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A Study on Molecular Systematics of the Antarctic *Bryum* Species and the Development of Microsatellite DNA Markers in *Bryum argenteum* Hedw.

A thesis
submitted in partial fulfilment
of the requirements for the Degree
of
Master of Science in Biological Sciences
at the
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by

Sarah A. Hunger

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of Waikato
Te Whare Wānanga
o Waikato

The University of Waikato
2000

This thesis is dedicated to my family and to Mr Cotter
for their endless love and support.

Antarctic *Bryum subrotundifolium* Jaeg.

"I shall point out once more that mosses and their allies present some remarkably promising but relatively untouched materials for experimental research on basic and fundamental problems of evolution and speciation in plants."

- W. C. Steere, 1958

ABSTRACT

The "silver" species *Bryum subrotundifolium* Jaeg. (Bryaceae) in Antarctica has undergone numerous taxonomic revisions. This research used the internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA (nrDNA) to estimate the phylogenetic relationships of silver *Bryum* specimens from Antarctica, the Subantarctic islands, New Zealand and Australia. *Bryum subrotundifolium B. argenteum* Hedw., *B. capillare* Hedw., and the non-silver species, *B. pseudotriquetrum* (Hedw.) Gaertn, Meyer et Scherb., which is also taxonomically difficult in Antarctica, were analysed with *B. pseudotriquetrum* used as an outgroup. Antarctic *B. subrotundifolium* formed a clade with the other *B. subrotundfolium* specimens and the *B. argenteum* specimens. This suggests that *B. subrotundifolium* is conspecific with *B. argenteum*. The Antarctic specimens formed a clade within the main *B. argenteum/B. subrotundifolium* clade. This combined with the variation in morphology observed in the Antarctic specimens suggest that the antarctic material is an ecotypical variant of *B. argenteum*.

ITS sequence data indicated genetic homogeneity between geographically isolated populations of antarctic *B. subrotundifolium* contrary to evidence detected by random amplified polymorphic DNA analyses (RAPDs). Contamination was observed in the Antarctic moss specimens and multiple bands were detected in polymerase chain reaction (PCR) products from these specimens. The contaminating bands were found to be from a fungal species from the genus *Phoma* using a combination of molecular data and morphological examination. This provides a possible explanation for the high levels of variation detected using RAPDs and indicates the usefulness of universal DNA sequences with length polymorphisms, such as ITS sequences, for detecting contaminants in DNA samples for RAPD analysis.

The development of microsatellite DNA markers in *B. argenteum* has commenced. This PCR-based technique is much less sensitive to contamination than RAPDs which use arbitrary primers to randomly amplify DNA throughout the genome. The primers used to amplify microsatellite DNA are targeted to a specific species and

close relatives of it. Microsatellite DNA markers are expensive and time-consuming to develop. However, once developed they are far more reliable than RAPDs, require minimal amounts of DNA, are simple to use and can be used to detect variation at different levels within a population depending on the amount of polymorphism present in a locus. These markers will be employed in future population-level research of these cosmopolitan moss species in Antarctica and temperate regions.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

CHAPTER ONE

INTRODUCTION

1.1 ANTARCTICA

1.1.1 Isolation of the Antarctic Continent

The Antarctic continent was once part of the supercontinent Pangaea (Wegener, 1915). Lauraisa and Gondwana were formed when Pangaea divided during the Triassic period approximately 220 million years ago. Gondwana drifted down to the Southern Hemisphere and began to fragment during the Jurassic era (approximately 200 million years ago; Ségoufin, 1978; Simpson et al., 1979; Lawver and Scotese, 1987) with Antarctica becoming completely isolated 172 million years later (Figure 1.1). Africa was first to shear away separating from the coast of Dronning Maud Land on the Antarctic continent approximately 140 million years ago (Lawver et al., 1985, Lawver et al., 1991). South America and the India/Sri Lanka landmass followed, rifting from Enderby Land and the Queen Mary Coast between 127 and 118 million years ago (Lawver et al., 1991). Australia separated from Wilkes Land approximately 110 to 90 million years ago (Cande and Mutter, 1982) followed by drifting of the North and South Islands of New Zealand from Victoria Land in the Ross Sea region and Marie Byrd Land approximately 72 million years ago (Laird, 1981; Stock and Molnar, 1982; Eittreim et al., 1985; Stock and Molnar, 1987; Bradshaw, 1988). Antarctica was completely isolated after the opening of the Drake Passage approximately 28 million years ago (Barker and Lawver, 1988), with the Antarctic Ice Sheet existing intermittently for 30 to 40 million years (Hambrey et al., 1989; Birkenmajer, 1987; Barrett et al., 1991). The ice sheet is estimated to have maintained its current configuration for the past 14 million years (Shackleton and Kennett, 1975; Sugden et al., 1993), although there have been substantial fluctuations over the last 2 million years (Inglølfsson et al., 1998)

1.1.2 Antarctic Botanical Zones

Antarctica, as it exists today, can be divided into three main botanical zones (Seppelt et al., 1998; Figure 1.2): The Subantarctic zone (cool), the Maritime Antarctic zone (cold), and the Continental Antarctic zone (frigid). The Subantarctic zone

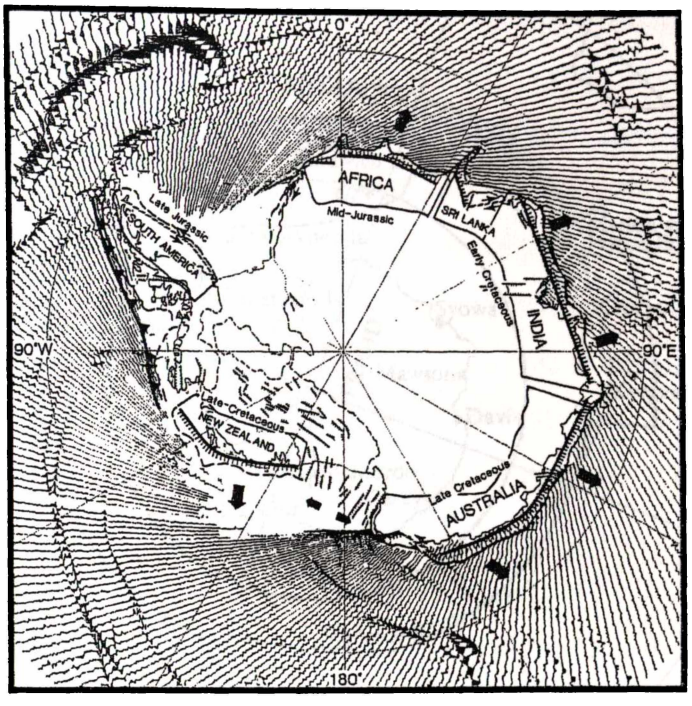

Figure 1.1. Antarctica as is exists today with the positions held by the major continents prior to the break up of Gondwana highlighted (Adapted from Lawver et al., 1991).

encompasses the oceans and islands from 60°S to 42°S including: South Georgia, Marion and Prince Edward Islands; Iles Crozet; Iles Kerguelen; Heard and Macdonald Islands; and Macquarie Island (Seppelt et al., 1998). Beneath this is the greater Antarctic continent, which includes all land south of 60°S latitude, incorporating the South Sandwich Islands and Bouvetøya Islands (Greene 1964). This zone is divided again into Maritime Antarctica and Continental Antarctica. Maritime Antarctica includes the South Orkney Islands, South Shetland Islands, South Sandwich and Bouvetøya Islands, and the north-west Antarctic peninsula. The eastern and southern Antarctic peninsula and the great mass of the Antarctic continent are considered to be Continental Antarctica (Seppelt et al., 1998). These last two zones are differentiated by climatic conditions, with the maritime Antarctic zone characterised by narrow seasonal temperature ranges, warmer mean monthly temperature ranges (0-3°C) during summer, frequent precipitation (often rain), and abundant free ground water, while Continental Antarctica has cooler summers, more severe winters, and extreme aridity (Holdgate, 1964).

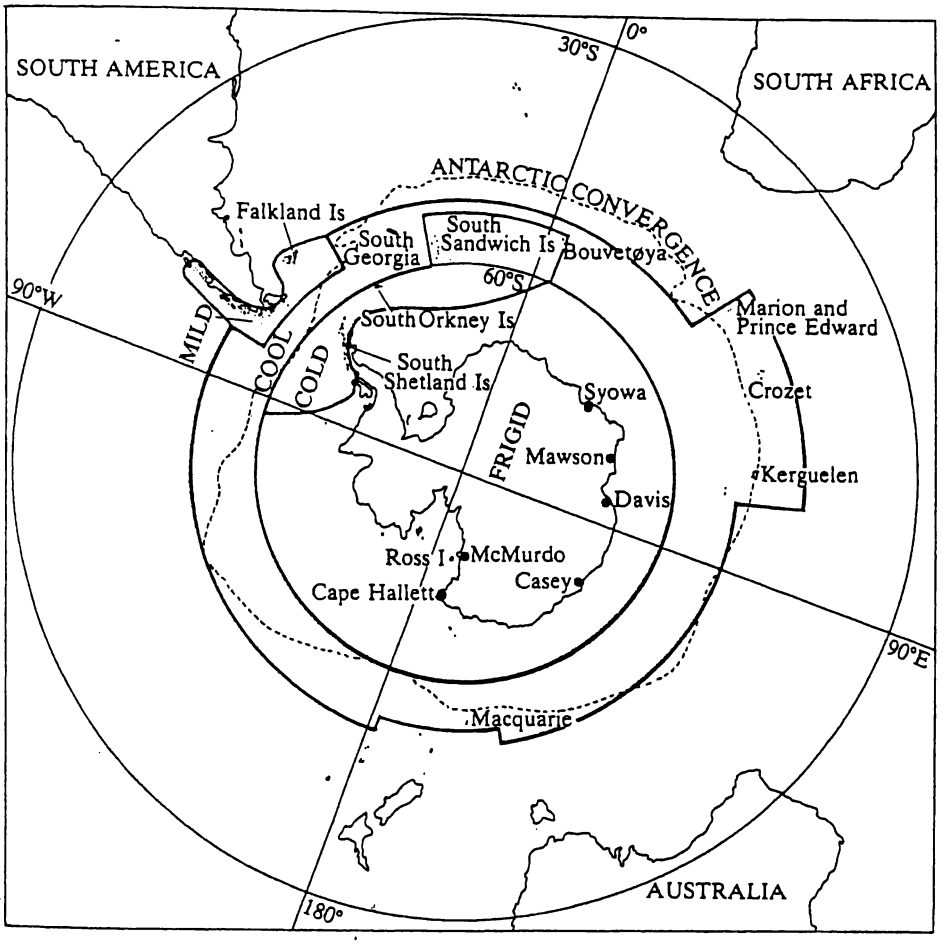

Figure 1.2. Map of the Antarctic continent illustrating the Antarctic botanical zones (from Longton, 1988). Boundaries as described by Greene (1964) and Longton (1985).

