green patches in foreground) on Beaufort Island, collection site of samples BI A/1 BS – BI G/7 BS. Scale: middle distance covers approximately 5 m width.

Random Amplified Polymorphic DNA (RAPD)

RAPD-PCR, despite the inherent simplicity of the idea, is a difficult technique to optimise and achieve reproducibility. Factors that are critical for consistent production of reproducible bands are magnesium chloride concentration, primer concentration, DNA polymerase (*Taq*) concentration and sample DNA concentration (Grosberg *et al.*, 1996). Also critical is the annealing temperature; Williams *et al.* (1990) in their original paper on this topic used an annealing temperature of 36°C, calculated on the basis of the Wallace rule: [T_M = 2 (number of A or T) + 4 (number of G or C)] (Wallace *et al.*, 1979). It has been found in subsequent studies, that this temperature is optimal for the majority of RAPD reactions (Operon Technologies Inc., 10-mer kits, product information). An annealing temperature of 45°C was used in the RAPD reactions for this study, which is 13° above the Wallace temperature and may help eliminate spurious banding patterns, however, see Figure 3.5 and comments. The optimal DNA concentration for RAPD reactions is 25 ng per reaction (Williams *et al.*, 1990), thus it is often convenient to make all stock DNA to a concentration of 10 ng μL^{-1}. It is essential for RAPD analysis of populations, that consistent conditions are used to produce and analyse the banding patterns. It is also essential that the banding

patterns be consistently amplified between reactions for the samples being analysed. It has been found, that using identical conditions, even down to the make of PCR machine used, that there are large inconsistencies in the production of banding patterns between laboratories and within laboratories (Meunier and Grimont, 1993). There can be also be differences in the bands resolved between individual workers (Jones *et al.*, 1997; Saunders *et al.*, 2001). Thus are many factors that can affect the banding patterns produced by RAPD-PCR, and therefore it is essential before any RAPD analysis is undertaken, to determine the reproducibility of the banding between reactions, and to score only those bands that are found to be consistently amplified. For example, compare lanes 1 and 5 in Figure 3.5, where the same moss DNA sample was used in the RAPD reaction, but lane 5 includes fungal DNA as well, very bright bands can be seen in lane 5 that are moss based, but are not seen in lane 1.

For determining the presence of a contaminant, as in this study, RAPDs can be a useful tool, provided bands can be consistently reproduced. However, Black IV (1996) found that arbitrarily primed-PCR analysis was "completely inappropriate" for detection of micro-organisms or minute organisms in host tissues, although no reasons for this statement are given. It was found that the reproduction of bands in moss DNA that also contained fungal DNA was problematic. This may have been because the binding temperature for the primer to the fungal DNA could not be optimised without shifting the moss DNA binding temperature from its optimum. Binding temperature in RAPD reactions is known to be critical for reproduction of banding patterns (Ellsworth *et al.*, 1993). This problem was never fully overcome; all RAPD-PCR products from moss DNA with fungal DNA present produced a high level of background and many un-resolvable bands. The 'spiked' samples however showed some characteristics of both the moss and fungal patterns. This may have been because the fungal DNA was at a sufficiently low concentration that only the brightest bands from its pattern showed up, while the moss DNA was at a sufficiently high concentration that the banding pattern was recognisable (Figure 3.5).

Contamination problems plague RAPD analyses (Figure 3.5). This is primarily due to the low specificity of the primers used in this reaction. It has been observed that even bacteria will produce RAPD patterns, which, with the small genome of these

organisms, is unexpected. Some authors take the patterns produced by bacteria as an indicator that the annealing temperatures commonly used for RAPD reactions are so low that non-specific binding occurs. This is a particular problem with short primer lengths and is one of the reasons that RAPD-PCR has such large reproducibility difficulties. It has also been observed that some reagents will produce banding patterns that cannot be eliminated. For example, Meunier and Grimont (1993) found that particular brands of *Taq* DNA polymerase produced natural contamination. Presumably, this is due to incomplete purification of the DNA polymerase, resulting in residual amounts of DNA in the enzyme. Meunier and Grimont (1993) also note that this is unlikely to affect an analysis as the banding pattern from the *Taq* DNA polymerase is unlikely to match any pattern produced in an analysis.

It is known that the species being studied for this project have low genetic variability for the ITS region of nrDNA (Hunger, 2000). In contrast to this, Skotnicki *et al.*, (1998a, b, c) in a variety of studies found high levels of polymorphism in RAPD (random amplified polymorphic DNA) studies, even at the shoot level on individual plants, there seemed to be some genetic variation. Their findings were that the intra-population variability accounted for up to 75% of the variation observed and inter-population variability was approximately 25%. An interesting point of note is that; some, if not a large proportion of this variability may be due to fungal-DNA contamination of the moss DNA during extraction. This phenomenon has been observed by Hunger *et al.* (in prep), and has resulted in successful attempts to isolate fungi from Antarctic mosses. How much of the variation observed is attributable to the fungal contamination is unclear, and further work is needed in this area for that reason.

MICROSATELLITE DEVELOPMENT

Microsatellites need to be isolated from an organism under study for the first time. As microsatellites are usually found in the non-coding regions of the genome, it is often difficult to design universal primers, such as those found for more conserved regions (e.g. the ITS region of ribosomal DNA). It is uncommon to be able to amplify sequences that have diverged more than 10 - 20 million years (Zane et al., 2002), but it is usually possible to amplify microsatellites in other closely related

species from within a genus and occasionally within the same family (Schlötterer *et al.*, 1991; Ellegren *et al.*, 1995; Dayanandan *et al.*, 1997; Karhu *et al.*, 2000).

Microsatellite Development Techniques

The number of microsatellites isolated using different techniques varies according to the species and the technique. At a basic level, the majority of microsatellite isolation protocols can be classified into four different types; 1) traditional protocols in which microsatellites are isolated from partial genome libraries, which have been selected for small insert size, such as the protocol used in this project (Glenn, 2001). 2) Enrichment protocols in which the frequency of microsatellite sequences in a library is enhanced by primer extension (and enrichment using uracil in the place of thiamine) (e.g. Ostrander *et al.*, 1992). 3) Selective hybridisation, in which the genomic DNA is digested, ligated to a known sequence and then hybridised to a filter-bound probe. The bound DNA fragments are then eluted from the filter and amplified by means of the known sequences. After this, further cloning and hybridisation steps are required to select the microsatellite sequences (e.g. Kandpal *et al.*, 1994). 4) RAPD-based methods, in which genomic DNA is amplified by a standard RAPD reaction, screened by hybridisation with microsatellite repeat containing oligonucleotide probes, and then using selective cloning of positive bands, isolate the microsatellite containing sequence (e.g. Weising *et al.*, 1995, Ender *et al.*, 1996). Each of these methods has advantages and disadvantages, based around the time involved, the complexity of the procedure, the efficiency of the procedure, and the expense involved. Traditional methods are costly to set up, time consuming and have a low efficiency, especially if the organism being investigated has a low level of microsatellite sequences (Zane *et al.*, 2002). Enrichment protocols are also expensive to set up, but the efficiency is relatively high and the time taken to isolate sequences is low. Selective hybridisation protocols are similar to enrichment protocols in all cost, efficiency and time respects. RAPD-based protocols are the cheapest and fastest method available, however the yield is variable and the time spent optimising the RAPD reactions before microsatellite isolation can be high. Thus, it can be seen that there is little point in performing a traditional protocol for microsatellite development in a species such as a moss, given the low incidence of microsatellite sequences found in these organisms.

In this study, attempts were made to isolate microsatellite sequences from New Zealand *B. argenteum* DNA for use on Antarctic *B. subrotundifolium*, as these species are thought to be conspecific (Hunger, 2000). The incidence of microsatellites in mosses is a little studied field, indeed only one group (to the knowledge of the author), has studied moss microsatellites at all. This study was performed by van der Velde *et al.* (2000, 2001a, b) and van der Velde and Bijlsma (2000, 2001) on the genus *Polytrichum*, producing a variety of papers relating to the genetic structure of the genus (van der Velde *et al.*, 2001b), the allodiploid origin of particular species (van der Velde and Bijlsma, 2001) and the reproductive biology of *P. formosum* (van der Velde *et al.*, 2001a). The findings of these studies were that the incidence of microsatellites and levels of polymorphism were low, when compared to those found in other higher plant groups (van der Velde *et al.*, 2001b), and that the inter-generic applicability of the markers developed was low (van der Velde and Bijlsma, 2001). van der Velde *et al.* (2001b) came to the conclusion that the low levels of genetic variability found in *P. formosum* were probably due to the haploid life style of this species. The low genetic variability of mosses in general, is probably due to the dominant haploid stage, which means that deleterious alleles are likely to be directly selected against (Longton, 1976; Shaw, 1991) and consequently removed from the population. The implications of this on the study undertaken as part of this thesis, are that there are low levels of microsatellites in mosses, thus the development of the markers is likely to be difficult as the chance of isolating a microsatellite from any organism is low (Zane *et al.*, 2002) but that a microsatellite isolated from a different *Bryum* species should amplify in the one being investigated. However, it has been found that while mosses are haploid for the majority of their life cycle, there is some evidence that a large proportion of mosses are ancient polyploids, thus it may be that the mosses are functionally diploid, while in a haploid stage (gametophyte) of the life cycle (Shaw, 1991).

In general, isolation of any particular sequence from a genome is dependant on the abundance of that sequence in the genome. Mosses with a haploid genome may have a lower frequency of microsatellite sequence due to the fact that half of the genome is missing, compared to higher plants. This may have an implication on the figures seen in the results of van der Velde *et al.* (2001b), in that the figures are a direct reflection of the amount of genome present. This finding is also backed up by the difficulty seen in isolating microsatellite sequences from the moss species

being studied for this project. Also underlying some of the difficulty is that not all microsatellite sequences isolated will be polymorphic.

The failure of this project to isolate microsatellite sequences, may not be due entirely to the low incidence of microsatellites in moss species, but may also be due to the haploid genetic content of the specimens studied. Mosses have a two-stage life cycle, with the dominant stage being the haploid gametophyte (Watson, 1968) This has the implication that there is half the genetic content of the organism present at the dominant stage, when compared to diploid organisms. This may have a bearing on this study in that given a low incidence of microsatellites in mosses, it may be that there is half the probability of isolating such a sequence when investigating a haploid organism as compared to a diploid organism. This possibility has not been investigated quantitatively to the knowledge of the author. Another reason for the failure may be that the protocol used suggests performing an entire genomic library screening procedure before attempting any enrichment. This may decrease the incidence of microsatellite isolation to lower than 0.04 to 12% positive clones isolated from genomic library (Zane *et al.*, 2002).

Ligation of Genomic DNA into the Vector

Experimental difficulties in the practical component of this study meant that the isolation of microsatellite sequences was not possible. The experimental difficulties experienced were at three critical stages; first of all, in the ligation steps, in which the digested genomic DNA of the mosses was inserted into the vector (pBluescript KS® +). Optimal DNA concentrations are required for this step; necessitating the quantitation of both the stock digested DNA and the prepared vector (Beckler *et al.*, 1996). This was performed on an EagleEye II DNA gel documentation system using the concentration calculation sub-programme on the Eaglesight software (version 3.2, Stratagene), based on the Saran™ wrap method of Sambrook *et al.* (1989). Because the amounts of DNA present in the solutions, as estimated by the calculation, were similar to those obtained from a DNA fluorometer at the University of Waikato Sequencing Unit, thus the EagleEye system was used for all remaining DNA concentration estimations. However, a recent study conducted in the same laboratory has found that the system used is inaccurate (P. Poletti and L. A. Hathaway, *pers. comm.*), thus the DNA levels present in the samples may have been wrongly estimated. According to the

protocol, the optimal ligation contained 200 ng of insert and 75 ng of vector, which would produce an approximately equal number of cohesive ends in both the insert and vector. If the concentrations estimated above were incorrect, lower amounts of each would have been present in the ligation reactions, thus making them sub-optimal and hence making the later steps more difficult.

Heat Shock Transformations

Despite the possibility of having sub-optimal ligations, the transformation efficiencies of the competent cells ranged from 9.1×10^5 to 1.5×10^7 with the number of white colonies on the library plates out-numbering the blue colonies, by approximately 2:1, as expected. The transformation step was the second problematic step in the development. Transformations took place using two methods, a modified Chung *et al.* (1989) and following Nishimura *et al.* (1991). For both of these protocols heat shock transformation was used to facilitate the uptake of the ligated plasmid and insert into the bacteria. It was found during initial trials that the use of electroporation was difficult to master, and that the unreliability of this procedure made it impractical to use this for the large number of transformations required. The modified Chung *et al.* (1989) method followed closely the system described in this paper, although there were differences in the procedures used (G. Jacobsen, *pers. comm.*). For example, the cells with the added ligation reaction products were not incubated for the suggested 10 minutes, but were incubated at 4°C for 20-30 minutes and then heat-shocked. It was found generally that the transformation efficiencies from this protocol were lower than those produced by the Nishimura *et al.* (1991) protocol. For this second method, it was found that the use of magnesium- and glucose-supplemented LB-broth gave the highest transformation efficiencies, as the cells were able to grow at a high rate and the magnesium enhanced the permeability of the cell membranes, allowing greater uptake of the DNA during the transformation (Hanahan, 1985).

Probing of Filters

Hybridisations, the third problematic step, and end-labelling of oligonucleotides with γ^{33}P-ATP were at first unsuccessful due to the probe lengths being too short to allow binding of the probes to the complementary sequences. For this reason, longer oligonucleotides were used in later reactions. It was found that when these longer sequences were hybridised to the nylon filters, that the incidence of

non-specific hybridisation was quite high, making it difficult to distinguish between positive clones and clones that had hybridised non-specifically to the oligonucleotides. It is not known why the level of non-specific hybridisation was so high. Experimentation with the washing procedure based on the protocol supplied in Sambrook *et al.* (1989) resulted in lower levels of non-specific hybridisation, although this was not quantifiable. It is presumed that the lower the stringency during the washing procedure, the higher the level of background and non-specific hybridisation will be (Sambrook *et al.*, 1989). Nylon filters are known to have lower noise: signal ratio (Sambrook *et al.*, 1989) and this may be reflected in the results obtained. The use of ^{33}P as a radioactive label was made on the premise that this isotope is more stable (half life of 25.14 days), and less dangerous to handle than ^{32}P. However, it was found that with this isotope the filters could not be washed efficiently to remove probes that had already been hybridised to them. This was because the weak β-emission of the ^{33}P isotope will not penetrate liquid layers or plastic, hence it was necessary to dry the filters before exposing them to film, to prevent the filters sticking to the film. Drying of the filters supposedly irreversibly binds the DNA probe to the filter (Sambrook *et al.*, 1989), however it was found that the majority of bound probe could be removed by washing the filters in boiling 0.5% SDS (Amersham International plc, Hybond™-N^+ product information sheet).

Double-Stranded DNA Extraction and Screening Putative Positive Clones

Further experimental difficulties were experienced with the amplification of the M13 multiple cloning site (M13 MCS), in which some samples that contained an insert failed to amplify either directly from the genomic DNA or from the lysed bacteria in water mixes. The failure to amplify in some cases may have been due to a low level of DNA in the DNA solution added to the PCR. However, in these cases all the samples that were picked as putative positive clones grew in fresh selective media, indicating that the amplification was not due to the bacteria losing the plasmid. Plasmid extracts from fresh cultures of the picked DNA resulted in some of the samples amplifying as expected, but other samples which had previously amplified from the water/bacteria mix produced double bands or failed to amplify again.

Double stranded DNA was extracted from the bacteria in the form of plasmids. The plasmid preparation procedures are designed to extract the plasmids by differential denaturation of the different types of DNA in the bacterial cell. This happens through the use of alkali, which disrupts the base pairing of the linear genomic DNA denaturing it, but the closed circular plasmid DNA are unable to separate from each other because they are topologically intertwined (Sambrook *et al.*, 1989). This allows the genomic DNA to be removed from the extraction in a dissolved form, leaving the plasmid DNA. As can be seen from Figure 3.10, the total removal of genomic DNA has not occurred, which is possibly acting as an inhibitor in the PCR of the M13 MCS, by being at a much greater concentration in the solution than the plasmid DNA, thus physically preventing the primers and *Taq* DNA polymerase from binding. It is possible that this result is due to inexperience in the preparation of plasmid DNA, as a common result of inexperience is that the DNA is resistant to cleavage by restriction enzymes (Sambrook *et al.*, 1989), an analogous situation to PCR where the polymerase also has to bind to the DNA.

In summary, the modifications and optimisations made to the procedure were the result of lack of experience of the various techniques attempted. To isolate microsatellite sequences from the mosses it would be more practical to use an enrichment procedure that follows either, a primer extension method or uracil enrichment, which should increase the likelihood of isolating a microsatellite sequence.

DISPERSAL

For the purposes of further research, microsatellites isolated from Antarctic *Bryum* species could potentially answer the question of dispersal (i.e. where the mosses come from), as well as proving or disproving the theory of refugia being present on the mainland during the last glacial maximum (Llano, 1965). If refugia did exist, the expected genetic diversity would be very low, given that there is no sexual reproduction, and that all the populations in a given area are likely to have dispersed from one refugium, rather than many. On the other hand, if the moss flora of Antarctica is due to more recent colonisation events, it is to be expected that there would be relatively high levels of genetic diversity among populations and localities, such as have been found in RAPD analyses of Antarctic samples (Skotnicki, *et al.*, 1997; 1998a, b, c)

Within continental Antarctica, there have been few studies on the dispersal of moss species, however, there have been several papers discussing this topic in the maritime Antarctic, especially Signy Island and the Ongul Islands (Lewis-Smith, 1997; Marshall and Convey, 1997). The findings of these papers are in general that mosses within Antarctica can disperse by two methods, first of all by spores, as is common amongst many moss species, and secondly by asexual means such as gemmae or easily detached axilliary buds (Steere, 1965; Ochi, 1979; Lewis-Smith, 1997; Seppelt and Green, 1998). Each of these types is probabaly primarily dispersed by a different method, spores are mostly dispersed by wind and propagules are mostly dispersed by water (Lewis-Smith, 1997). This was studied on James Ross Island, for several species. Those that reproduced sexually and were able to develop mature sporophytes, showed a correlation with wind direction, in the number of plants found down-wind of the point source for the mosses (a seal carcass). Those that were found to be reproducing entirely asexually showed a correlation for dispersal with the gentle slope on which the carcass lay Lewis-Smith, 1997).

The question of dispersal structures in Antarctica is interesting, as the climate is harsh, it is to be expected that, unless an organism uses a very resistant dispersal structure, then the chances of it dispersing a large distance are small (Broady *et al.*, 1987). From the studies of Lewis-Smith (1997) it is likely that mosses within Antarctica are mostly dispersing via propagules such as axilliary buds or bulbils (but see van der Velde *et al.*, 2001a), fairly heavy structures, that are quite resistant and could potentially be transported on the feet and feathers of skuas (Lewis-Smith, 1997), although no studies of this have been performed. Wind and water are likely to be the most effective dispersal mechanisms, though no known fresh water sources on continental Antarctica travel more than a few kilometres (e.g. the Onyx river, Taylor Valley) and salt water is an unlikely prospect, as the salt content would kill the propagules, although rafting on sea ice is a possibility. All the same, the study of Lewis-Smith (1997) found that moss dispersal via propagules was closely correlated to water flow patterns along slopes. Dispersal by spores is possible for those species that can produce spores under the conditions found in continental Antarctica. Spore dispersal was found to be strongly correlated with wind patterns (Lewis-Smith, 1997). It is to be expected that spore dispersal is not

the main mechanism of gene flow within continental Antarctica as the incidence of sporophytes is at best, infrequent, and maturation of the sporophytes such that the spores are still viable is very rare (Greene, 1962).

There is evidence for wind dispersal of vegetative propagules within Antarctica (Marshall and Convey, 1997; M. I. Stevens, *pers. comm.*), although the distances over which these can travel is not known, it is presumably related to the wind velocity. A quantitative study of this, similar to that of Stevens et al. (2002 in press) for Collembola, would establish the frequency of such events as well as the amounts of propagules being dispersed over a given time. Lewis-Smith (1997) and van der Velde *et al.* (2001b) found that the distribution of spores and propagules from a source followed a leptocurtic curve, with the vast majority of dispersal structures falling close to the source, and only a tiny proportion landing far away from the source. Obviously these curves were substantially different for the type of structure being dispersed, spores being less dense covered much greater distances than those travelled by propagules (Lewis-Smith, 1997).

MP-PCR DEVELOPMENT

The microsatellite-primed PCR failed to amplify any distinct bands in the mosses examined; instead smears were observed on the gel with the majority of the molecular weight being over 2000 bp in size. Some faint and indistinct bands of smaller size, resembling RAPD patterns were seen in the lower half of the gels run. These bands are presumably the equivalent of the banding patterns produced in a RAPD reaction, but at a lower incidence of occurrence as the microsatellite oligonucleotide used to prime these reactions is longer in sequence and less likely to have complementary sequences in the genomic to which to bind. Gupta *et al.* (1994) found that some samples produced smears with di-nucleotide and some tri-nucleotide oligonucleotides used as primers, and there was little polymorphism exhibited in those species that did produce banding patterns with these primers.

The specimens used for this were the New Zealand *Bryum argenteum* samples that were also used for the microsatellite development. There may be many reasons why the MP-PCR failed to amplify any bands in these samples. Primarily it was probably due to inappropriate methodology, which was unable to be resolved due to time constraints.

Other reasons why this experiment failed to work may be that the incidence of microsatellites in the mosses is particularly low, as found by van der Velde *et al.* (2000; 2001), this would mean that the incidence of binding of the microsatellite oligonucleotide used as a primer to the genomic DNA was very low, resulting in the lack of bands seen on the agarose gels after electrophoresis. The number of binding sites for a random primer of 14 bp length is expected to be one every 4^{14} bp which equals one every 268435456 bp, hence it is to be expected that there are approximately 18 binding sites in a genome of 5×10^9 bp, which is approximately average size for a diploid plant genome) thus the chance of the genome containing several binding sites less than 3000 bp apart that are in an inverted orientation (Hadrys *et al.*, 1992) is low, suggesting that shorter oligonucleotide primers (10 bp or less) should have been used.

The primers used in these cases were $(GA)_7$ and $(CAA)_6$, which from the literature may be at a low incidence in plants, however, similar sequences of microsatellite oligonucleotide have been successfully used in other studies on plants (Matioli and deBrito, 1995; Weising *et al.*, 1995; Ender *et al.*, 1996; Ramser *et al.*, 1997a; Ramser *et al.*, 1997b; Bornet and Branchard, 2001). Despite the difficulties observed in this study, van der Velde *et al.* (2000) managed to isolate microsatellites from the genus *Polytrichum* using a $(GA)_7$ oligonucleotide primer. Which may give an indication that the PCR conditions were not correct for the MP-PCR performed using the $(GA)_7$ sequence.

FUTURE RESEARCH

It is to be recommended that in the future, this work be carried to completion as a part of a higher degree such as part of a PhD or an MPhil. This is more likely to be successful as the methodological development stage is particularly long and difficult. In addition, at this stage, very few Masters students will have had any laboratory experience with cloning, hybridisations or worked with phage-transfection of bacteria, all of which require extensive knowledge of the various difficulties and inherent problems with such techniques. Also acquiring the knowledge and experience necessary to work with these techniques is time consuming, which makes completion of a difficult project such as this in the time required for an MSc, very difficult.

Completion of the Glenn (2001) protocol will involve obtaining positive clones from which useful microsatellites can be obtained, this requires the microsatellite to be surrounded by enough flanking regions such that primers can be designed. Once primers are designed, it is possible to test the microsatellite for variation within the populations. If the microsatellites developed are polymorphic, they can be used for full-scale population genetic analysis. Data can be collected from polyacrylamide gels similar to those used for DNA sequencing, in which the products are denatured so that the mutations in sequence length (alleles) can be observed. Data can be analysed using appropriate data analysis packages, such as PAUP* (Swofford, 1998) or Phylip (Felsenstein, 1993) to produce dendrograms of the relatedness of the populations.

It is also to be recommended that different methods of isolation be attempted, such as the MP-PCR method discussed above. Such a method has the potential to be a rapid, efficient and ultimately successful method of finding and isolating microsatellites from species that have inherently low levels of microsatellites. Such a method also would require less knowledge of many different processes, making the task easier and more manageable for a Masters student. There are many other methods of microsatellite development, that vary in the number and complexity of the steps required for isolation of the sequences. The Glenn (2001) protocol is laborious and of low efficiency (Zane *et al.*, 2002), making the isolation of microsatellites from microsatellite impoverished species such as mosses, difficult.

CHAPTER 5

SUMMARY

Mosses are the most abundant vascular plant group in continental Antarctica. Much taxonomic confusion has occurred in the classification of *Bryum* species in Antarctica, especially regarding *B. subrotundifolium* (*sensu lato*) and how this relates to *B. argenteum*, a morphologically very similar species. ITS studies on Antarctic *Bryum* samples have found that samples from Antarctica classified as *B. subrotundifolium* and *B. argenteum* form one clade with samples of both species from Australia and New Zealand, thus suggesting that *B argenteum* and *B. subrotundifolium* are morphological variants of the same species, at least as far as Antarctic specimens are classified (Hunger, 2000). This should be investigated further to ascertain the taxonomic status of these taxa.

Dispersal of mosses within Antarctica has not been investigated closely, but it is hypothesised that the main means of dispersal by non-fruiting mosses such as *B. subrotundifolium*, is via propagules, which are dispersed by wind and water. This mechanism of dispersal implies that unless there has been long-term separation of sites where mosses are found, all the populations are likely to be closely related, provided they are from one founding plant. The question of whether the present-day flora is composed of recent elements or is a relictual flora that has recently dispersed from refugia after the end of the last glacial maximum is also unanswered. Because of these unknown factors, the population genetics of Antarctic mosses are of interest. Examination of the microsatellite variability in these populations could potentially resolve these questions.

Development of microsatellite markers is a time-consuming and demanding process that requires the screening of a genomic library of bacterial clones. For this process, it is necessary that the DNA used for developing the markers is free of contaminating DNA from other organisms that were extracted at the same time as the DNA from the sample. An example of co-extracted DNA can be found in Hunger (2000), where fungal DNA was found in DNA extracted from Antarctic moss samples. Detection of such contaminants is difficult, but may be performed by the amplification of specific regions such as the internal transcribed spacer

region (ITS) of nuclear ribosomal DNA producing multiple bands on a gel, or by random amplified polymorphic DNA (RAPD) amplification of extracted samples, which are then electrophoresed next to RAPD profiles of known or potential contaminants and samples that are known not to contain contaminants. A comparison of the patterns produced should enable identification of those samples that contain a contaminant

The level of genetic variation in mosses has been found to vary widely between the genera studied and the type of genetic analysis performed. For example, isozyme analyses have reported levels of genetic variation similar to those found in higher plants, while studies ITS have found low levels of genetic variability (Hunger, 2000) and RAPD-PCR has found extreme hyper-variability (Skotnicki *et al.*, 1998a, b, c), admittedly these studies have been performed on different species and genera, however the large variation in results is a reflection of the choice of genetic marker. Levels of genetic diversity have been observed to vary between populations living in stressed habitats, such as Arctic and alpine regions, and those found in temperate climates, this is attributed to the low levels of sexual reproduction occurring in the stressed localities (Montagnes *et al.*, 1993). Continental Antarctic moss populations have only infrequently been observed to produce sporophytes, thus there is little evidence for complete sexual reproduction occurring in this area (Steere, 1965; Horikawa and Ando, 1967; Ochi, 1979; Ochi and Ochyra, 1985; Ochyra, and Ochi, 1986; Seppelt, 1986), also the mosses are highly stressed in these localities (Seppelt and Selkirk, 1984). These findings would indicate that the genetic diversity of Antarctic moss populations is low. RAPD analysis of several moss species from Victoria Land, in the continental Antarctic have found hyper-variability in moss populations (Skotnicki *et al.*, 1998a, b, c), however, some of this variability could be due to fungal contamination of the DNA used for these analyses (Hunger 2000).