1.1.3 Ecology of the Antarctic Botanical Zones

The vegetation ecology of the Antarctic botanical zones is unique in that it consists mainly of cryptogamic species. The limited species diversity in this system results in distribution of these species through a variety of habitats with little fidelity to specific communities (Longton, 1979). The Maritime Antarctic flora is impoverished compared to equivalent ecosystems in the Arctic (Brown, 1906) and is closer in composition to vegetation in the middle to high Arctic (Polunin, 1951). Lower summer temperatures, rather than geographic isolation, have been postulated as an explanation for the predominance of cryptogaminc flora at this longitude (Longton and Holdgate, 1967). Vegetation distribution in Continental Antarctica is thought to be governed primarily by free water availability (Llano, 1965; Rudolph, 1971), with composition of the vegetation influenced primarily by the amount of free water available and the chemical constituents of the substratum (Longton, 1979).

Distribution is also influenced by snow cover and prevailing wind direction due to the effect of these factors on moisture levels and temperature (Longton, 1979).

Reduced free water availability in Continental Antarctica decreases the range of cryptogamic communities in this zone (Llano, 1965; Rudolf, 1971). The four main moss communities occur in Antarctica: The short moss turf and cushion, the tall moss turf, the bryophyte carpet and mat, and the moss hummock subformations (Longton, 1979). The type of community in an area relates to water availability with the latter communities occurring in drier habitats. This is thought to be due to the different water absorption and retention capacities of each community (Gimingham, 1967; Gimingham and Smith, 1971).

Vegetation in Continental Antarctica consists of the latter three moss subformations characteristic of the drier areas in the Maritime Antarctic, although the short moss turf and cushion formation is reported to occur in coastal areas (Rudolph 1963; Llano, 1965; Filson, 1966; Kuc, 1968-1969) and on ice free nunataks (Siple, 1938; Perkins, 1945; Bowra et al., 1966). Rich communities of mosses and algae are also reported to grow beneath lakes in both Continental and Maritime Antarctica (Light and Heywood, 1973; 1975; Heywood, 1977). Mosses and lichens have not been reported from the ice on the central plateau region, although they have been recorded as far south as 84°35'S and 87°21'S (Wise and Gressit, 1965; Cameron et al., 1971).

1.1.4 Biodiversity in Antarctica

Two species of angiosperms (Greene, 1970), approximately 130 bryophyte species (Steere, 1961; Greene, 1968) including 25 - 30 hepatics (Grolle, 1972), at least 250 species of lichen (Dodge, 1973) and approximately 700 terrestrial and aquatic algae constitute the Antarctic terrestrial flora (Seppelt et al., 1998). This vegetation is restricted to ice-free areas which constitute 2% of the 14 million km^2 continent (Melick and Seppelt, 1997; Seppelt et al., 1998). Highest concentrations of vegetation occur in the northern Antarctic Peninsula while the areas further south and inland and the peripheral islands are less densely vegetated (Melick et al., 1994). Due to physiological requirements mosses are generally confined to coastal areas exposed to

run-off from summer melt-water (Kennedy, 1993).

Biodiversity is greatly reduced in Continental Antarctica (Greene, 1967). Approximately 90 species of moss are reported from Maritime Antarctica, declining to 12 species along the Antarctic coastline and to four species inland in the Dry Valleys (Seppelt, 1984). Eight genera, *Bryoerythrophyllum* Chen, *Bryum* Hedw., *Ceratodon* Brid., *Didymodon* Hedw., *Grimmia* Hedw., *Hennediella* Paris, *Sarconeurum* Bryhn and *Schistidium* Bruch et Schimp., are represented in the Continental Antarctic flora. Only eight moss species are found in Southern Victoria Land: *Bryum subrotundifolium* Jaeg., *B. pseudotriquetrum* (Hedw.) Gaertn., Meyer et Scherb., *Ceratodon purpureus* (Hedw.) Brid., *Didymodon gelidus* Card., *Grimmia Antarctici* Card., *G. plagiopodia* Hedw., *Hennediella heimii* (Hedw.) Zander, and *Sarconeurum glaciale* (C. Müll.) Card. et Bryhn, (Seppelt et al., 1998).

1.2 THE GENUS *BRYUM*

1.2.1 Main Taxonomic Features of the Genus *Bryum*

The genus *Bryum* Hedw. (Bryaceae) is the largest and most polymorphic of all acrocarpous mosses (Brown, 1899; Spence, 1987). This genus is an artificial construct and probably represents a polyphyletic group as indicated in recent phylogenetic analyses (Cox, 1999). Plants in this genus are perennial, small to robust, dioicous or monoicous, and grow in terrestrial turves or tufts. The main features of this genus include hexagonal to rhombic hexagonal upper lamina cells, vegetative reproductive structures, such as bulbiform leaf gemmae, rhiziodal tubers, and filiform leaf gemmae, and pendulous to horizontal pyriform capsules (Spence, 1987). It is these sporophytic characters which distinguish *Bryum* species from members of the genus *Brachymenium* Schwaegr. (Spence, 1987). The "perfect" double peristome is also a key feature in *Bryum* species (Brown, 1899).

1.2.2 The "Silver" *Bryum* Species

The "silver" *Bryum* species include *B. albopulvinatum* C. Muell., *B. argenteum* Hedw., *B. bicolor* Brid., *B. caespiticium* Hedw., *B. capillare* Hedw., *Bryum cellulare* Hook. in Schwaegr., *B. ellipsifolium* C. Muell., *B. radiculosum* Brid., *B. rigidicuspis*

Dix., and *B. subrotundifolium* Jaeg. This is an artificial group within the genus *Bryum* with members collectively having a silvery appearance due to the presence of hyaline cells in the upper part of the stem leaves. This feature varies in most of the members of this group and is only invariable persistent in *B. argenteum*.

1.2.3 *Bryum* in Antarctica

Bryum is the most specious moss genus in Antarctica (Seppelt et al., 1998); it is also the most taxonomically confusing (Ochi, 1979). This problem is exacerbated by the morphological variation induced by the harsh Antarctic environment and makes taxonomy very difficult (Nakanishi, 1979). Fifteen species of *Bryum* have been reported from Continental Antarctica (Ochyra and Ochi, 1986) since the earliest reported botanical collections by Edward Bransfield's expedition in 1820 (Literary Gazette and Journal of Belles Lettres, Nov 10, 1821, pp712-713). Numerous morphology-based revisionary studies (Savich-Lyubitskaya and Smirnova, 1959; Ochi, 1979; Kanda, 1981; Seppelt 1983; Kanda and Ochi, 1986; Seppelt and Kanda, 1986; Ochi and Kanda, 1991; Seppelt and Green, 1998) lead to the recognition of only two *Bryum* species in Continental Antarctica: *B. subrotundifolium* and *B. pseudotriquetrum*.

1.3 POPULATION GENETICS OF BRYOPHYTES

1.3.1 Population Biology

Antarctic mosses are an interesting study group from a population biology perspective. They have been presumed to be largely sterile (but see Kanda and Ochi, 1986; Seppelt et al., 1992), reproducing primarily via asexual propagules, although sporophytes have been reported in continental Antarctic populations of *B. pseudotriquetrum* (as *B. antarcticum* and *B. algens*) and *Hennediella*. Inefficacy of sexual reproduction and a haploid-dominant life cycle suggests little opportunity for the introduction of genetic variation.

Mosses tend to colonise in spatially and temporally separated patches, with growth beyond these transient "habitat islands" (Herben, 1994) restricted due to unfavourable conditions (Söderström and Herben, 1997). Population biology of bryophytes can

therefore be addressed at two-levels; within and between islands (Levins, 1969). Within-island variation is influenced by growth, reproduction and mortality, while between-island variation involves external factors influencing migration potential between islands, establishment prospects for migrant propagules, and the risks of migration between islands (Levins, 1969).

The growth of mosses in small, disjunct populations (as islands) has interesting ramifications for random genetic drift (chance fluctuations in allele frequencies) within these islands. Genetic drift may occur in all natural populations however it occurs much more rapidly in small populations (Hartl, 1988). The influences of migration, mutation, and natural selection on a small population become more pronounced as the effective gene pool for that population is reduced (Hartl, 1988). This effect is compounded in Antarctic mosses due to asexual reproduction and the dominant haploid gametophyte which results in only one copy of each allele at a locus per individual being retained, effectively half the genetic variation retained in a diploid population. This would restrict genetic variation occurring through mutation in Antarctic mosses as novel phenotypes are expressed immediately with subsequent exposure to natural selection.

1.3.2 Genetic Variation of Mosses

Bryophytes appear to exhibit genetic stability in that they have retained their primitive morphological structure, with little apparent evolutionary advances in comparison to angiosperm species. Geographically isolated species also follow this trend exhibiting little apparent speciation post separation (Scott, 1988). Scott (1988) postulated highly efficient dispersal of asexual spores and vegetative propagules from bryophyte populations as an explanation for geographic homogeneity, a postulate for which there is some evidence (Van Zanten, 1976, 1978, 1984; Van Zanten and Gradstein, 1988). Immediate selection against lethal or detrimental mutations expressed in the dominant haploid gametophyte was also thought to contribute to the lack of observed morphological variation (Gemmell, 1950; Steere, 1954; Anderson, 1963; Schuster, 1966; Crum, 1972).

Genetic studies have indicated the presence of much higher levels of variation in

these "evolutionary failures" (Crum, 1972) than was originally expected. Isozyme analysis of several temperate moss species has revealed levels of variation comparable to those found in angiosperms (Hamrick, 1979; Cummins and Wyatt, 1981; Vries et al., 1983; Bramen, 1986; Hofman, 1988 Wyatt et al., 1989a; Wyatt et al., 1989b; Derda and Wyatt, 1990; Stoneburner et al., 1991; Derda and Wyatt, 1999a; Derda and Wyatt, 1999b; Derda et al., 1999). This genetic variation is not reflected in morphological differences suggesting that bryophyte species may evolve at a physiological level (Wyatt, 1985; Dewey, 1989) or with morphological evolution so subtle it is undetectable (Wyatt, 1985).

Randomly amplified polymorphic DNA analysis (RAPDs; Adam et al., 1997; Skotnicki et al., 1997, 1998a, b, c) have detected the presence of even higher levels of genetic variation in Antarctic moss species. In a study comparing populations of Australian and New Zealand *B. argenteum* and Antarctic *B. argenteum s.l.* (Skotnicki et al., 1998a), 7% of the total variation occurred between the Australian and New Zealand populations and 19% of the variation occurred between these two populations and the Antarctic populations. The majority (75%) of the variation in this study occurred within these populations, with genetic differences detected between adjacent moss shoots. However, RAPDs are difficult to reproduce (e.g. Jones et al., 1997) and it is possible that these levels of variation is due to an artifact of the technique.

1.4 BIOGEOGRAPHY

The progressive isolation of Antarctica (Section 1.1.1) has some interesting biogeographical repercussions. As land masses moved away from Antarctica, geographical barriers would have formed, separating the populations of mosses in these areas. If the Antarctic moss populations did arise through vicariance then we would expect moss populations from Victoria Land to exhibit divergence from populations on adjacent landmasses, but be most closely related to moss populations in New Zealand from the past configuration of Gondwana. Alternatively, if these populations have established more recently through long distance dispersal, we would anticipate little or no apparent divergence from moss populations on adjacent

landmasses.

Molecular data has been used to facilitate our understanding the colonisation history of many disjunct species (e.g. Carr and Colemann, 1974; Eanes and Koehn, 1978; Brown, 1980; Brower and Boyce, 1991; Wyatt, 1997). It has been suggested that these data could be extrapolated in the form of a molecular clock to estimate the arrival times of colonising species (Zuckerandl and Pauling, 1965). This concept has interesting potential, although the reliability, accuracy and usefulness of molecular clocks has yet to be proven and is still highly criticised (see review by Thorpe, 1982 and references therein). Recent attempts to interpret the colonisation history of Antarctic moss populations using random amplified polymorphic DNA analysis (RAPDs) have not been successful (Adam et al., 1997; Stoknicki et al., 1997, 1998a, b, c), concluding that further molecular research using DNA sequence information is required. Slowly evolving multicopy gene sequences in chloroplast and ribosomal DNA have already proven useful in bryophytes at the species level (Mischler et al., 1992; Waters et al., 1992), with the more rapidly evolving spacers in these regions resolving relationships at the intraspecific level (Stech and Frahm, 1999).

1.5 DISPERSAL MECHANISMS

Airborne pollen and fungal spores have been reported in the airstream above Antarctica (Marshall, 1996) with pollen from 27 plant species being identified in moss samples from coastal Victoria Land (Linskens et al., 1993). These observations suggest possible mechanisms for the introduction of moss spores to Antarctica lending support to the theory of colonisation through dispersal. Dispersal of moss propagules within Antarctica relies on similar mechanisms including primarily wind, water, and avian dispersal, along with increasing human activity. The potential of wind dispersal of moss propagules has been demonstrated experimentally, with greater dispersal distances of lighter moss propagules offset by proportionally diminished survival capacity (Van Zanten, 1978). Seppelt and Kanda (1986) suggest the activities of man as another possible vector, particularly in Continental Antarctic localities, as *Bryum subrotundifolium* Jaeg. populations are generally found close to manned sites. However, this may be due to the coincidental location of these sites in

coastal areas rather than having any anthropogenic connection (R. Seppelt, *pers. comm.*).

1.6 OBJECTIVES

This research had three objectives: **1)** To address the phylogenetic relationships between the silver *Bryum* species including *B. subroundifolium* from Antarctica, the Subantarctic islands and Australia, *B. argenteum* from New Zealand and Australia, *B. capillare* from New Zealand, and the non-silver species *B. pseudotriquetrum* from Antarctica, New Zealand and Australia using the internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA (nrDNA). Phylogenies were used to answer the following taxonomic, evolutionary and biogeographic questions: **a)** Are the Antarctic populations referred to by Spence as *B. subrotundifolium* more genetically similar to *B. subrotundifolium* from Australia or *B. argenteum* from New Zealand? **b)** Do the phylogenetic relationships of these populations reflect the expected biogeographic history? **c)** Is Antarctic *B. subrotundifolium* a relictual species or the result of recent colonisation via long distance dispersal?

2) To determine if fungal symbionts associated with Antarctic mosses identified in phylogenetic analyses of this research could provide an explanation for the high-levels of variation detected using RAPDs.

3) To develop microsatellite DNA markers in the cosmopolitan moss species, *B. argenteum* for future use in comparative population genetics studies between populations of Antarctic mosses and those in temperate regions. A continuation of this research will implement microsatellite DNA markers, focussing on population-level studies on a fine scale within and between populations of *B. argenteum* and hopefully between related taxa.

CHAPTER TWO

PHYLOGENY OF CONTINENTAL ANTARCTIC *BRYUM* (BRYACEAE) SPECIES

2.1 INTRODUCTION

2.1.1 Overview of Systematics of the Genus *Bryum*

Bryum species are distinguished from other members of the subfamily Bryoideae (Table 2.1), by horizontal to pendulous capsules and perfect to reduced peristomes (Spence, 1987). Gametophytic and sporophytic diversity within this genus has made assigning ancestral traits difficult. The presence of filiform axial gemmae is thought to be the only synapomorphic trait uniting this genus (Spence, 1987). However, this characteristic is featured in only a few species (Spence, 1987). Identical gametophytic character states in *Bryum* and *Brachymenium* Schwaegr., intermediary character states between *Bryum* and *Anomobryum* Schimp., and shared character states with *Pohlia* Hedw. (such as rhizoidal tubers, axial bulbils, and reduced peristomes) negate the phylogenetic usefulness of many potentially informative traits (Spence, 1987).

Table 2.1. Genera in the subfamily Bryoideae (Bryaceae).

The Bryoideae	
Acidodontium Schwaegr.	*Leptobryum* (B.S.G.) Wils.
Anomobryum Schimp.	*Plagiobryum* Lindb.
Brachymenium Schwaegr.	*Pohlia* Hedw.
Bryum Hedw.	*Rhodobryum* (Schimp.)Hampe
Epipterygium Lindb.	*Roellia* Lindb.

Purported "natural" classifications within the Bryoideae have used morphological data in the past and are largely speculative (Ochi, 1992). Ochi (1992) had previously presented an infrageneric classification of the genus *Bryum* based on morphological similarities. Three subgeneric groups, Anomobryum, Bryum and Rhodobryum, were recognised in this treatment, which included 181 recognised *Bryum* species and 13 species of uncertain taxonomic affinity (Table 2.2), with the majority (142) of these

taxa placed in the subgenus Bryum. This treatment was thought to represent the "natural" groups within this genus. Recent phylogenetic estimates of the relationships within the Bryoideae based on *rps*4 and *trn*L sequences from chloroplast DNA (cpDNA) represented the genus *Bryum* as a polyphyletic group interspersed throughout the subfamily Bryoideae (Cox, 1998). This is not surprising as *Bryum* is an artificial construct, including many otherwise indeterminable taxa. This indicates the need for a thorough taxonomic revision of this subfamily incorporating molecular data so that phylogenetic relationships are better represented in the classification (Cox, 1998).

Table 2.2. Distribution of the 181 *Bryum* species and 13 species of uncertain taxonomic affinity after Ochi's (1981) infrageneric classification of the genus *Bryum*.