Microsatellite sequences are a common part of all eukaryote genomes studied so far. They consist of short sequences, 1 – 6 bp in length, which can be repeated many hundreds of times within the genome (Tautz, 1989). Microsatellites exhibit a high degree of length polymorphism due to slippage synthesis events and are co-dominantly inherited and usually selectively neutral. For these reasons microsatellites are ideal markers for population genetic studies. The level of

microsatellites in mosses has been little studied, but the findings of the studies by van der Velde *et al.* (2000, 2001a, b) and van der Velde and Bijlsma (2000, 2001) were that microsatellites had a low incidence in the genus studied (*Polytrichum*), and that the degree of polymorphism in the microsatellites isolated was particularly low.

Isolation of microsatellite sequences relies on screening of genomes for microsatellite containing sequences, generally through the use of genomic DNA libraries. The number of microsatellites that can be isolated depends on the frequency at which they are found in the organism being studied. Mosses, with a low incidence of microsatellites, are likely to require screening of many thousands of clones to isolate a small number of microsatellite sequences (Zane *et al.*, 2002). As these processes are time-consuming and expensive to set up, it is recommended that a high efficiency protocol such as the Kandpal *et al.* (1994) selective hybridisation protocol be used to increase the likelihood of isolating microsatellite sequences.

Difficulties in the development of microsatellites by the majority protocols are likely to be experienced at three key places, these are: 1) ligation of insert DNA into a vector, where determination of the correct ratio of genomic DNA to vector, as estimated from the DNA concentrations in solution, is critical to achieve high levels of insert-containing clones, 2) transformation of the ligated vector into bacterial cells, this can vary according to the method of preparations use and whether the cells have been stored or not, and 3) probing of the clones with oligonucleotide probes to isolate those sequences that contain microsatellites, in this step there are several parts that can lead to spurious results, these include the probe length used in the hybridisation, the hybridisation and wash solutions used (degree of stringency in the wash), hybridisation temperatures and hybridisation times. All of these three steps will variously alter the results obtained and the efficiency of extraction of the microsatellite sequences.

Future work should focus on isolation of microsatellites from the mosses, and investigating the population genetics of *Bryum* species within Antarctica using the markers developed. Such a study could potentially elucidate important questions on the age and adaptability of the native Antarctic moss flora.

REFERENCES

Adam, K. D., Selkirk, P. M., Connett, M. B., Walsh, S. M., 1997. Genetic variation in populations of the moss *Bryum argenteum* in East Antarctica. In: *Antarctic Communities: Species, Structure and Survival.* B. Battaglia, J. Valencia, D. W. H. Walton (eds.), Cambridge University Press, Cambridge, United Kingdom.

Akkaya, M. S., Bhagwat, A. A., Cregan, P. B., 1992. Length polymorphisms of simple sequence repeat DNA in soybean. *Genetics* **132**: 1131 – 1139.

Armour, J. A. L., Neumann, R., Gobert, S., Jeffreys, A. J., 1994. Isolation of human simple repeat loci by hybridisation selection. *Human Molecular Genetics* **3**: 599 – 605.

Ashley, M. V., Dow, B. D., 1994. The use of microsatellite analysis in population biology: background, methods and potential applications. In: *Molecular Ecology and Evolution: Approaches and Applications.* B. Schierwater, B. Streit, G. P. Wagner, R. DeSalle (eds.), Birkhäuser Verlag, Basel, Switzerland.

Balakrishna, P., 1995. Evaluation of intra-specific variability in *Avicennia marina* Forsk. using RAPD markers. *Current Science* **69**: 926 – 929.

Baldwin, G. B., 1992. Phylogeny of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the Compositae. *Molecular Phylogenetics and Evolution* **1**: 3 – 16.

Baldwin, G. B., Sanderson, M. J., Porter, J. M., Wojciechowski, M. F., Campbell, C. S., Donoghue, M. J., 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden* 82: 247 - 277.

Barrett, P. J., 1991. Antarctica and global climatic change: a geological perspective. In: *Antarctica and Global Climatic Change*. C. M. Harris, B. Stonehouse (eds.), Belhaven Press, London, United Kingdom.

Barrier, M., Friar, E., Robichaux, R., Purugganan, M., 2000. Interspecific evolution in plant microsatellite structure. *Gene* **241**: 101 – 105.

Beckler, G., Bahtia, A., Brisco, P., Brondyk, W., Butler, B., DeMars, S., Doers, M., Doyle, K., Goueli, S., Harper, T., Knoche, K., Knuth, M., Lepinske, M., Lesley, S., Marcus, E., Murray, E., Okragly, A., Otto, P., Romanin, D., Romanin, J., Ryan, A., Salatino, R., Schaefer, E., Schenborn, E., Sherf, B., Slater, M., Smith, C., Sobol, M., Stock, C., Storts, D., Sveinsson, D., Tereba, A., 1996. Cloning in plasmid vectors. In: *Protocols and Applications Guide, 3rd Edition*. K. Dolye (ed.), Promega, Madison, U.S.A.

Beckmann, J. S., Weber, J. L., 1992. Survey of human and rat microsatellites. *Genomics* **12**: 627 – 631.

Bell, C. J., Ecker, J. R., 1994. Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis*. *Genomics* **19**: 137 – 144.

Black IV, W. C., 1993. PCR with arbitrary primers: approach with care. *Insect Molecular Biology* **2**: 1 – 6.

Black IV, W. C., 1996. Statistical analysis of arbitrarily primed PCR patterns in molecular taxonomic studies. In: *Methods in Molecular Biology, Volume 50: Species Diagnostics Protocols: PCR and Other Nucleic Acid Methods*. J. P. Clapp (ed.), Humana Press Inc., Totowa, U.S.A.

Block, W., 1984. Terrestrial microbiology, invertebrates and ecosystems. In: *Antarctic Ecology, Volume 1*. R. M. Laws (ed.), Academic Press, London, United Kingdom.

Bornet, B., Branchard, M., 2001. Nonanchored inter simple sequence repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting. *Plant Molecular Biology Reporter* **19**: 209 – 215.

Broady, P., Given, D., Greenfield, L., Thompson, K., 1987. The biota and environment of fumaroles on Mount Melbourne, northern Victoria Land. *Polar Biology* **7**: 97 – 113.

Brown, T. A., Ikemura, T., McClelland M., Roberts, R. J., 1991. *Molecular Biology Labfax.* T. A. Brown (ed.), BIOS Scientific Publishers Limited, Oxford, United Kingdom.

Bruford, M. W., Wayne, R. K., 1993. Microsatellites and their application to population genetic studies. *Current Opinion in Genetics and Development* **3**: 1 - 4.

Bull, C., 1966. Climatological observations in ice-free areas of southern Victoria Land, Antarctica. *Antarctic Research Series* **9**: 177 – 194.

Campbell, I. B., Claridge, G. G. C., 2000. Soil temperature, moisture and salinity patterns in Transantarctic Mountain cold desert ecosystems. In: *Antarctic Ecosystems: Models for Wider Ecological Understanding.* W. Davison, C. Howard-Williams, P. Broady (eds.), Canterbury University, Christchurch, New Zealand.

Cardot, J., 1908. La flore bryologique des Terres Magellaniques, de la Géorgie du Sud et de l'Antarctide. *Wissenschaftliche Ergebnisse der Schwedischen Südpolarexpedition* 1901-03, **4**: 1 - 298.

Castello, M., Nimis, P. L., 1997. Diversity of lichens in Antarctica. In: *Antarctic Communities: Species, Structure and Survival.* B. Battaglia, J. Valencia, D. W. H. Walton (eds.), Cambridge University Press, Cambridge, United Kingdom.

Catcheside, D. G., 1980. Mosses of South Australia. In: *Handbook of the Flora and Fauna of South Australia.* Issued by the Handbooks Committee on behalf of the South Australian Government. D. J. Woolman, Government Printer, South Australia, Australia.

Chung, C. T., Niemela, S. L., Miller, R. H., 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proceedings of the National Academy of Sciences of the United States of America* **86**: 2172 - 2175.

Clark, A. G., Lanigan, C. M. S., 1993. Prospects for estimating nucleotide divergence with RAPDs. *Molecular Biology and Evolution* **10**: 1096 – 1111.

Clarke, G. C. S., Greene, S. W., 1970a. Reproductive performance of two species of *Pohlia* at widely separated stations. *Transactions of the British Bryological Society* **6**: 114 – 128.

Clarke, G. C. S., Greene, S. W., 1970b. Reproductive performance of two species of *Pohlia* from temperate and sub-Antarctic stations under controlled environmental conditions. *Transactions of the British Bryological Society* **6**: 278 – 295.

Condit, R., Hubbell, S. P., 1991. Abundance and DNA sequence of two-base repeat regions in tropical tree genomes. *Genome* **34**: 66 – 71.

Convey, P., 1997. Comparative studies of Antarctic arthropod and bryophyte life cycles: do they have a common strategy? In: *Antarctic Communities: Species, Structure and Survival.* B. Battaglia, J. Valencia, D. W. H. Walton (eds.), Cambridge University Press, Cambridge, United Kingdom.

Cox, C. J., 1998. Phylogenetic relationships of the Eubryalean mosses (Bryineae, Musci). *PhD Thesis.* University of Reading, Reading, United Kingdom.

Craddock, C., 1977. Antarctica and Gondwanaland. In: *Antarctic Geoscience.* C. Craddock (ed.), University of Wisconsin Press, Madison, U.S.A.

Dahl, E., 1946. On different types of unglaciated areas during the Ice Ages and their significance to phytogeography. *New Phytologist* **45**: 225 – 242.

Daniels, R. E., 1993. Phenotypic and genotypic variation in *Sphagnum. Advances in Bryology* **5**: 31 – 60.

Dayanandan, S., Bawa, K. S., Kesseli, R., 1997. Conservation of microsatellites among tropical trees (Leguminosae). *American Journal of Botany* **84**: 1658 – 1663.

Dellaporta, S. L., Wood, J., Hicks, J. B., 1983. A plant DNA minipreparation: version II. *Plant Molecular Biology Reporter* **1**: 19 – 21.

Du Toit, A. L., 1937. *Our Wandering Continents, an Hypothesis of Continental Drifting.* Hafner Publishing Company, New York, U.S.A.

Ellegren, H., 2000. Microsatellite mutations in the germline: implications for evolutionary inference. *Trends in Genetics* **16**: 551 – 558.

Ellegren, H., Primmer, C. R., Sheldon, B. C., 1995. Microsatellite 'evolution': directionality or bias? *Nature Genetics* **11**: 360 - 362.

Elliot, D. H., 1985. Physical geography- geological evolution. In: *Key Environments: Antarctica.* W. N. Bonner, D. W. H. Walton (eds.), Pergamon Press, Oxford, United Kingdom.

Ellsworth, D. L., Rittenhouse, K. D., Honeycutt, R. L., 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. *Biotechniques* **14**: 214 – 217.

Ender, A., Schwenk, K., Städler, T., Streit, B., Schierwater, B., 1996. RAPD identification of microsatellites in *Daphnia*. *Molecular Ecology* **5**: 437 - 441.

Gaggiotti, O. E., Lange, O., Rassmann, K., Gliddons, C., 1999. A comparison of two indirect methods for estimating average levels of gene flow using microsatellite data. *Molecular Ecology* **8**: 1513 – 1520.

Goldstein, D. B., Pollock, D. D., 1998. Launching microsatellites: A review of mutation processes and methods of phylogenetic inference. *Journal of Heredity* **88**: 335 – 342.

Greene, S. W., 1962. Problems and progress in Antarctic bryology. In: *Biologie Antarctique*. R. Carrick, M. Holdgate, J. Prévost (eds.), Hermann, Paris, France.

Grosberg, R. K., Levitan, D. R., Cameron, B. B., 1996. Characterisation of genetic structure and genealogies using RAPD-PCR markers: a random primer for the novice and nervous. In: *Molecular Zoology: Advances, Strategies and Protocols*. J. D. Ferraris, S. R. Palumbi (eds.), Wiley-Liss Inc., New York, U.S.A.

Gupta, P. K., Balyan, H. S., Sharma, P. C., Ramesh, B., 1996. Microsatellites in plants: A new class of molecular markers. *Current Science* **70**: 45 – 54.

Gupta, M., Chyi, Y.-S., Romero-Severson, J., Owen, J. L., 1994. Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple sequence repeats. *Theoretical and Applied Genetics* **89**: 998 – 1006.

Gurnis, M., 1988. Large-scale mantle convection and the aggregation and dispersal of supercontinents. *Nature* **332**: 695 – 699.

Hadrys, H., Balick, M., Schierwater, B., 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Molecular Ecology* **1**: 55 – 63.

Hambrey, M. J., Larsen, B., Ehrmann, W. U., ODP Leg 119 Shipboard Party, 1989. Forty million years of Antarctic glacial history yielded by Leg 119 of the Ocean Drilling Program. *Polar Record* **25**: 99 – 106.

Hamby, R. K., Zimmer, E. A., 1992. Ribosomal RNA as a phylogenetic tool in plant systematics. In: *Molecular Systematics of Plants.* P. S. Soltis, D. E. Soltis, J. J. Doyle (eds.), Chapman Hall, New York, U.S.A.

Hanahan, D., 1985. Techniques for transformation of *E. coli.* In: *DNA Cloning- A Practical Approach Volume 1.* D. M. Glover, (ed.), IRL Press Ltd., Oxford, United Kingdom.

Hansom, J. D., Gordon, J. E., 1998. *Antarctic Environments and Resources, a Geographical Persepective.* Addison Wesley Longman Ltd., New York, U.S.A.