Subgenera	*Bryum* spp.	Undetermined spp.
Amonobryum	21	1
Bryum	142	4
Rhodobryum	18	0
Undetermined	-	8
Totals	**181**	**13**

2.1.2 The Genus *Bryum*

The genus *Bryum* Hedw. (Bryaceae) is the largest and most polymorphic of all acrocarpous mosses (Brown, 1899; Spence, 1987). *Bryum* consists of small to robust, dioicous or monoicous, perennial plants, growing in terrestrial turves or tufts. Stems have abundant rhizoids, which may bear tubers (Spence, 1987). Imbricate leaves are generally comose, and can be ovate, obovate, spathulate, elliptic, lanceolate, or round, with an acute, acuminate or rarely rounded leaf apex and a strong percurrent to excurrent costa (Sainsbury, 1955). Margins are toothed or entire, bordered by elongate, incrassate cells. Upper lamina cells are hexagonal or rhombic hexagonal, thin- or firm-walled, becoming more oblong in the lower leaf. Pyriform capsules, containing spherical spores (<25μm), have a well-developed neck and annulus, and are horizontal to pendent on elongated, often red, setae (Sainsbury, 1955; Spence, 1987). The "perfect" double peristome has 16 outer teeth, free to the base, and an

inner membrane divided to the middle into 16 keeled segments, with or without intermediate cilia and having a cucullate calyptra (Brown, 1899). The operculum is convex-conic, with abundant, superficial stomata restricted to the neck of the capsule (A. Fife, unpub. data). Vegetative reproductive structures, such as bulbiform leaf gemmae, rhiziodal tubers, and filiform leaf gemmae are often present and are variable in form and distribution (Spence, 1987).

2.1.3 The "Silver" *Bryum* Species

The "silver" *Bryum* species are an informal grouping within the genus *Bryum* collectively having a silvery appearance due to the presence of hyaline cells in the upper part of the stem leaves. This feature is transient in most members of this group and is only constant in *B. argenteum* Hedw. In the Southern Hemisphere, this group includes *B. albopulvinatum* C. Muell., *B. argenteum*, *B. bicolor* Brid., *B. caespiticium* Hedw., *B. capillare* Hedw., *Bryum cellulare* Hook. in Schwaegr., *B. ellipsifolium* C. Muell., *B. radiculosum* Brid., *B. rigidicuspis* Dix., and *B. subrotundifolium* Jaeg. (Table 2.3).

2.1.4 Morphological Variation in Antarctic Mosses

Identification of Antarctic mosses is difficult due to morphological variation induced by the harsh environment. Longton (1981) investigated the morphological variation present in cosmopolitan populations of *B. argenteum* and Antarctic populations of *B. subrotundifolium*. Stem length, leaf length, and costa excurrence were observed to be variable in specimens from different localities in the field, but reverted to an identical state when cultivated collaterally. These characters exhibit environmentally induced phenotypic plasticity and are therefore not useful characters for taxonomy or phylogenetic analyses. Variations in leaf shape, shoot arrangement, and protonema morphology were also investigated in Longton's (1981) study. Distinctions in these features between *B. argenteum* and *B. subrotundifolium* were retained in parallel cultivation experiments suggesting that they are genetically determined and therefore useful for taxonomic differentiation. Nakanishi (1979) observed similar variation in leaf size and shape in *B. pseudotriquetrum*. Leaf dimensions were found to decrease with scarcity of free water, while leaf shape appeared to be constant. Leaves along

innovation shoots showed significant position-dependent variation in both size and shape, and costa thickness and length. These variations are also attributed to environmental influences.

Table 2.3. The "silver" *Bryum* species (*sensu* R. D. Seppelt, pers. comm.) and their distribution (Scott and Stone, 1973).

Taxon	Distribution
Bryum albopulvinatum C. Muell.	Africa
B. argenteum Hedw.	Cosmopolitan
B. bicolor Brid.	Europe, Asia, Africa, America and Australia
B. caespiticium Hedw.	Cosmopolitan
B. capillare Hedw.	Cosmopolitan
B. cellulare Hook. in Schwaegr.	Australia
B. ellipsifolium C. Muell.	South Africa
B. radiculosum Brid.	Europe, Asia and Africa
B. rigidicuspis Dix.	Africa
B. subrotundifolium Jaeg.	Australia, the Subantarctic islands and Antarctica

2.1.5 Introduction to Historical Taxonomy of the Antarctic Mosses

The first reported bryological collections are thought to have been made by Edward Bransfield's team in January 1820 (Literary Gazette and Journal of Belles Lettres, Nov 10, 1821, pp712-713). Nearly two centuries later, despite numerous reviews, the taxonomy of the Antarctic mosses is still not completely understood. Early bryologists, such as Müller and Cardot, believed that islands should be treated as a discrete regions, each with a unique flora of endemic species (Steere, 1965). This resulted in the description of numerous redundant taxa based on inadequate holotype material (Steere, 1965). Many bryophyte species which bear similar vegetative features can be differentiated at the generic level only by capsule characters making the systematic position of taxa without sexual reproductive structures unreliable

(Greene, 1962). Sterility (but see Kanda and Ochi, 1986; Seppelt et al., 1992) and the extreme range of morphological variation makes taxonomy in Antarctic mosses difficult, with *Bryum*, *Grimmia* Hedw., *Hennediella* Paris, and *Schistidium* Bruch. et Schimp. being particularly problematic.

2.1.6 Taxonomic Revisions of the Genus *Bryum* in Antarctica

Numerous taxonomic revisions (Cardot, 1908; Dixon and Watts, 1918; Bartram, 1957; Clifford, 1957; Horikawa and Ando, 1961; Steere, 1961; Horikawa and Ando, 1967; Greene, 1968; Ochi, 1970; Robinson, 1972; Ochi, 1979; Kanda, 1981; Kaspar et al., 1982; Seppelt, 1983; Zander, 1993) have reduced the number of *Bryum* species recognised in Continental Antarctica to only two species, *B. subrotundifolium* and *B. pseudotriquetrum*. These revisions have been summarised by Seppelt and Kanda (1986; Table 2.4) who recognised *B. pseudotriquetrum* and *B. argenteum*.

2.1.7 The *Bryum subrotundifolium* Debate

J. Spence (unpub. data) was the first to refer Antarctic *B. argenteum* to Australian alpine species *B. subrotundifolium,* an Australian alpine species, with this change officially recognised by Seppelt and Green (1998). This research observes the change of classification to *B. subrotundifolium*, however, other recent publications refer to this taxon as *B. argenteum* (Adam et al., 1997; Skotnicki et al, 1998a).

Bryum subrotundifolium and *B. argenteum* exhibit similar characteristics and are differentiated largely the development of chlorophyllose lamina cells in *B. subrotundifolium*, which are absent in *B. argenteum*, and foliage colour (Table 2.5). Antarctic *B. subrotundifolium* is yellowish-green to pale green, with hyaline lamina cells in the upper 1/3 to 1/2 of the outer apical leaves. When cultured under low-light conditions on nutrient agar media the shoots become darker green with the hyaline cells becoming chlorophyllose, as seen in the shade form of this species in Antarctica. Temperate populations attributed to *B. argenteum* are silver-green in colour and also exhibit hyaline lamina cells in the upper 1/3 to 1/2 of the outer apical leaves. Cultivation of the *B. argenteum* specimen under identical, low-light

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Table 2.4. Continental Antarctic *Bryum* species subsumed into *B. subrotundifolium* Jaeg and *B. pseudotriquetrum* (Hedw.) Gaertn. Meyer et Scherb. after taxonomic appraisal of this genus (Seppelt and Kanda, 1986)

B. subrotundifolium	***B. pseudotriquetrum***
B. ablyolepis Card.	*B. algens* Card.
B. argenteum Hedw.	*B. austropolare* Card.
B. cephaloziodes Card.	*B. crateris* Dix.
B. siplei Bartr.	*B. gerlachei* Card.
	B. imperfectum Card.
	B. inconnexum Card.
	B. inconnexum var. *tomentosum* Card.
	B. korotkevicziae Sav. et Smirn.
	B. korotkevicziae var. *hollerbachii* Sav. et Smirn.
	B. ongulense Hor. et Ando
	B. perangustidens Card.
	B. stenotrichum C. Müll.
	Webera racovitzae var. *laxiretis* Card.

conditions induces the dark-green shade-form shoots. However, unlike the Antarctic *B. subrotundifolium*, the chloroplast-free hyaline lamina cells in the upper 1/3 to 1/2 of the leaves are retained (R. Seppelt, pers. comm.). Distinct protonema characteristics between these two taxa were also observed in these collateral growth experiments with Antarctic *B. subrotundifolium* exhibiting fewer axillary shoots and shorter distances between branches (Seppelt and Green, 1998). Similar experiments carried out by Longton (1981) in morphologically variable specimens of *B. argenteum* collected from a variety of temperate (Indiana, U.S.A.), tropical (Hawaii, U.S.A. and Costa Rica) and polar (Arctic) locations, revealed similar results. Differences between Arctic and Antarctic material were also demonstrated, with the Arctic material resembling *B. argenteum s.s.* This negates the possibility that the distinct features observed in the Antarctic material (Longton, 1981; Seppelt and Green, 1998) are attributable to residual effects from the harsh polar environment (Longton, 1981). If these effects were a reflection of the conditions experienced prior

to cultivation, they would have been common to both polar species.

Table 2.5. Distinguishing features of *B. subrotundifolium* vs *B. argenteum* in the field and in culture (Longton, 1981; Seppelt and Green, 1998). Measurements given are adapted from Longton (1981).

Character	***Bryum subrotundifolium***	***Bryum argenteum***
In the field:		
Foliage	Yellow-green	Silver-green
Shoots	2.2mm	3.9mm
Stems	0.11mm	0.17mm
Leaves	0.46 by 0.32mm	0.78 by 0.48mm
Apex	0.06mm	0.15mm
Nerve	2.5mm	4.0mm
Vegetative Structures	Bulbils abundant at apices of long flagelliform shoots branching from the stems of leafy shoots.	Bulbils abundant at the apices of short, slender branching shoots from the axils of leaves on the main shoots.
In culture:		
Photosynthetic filaments	erect, growing as aerial branches, shorter distances between branches.	growing as branches from horizontal filaments, fewer axial filaments
Leaves	Spreading with short chlorophyllose apices	Imbricate with hyaline apices.