Hillis, D. M., Mable, B. K., Larson, A., Davis, S. K., Zimmer, E. A., 1996. Nucleic acids IV: sequencing and cloning. In: *Molecular Systematics 2nd Edition.* D. M. Hillis, C. Moritz, B. K., Mable (eds.), Sinauer Associates Inc., Sunderland, U.S.A.

Holdgate, M. W., 1967. Signy Island. *Philosophical Transactions of the Royal Society of London, series B, Biological Sciences* **252**: 173 – 177.

Holmes, A., 1965. *Principles of Physical Geography.* Thomas Nelson Ltd., London, United Kingdom.

Horikawa, Y., Ando, H., 1960. Mosses of the Ongul Islands collected during the 1957 - 1960 Japanese Antarctic Research Expedition. *Hikobia* **2**: 160 - 178.

Horikawa, Y., Ando, H., 1967. The mosses of Ongul Islands and adjoining coastal areas of the Antarctic continent. *Japanese Antarctic Research Expedition Scientific Reports, Special Issues* **1**: 245 – 252.

Hunger, S. A., 2000. A study on molecular systematics of the Antarctic *Bryum* species and the development of microsatellite DNA markers in *Bryum argenteum* Hedw. *MSc Thesis.* University of Waikato, Hamilton, New Zealand.

Jarne, P., Lagoda, P. J. L., 1996. Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution* **11**: 424 – 429.

Jones, C. J., Edwards, K. J., Castaglione, S., Winfield, M. O., Sala, F., van de Wiel, C., Bredemeijer, G., Vosman, B., Matthes, M., Daly, A., Brettschneider, R., Bettini, P., Buiatti, M., Maestri, E., Malcevschi, A., Marmiroli, N., Aert, R., Volckaert, G., Rueda, J., Linacero, R., Vazquez, A., Karp, A., 1997. Reproducibility testing of RADP, AFLP and SSR markers in plants by a network of European laboratories. *Molecular Breeding* **3**: 381 – 390.

Kanda,H., 1987. New localities and taxonomic remarks for some mosses in continental Antarctica. *Proceedings of the National Institute for Polar Research Symposium on Polar Biology* **1**: 265 - 272.

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

Kandpal, R. P., Kandpal, G., Weissman, S. M., 1994. Construction of libraries enriched for sequence repeats and jumping clones, and hybridisation selection of region-specific markers. *Proceedings of the National Academy of Sciences of the United States of America* **91**: 88 – 92.

Karhu, A., Dieterich, J.-H., Savolainen, O., 2000. Rapid expansion of microsatellite sequences in pines. *Molecular Biology and Evolution* **17**: 259 - 265.

Kennedy, A. D., 1993. Water as a limiting factor in the Antarctic terrestrial environment: a biogeographical synthesis. *Arctic Alpine Research* 25: 308 – 315.

Kruglyak, S., Durrett, R. T., Schug, M. D., Aquadro, C. F., 1998. Equilibrium distributions of microsatellite repeat length resulting from a balance between slippage events and point mutations. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 10774 – 10778.

Lagercrantz, U., Ellegren, H., Andersson, L., 1993. The abundance of various polymorphic microsatellite motifs differs between plants and vertebrates. *Nucleic Acids Research* **21**: 1111 – 1115.

Lamb, I. M., 1970. Antarctic terrestrial plants and their ecology. In: *Antarctic Ecology, Volume 2.* M. W. Holdgate (ed.), Academic Press, London, United Kingdom.

Levinson, G., Gutman, G. A., 1987. Slipped-strand mispairing: a major mechanism for DNA sequence evolution. *Molecular Biology and Evolution* **4**: 203 – 221.

Lewis-Smith, R. I., 1984. Terrestrial plant biology of the sub-Antarctic and Antarctic. In: *Antarctic Ecology, Volume 1.* R. M. Laws (ed.), Academic Press, London, United Kingdom.

Lewis-Smith, R. I., 1997. Oases as centres of high plant diversity and dispersal in Antarctica. In: *Ecosystem Processes in Antarctic Ice-Free Landscapes.* W. B. Lyons, C. Howard-Williams, I. Hawes (eds.), A.A. Balkema, Rotterdam, Netherlands.

Litt, M., Luty, J. A., 1989. A hypervariable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac muscle actin gene. *American Journal of Human Genetics* **44**: 397 – 401.

Linskens, H. F., Bargagli, B., Cresti, M., Focardi, S., 1993. Entrapment of long distance transported pollen grains by various moss species in coastal Victoria Land, Antarctica. *Systematic Botany* **21**: 109 – 121.

Llano, G. A., 1965. The flora of Antarctica. In: *Antarctica*. T. Hatherton (ed.), A. H. and A. W. Reed, Wellington, New Zealand

Li, Q.-B., Cai, Q., Guy, C. L., 1994. A DNA extraction method for RAPD analysis from plants rich in soluble polysaccharides. *Plant Molecular Biology Reporter* **12**: 215 – 220.

Longton, R. E., 1973. The occurrence of radial infection patterns in colonies of polar bryophytes. *British Antarctic Survey Bulletin* **32**: 41 – 49.

Longton, R. E., 1976. Reproductive biology and evolutionary potential in bryophytes. *Journal of the Hattori Botanical Laboratory* **41**: 205 – 223.

Longton, R. E., 1985. Terrestrial habitats - Vegetation. In: *Key Environments Series: Antarctica*. W. N. Bonner, D. W. H. Walton (eds.), Pergamon Press, Oxford, United Kingdom.

Longton, R. E., Greene, S. W., 1967. The growth and reproduction of *Polytrichum alpestre* Hoppe on South Georgia. *Philosophical Transactions of the Royal Society of London, series B, Biological Sciences* **252**: 295 – 322.

Marshall, W. A., 1996. Biological particles over Antarctica. *Nature* **383**: 680.

Marshall, W. A., Convey, P., 1997. Dispersal of propagules on Signy Island, maritime Antarctic. *Polar Biology* **18**: 376 – 383.

Matioli, S.R., de Brito, R. A., 1995. Obtaining genetic markers by using double-stringency PCR with microsatellites and arbitrary primers. *Biotechniques* **19**: 752 – 758.

McCouch, S. R., Chen, X., Panaud, O., Temnykh, S., Xu, Y., Gu Cho, Y., Huang, N., Ishii, T., Blair, M., 1997. Microsatellite marker development, mapping and applications in rice genetics and breeding. *Plant Molecular Biology* **35**: 89 – 99.

Melick, D. R., Seppelt. R. D., 1997. Vegetation patterns in relation to climate and endogenous changes in Wilkes Land, continental Antarctica. *Journal of Ecology* **85**: 43 – 56.

Meunier, J.-R., Grimont, P. A. D., 1993. Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. *Research in Microbiology* **144**: 373 – 379.

Montagnes, R. J. S., Bayer, R. J., Vitt, D. H., 1993. Isozyme variation in the moss *Messia triquetra* (Messiaceae. *Journal of the Hattori Botanical Laboratory* **74**: 155 – 170.

Morgante, M., Olivieri A. M., 1993. PCR – amplified microsatellites as markers in plant genetics. *The Plant Journal* **3**: 175 – 182.

Murray, H. G., Thompson, W. F., 1980. Rapid isolation of high molecular weight DNA. *Nucleic Acids Research* **8**: 4321 – 4325.

Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., Matrin, C., Fujimoto, E., Hoff, M., Kumlin, E., White, R., 1987. Variable number tandem repeat (VNTR) markers for human genome mapping. *Science* **235**: 1616 – 1622.

Nishimura, A., Morita, M., Nishimura, Y., Sugino, Y., 1990. A rapid and highly efficient method for preparation of competent *Escherichia coli* cells. *Nucleic Acids Research.* **18**: 6169.

Nybom, H., Ramser, J., Kaemmer, D., Kahl, G., Weising, K., 1992. Oligonucleotide DNA fingerprinting detects a multiallelic locus in box elder (*Acer negundo*). *Molecular Ecology* **1**: 65 – 67.

Ochi, H., 1979. A taxonomic review of the genus *Bryum*, Musci in Antarctica. In: *Proceedings of the Symposium on Terrestrial Ecosystems in the Syowa Station Area – Memoirs of the National Institute of Polar Research, Special*

Issue No. 11. T. Matsuda, T. Hoshiai (eds.), National Institute of Polar Research, Tokyo, Japan.

Ochi, H., 1992. A revised infrageneric classification of the genus *Bryum* and related genera (Bryaceae, Musci). *Bryobrothera* **1**: 231 - 244.

Ochi, H., Kanda, H., 1991. New additions of Bryaceous mosses (Musci, Bryaceae) to the Antarctic flora. *Proceedings of the National Institute for Polar Research Symposium on Polar Biology* **4**:123 - 126.

Ochi, H., Ochyra, R., 1985. *Bryum dichotomum* Hedw., a species new to the Antarctic. *Lindbergia* **11**: 157 - 160.

Ochyra, R., Ochi, H., 1986. New or otherwise interesting species of the genus *Bryum* (Musci, Bryaceae) in the Antarctic. *Acta Botanica Hungarica* **32**: 209 - 219.

Ortí, G., Pearse, D. E., Avise, J. C., 1997. Phylogenetic assessment of length variation at a microsatellite locus. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 10745 – 10749.

Ostrander, E. A., Jong, P. M., Rine, J., Duyk, G., 1992. construction of small-insert genomic libraries highly enriched for microsatellite repeat sequences. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 3149 – 3424.

Porebski, S., Bailey, L. G., Baum, B. R., 1997. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Molecular Biology Reporter* **15**: 8 – 15.

Primmer, C. R., Ellegren, H., Saino, N., Møller, A. P., 1996. Directional evolution in germline microsatellite mutations. *Nature Genetics* **13**: 391 – 393.

Ramser, J., Weising, K., Chikaleke, V., Kahl, G., 1997a. Increased informativeness of RAPD analysis by detection of microsatellite motifs. *Biotechniques* **23**: 285 - 290.

Ramser, J., Weising, K., Lopez-Peralta, C., Terhalle, W., Terauchi, R., Kahl, G., 1997b. Molecular marker based taxonomy and phylogeny of Guinea yam (*Dioscorea rotundata – D. cayenensis*). *Genome* **40**: 903 – 915.

Robin, G. de Q., 1988. The Antarctic ice sheet, its history and response to sea level and climatic changes over the past 100 million years. *Palaeogeography, Palaeoclimatology, Palaeoecology* **67**: 31 – 50.

Rogers, S. O., Bendich, A. J., 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Molecular Biology* **5**: 69 – 76.

Rosenbaum, H. C., Deinard, A. S., 1998. Caution before claim: an overview of microsatellite analysis in ecology and evolutionary biology. In: *Molecular Approaches to Ecology and Evolution.* R. DeSalle, B. Schierwater (eds.), Birkhäuser Verlag, Basel, Switzerland.

Rossetto, M., Harriss, F. C. L., Mclauchlan, A., Henry, R. J., Baverstock, P. R., Lee, L. S., 2000. Interspecific amplification of tea tree (*Melaleuca alternifolia* – Myrtaceae) microsatellite loci - potential implications for conservation studies. *Australian Journal of Botany* **48**: 367 – 373.

Rychlik, P., Rychlik, W., 2002. Oligo, Version 6.61. *Molecular Biology Insights.*

Sambrook, J., Fritsch, E. F., Maniatis, T., 1989. *Molecular Cloning, A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York, U.S.A.

Sanger, F., Nicklen, S., Coulson, A. R., 1977. DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America.* **74**: 5463 – 5467.

Saunders, G. C., Dukes, J., Parkes, H. C., Cornett, J. H., 2001. Interlaboratory study on thermal cycler performance in controlled PCR and random amplified polymorphic DNA analyses. *Clinical Chemistry* **47**: 47 – 55.

Schlötterer, C., Amos, W., Tautz, D., 1991. Conservation of simples sequence loci in cetacean species. *Nature* **354**: 63 - 65.

Schlötterer, C., Pemberton, J., 1994. The use of microsatellites for genetic analysis of natural populations. In: *Molecular Ecology and Evolution: Approaches and Applications*. B. Schierwater, B. Streit, G. P. Wagner, R. DeSalle (eds.), Birkhäuser Verlag, Basel, Switzerland.

Schlötterer, C., Pemberton, J., 1998. The use of microsatellites for genetic analysis of natural populations – a critical review. In: *Molecular Approaches to Ecology and Evolution*. R. DeSalle, B. Schierwater (eds.), Birkhäuser Verlag, Basel, Switzerland.

Schlötterer, C., Tautz, D., 1992. Slippage synthesis of simple sequence DNA. *Nucleic Acids Research* **20**: 211 – 215.

Seppelt, R. D., 1986. Bryophytes of the Vestfold Hills. In: *Antarctic Oasis, Terrestrial Environments and History of the Vestfold Hills*. J. Pickard (ed.), Academic Press, Australia.

Seppelt, R. D., Green, T. G. A., 1998. A bryophyte flora for southern Victoria Land, Antarctica. *New Zealand Journal of Botany* **36**: 617 - 635.

Seppelt, R. D., Selkirk, P. M., 1984. Effects of submersion on morphology and the implications of induced environmental modification on the taxonomic interpretation of selected Antarctic moss species. *Journal of the Hattori Botanical Laboratory* **55**: 273 – 279.

Shackelton, N. J., Kennett, P. J., 1975. Paleotemperature history of the Cenozoic and the initiation of Antarctic glaciation: oxygen and carbon isotope

analyses in DSDP sites 277, 279 and 281. In: *Initial Reports of the Deep Sea Drilling Project.* P. J. Kennett, R. Houtz (eds.) **29**: 743 – 755.

Shaw, A. J., 1991. Ecological genetics, evolutionary constraints and the systematics of Bryophytes. *Advances in Bryology* **4**: 29 – 74.

Sibly, R. M., Whittaker, J. C., Talbot, M., 2001. A maximum-likelihood approach to fitting equilibrium models of microsatellite evolution. *Molecular Biology and Evolution* **18**: 413 – 417.

Skotnicki, M. L., Selkirk, P. M., Dale, T. M., 1997. RAPD profiling of genetic variation in Antarctic mosses. In: *Ecosystem Processes in Antarctic Ice-Free Landscapes.* W. B. Lyons, C. Howard-Williams, I. Hawes (eds.) A. A. Balkema, Rotterdam, Netherlands.

Skotnicki, M. L., Ninham, J. A., Selkirk, P. M., 1998a. High levels of RAPD diversity in the moss *Bryum argenteum* in Australia, New Zealand and Antarctica. *The Bryologist* **101**: 412 – 421.

Skotnicki, M. L., Selkirk, P. M., Beard, C., 1998b. RAPD profiling of genetic diversity in two populations of the moss *Ceratodon purpureus* in Victoria Land, Antarctica. *Polar Biology* **19**: 172 – 176.

Skotnicki, M. L., Selkirk, P. M., Ninham, J. A., 1998c. RAPD analysis of genetic variation and dispersal of the moss *Bryum pseudotriquetrum* from southern Victoria Land, Antarctica. *Polar Biology* **20**: 121 – 126.

Slatkin, M., 1995. A measure of population subdivision based on microsatellite allele frequencies. *Genetics* **139**: 457 – 462.

Steere, W. C., 1961. A preliminary review of the bryophytes of Antarctica. In: *Science in Antarctica, Part 1. The Life Science of Antarctica.* National Academy of Science, National Research Council. Publication **839**: 20 – 33, Washington, U.S.A.

Steere, W. C., 1965. Antarctic Bryophyta. *BioScience* 283-285.

Stoneburner, A., Wyatt, R., Odrzykoski, I. J., 1991. Applications of enzyme electrophoresis to bryophyte systematics and population biology. *Advances in Bryology* **4**: 1 – 28.

Strassmann, J. E., Solís, C. R., Peters, J. M., Queller, D. C., 1996. Strategies for finding and using highly polymorphic DNA microsatellite loci for studies of genetic relatedness and pedigrees. In: *Molecular Zoology: Advances, Strategies and Protocols*. J. D. Ferraris, S. R. Palumbi (eds.), Wiley – Liss Inc., New York, U. S. A.

Swofford, D. L., 1998. PAUP* Version 4.0b2a: Phylogenetic Analysis using Parsimony (* And Other Methods). Sinauer Associates Inc., Sunderland, Massachusetts, U.S.A.

Takezaki, N., Nei, M., 1996. Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics* **144**: 389 – 399.

Tautz, D., 1989. Hypervariability of simple sequence repeats as a general source for polymorphic DNA markers. *Nucleic Acids Research* **17**: 6463 - 6471.

Tautz, D., 1993. Notes on the definition and nomenclature of tandemly repetitive DNA sequences. In: *DNA Fingerprinting: State of the Science*. S. D. J. Pena, R. Chakraborty, J. T. Epplen, A. J. Jeffreys (eds.), Birkhäuser Verlag, Basel, Switzerland.

Tautz, D., Renz, M., 1984. Simple sequence repeats are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acids Research* **12**: 4127 – 4138.

Ugolini, F. C., 1970. Antarctic soils and their ecology. In: *Antarctic Ecology, Volume 2*. M. W. Holdgate (ed.), Academic Press, London, United Kingdom.

Valentine, J. W., Moores, E. M., 1970. Plate-tectonic regulation of faunal diversity and sea level: a model. *Nature* **228**: 657 – 659.

van der Velde, M., Bijlsma, R., 2000. Amount and structure of intra- and interspecific genetic variation in the moss genus *Polytrichum*. *Heredity* **85**: 328 - 337.

van der Velde, M., Bijlsma, R., 2001. Genetic evidence for the allodiploid origin of the moss species *Polytrichum longisetum*. *Plant Biology* **3**: 379 – 385.

van der Velde, M., During, H. J., van de Zande, L., Bijlsma, R., 2001a. The reproductive biology of *Polytrichum formosum*: clonal structure and paternity revealed by microsatellites. *Molecular Ecology* **10**: 2423 – 2434.

van der Velde, M., van der Strate, H. J., van de Zande, L., Bijlsma, R., 2000. Isolation and characterisation of microsatellites in the moss species *Polytrichum formosum*. *Molecular Ecology* **9**: 1678 – 1680.

van der Velde, M., van de Zande, L., Bijlsma, R., 2001b. Genetic structure of *Polytrichum formosum* in relation to the breeding system as revealed by microsatellites. *Journal of Evolutionary Biology* **14**: 288 – 295.

Van Treuren, R., Kuittinen, H., Karkkainen, K., Baena-Gonzalez, E., Savolainen, O., 1997. Evolution of microsatellites in *Arabis petraea* and *Arabis lyrata*, outcrossing relatives of *Arabidopsis thaliana*. *Molecular Biology and Evolution* **14**: 220 – 229.

Vincent, W. F., 1997. Polar desert ecosystems in a changing climate: a North-South perspective. In: *Ecosystem Processes in Antarctic Ice-Free Landscapes*. W. B. Lyons, C. Howard-Williams, I. Hawes (eds.), A. A. Balkema, Rotterdam, Netherlands.

Wallace, R. B., Shaffer, J., Murphy, R. F., Bonner, J., Hirose, T., Itakura, K., 1979. Hybridization of synthetic oligodeoxyribonucleotides to phi chi 174 DNA:

the effect of single base pair mismatch. *Nucleic Acids Research* **6**: 3543 - 3557

Walton, D. W. H., 1984. The terrestrial environment. In: *Antarctic Ecology, Volume 1.* R. M. Laws (ed.), Academic Press, London United Kingdon.

Walton, D. W. H., Morris, E. M., 1990. Science, environment and resources in Antarctica. *Applied Geography* **10**: 265 – 286.

Wang, Z., Weber, J. L., Zhong, G., Tanksley, S. D., 1994. Survey of plant short tandem DNA repeats. *Theoretical and Applied Genetics* **88**: 1 – 6.

Watson, E. V., 1968. *British Mosses and Liverworts.* Cambridge University Press, Cambridge, United Kingdom.

Weber, J. L., 1990. Informativeness of human $(dC\text{-}dA)_n$ $(dG\text{-}dT)_n$ polymorphisms. *Genomics* **7**: 524 – 530.

Weber, J. L., May, P.E., 1989. Abundant class of human DNA polymorphisms, which can be typed using the polymerase chain reaction. *American Journal of Human Genetics* **44**: 388 – 396.

Weber, J. L., Wong, C., 1993. Mutation of human short tandem repeats. *Human Molecular Genetics* **2**: 1123 - 1128.

Wegener, A., 1924. *Die Entstehung der Kontinente und Ozeane* (The Origin of Continents and Oceans: translated from the 3rd German ed. by J. G. A. Skerl). Methuen, London, United Kingdom.

Weising, K., Atkinson, R. G., Gardner, R. C., 1995. Genomic fingerprinting by microsatellite-primed PCR: a critical evaluation. *PCR Methods and Applications* **4**: 249 - 255.

White, T. J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR*

Protocols: A Guide to Methods and Applications. M. Innis, D. Gelfand, J. Sinisky, T. White (eds.), Academic Press, San Diego U.S.A.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., Tingey, S. V., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* 18: 6531 - 6535.

Wise, K. A. J., Gressitt, J. L., 1965. Far southern animals and plants. *Nature* **207**: 101 – 102.

Wyatt, R., Derda, G. S., 1997. Population biology of the Polytrichaceae. *Advances in Bryology* **6**: 265 – 296.

Zane, L., Bargelloni, L., Patarnello, T., 2002. Strategies for microsatellite isolation: a review. *Molecular Ecology* **11**:1 - 16.

Web Site References:

Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5. Distributed by the author. Department of Genetics, University of Washington, Seattle, U.S.A. (http://evolution.genetics.washington.edu/phylip.html). Extracted from website, March 2000.

Glenn, T. C. 2001. Microsatellite Manual, Version 7.0.1. Savannah River Ecology Laboratory, University of Georgia, USA. (http://www.uga.edu/srel/DNA_Lab). Extracted from website, March 2001.

Schnieder, S., Roessli, D, Excoffier, L., 2000. Arlequin Version 2.000. A software for population genetic analysis. Genetics and Biometry Laboratory, Department of Anthropology and Ecology, University of Geneva, Switzerland. (http://anthro.unige.ch/arlequin) extracted from website March 2000.

Taxon	ID	Source	Island	Date	Locality
Hennidiella heimii	RH 1/1 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 1/2 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 1/3 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 1/4 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 1/5 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 2/1 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 2/2 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 2/3 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 3/1 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 3/2 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 3/3 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 3/4 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 3/5 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 3/6 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 3/7 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 3/8 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 3/9 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 3/10 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 3/11 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 3/12 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 3/13 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 3/14 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 3/15 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 4/1 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica

Appendix 1

Taxon	ID	Source	Island	Date	Locality
Hennidiella heimii	RH 4/2 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 4/3 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 4/4 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 5/1 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 6/1 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 6/2 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 6/3 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 6/4 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 6/5 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 6/6 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 7/1 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 8/1 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 8/2 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 8/3 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 8/4 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 8/5 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 8/6 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 8/7 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 8/8 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 9/1 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 9/2 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 9/3 HH	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 10/1 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 10/2 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica

Appendix 1

Taxon	ID	Source	Island	Date	Locality
Hennidiella heimii	RH 11/1 HH	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 12/1 HH	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 12/2 HH	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 12/3 HH	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 12/4 HH	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 13/1A BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 13/1B BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 13/2 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 13/3 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 14/1 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 14/2 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 14/3 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 15/1 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 15/2 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 15/3 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 15/4 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 15/5 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 15/6 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 15/7 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 15/8 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 15/9 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 15/10 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 15/11 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 15/12 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica

Taxon	ID	Source	Island	Date	Locality
Bryum subrotundifolium	RH 15/13 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 16/1 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 17/1 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 17/2 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 17/3 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 17/4 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 17/5 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 18/1A BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 18/1B BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 18/2A BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 18/2B BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 18/3A BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 18/3B BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 18/4 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 18/5 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 18/6 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/1 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/2 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/3 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/4 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/5 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/6 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/7 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/8 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica

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Taxon	ID	Source	Island	Date	Locality
Bryum pseudotriquetrum	RH 19/9 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/10 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/11 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/12 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/13 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/14 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/15 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/16 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/17 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/18 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/19 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/20 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/21 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/22 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/23 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/24 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/25 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/26 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/27 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/28 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/29 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/30 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/31 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/32 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica

Taxon	ID	Source	Island	Date	Locality
Bryum pseudotriquetrum	RH 19/33 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/34 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/35 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 19/36 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 20/1 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 20/2 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 20/3 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 20/4 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 20/5 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 20/6 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 20/7 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 20/8 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 20/9 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 20/10 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 21/1 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 22/1 HH	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 22/2 HH	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 22/3 HH	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 22/4 HH	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 22/5 HH	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 22/6 HH	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	RH 22/7 HH	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 23/1 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 23/2 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica

Taxon	ID	Source	Island	Date	Locality
Bryum subrotundifolium	RH 23/3 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 23/4 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 23/5 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 23/6 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 23/7 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 23/8 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 23/9 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 23/10 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 23/11 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 23/12 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 23/13 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 23/14 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 23/15 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 24/1 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 24/2 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 24/3 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 25/1 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 25/2 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 25/3 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 25/4 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 25/5 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 25/6 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 25/7 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 25/8 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica

Taxon	ID	Source	Island	Date	Locality
Bryum subrotundifolium	RH 25/9 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 25/10 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 25/11 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 25/12 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 25/13 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 26/1 BS	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum pseudotriquetrum	RH 27/1 BP	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	RH 28/1 HH	Wild	Ross Is	26-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 1 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 2 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 3 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 4 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 5 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 6 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 7 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 8 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 9 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 10 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 11 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 12 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 13 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 14 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 15 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 16 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica

Taxon	ID	Source	Island	Date	Locality
Bryum subrotundifolium	ST A 17 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 18 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 19 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Bryum subrotundifolium	ST A 20 BS	Wild	Ross Is	25-Jan-00	Cape Bird, Antarctica
Hennidiella heimii	BI HH	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI 1A BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI 1C BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI A1 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI A2 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI A3 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI A4 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI A5 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI A6 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI A7 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI B1 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI B2 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI B3 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI B4 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI B5 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI B6 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI B7 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI C1 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI C2 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI C3 BS	Wild	Beaufort Island	27-Jan-00	Antarctica

Taxon	ID	Source	Island	Date	Locality
Bryum subrotundifolium	BI C4 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI C5 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI C6 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI C7 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI D1 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI D2 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI D3 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI D4 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI D5 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI D6 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI D7 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI E1 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI E2 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI E3 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI E4 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI E5 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI E6 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI E7 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI F1 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI F2 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI F3 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI F4 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI F5 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI F6 BS	Wild	Beaufort Island	27-Jan-00	Antarctica

Taxon	ID	Source	Island	Date	Locality
Bryum subrotundifolium	BI F7 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI G1 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI G2 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI G3 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI G4 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI G5 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI G6 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI G7 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 1 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 2 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 3 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 4 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 5 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 6 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 7 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 8 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 9 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 10 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 11 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 12 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 13 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 14 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 15 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 16 BS	Wild	Beaufort Island	27-Jan-00	Antarctica

Taxon	ID	Source	Island	Date	Locality
Bryum subrotundifolium	BILT 17 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 18 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 19 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 20 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 21 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 22 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BILT 23 BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 1A BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 1B BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 1C BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 1D BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 1E BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 2A BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 2B BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 2C BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 2D BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 2E BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 3A BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 3B BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 3C BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 3D BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 3E BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 4A BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 4B BS	Wild	Beaufort Island	27-Jan-00	Antarctica