The presence of *B. argenteum s.s.* in Continental Antarctica has yet to be confirmed. Collections from Cape Hallett revealed the presence of two morphotypes of *B. subrotundifolium* (Figure 2.1), one of which bears characteristics similar to that of cosmopolitan *B. argenteum*. This specimen is silvery-green with a hyaline hair-point at the leaf apices. The second specimen exhibits characteristics typical of *B. subrotundifolium* with yellow-green foliage and rounded leaf apices. This specimen was later confirmed to be *B. subrotundifolium* (R. Seppelt, pers. comm.). This *B. argenteum*-type variation in leaf morphology has also been found in other populations attributed to *B. subrotundifolium* from Southern Victoria Land (Taylor Valley, Garwood Valley, Botany Bay, Cape Royds, Cape Bird, Beaufort Island; S. Hunger pers. obs.; R. Seppelt, pers. comm.).

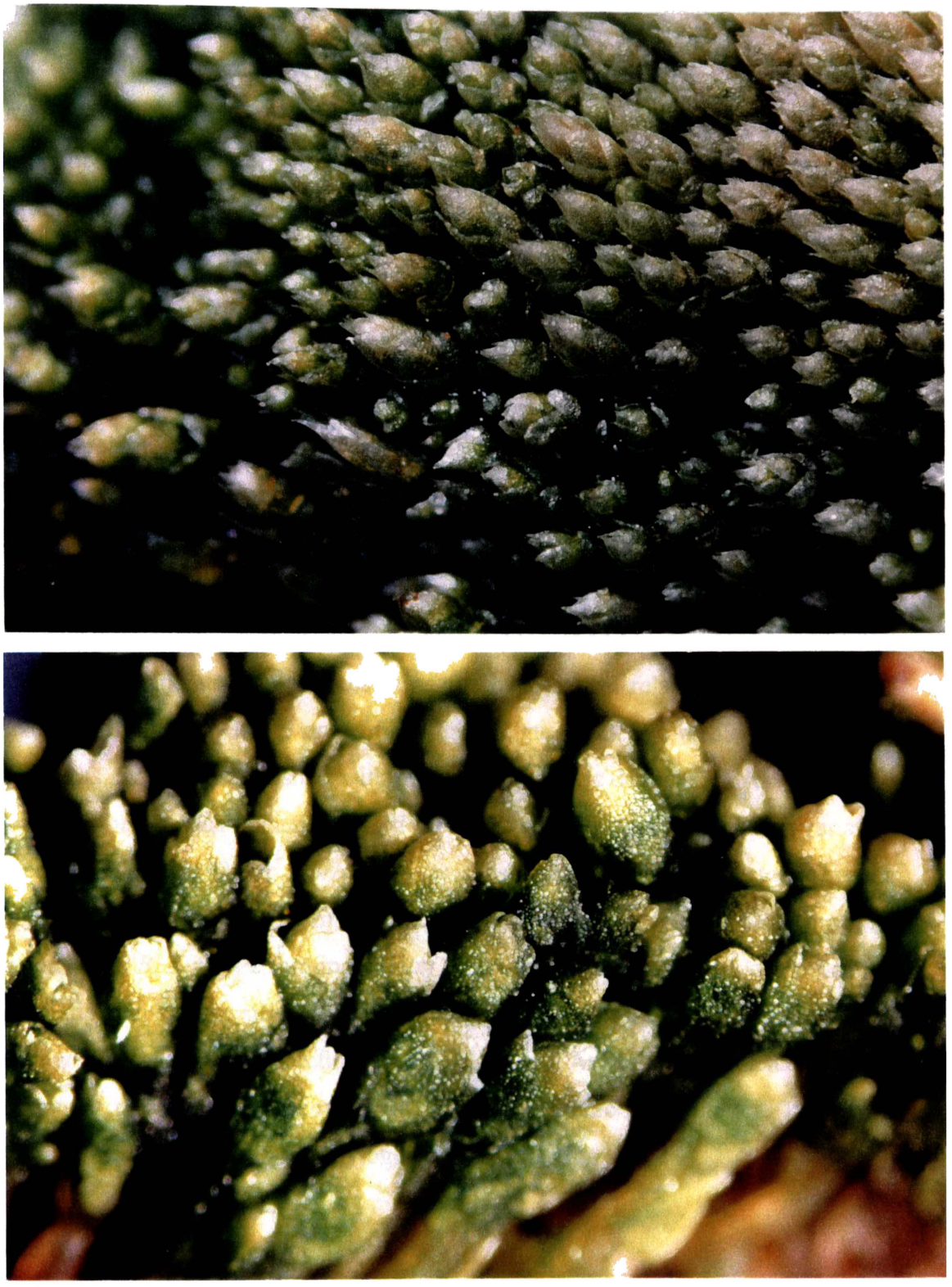


Figure 2.1. Two morphologically distinct specimens of *B. subrotundifolium* collected from Cape Hallett, Antarctica. One specimen (Accession number SH21; top) exhibits the silvery-green foliage and hyaline hairpoints at the leaf apices characteristic of cosmopolitan *B. argenteum* (3X magnification). The second specimen (Accession number SH27-1; bottom) has yellow-green foliage characteristic of *B. subrotundifolium* with rounded leaf apices (3X magnification). These specimens, are denoted 1 and 2, respectively, in this research.

Antarctic moss populations identified as *B. argenteum s.l.* from the Garwood Valley, Lake Fryxell and Granite Harbour have been compared to *B. argenteum* populations from Australia and New Zealand using RAPDs (Skotnicki et al., 1998a). The Antarctic populations formed a separate clade on the dendrogram with 19% of the variation occurring between these populations and the Australian and New Zealand populations and 7% of the variation in the tree occurred between the Australian and New Zealand populations. The rest of the variation in the tree (75%) occurred within the populations with the Australian and New Zealand populations forming a clade. The short branch lengths separating these populations suggest that these populations are conspecific and that *B. argenteum* is present in Antarctica.

This study focussed on a comparison of *B. subrotundifolium* from Antarctica, the Subantarctic region and Australia, *B. argenteum s.s.* from Australia and New Zealand and *B. capillare* from New Zealand. Maclade 3.0 (Maddison and Maddison, 1992) was used to generate a tree representing the hypothetical relationships of the taxa included in this study based on artificial data (Figure 2.2). We would anticipate the specimens from different sites within Antarctica (Cape Hallett and the Garwood Valley) to be genetically distinct based on the levels of between-population variation detected in RAPDs. The *B. subrotundifolium* specimens would be expected to form a single clade with a clade containing the *B. argenteum* specimens as a sister group to this and with *B. capillare* forming a sister group to the *B. argenteum* clade reflecting the relationships of the silver *Bryum* species. Specimens of the non-silver species, *B. pseudotriquetrum* from Antarctica, New Zealand and Australia were included in the analysis as this taxon has been taxonomically problematic in Antarctica. This taxon was used as an outgroup in the phylogenetic analysis.

2.1.8 Biogeography

The colonisation of Antarctica by mosses is hypothesised to have occurred through vicariance or long distance dispersal. The hypothesis tree (Figure 2.2) reflects colonisation of Antarctica through vicariance with the Antarctic *B. subrotundifolium* populations represented as diverged from the Australian populations. The Subantarctic populations are anticipated to form a sister group to the Antarctic

populations. *Bryum subrotundifolium* was thought to be an Australian endemic prior to its recognition in Antarctica, therefore, we would anticipate that the Antarctic populations would have diverged first, followed by the Subantarctic populations as the continents drifted into the current configuration.