Taxon	ID	Source	Island	Date	Locality
Bryum subrotundifolium	BI2C 4C BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 4D BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 4E BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 5A BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 5B BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 5C BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 5D BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Bryum subrotundifolium	BI2C 5E BS	Wild	Beaufort Island	27-Jan-00	Antarctica
Hennidiella heimii	MV 1/1 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 1/2 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 1/3 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 1/4 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 1/5 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 1/6 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 1/7 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 1/8 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 1/9 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 1/10 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 1/11 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 1/12 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 1/13 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 1/14 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 1/15 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 2/1 HH	Wild		30-Jan-00	Miers Valley, Antarctica

Appendix 1

Taxon	ID	Source	Island	Date	Locality
Bryum subrotundifolium	MV 2/2 BS	Wild		30-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 2/3 BS	Wild		30-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 2/4 BS	Wild		30-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 2/5 BS	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 3/1 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 3/2 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 3/3 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 3/4 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 3/5 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 4/1 BS	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 5/1 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 6/1 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MV 7/1 BP	Wild		30-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MV 7/2 BP	Wild		30-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MV 7/3 BP	Wild		30-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MV 7/4 BP	Wild		30-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MV 7/5 BP	Wild		30-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 8/1 HH	Wild		30-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MV 9/1 BP	Wild		30-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 10/1BS	Wild		30-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 11/1 BS	Wild		30-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/1 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/2 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/3 BS	Wild		31-Jan-00	Miers Valley, Antarctica

Taxon	ID	Source	Island	Date	Locality
Bryum subrotundifolium	MV 13/4 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/5 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/6 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/7 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/8 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/9 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/10 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/11 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/12 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/13 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/14 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/15 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/16 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/17 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/18 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/19 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/20 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/21 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/22 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/23 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/24 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/25 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/26 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/27 BS	Wild		31-Jan-00	Miers Valley, Antarctica

Taxon	ID	Source	Island	Date	Locality
Bryum subrotundifolium	MV 13/28 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/29 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/30 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/31 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/32 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/33 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/34 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/35 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/36 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/37 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/38 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/39 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/40 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/41 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/42 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/43 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/44 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/45 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/46 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/47 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/48 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/49 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/50 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/51 BS	Wild		31-Jan-00	Miers Valley, Antarctica

Taxon	ID	Source	Island	Date	Locality
Bryum subrotundifolium	MV 13/52 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/53 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/54 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/55 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MV 13/56 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 14/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 15/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 16/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 17/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MV 18/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 1/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 2/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 3/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 4/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 5/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 6/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 7/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 8/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 9/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 9/2 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 9/3 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 9/4 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 9/5 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 9/6 HH	Wild		31-Jan-00	Miers Valley, Antarctica

Taxon	ID	Source	Island	Date	Locality
Hennidiella heimii	MG 9/7 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 9/8 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 9/9 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 9/10 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 9/11 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 9/12 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 9/13 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 9/14 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 9/15 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MG 10/1 BP	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MG 10/2 BP	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MG 10/3 BP	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MG 10/4 BP	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MG 10/5 BP	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MG 10/6 BP	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MG 10/7 BP	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MG 10/8 BP	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MG 10/9 BP	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MG 10/10 BP	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MG 10/11 BP	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MG 10/12 BP	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MG 10/13 BP	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MG 10/14 BP	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MG 10/15 BP	Wild		31-Jan-00	Miers Valley, Antarctica

Taxon	ID	Source	Island	Date	Locality
Hennidiella heimii	MG 11/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 12/1 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 13/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 14/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 15/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 16/1 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 17/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 18/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 18/1A BS	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/2 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/3 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/4 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/5 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/6 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/7 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/8 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/9 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/10 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/11 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/12 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/13 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/14 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/15 HH	Wild		31-Jan-00	Miers Valley, Antarctica

Taxon	ID	Source	Island	Date	Locality
Hennidiella heimii	MG 19/16 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/17 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/18 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/19 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/20 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/21 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/22 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/23 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/24 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/25 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 19/26 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/1 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/2 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/3 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/4 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/5 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/6 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/7 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/8 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/9 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/10 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/11 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/12 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/13 BS	Wild		31-Jan-00	Miers Valley, Antarctica

Appendix 1

Taxon	ID	Source	Island	Date	Locality
Bryum subrotundifolium	MG 20/14 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/15 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/16 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/17 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/18 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/19 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/20 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 20/21 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 21/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 21/2 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 21/3 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 21/4 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 21/5 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 21/6 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 21/7 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 21/8 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 21/9 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 21/10 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 22/1 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 23/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 24/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Hennidiella heimii	MG 25/1 HH	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 26/1 BS	Wild		31-Jan-00	Miers Valley, Antarctica
Bryum subrotundifolium	MG 27/1 BS	Wild		31-Jan-00	Miers Valley, Antarctica

Taxon	ID	Source	Island	Date	Locality
Hennidiella heimii	AG 1/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 1/2 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 2/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 2/2 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 3/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 4/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 5/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 5/2 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 6/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 6/2 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 7/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 7/2 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	AG 8/1 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	AG 8/2 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	AG 8/3 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 9/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	AG 10/1 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 11/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 11/2 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	AG 12/1 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	AG 12/2 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	AG 12/3 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 13/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	AG 14/1 BS	Wild		01-Feb-00	Miers Valley, Antarctica

Taxon	ID	Source	Island	Date	Locality
Hennidiella heimii	AG 15/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	AG 16/1 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 17/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 18/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 19/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 20/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 21/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	AG 22/1 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 23/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	AG 24/1 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 25/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	AG 26/1 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	AG 26/2 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	AG 26/3 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	AG 26/4 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	AG 26/5 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 27/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 27/2 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 27/3 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 27/4 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 27/5 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 27/6 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 27/7 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 27/8 HH	Wild		01-Feb-00	Miers Valley, Antarctica

Taxon	ID	Source	Island	Date	Locality
Hennidiella heimii	AG 27/9 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 27/10 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 27/11 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 27/12 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 27/13 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 27/14 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	AG 27/15 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 1/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 1/2 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 2/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 3/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 3/2 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 4/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 5/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 6/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MS 7/1 BP	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 8/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 9/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 10/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 11/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 12/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 13/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MS 14/1 BP	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 15/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica

Taxon	ID	Source	Island	Date	Locality
Hennidiella heimii	MS 16/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 17/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum pseudotriquetrum	MS 18/1 BP	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 19/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 20/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 21/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 22/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 23/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 24/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 25/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 26/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 27/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	MS 28/1 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 29/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 30/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 31/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 32/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 33/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 34/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 35/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 36/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 37/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 38/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 39/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica

Taxon	ID	Source	Island	Date	Locality
Hennidiella heimii	MS 40/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 41/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 42/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 43/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 44/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	MS 45/1 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 46/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 47/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 48/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	MS 49/1 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 50/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	MS 51/1 BS	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/1 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/2 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/3 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/4 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/5 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/6 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/7 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/8 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/9 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/10 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/11 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/12 HH	Wild		01-Feb-00	Miers Valley, Antarctica

Taxon	ID	Source	Island	Date	Locality
Hennidiella heimii	MS 52/13 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/14 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/15 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/16 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/17 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/18 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/19 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Hennidiella heimii	MS 52/20 HH	Wild		01-Feb-00	Miers Valley, Antarctica
Bryum subrotundifolium	CC 1/1 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 1/2 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 1/3 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 2/1 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 2/2 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 3/1 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 3/2 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 3/3 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 4/1 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 5/1 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 5/2 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 5/3 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 5/4 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 6/1 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 7/1 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 7/2 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica

Taxon	ID	Source	Island	Date	Locality
Bryum subrotundifolium	CC 8/1 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 8/2 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 8/3 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 8/4 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 9/1 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 9/2 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	CC 9/3 BS	Wild	Ross Is	13-Jan-01	Cape Crozier, Antarctica
Bryum subrotundifolium	GH 1/1 BS	Wild		18-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 1/2 BS	Wild		18-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 1/3 BS	Wild		18-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 1/4 BS	Wild		18-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 1/5 BS	Wild		18-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 1/6 BS	Wild		18-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 1/7 BS	Wild		18-Jan-01	Granite Harbour, Antarctica
	CG 422	Wild		18-Jan-01	Granite Harbour, Antarctica
	CG 423-1	Wild		18-Jan-01	Granite Harbour, Antarctica
	CG 423-2	Wild		18-Jan-01	Granite Harbour, Antarctica
	CG 423-3	Wild		18-Jan-01	Granite Harbour, Antarctica
	CG 423-4	Wild		18-Jan-01	Granite Harbour, Antarctica
	CG 424	Wild		18-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 2/1 BS	Wild		18-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 2/2 BS	Wild		18-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 2/3 BS	Wild		18-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 2/4 BS	Wild		18-Jan-01	Granite Harbour, Antarctica

Taxon	ID	Source	Island	Date	Locality
Bryum subrotundifolium	GH 2/5 BS	Wild		18-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 3/1 BS	Wild		19-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 4/1 BS	Wild		19-Jan-01	Granite Harbour, Antarctica
Bryum pseudotriquetrum	GH 5/1 BP	Wild		19-Jan-01	Granite Harbour, Antarctica
Bryum pseudotriquetrum	GH 6/1 BP	Wild		19-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 7/1 BS	Wild		19-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 7/2 BS	Wild		19-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 7/3 BS	Wild		19-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 8/1 BS	Wild		18-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 8/2 BS	Wild		18-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 8/3 BS	Wild		18-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	GH 8/4 BS	Wild		18-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	ME 1/1 BS	Wild		20-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	ME 1/2 BS	Wild		20-Jan-01	Granite Harbour, Antarctica
Bryum subrotundifolium	MP 1/1 BS	Wild		22-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 1/2 BS	Wild		22-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 1/3 BS	Wild		22-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 1/4 BS	Wild		22-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 2/1 HH	Wild		22-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 2/2 HH	Wild		22-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 2/3 HH	Wild		22-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 2/4 HH	Wild		22-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 3/1 HH	Wild		22-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 3/2 HH	Wild		22-Jan-01	Marble Point, Antarctica

Taxon	ID	Source	Island	Date	Locality
Hennidiella heimii	MP 3/3 HH	Wild		22-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 4/1 BS	Wild		22-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 4/2 BS	Wild		22-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 4/3 BS	Wild		22-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 4/4 BS	Wild		22-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 4/5 BS	Wild		22-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 5/1 HH	Wild		22-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 5/2 HH	Wild		22-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 5/3 HH	Wild		22-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 5/4 HH	Wild		22-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 5/5 HH	Wild		22-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 6/1 BS	Wild		22-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 6/2 BS	Wild		22-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 6/3 BS	Wild		22-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 6/4 BS	Wild		22-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 7/1 BS	Wild		23-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 7/2 BS	Wild		23-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 7/3 BS	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 8/1 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 8/2 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 8/3 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 8/4 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 9/1 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 9/2 HH	Wild		23-Jan-01	Marble Point, Antarctica

Appendix 1

Taxon	ID	Source	Island	Date	Locality
Hennidiella heimii	MP 9/3 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 10/1 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 10/2 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 10/3 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 10/4 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 10/5 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 10/6 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 10/7 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 10/8 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 10/9 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 10/10 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 10/11 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 10/12 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 10/13 HH	Wild		23-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 10/14 HH	Wild		23-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 11/1 BS	Wild		24-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 11/2 BS	Wild		24-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 11/3 BS	Wild		24-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 11/4 BS	Wild		24-Jan-01	Marble Point, Antarctica
Bryum subrotundifolium	MP 11/5 BS	Wild		24-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 12/1 HH	Wild		24-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 12/2 HH	Wild		24-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 12/3 HH	Wild		24-Jan-01	Marble Point, Antarctica
Hennidiella heimii	MP 12/4 HH	Wild		24-Jan-01	Marble Point, Antarctica

Taxon	ID	Source	Island	Date	Locality
Hennidiella heimii	MP 12/5 HH	Wild		24-Jan-01	Marble Point, Antarctica
	M1 Anderson Gl	Wild		13-Jan-01	Taylor Valley, Antarctica
	M1 Von Gl	Wild		16-Jan-01	Taylor Valley, Antarctica
	M2 Von Gl	Wild		16-Jan-01	Taylor Valley, Antarctica
	M3 Von Gl	Wild		16-Jan-01	Taylor Valley, Antarctica
	M1 Bowles Gl	Wild		16-Jan-01	Taylor Valley, Antarctica
	M2 Bowles Gl	Wild		16-Jan-01	Taylor Valley, Antarctica
	M3 Bowles Gl	Wild		16-Jan-01	Taylor Valley, Antarctica
	M1 Huey Gl	Wild		18-Jan-01	Taylor Valley, Antarctica
	M3 Huey Gl	Wild		18-Jan-01	Taylor Valley, Antarctica
	M1 Canada Gl	Wild		18-Jan-01	Taylor Valley, Antarctica
	M2 Canada Gl	Wild		18-Jan-01	Taylor Valley, Antarctica
	M3 Canada Gl	Wild		18-Jan-01	Taylor Valley, Antarctica
Bryum argenteum	HR 1/1 BA	Wild	North Island	15-Feb-01	Hillcrest road Ham.
Bryum argenteum	HR 1/2 BA	Wild	North Island	15-Feb-01	Hillcrest road Ham.
Bryum argenteum	HR 2/1 BA	Wild	North Island	15-Feb-01	Hillcrest road Ham.
Bryum argenteum	HR 2/2 BA	Wild	North Island	15-Feb-01	Hillcrest road Ham.
Bryum argenteum	HR 3/1 BA	Wild	North Island	15-Feb-01	Hillcrest road Ham.
Bryum argenteum	HR 3/2 BA	Wild	North Island	15-Feb-01	Hillcrest road Ham.

APPENDIX 2

REAGENT PROTOCOLS AND SUPPLIES

This appendix contains protocols from the making of solutions and other materials used in the microsatellite development protocol. There is a section on equations used to make molar solutions and for diluting stock solutions to the correct concentrations at the end of this appendix. Many of the chemicals and reagents used in these protocols are extremely hazardous and often very expensive, all of them should be handled with care and the Material Safety Data Sheets (MSDS) consulted before using for the first time.

Please note:

All solutions should be autoclaved unless otherwise instructed.

This list is not comprehensive, some other reagents may be required for completion of the whole protocol, useful references are: Sambrook *et al.* (1989), Promega Applications Guide and Molecular Biology Labfax.

REAGENT PROTOCOLS

30% Acrylamide: 19:1, acryalamide: bisacrylamide
For 100 mL mix in a fumehood or Captair cabinet

29.0 g of acrylamide
1.0 g *N*, *N'*-methylenebisacrylamide (bisacrylamide)
42.0 g Urea

Add to 60 mL of ultrapure water (Milli-Q or RO), heat to 37°C to dissolve chemicals, make up to 100 mL with Milli-Q water. Filter solution and check pH is close to 7.0, store at room temperature in a dark bottle. CARE REQUIRED; POTENT NEUROTOXIN AND TERATOGEN. Do not autoclave.

10% Ammonium persulphate (APS)
For 10 mL mix

1.0 g APS
9.0 mL Milli-Q water

Dissolve APS and then make up to 10 mL with Milli-Q water. Store at 4°C, will keep for several weeks. Do not autoclave. CARE REQUIRED; RADICAL GENERATOR.

Ampicillin (50 mg mL^{-1})
Add the following to a 15 mL foil covered tube

0.50 g Ampicillin
10.0 mL dH_2O

Mix by shaking until Ampicillin has dissolved. Aliquot 1.0 mL into 1.5 mL tubes and store at -20°C. CARE REQUIRED; ANTIBIOTIC. Do not autoclave.

B1 (Pre-lysis buffer): 25 mM Tris-HCl, 10 mM EDTA, 0.2 mg mL^{-1} RNAse A
For 50 mL mix

1.25 mL 1.0 M Tris-HCl (pH 8.0)

1.0 mL 0.5 M EDTA (pH 8.0)
1.0 mL RNAse A (10 mg mL^{-1})

Make up to 50 mL with dH_2O in a 50 mL centrifuge tube. Store at 4°C. Do not autoclave.

B2 (Lysis buffer): 1.0% SDS (v/v), 0.2 M NaOH
For 30 mL mix

26.4 mL dH_2O
3.0 mL 10% SDS (Sodium dodecyl sulphate)
600 µL 10 N NaOH

Mix in a 50 mL centrifuge tube and adjust pH to 12.0. Store at 4°C. Do not autoclave.

B3 (Neutralising solution): 1.0 M Potassium acetate
For 30 mL mix

8.84 g Potassium acetate

Add to 25 mL dH_2O in a 50 mL centrifuge tube, adjust pH to 5.5 and make up to 30 mL. Store at 4°C. Do not autoclave.

C:I: Chloroform: Isoamyl alcohol, 24:1 (v/v)
Mix 24 parts Chloroform with 1 part Isoamyl alcohol. Do not autoclave. CARE REQUIRED; CHOLOFROM IS HIGHLY TOXIC.

1× CTAB extraction buffer: 1.0% CTAB (w/v), 100mM Tris, 20 mM EDTA, 1.4 M NaCl, 1.0% PVP (w/v)
For 1 L mix

10.0 g CTAB (Cetyltrimethylammonium bromide)
12.1 g Tris-base
7.45 g EDTA (Ethylenediaminetetraacetic acid, disodium salt)
81.8 g NaCl
10.0 g PVP (Polyvinylpyrrolidone, MW 40 000)

Add to 800 mL distilled H_2O (dH_2O), stir until dissolved (heating may be necessary), bring volume to nearly 1 L and adjust pH to 8.0, make to final volume of 1 L. CARE REQUIRED; CTAB WILL DISSOLVE CELLULAR MEMBRANES.

10% CTAB buffer: 10% CTAB (w/v), 0.7 M NaCl
For 100 mL mix

10.0 g CTAB
4.09 g NaCl

Make up to 100 mL with dH_2O in volumetric flask. CARE REQUIRED; CTAB WILL DISSOLVE CELLULAR MEMBRANES.

CTAB precipitation buffer: 1.0% CTAB (w/v), 50 mM Tris, 10 mM EDTA
For 1 L mix

10.0 g CTAB
6.06 g Tris-base
20.0 mL 0.5 M ETDA (pH 8.0)

Add 950 mL dH_2O, adjust pH to 8.0, make up to 1 L. CARE REQUIRED; CTAB WILL DISSOLVE CELLULAR MEMBRANES.

Denaturing solution: 0.5 N † NaOH, 1.5 M NaCl
For 1 L mix

20.00 g NaOH (add slowly to water, evolves heat when dissolved)
87.66 g NaCl

Add to 700 mL dH_2O, dissolve solids and make up to final volume of 1000 mL. CARE REQUIRED; CORROSIVE.

50× Denhardt's solution: 1.0% BSA (w/v), 1.0% Ficoll (w/v), 1.0% PVP (w/v)
For 50 mL mix in a 50 mL centrifuge tube

0.5 g Bovine Serum Albumin, fraction V (BSA)
0.5 g Ficoll
0.5 g PVP

Add Milli-Q water up to 50 mL and mix until all solids have dissolved, filter sterilise and store at 4°C for short-term or -20°C for long-term. Do not autoclave.

dNTP (1 mM each)
For 10 mL mix

100.0 µL 100 mM dATP
100.0 µL 100 mM dGTP
100.0 µL 100 mM dCTP
100.0 µL 100 mM dTTP

Add to 9.60 mL Milli-Q H_2O, mix thoroughly, aliquot 1.0 mL into 1.5 mL tubes, store at -20°C. Do not autoclave.

EDTA (0.5 M)
For 500 mL mix

93.1 g EDTA (disodium salt)

EDTA will not dissolve unless the pH of the solution is 8.0.
Dissolve approximately 7.0 g of NaOH in about 300 mL dH_2O then add the EDTA, continue adding NaOH until the pH is close to 8.0. The EDTA should now dissolve slowly, bring volume close to 500 mL and adjust pH to 8.0 with 10 M NaOH, adjust final volume to 500 mL and check pH again, adjust if needed.

Ethidium bromide (5 mg mL^{-1})
For 5 mL

In a brown glass bottle dissolve
25 mg Ethidium bromide
5.0 mL dH_2O

Mix until solids are dissolved, store at room temperature. CARE REQUIRED; TERATOGEN, USE A CAPTAIR CABINET TO WEIGH SOLID. Do not autoclave.

6× Gel Loading Dye: 0.05% Bromophenol Blue/Xylene cyanol (v/v), 15% Ficoll (v/v), 30 mM EDTA
For one millilitre mix

50.0 µL 1.0% Bromophenol Blue/Xylene cyanol mix (BBXC)

† Normal solutions (N) are solutions that contain one 'gram equivalent weight' (gEW) of solute per litre of solution. The gEW is equal to the molar mass of the solute divided by the valency of the solute. For example, for a 1.0 N solution of Magnesium hydroxide ($Mg(OH)_2$) M_r=58.33 g mol^{-1}. $Mg(OH)_2$ contains two hydroxyl groups so half a mole of $Mg(OH)_2$ will accept one mole of protons (H^+), therefore the valency is two. Thus 58.33/2 = 29.165 g, dissolve this in one litre of water to make a 1.0 N solution.

750.0 µL 70 % Ficoll
60.0 µL 0.5 M EDTA

Add to 140.0 µL dH_2O in a 1.5 mL tube, store at room temperature or at 4°C. Do not autoclave. CARE REQUIRED, BBXC IS HIGHLY TOXIC IN POWDERED FORM

IPTG (Isopropyl β-D-thiogalactopyranoside) (100 mM)
Add to a foil covered 15 mL tube

0.50 g IPTG
12.25 mL dH_2O

Mix by shaking, until the solids have dissolved. Aliquot 1.0 mL into 1.5 mL tubes and store at -20°C. Do not autoclave.

LB-broth (Luria-Bertani broth): 1.0% Tryptone (w/v), 0.5% Yeast extract (w/v), **1.0% NaCl (w/v)**

For 1 L mix

10.0 g Tryptone
5.0 g Yeast extract
10.0 g NaCl

Add to 990 mL dH_2O, adjust pH to 7.0, make up to 1000 mL. Autoclave and allow to cool to at least 50 °C before adding antibiotics.

LB-agar plates: 1.0% Tryptone (w/v), 0.5% Yeast extract (w/v), 1.0% NaCl (w/v), 1.5% Agar (w/v)

Make LB-Broth as above, but add 15 g L^{-1} agar before autoclaving. If making one litre, divide into two 500 mL flasks and allow to cool to 50°C before adding antibiotics and pouring into plates.

Neutralising solution: 0.5 M Tris, 1.5 M NaCl
For 1 L mix

60.55 g Tris-base
87.66 g NaCl

Make up to 970 mL with dH_2O, adjust pH to 7.7, make to final volume of 1000 mL.

P:C:I: Phenol: Chloroform: Isoamyl alcohol, 25:24:1 (v/v)
To make P:C:I , measure a volume of TE saturated Phenol into a glass measuring cylinder and take the volume of the organic layer, then add an equal volume of C:I, transfer to a separating funnel and shake well, venting the funnel frequently. Finally let the funnel stand until the layers have separated and drain the organic layer, with a little of the aqueous into a brown bottle and discard the remainder, repeat twice more and check the pH, when it is between 7.0 and 8.0, the equilibration is finished, top off the bottle with a layer of 100 mM Tris (pH 8.0) and 100 µL β-Mercaptoethanol. Store at 4 °C in a brown glass bottle. Do not autoclave. CARE REQUIRED; PHENOL AND CHLOROFORM PRODUCE TOXIC VAPOUR.

PEG: 20% PEG 8000 (w/v), 2.5 M NaCl
For 50 mL mix

10.0 g PEG 8000
7.305 g NaCl

Dissolve in 25 mL dH_2O and make up to final volume of 50 mL. Store at 4°C. Do not autoclave.

10% SDS (Sodium dodecyl sulfate) (w/v)
For 100 g L^{-1}, dissolve 100g SDS and dilute to 1000 mL with dH_2O. Do not autoclave. CARE REQUIRED; IRRITANT, WEAR MASK OR WORK IN FUMEHOOD WHEN WEIGHING.

Sequencing loading dye: 80% Formamide, 0.01% BBXC
For 1 mL mix

800 µL Formamide (deionised)
200 µL 0.5% BBXC solution

Mix in a foil covered bijou or dark glass bottle and cap firmly. Store in a vented cupboard or in a fume hood. ALL WORK WITH FORMAMIDE (TERATOGEN) MUST BE DONE IN A FUMEHOOD WITH NITRILE GLOVES OR OTHER SUITABLE PROTECTION. BBXC IS VERY TOXIC, TAKE CARE AROUND POWDER FORM.

Sodium acetate (3 M)
For 1 L mix

246.09 g Sodium acetate (anhydrous)

Add to 900 mL dH_2O, mix until all solids have dissolved and make up to final volume of 1000 mL.

Sodium hydroxide (10 N)
For 100 mL

40.0 g NaOH (pellets)

Add slowly to 90 mL dH_2O. Heat is evolved during this step, cool flask under running water. When all pellets are dissolved, make up to final volume of 100 mL. CARE REQUIRED, VERY CORROSIVE.

20× SSC (standard saline citrate): 3 M NaCl, 0.3 M Sodium citrate
For 2 L mix

351.0 g NaCl
176.0 g Sodium citrate

Dissolve in 1800 mL dH_2O and bring to final volume of 2000 mL.

1× STE buffer (high salt TE): 10 mM Tris, 1.0 mM EDTA, 1.0 M NaCl
For 1 L mix

10.0 mL 1.0 M Tris-base (pH 8.0)
2.0 mL 0.5 M EDTA (pH 8.0)
58.44 g NaCl

Bring to final volume of 1000 mL with dH_2O, after adjusting pH to 8.0.

5× TBE: 445 mM Tris, 444 mM Orthoboric acid, 11 mM EDTA
For 2 L mix

108.0 g Tris-base
55.0 g Orthoboric acid
8.3 g EDTA (disodium salt)

Add to 1950 mL dH_2O, adjust pH to 8.0 (± 0.3), and make up to final volume of 2000 mL.

1× TE buffer: 10 mM Tris, 1.0 mM EDTA
For 1 L mix

10.0 mL 1 M Tris-base (pH8.0)
2.0 mL 0.5 M EDTA

Bring to final volume of 1000 mL with dH_2O.

Tetracycline (13 mg mL^{-1})

Add to a 15 mL foil covered tube

0.13 g Tetracycline
10 mL 50% ethanol

Mix until Tetracycline is dissolved, store at –20°C. Inspect for precipitation of solids, and discard when present. CARE REQUIRED; ANTIBIOTIC. Do not autoclave.

Tris-HCl (1 M)

For 1 L mix

40.37 g Tris-base
105.07 g Tris-HCl

Dissolve in 700 mL dH_2O, and adjust pH to 8.0 using concentrated HCl. Make up to final volume of 1000 mL, double-check pH and adjust if necessary.

1× TSS: 10% (w/v) PEG 8000, 5.0% (v/v) DMSO, 50 mM $MgSO_4$

For 50 mL, add to a 40 mL LB-broth in a 50 mL tube

5.0 g PEG (Polyethylene glycol) 8000
5.0 mL DMSO (Dimethyl sulphoxide)
2.5 mL 1.0 M $MgSO_4$

Adjust pH to 6.5 (if necessary) and make up to final volume of 50 mL using LB-broth. Filter-sterilise and store at 4°C. Do not autoclave.

X-Gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside) (50 mM)

In a 15 mL Tube

0.20 g X-Gal
10.0 mL Dimethyl formamide (DMF) or 50% DMSO

Wrap tube in foil and mix by shaking until the X-Gal has dissolved completely, store in freezer at -20°C. Do not autoclave. CARE REQUIRED; DMF AND DMSO ARE POTENTIAL TERATOGENS

2 X YT-Broth: 1.6% Tryptone (w/v), 1.0% Yeast extract (w/v), 0.5% NaCl (w/v)

For 1 L mix

16.0 g Tryptone
10.0 g Yeast extract
5.0 g NaCl

Add to 900 mL of dH_2O, stir until all solutes have dissolved. Adjust pH to7.0 with 5 N NaOH and make up to 1 L with dH_2O.