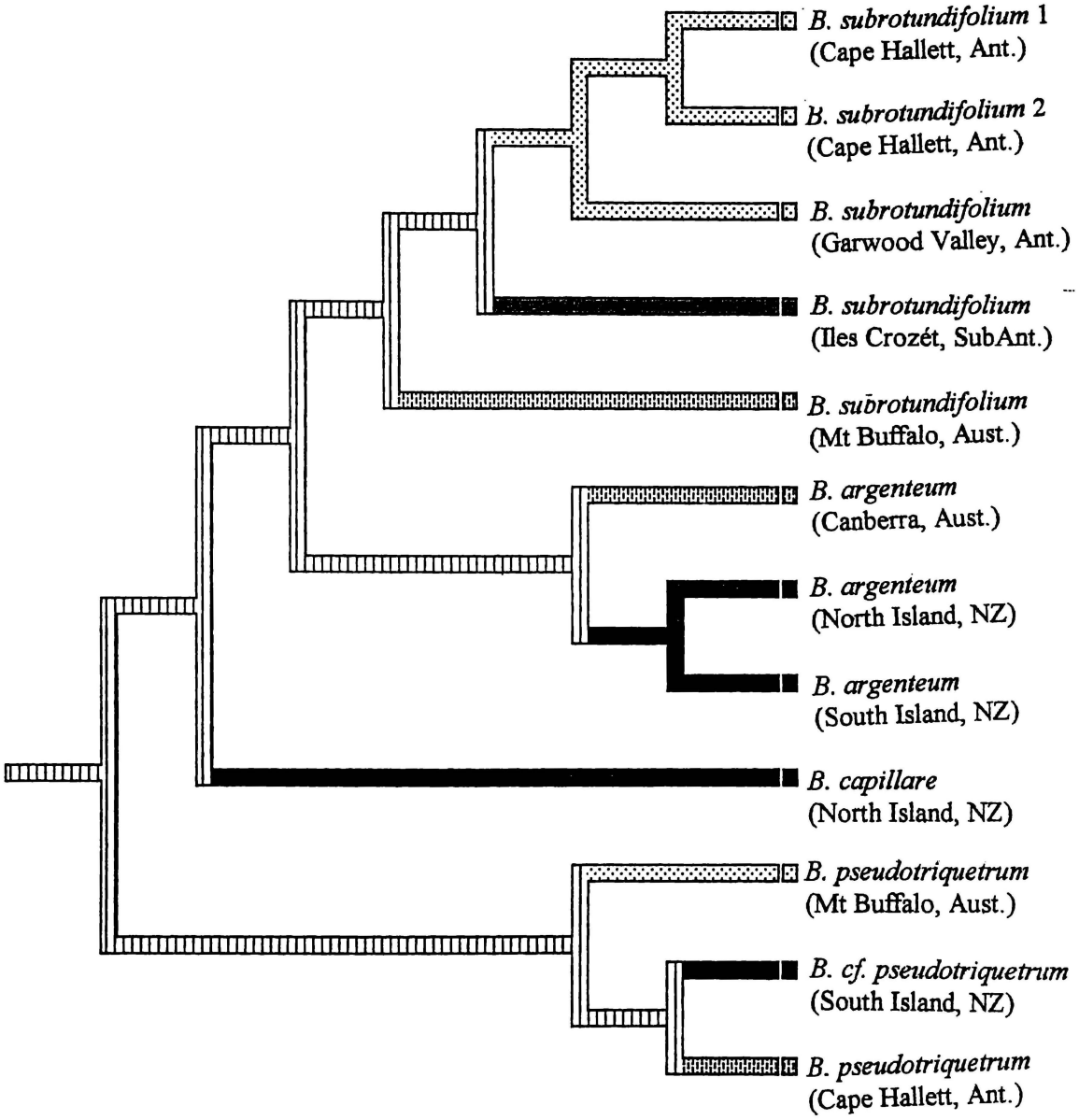


Figure 2.2. Graphical depiction of the hypothesised relationships among the *Bryum* taxa investigated in this research.

2.1.9 Internal Transcribed Spacer Sequences

Internal transcribed spacer (ITS) sequences are non-coding regions of nuclear ribosomal DNA (nrDNA; Baldwin 1993) which exist in high copy numbers throughout the genome. The two spacer regions are situated between the 18S and 5.8S ribosomal genes (ITS 1) and the 5.8S and 26S ribosomal genes (ITS 2) as illustrated in Figure 2.2 (Baldwin et al., 1995). The ITS regions are highly mutable and exhibit concerted evolution (Arnheim et al., 1980; Zimmer et al., 1980; Appels and Dvorak, 1982; Arnheim, 1983 and Hillis et al., 1991) in the form of uneven crossing-over and gene conversion during recombination (Baldwin et al., 1995). Primers are targeted to specific sites on these genes (Figure 2.3) and are used in the polymerase chain reaction (PCR) to amplify the regions between the primers.

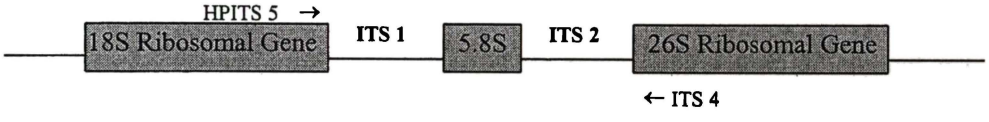


Figure 2.3. The repeat unit of 18-26S nuclear ribosomal DNA. Arrows indicate the approximate positions occupied by the primers used to amplify double-stranded DNA for sequencing (adapted from Baldwin et al., 1995).

ITS sequences have proven to be useful in deciphering phylogenetic relationships between many higher plants species (Baldwin, 1992; Baldwin, 1993; Wojciechowski et al., 1993; Baldwin et al., 1995; Sang et al., 1995; Downie and Katz-Downie, 1996; Gielly et al., 1996; Liston et al., 1996; Manos, 1997; Kelly, 1998; Kornkven et al., 1998; Ainouche and Bayer, 1999; Alice and Campbell, 1999; Li et al., 1999; Noyes and Rieseberg, 1999; Starr et al., 1999; Vargas et al., 1999), and are now being employed in phylogenetic studies of mosses (Stech and Frahm, 1999). This region exhibits greater sequence diversity than chloroplast DNA (cpDNA) in bryophytes providing more informative sites for species-level phylogenies (Stech and Frahm, 1999).

2.1.10 Objectives

This research addressed the phylogenetic relationships between the silver *Bryum* species including *B. subroundifolium* from Antarctica, the Subantarctic islands and

Australia, *B. argenteum* from New Zealand and Australia, *B. capillare* from New Zealand, and the non-silver species *B. pseudotriquetrum* from Antarctica, New Zealand and Australia using the internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA (nrDNA). Phylogenies were used to answer the following taxonomic, evolutionary and biogeographic questions: **a)** Are the Antarctic populations referred to by Spence as *B. subrotundifolium* more genetically similar to *B. subrotundifolium* from Australia or *B. argenteum* from New Zealand depicted in Figure 2.2? **b)** Do the phylogenetic relationships of these populations reflect the expected biogeographic history? **c)** Is Antarctic *B. subrotundifolium* a relictual species or the result of recent colonisation via long distance dispersal? One would expect the Antarctic populations to be related to, but diverged from *B. subrotundifolium* from Australia if the Antarctic populations are derived from relict population. This species is not reported in New Zealand which makes the Australian populations the nearest neighbours. This relationship would suggest establishment also involved dispersal as Australia drifted away from Wilkes Land while these samples were collected from Victoria Land. We would expect to observe little or no divergence between these disjunct populations if colonisation has occurred via long distance dispersal as interchange of genetic information through propagules and spores between these populations is likely to be a continual process.

2.2 MATERIALS AND METHODS

2.2.1 Plant Material

Twelve specimens (Table 2.6) were sampled in this study, representing populations of *B. argenteum*, *B. subrotundifolium*, *B. capillare*, and *B. pseudotriquetrum* from Antarctica, Australian and New Zealand (Figure 2.4). Specimens were obtained from herbarium collections (Landcare Research Christchurch, CHR) and from fresh field samples (Appendix I). Samples collected in the field were placed in paper envelopes and air dried as quickly as possible. Specimens for DNA analysis were stored at -74°C with voucher specimens deposited in the University of Waikato Herbarium (WAIK).

Table 2.6. List of specimens included in phylogenetic reconstructions for this study. Sectional classifications taken from Ochi (1992). *Bryum subrotundifolium* was not included in Ochi's (1992) treatment, but the similarities between this taxon and *B. argenteum* suggest it could accurately be placed in section *Bryum*.

Taxon Subgenus: Bryum	Source	Accession Number
sect. *Bryum*		
Bryum argenteum Hedw.	Australia:	
	Canberra (35°18'S, 149°08'E)	SH42
	New Zealand:	
	Hamilton (35°53'S, 175°28'E)	SH16
	Christchurch (43°5'S, 172°7'E)	SH43
Bryum subrotundifolium Jaeg.	Antarctica:	
	Cape Hallett 1 & 2 (72°18'S, 170°18'E)	SH21 & SH27-1
	Garwood Valley (78°03'S, 164°10'E)	SH34
	Granite Harbour (77°00'S, 162°34'E)	KMG2
	Subantarctica:	
	Iles Crozet (46°27'S, 52°00'E)	SH48
	Australia:	
	Mt. Buffalo (36°47'S, 146°03'E)	SH56-1
sect. *Capillaria* sub-sect. *Capillaria*		
Bryum capillare Hedw.	New Zealand:	
	Hamilton (35°53'S, 175°28'E)	SH38
sect. *Caesiticia* sub-sect *Caespiticia*		
Bryum cf. pseudotriquetrum (Hedw.) Gaertn., Meyer et Scherb.	New Zealand:	
	Canterbury (43°09'S, 171°40'E)	CHR515077
Outgroup Taxon		
sect. *Caesiticia* sub-sect *Caespiticia*		
Bryum pseudotriquetrum (Hedw.) Gaertn., Meyer et Scherb.	Antarctica:	
	Cape Hallett (72°18'S, 170°18'E)	SH23-1
	Australia:	
	Mt Buffalo (36°47'S, 146°03'E)	SH69

2.2.2 DNA Extraction and PCR Amplification

Total genomic DNA was extracted from 100 - 500mg of clean, dried, herbarium or frozen moss material following the method described by Rogers and Bendich (1985) (Appendix II). PCR was performed in 0.5mL thin-walled microcentrifuge tubes using 20-100ng of template DNA, 1X PCR buffer ([10mM Tris-HCl, 50mM KCl, pH 8.3] Boehringer Mannheim), 2.5mM magnesium chloride, 1μM HPITS 5 [5'-GGA AGG AGA AGT CGT AAC AAG G-3'] (LMS, Smithsonian Institute), 1μM ITS 4 [5'-TCC TCC GCT TAT TGA TAT GC-3'] (White et al., 1990), 0.25mM each of dATP, dTTP, dCTP, dGTP (Boehringer Mannheim), 2-3 units *Taq* DNA polymerase (Boehringer Mannheim)in a total volume of 100μL. Amplification was performed in an Eppendorf Mastercycler Gradient thermal cycler using the following protocol: 5 minutes at 96°C, followed by 29 cycles of 30 seconds at 95°C, 30 seconds at 55°C

and 45 seconds at 72°C. PCR products were electrophoresed on a 1.0% agarose (SeaKem LE) minigel to confirm amplification success.