USEFUL EQUATIONS:

1. $C = n / V$

Where 'C' is the concentration in mol L^{-1}, 'n' is the number of mole of substance you have, and 'V' is the final volume in litres. Thus if I need to make 250 mL of a two-molar solution of NaCl (Sodium chloride), I need to know the number of mole required for that volume

C = mol L^{-1}, V = 0.25 L

Make 'n' the subject of the equation:

$n = C \times V$
n = 2.0 mol $L^{-1} \times 0.25$ L
n = 0.5 mole

Thus, I need half a mole of NaCl to make 250 mL of a two-molar solution.

2. $n = m/ m_r$

Where 'n' is the number of mole of substance you have, 'm' is the mass you have, and 'm_r' is the molar mass of the substance in g mol^{-1}. For example, for the 250 mL, two-molar solution of NaCl (m_r= 58.44) above, I need to work out the mass of NaCl required for this solution:

n = 0.5 mole, m_r = 58.44 g mol^{-1}.

Make 'm' the subject of the equation

$m = n \times m_r$
m = 0.5 mol $\times$ 58.44 g mol^{-1}
m = 29.22 g

Thus, I need 29.22g of NaCl to make 250 mL of a two-molar solution of NaCl.

3. $C_1V_1 = C_2V_2$

Where 'C_1' is the final concentration of a solution you want, 'V_1' is the final volume you want, 'C_2' is the initial concentration you have and 'V_2' is the initial volume you have. For example, if I need to make 50 mL of 100 mM NaCl solution and I have the two-molar solution made above:

V_1 = 50 mL, C_1 = 0.1 mol L^{-1}, C_2 = 2.0 mol L^{-1}

Make 'V_2' the subject of the equation

$V_2 = C_1V_1/C_2$
V_2 = 0.1 mol $L^{-1} \times$ 50 mL/2.0 mol L^{-1}
V_2 = 2.5 mL

Thus to make the 100 mM solution above, I need to take 2.5 mL of 2.0 mol L^{-1} NaCl and make up to a final volume of 50 mL.

N.B. Cancellation of the units in (3) above is correct as this is a ratio, thus it is possible to mix 'mL' with 'mol L^{-1}' for this equation, but it is necessary to work with litres and grams as units for volumes and masses to be consistent with the g mol^{-1} and mol L^{-1} in equations one and two.

SUPPLIES

This section of the Appendix contains chemical and expendable equipment used for the microsatellite development protocol. Reagents and equipment are catalogued by name, supplier and catalogue number

Chemical	Formula	Supplier	Catalogue #
Acrylamide	C_3H_3NO	BDH	44313 2V
Agar	-	Applichem	A3477 0250
Agarose	-	Roche	1 388 991
Ampicillin	$C_{16}H_{18}N_3O_4SNa$	Roche	835 242
APS	$(NH_4)_2S_2O_2$	Sigma	A-9164
^{33}P-dATP	-	Amersham	BF1000-250μCi
BBXC	-	Sigma	B-3269
Bisacrylamide	$C_7H_{10}N_2O_2$	Sigma	M-2022
BSA (Fraction V)	-	Sigma	A-4503
Chloramphenicol	$C_{11}H_{12}Cl_2N_2O_5$	BDH	44204 2Q
Chloroform	CH_3Cl	BDH	10077 6B
CTAB	$C_{19}H_{42}NBr$	Sigma	H-6269
Dithiothreitol (DTT)	$C_4H_{10}S_2O_2$	GibcoBRL	15508-013
DMSO	C_2H_6SO	Sigma	D-8418
DNTP	-	Roche	1 277 049
EDTA	$C_{10}H_{14}N_2O_8Na_2$	Sigma	E-5134
Ethidium bromide	$C_{21}H_{20}N_3Br$	Sigma	E-7637
Ficoll	-	Sigma	F-4375
Formamide	$HCONH_2$	BDH	444472T
Glucose	$C_6H_{12}O_6$	BDH	100117 4Y
Glycerol	$C_3H_8O_3$	ICN Biomedicals	193996
Hydrochloric acid	HCl	BDH	45002 0H
IPTG	$C_9H_{18}O_2S$	Sigma	I-5502
iso-Amyl alcohol	$C_5H_{11}OH$	BDH	10038 3L
Kanamycin sulphate	$C_{18}H_{36}N_4O_{11}.H_2SO_4$	GibcoBRL	11815-024
Magnesium chloride	$MgCl_2$	Scharlau	Ma 0037
Magnesium sulphate	$MgSO_4.7H_2O$	Sigma	M-2773
Malt extract	-	ICN Biomedicals	1006917
β-Mercaptoethanol	C_2H_6OS	Sigma	M-6250
Orthoboric acid	H_3BO_3	BDH	10058 3R
P:C:I	-	Sigma	P-3803
PEG 8000	-	Sigma	P-5413
Potassium acetate	$KC_2H_3O_2$	Sigma	P-1190
Potassium chloride	KCl	BDH	43702 3F
PVP 40000	-	Sigma	PVP-40T
Sodium acetate	$C_2H_3O_2Na$	Sigma	S-2889
Sodium chloride	NaCl	BDH	44382 4T
Sodium citrate	$C_6H_5Na_3O_7$	BDH	43607 5N
Sodium dodecyl sulphate	$C_{12}H_{25}O_4Sna$	Gibco BRL	15525-017
Sodium hydroxide	NaOH	Scharlau	So 0420
TEMED	$C_6H_{16}N_2$	Sigma	T-7024
Tetracycline	$C_{22}H_{24}N_2O_8$	Sigma	T-3258

(Freebase)			
Tris-base	$C_4H_{11}NO_3$	Applichem	A1379 1000
Trizma-HCl (Tris-HCl)	$C_4H_{11}NO_3.HCl$	Sigma	T-3253
Tryptone	-	ICN Biomedicals	1010817
UTP (Li Salt)	-	Roche	3 420 470
X-gal	$C_{14}H_{15}BrClNO_6$	Sigma	B-4252
Yeast extract	-	Serva	24540

Hardware

Item	Supplier	Catalogue #
10 µL pipette tips	Scientific Specialities Inc. Mettler toledo	PT-01-N
250 µL pipette tips	Scientific Specialities Inc. Molecular Bioproducts Inc.	4220-00
1000 µL pipette tips	Scientific Specialities Inc. Molecular Bioproducts Inc.	4330-00
0.2 mL PCR tubes	Scientific Specialities Inc.	3210-00
0.5 mL PCR tubes	Scientific Specialities Inc.	3320-00
1.5 mL Eppendorf tubes	Scientific Specialities Inc.	1211-00
15 mL tubes	Scientific Specialities Inc.	2835-SP-50100
50 mL tubes	Scientific Specialities Inc.	2935-SP-50100
Developer	Kodak	400 9510
Film	Kodak	165 4545
Fixer	Kodak	400 9478
Gel Extraction Kit	Life Technologies	11456-019
Plasmid preparations	Eppendorf Perkin-Elmer Applied Biosystems	

Primer Sequences

Primer	Sequence
ITS4	TCC TCC GTC TAT TGA TAT GC
ITS5HP	GGA AGG AGA AGT CGT AAC AAG G
M13 universal forward	TGT AAA ACG ACG GCC AGT
M13 universal reverse	GGA AAC AGC TAT GAC CAT G
OPA3	AGT CAG CCA C

Melting Temperature Calculations

For oligonucleotides greater than 10 bp in length and 1 M salt (annealing conditions):

$T_M = 81.5 + 41(\%G/C) - (675/\text{primer length})$ (Equation 1)

For primers greater than 10 bp in length in 50 mM salt solution (PCR conditions):

$T_M = 59.9 + 41(\%G/C) - (675/\text{primer length})$ (Equation 2)

Where percent G/C values are the decimal value (e.g. 46% G/C = 0.46) and the primer length is in base pairs.

APPENDIX 3

SUPPLEMENTARY PROTCOLS

AGAROSE GEL ELECTROPHORESIS

To make a 1.5% agarose gel in 1× TBE buffer, 1.5 % (weight per volume) of agarose was weighed out and added to the corresponding volume of 1× TBE buffer in a conical flask (e.g., for a 1.5% agarose gel of 50 mL volume, use 0.75 g agarose in 50 mL of 1× TBE buffer). This was then weighed and heated to boiling point in a microwave, at which point the solution was taken out and swirled. It was then re-weighed and distilled water added to make up the volume lost, with a little extra to account for evaporation during further heating and cooling. The solution was then re-heated in the microwave until the agarose was completely dissolved. A gel mould of suitable size was set up with an appropriate comb while the agarose/TBE solution was cooling to approximately 50°C. When the agarose solution had cooled, 0.1 ng mL^{-1} ethidium bromide was added and thoroughly mixed by swirling. The solution was then poured into the mould, any bubbles removed and the gel allowed to set. Once set, the comb was removed to form the wells, and the gel was placed in a horizontal, submarine-gel-electrophoresis tank containing 1× TBE buffer so that the gel was completely submerged under 0.5 - 1.0 cm of buffer. To submarine load DNA into the gel, an aliquot of DNA solution was mixed with one tenth of the aliquot volume of loading buffer (bromophenol blue/ xylene cyanol). This mixture was then pipetted into the wells on the gel. Gels were electrophoresed at between 2.4 and 4.0 Watts per centimetre of gel length.

PLASMID MINI-PREP (MODIFIED X-GEN PROTOCOL)

1. From an overnight culture of cells, aliquot 1.5 mL of cells into a labelled Eppendorf tube, centrifuge at G_{max} for two minutes on a bench-top centrifuge.
2. Aspirate away supernatant and add 100 µL B1 using a wide bore pipette tip, re-suspend the pellet.
3. Add 100 µL B2 to each tube and mix by inversion for one to three minutes. Clearing of the solution indicates complete mixing.

4. Add 100 µL of B3 and mix by inversion. When a white precipitate is observed (two phases obvious) vigorously shake the tube to break up the precipitate.
5. Centrifuge the tubes at G_{max} for five minutes and transfer the supernatant to a new tube, making sure none of the white precipitate is transferred.
6. Add 1.0 mL of -20°C, 95% Ethanol and incubate at -20°C for 1 hour or more.
7. Centrifuge tubes at G_{max} for five minutes and aspirate away supernatant. A white pellet should now be visible, dry the pellet completely in a DNA speed-vac (Savant)
8. Re-suspend the pellet in 50-80 µL TLE buffer or Milli-Q water, centrifuge at G_{max} for two minutes and transfer to a new tube.
9. Quantify the plasmid DNA on a 1.5 % agarose gel in TBE buffer.

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

To make a polyacrylamide gel for sequencing or running microsatellite amplification products, it is necessary to have the solutions pre-prepared, this requires linear acrylamide (30%), *N*, *N'*-methylenebisacrylamide, 10% APS (ammonium persulphate) and TEMED (*N*, *N*, *N'*, *N'*-tetramethylethylenediamine). Care is required in the manufacture of all these solutions as all are suspected carcinogens or neurotoxins. Linear acrylamide especially is a neurotoxin, and suspected carcinogen. It must be handled with care; all preparations involving the powdered from must be carried out in a fume hood or Captair cabinet.

Make sure all the glassware is clean, especially the plates for the gel. These can be cleaned using KOH/methanol (~5 g KOH pellets in 100 mL methanol), then washed with detergent and rinsed under tap water, then distilled water. Make sure that the plates are held by the edges so that grease from the fingers does not get on to working surfaces. Finally, rinse with ethanol and allow to dry. Treat one surface of each plate with a silicone solution (e.g. Sigmacote, care, work in a fumehood) by wiping gently over the surface with Kim-wipes and rinsing in de-ionised water, then dry with a hair dryer. The silicone prevents the gel from sticking to the glass when removed from the electrophoresis apparatus.

Set up a gel mould with 0.4 mm spacers on either side, making sure all the sides are sealed adequately. This is done by laying the large glass plate down and placing the spacers down the sides with a minute amount of Vasoline to keep them in place. Lay the smaller plate on top of the spacers and align with one end of the big plate. Tightly tape the two sides and bottom together for a watertight seal.

For this protocol, 6% denaturing polyacrylamide gels are used. To prepare a 6% gel, mix in vacuum flask: 20 mL acrylamide solution, 20 mL 5× TBE buffer and add deionised water to 100 mL, then add 500 µL 10% APS and de-gas the solution with swirling until the solution stops bubbling. Degassing is an important step as the formation of the cross-linking bonds in polyacrylamide is inhibited in the presence of oxygen. Wearing gloves and working over a spill tray, add 35 µL TEMED to 100 mL of the 6% acrylamide solution and mix.

Draw the solution into a 50 mL syringe and expel any air drawn up at the same time. Place the nozzle of the syringe at the opening. Fill the gel mould almost to the top making sure no air bubbles are present in the gel, and keep remaining solution at 4°C to slow polymerisation. Place the gel mould at a 10° tilt and insert a comb (sharkstooth for microsatellites), making sure there is no air trapped between the comb and the gel. If there is a gap between the top of the gel and the top of the mould, fill completely with some of the remaining gel solution. Clamp the comb in place and check that there is no acrylamide leaking from the mould. Allow the gel to polymerise for at least 2 hours, longer is preferable.

Once gel is polymerised remove the comb and wash the wells out immediately with MilliQ water, and remove the tape from the sides and bottom of the gel mould. Clamp the gel into the electrophoresis tank; the smaller plate should face the buffer reservoir. Fill the reservoirs with 1× TBE, or the same buffer as used to make the gel and remove any air bubbles trapped under the gel mould. Flush the wells of the gel with more 1× TBE buffer.

Load the gel, making sure that samples do not spill over into the next well, connect the electrodes to a power pack and run at 1 – 8 V cm^{-1} for the times shown in Table 2.3.