Multiple bands amplified in some samples which were excised individually with a sterile scalpel blades. The gel slice was melted at 96°C for 10 minutes then 10-15µL of this added directly to the reaction mix above and reamplified. Amplification products were purified for sequencing using a QIAquick PCR Purification Kit (Qiagen).

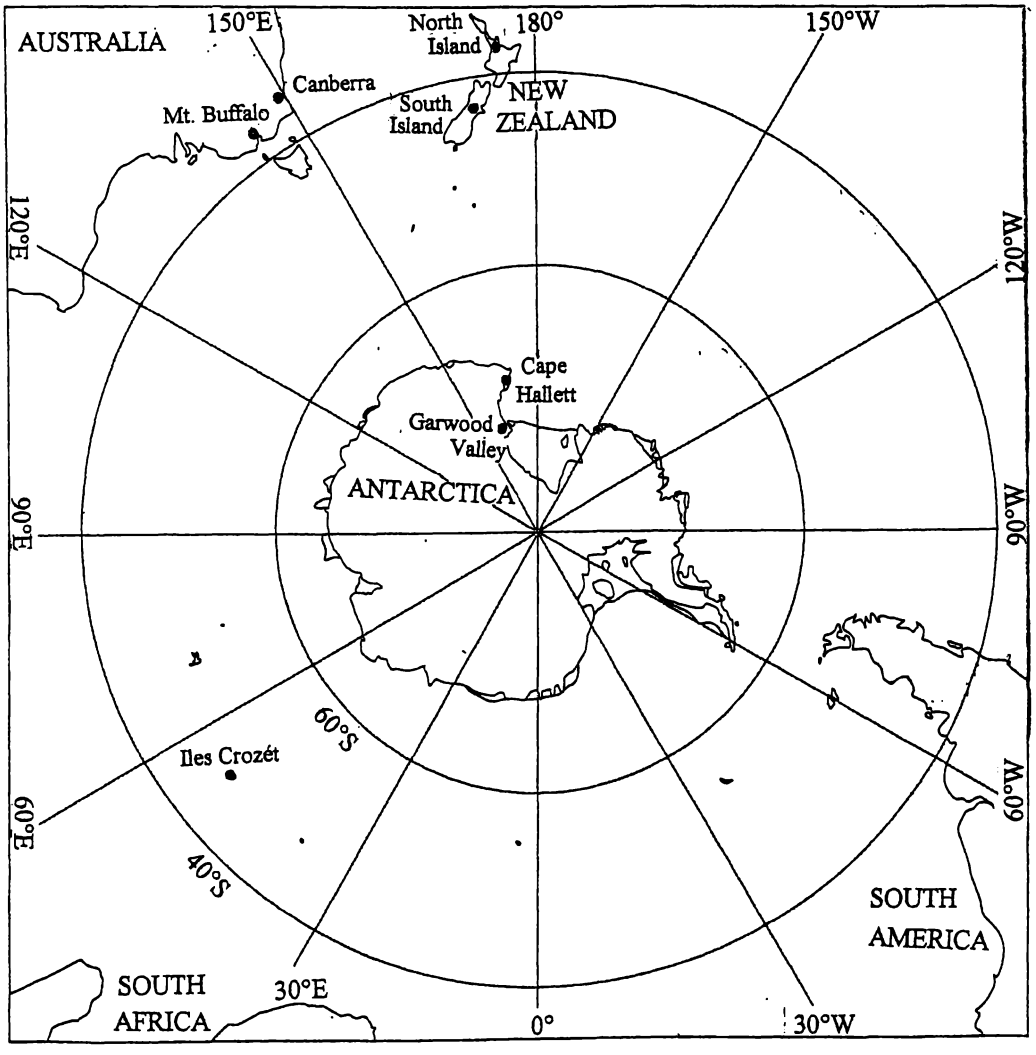


Figure 2.4. Sampling localities for the specimens included in this phylogenetic analysis.

2.2.3 DNA Sequencing

Purified PCR products were quantified with a Hoefer DNA Fluorometer (Hoefer Scientific Instruments) and sequenced at the University of Waikato DNA Sequencing Facility. Forward and reverse sequences were generated using BigDye™ Terminator chemistry (Perkin-Elmer Applied Biosystems) for all sequences. This facilitated base-calling with ambiguous data and ensured complete sequence information was obtained for the entire ITS region, including the 5.8S ribosomal gene. Five percent dimethylsulfoxide (DMSO) was added to sequencing reactions to prevent the formation of secondary structures. The products of these reactions were then separated on a 4.5% polyacrylamide gel using an ABI 377 Automated DNA Sequencer (Perkin-Elmer Applied Biosystems) and data collected in electropherograms.

2.2.4 Alignment and Phylogenetic Analysis

Electropherograms of the ITS sequences were edited and aligned using Sequencher version 3.0 (Genecodes). Data matrices from the aligned sequences were imported into the beta-test version of the computer programme PAUP* 4.0b2a (Swofford, 1998). Gaps were treated as missing and all characters weighted equally. These data were analysed using parsimony with a branch and bound search and character changes interpreted with ACCTRAN (Accelerated Transition State) optimisation. Bootstrap analyses (Felsenstein, 1985) of 1000 replicates using TBR (Tree Bisection and Reconnection) branch swapping of the heuristic search were also conducted to obtain a measure of support for putative clades. *Bryum pseudotriquetrum* specimens from Australia and Antarctica were used as an outgroup to root the tree as this taxon is not a silver *Bryum* species. The New Zealand specimen designated as *B.* cf. *pseudotriquetrum* was retained in the ingroup due to dubious identification based on sterile gametophyte characters.

2.3 RESULTS

2.3.1 Sequence Alignment and Characteristics

PCR amplification products were 1000bp long in *Bryum subrotundifolium* and 1100bp long in *B. pseudotriquetrum* (Figure 2.5). A second band approximately

550bp long was consistently amplified in specimens collected from Antarctica, revealing the presence of a persistent contaminant associated with these samples. Aligned sequence data were truncated at the shortest read length sequence to prevent bias due to data absence in some taxa (Figure 2.6) Several large gaps (approximately 20bp) were inserted into sequences to accommodate the length polymorphisms throughout *B. pseudotriquetrum*. The intergenic spacers, ITS 1 and ITS 2, prior to truncation, ranged in length from 436bp to 450bp (average 443bp) and from 146bp to 205bp (average 166bp), respectively (Table 2.7). ITS 1 was shortest in Antarctic *B. subrotundifolium* (436bp) and longest in *B. argenteum* from Australia and the North Island of New Zealand. Antarctic *B. subrotundifolium* also had the shortest ITS 2 sequence (146bp) while this sequence was longest in *B. pseudotriquetrum* from Australia. The ITS 1 spacer was more than twice as long as the ITS 2 spacer in all specimens. The 5.8S gene was 95bp long and highly conserved compared to the intergenic spacers with only one transition from an adenosine residue to a guanosine evident between *B. pseudotriquetrum* and *B. capillare*, and the other taxa.

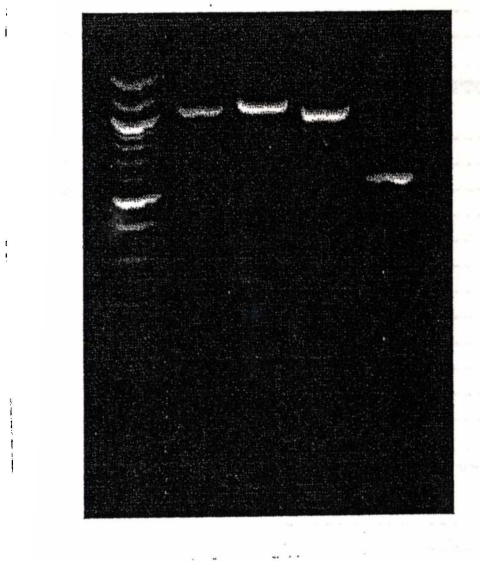


Figure 2.5. PCR amplification products from Antarctic *B. subrotundifolium* (Lanes 2 [SH27-1] & 4 [SH21]), *B. pseudotriquetrum* (Lane 3 [SH23-1]), and the contaminating band (Lane 5) isolated from the Antarctic specimens. Lane 1: 100bp DNA ladder.

Figure 2.6. Truncated aligned sequence data for ITS region of nrDNA. 5.8S and 26S (partial) ribosomal gene sequences are underlined. Sample abbreviations: Ant - Antarctica (GV - Garwood Valley, CH - Cape Hallett), Aus - Australia, NZ - New Zealand (SI - South Island, NI - North Island), SubAI - Subantarctic islands. Sequence codes (Standard IUPAC/IUB annotation): A or G = R, C or T = Y, A or C =M, G or T = K, A or T = W, C or G = S, A or C or G (not T) = V, A or C or T (not G) = H, A or G or T (not C) = D, C or G or T (not A) = B, N = unknown.

```
                                ITS 1 ->
B. subrotundifolium Ant GV      ---------- ---------C ---------- ----------
B. subrotundifolium AntCH1      ---------- ---------C ---------- ----------
B. subrotundifolium AntCH2      ---------- ---------C ---------- ----------
B. subrotundifolium Aus         ---------- -----:---- ---------- ----------
B. argenteum NZ SI              ---------- -----:---- ---------- ----------
B. subrotundifolium SubAI       ---------- -----:---- ---------- ----------
B. argenteum NZ NI              ---------- ---------- ---------- ----------
B. argenteum Aus                ---------- ---------- ---------- ----------
B. pseudotriquetrum Aus         CTGC-G-GTG A-TT-G-GC- -----A--A- TA---C----
B. pseudotriquetrum Ant         CTGC-G-GTG A-TT-G-GC- -----A--A- TA---C----
B. capillare NZ NI              ATGC-G-GTG A-TC-G-GC- -----A--A- TA---A----
B. cf. pseudotriquetrum NZ SI   -GGCCTCGTG AAAGCA-G-- ----CA--A: ----------
 #1                             ........................................
                                TCATGAGAAT GCGAGTTTTT ACCTTTACCA ::TTCTGAAG

B. subrotundifolium Ant GV      ----C----- ---------- -:-------- AA--------
B. subrotundifolium AntCH1      ----C----- ---------- -:-------- AA--------
B. subrotundifolium AntCH2      ----C----- ---------- -:-------- AA--------
B. subrotundifolium Aus         ---------- ---------- -T---C---- :A----G---
B. argenteum NZ SI              ---------- GC-------- -T---C---- :A----G---
B. subrotundifolium SubAI       ---------- ---------- -T---C---- :A----G---
B. argenteum NZ NI              ---------- ---------T T---------- -GG-------
B. argenteum Aus                ---------- ---------T T---------- -GG-------
B. pseudotriquetrum Aus         ---------- ----::---- T----::::G -G-T----::
B. pseudotriquetrum Ant         ---------- ----::---- T----::::G -G-T----::
B. capillare NZ NI              ---------- ----::--:- -----::::G -G-T----G-
B. cf. pseudotriquetrum NZ SI   ---------- ---:::--:- -----::::G -G-T----GT
 #41                            ........................................
                                CTCTTCGGGT :GCTGCTGC: :CTTCTGAGC GRACACTCCG

B. subrotundifolium Ant GV      --G--::--- :--------- ---------- ----------
B. subrotundifolium AntCH1      --G--::--- :--------- ---------- ----------
B. subrotundifolium AntCH2      --G--::--- :--------- ---------- ----------
B. subrotundifolium Aus         --C------- ---------- ---------- ----------
B. argenteum NZ SI              --C------- ---------- ---------- ----------
B. subrotundifolium SubAI       --C------- ---------- ---------- ----------
B. argenteum NZ NI              --C------- :--------- ---------- ----------
B. argenteum Aus                --C------- :--------- ---------- ----------
B. pseudotriquetrum Aus         :-G--TACTT ----T----- ---------- ----------
B. pseudotriquetrum Ant         :-G--TACTT ----T----- ---------- ----------
B. capillare NZ NI              TATC-CGGGG G---T----- ---------- ----------
B. cf. pseudotriquetrum NZ SI   GTACCTTCGG G---T--T-- ---------- ----------
 #81                            ........................................
                                CGSGGGC::: TACTCGG:GC GTCCTCCTCC TCTTCAAGGC

B. subrotundifolium Ant GV      ---------- ---------- ---------- ----------
B. subrotundifolium AntCH1      ---------- ---------- ---------- ----------
B. subrotundifolium AntCH2      ---------- ---------- ---------- ----------
B. subrotundifolium Aus         ---------- -------A-- ---------- ---------G
B. argenteum NZ SI              ---------- -------A-- ---------- ---------G
B. subrotundifolium SubAI       ---------- -------A-- ---------- ---------G
B. argenteum NZ NI              ---------- -------A-- ---------- ----------
B. argenteum Aus                ---------- -------A-- ---------- ----------
B. pseudotriquetrum Aus         -------:-- ---------- ---------- ----------
B. pseudotriquetrum Ant         -------:-- ---------- ---------- ----------
B. capillare NZ NI              -------:-- ---------- ---------- ----------
B. cf. pseudotriquetrum NZ SI   ---G---:-- ---------- ---------- ----------
 #121                           ........................................
                                TGCATTCCAG CTCCTCA:GC CTGTCGCCCC CGGCCGGAAC
```

```
B. subrotundifolium Ant GV    ---------- ---------- ---------- ----------
B. subrotundifolium AntCH1    ---------- ---------- ---------- ----------
B. subrotundifolium AntCH2    ---------- ---------- ---------- ----------
B. subrotundifolium Aus       ---------- -T----G--- ---------- ----------
B. argenteum NZ SI            ---------- -T----G--- ---------- ----------
B. subrotundifolium SubAI     ---------- -T----G--- ---------- ----------
B. argenteum NZ NI            ---------- -T----G--- -----T---- ----------
B. argenteum Aus              ---------- -T----G--- -----T---- ----------
B. pseudotriquetrum Aus       ----G-G--- :--------- ---------- ----------
B. pseudotriquetrum Ant       ----G-G--- :--------- ---------- ----------
B. capillare NZ NI            ----G-G--- :--------- ---------- ----------
B. cf. pseudotriquetrum NZ SI ----G-G--- :--------- ---------- ----------
 #161                         ........................................
                              TTTGCCTCGC T:GGTG:CTC GGTCTAGGCC GAATCCACTT


B. subrotundifolium Ant GV    ---------- ---------- ---------- ----------
B. subrotundifolium AntCH1    ---------- ---------- ---------- ----------
B. subrotundifolium AntCH2    ---------- ---------- ---------- ----------
B. subrotundifolium Aus       --T------- -C-------- ---------- ----------
B. argenteum NZ SI            --T------- -C-------- ---------- ----------
B. subrotundifolium SubAI     --T------- -C-------- ---------- ----------
B. argenteum NZ NI            ---------- -C-------- ---------- ----------
B. argenteum Aus              ---------- -C-------- ---------- ----------
B. pseudotriquetrum Aus       :----:---- ---------- ---------- ----------
B. pseudotriquetrum Ant       :----:---- ---------- ---------- ----------
B. capillare NZ NI            :----:---- ---------- ---------- ----------
B. cf. pseudotriquetrum NZ SI :----:---- ---------- ---------- ----------
 #201                         ........................................
                              AGCGGGTCAC GGGACTGGAT CCGGGATTAG TACCGGCAGG


B. subrotundifolium Ant GV    ---------- ---------- ---------- ----------
B. subrotundifolium AntCH1    ---------- ---------- ---------- ----------
B. subrotundifolium AntCH2    ---------- ---------- ---------- ----------
B. subrotundifolium Aus       ---------- ---------- ---------- -----A----
B. argenteum NZ SI            ---------- ---------- ---------- -----A----
B. subrotundifolium SubAI     ---------- ---------- ---------- -----A----
B. argenteum NZ NI            ---------- ---------- ---------- -----A----
B. argenteum Aus              ---------- ---------- ---------- -----A----
B. pseudotriquetrum Aus       -:-------- ---------- ---------- T---------
B. pseudotriquetrum Ant       -:-------- ---------- ---------- T---------
B. capillare NZ NI            -:-------- ---------- ---------- T---------
B. cf. pseudotriquetrum NZ SI -:-------- ---------- ---------- -------:--
 #241                         ........................................
                              CTGGGGAGTG CTACCGCGGG AGGCTAGGGA :GGGTTCCAG


B. subrotundifolium Ant GV    ---------- --------:: ::--------- ----------
B. subrotundifolium AntCH1    ---------- --------:: ::--------- ----------
B. subrotundifolium AntCH2    ---------- --------:: ::--------- ----------
B. subrotundifolium Aus       ---------- ---------- -T:------: ----------
B. argenteum NZ SI            ---------- ---------- -T:------: ----------
B. subrotundifolium SubAI     ---------- ---------- -T:------: ----------
B. argenteum NZ NI            ---------- ---------- ---------- ----------
B. argenteum Aus              ---------- ---------- ---------- ----------
B. pseudotriquetrum Aus       ---------- --C---ACCA C---T-CCT- C-----TGCA
B. pseudotriquetrum Ant       -----A---- --C---ACCA C---T-CCT- C-----TGCA
B. capillare NZ NI            -----A---G --C--GACCA C-A-T-CCT- C-C------C
B. cf. pseudotriquetrum NZ SI -----A---G C----GGCCA C-A-T-CCT- C--------C
 #281                         ........................................
                              CGCCCGAGTA :CTCGTTGTG GGGGGTGGCT GC:GAG::::
```

```
B. subrotundifolium Ant GV     ---------- ---------- ---------- ----------
B. subrotundifolium AntCH1     ---------- ---------- ---------- ----------
B. subrotundifolium AntCH2     ---------- ---------- ---------- ----------
B. subrotundifolium Aus        -:::--CCAG- ---------- ---------- ----------
B. argenteum NZ SI             -:::--CCAG- ---------- ---------- ----------
B. subrotundifolium SubAI      -:::--CCAG- ---------- ---------- ----------
B. argenteum NZ NI             -:::--CCAG: ---------- ---------- ----------
B. argenteum Aus               -:::--CCAG: ---------- ---------- ----------
B. pseudotriquetrum Aus        A--------: ::::::::-- ---C------ ---AAG----
B. pseudotriquetrum Ant        A--------: ::::::::-- ---C------ ---AAG----
B. capillare NZ NI             A---:----: ::::::::-- ---C------ ---AAG----
B. cf. pseudotriquetrum NZ SI  AC--:----: ::::::::-- ---C------ ----------
 #321                          ........................................
                               :GTGG::::C CAGCAGTACT CGTTGGGTGG AAG:::CCCG


B. subrotundifolium Ant GV     ---------- --A------- ---------- ----------
B. subrotundifolium AntCH1     ---------- --A------- ---------- ----------
B. subrotundifolium AntCH2     ---------- --A------- ---------- ----------
B. subrotundifolium Aus        ---------- ------C--- ---------- ----------
B. argenteum NZ SI             ---------- ------C--- ---------- ----------
B. subrotundifolium SubAI      ---------- ---------- ---------- ----------
B. argenteum NZ NI             ---------- ------C--- ---------- ----------
B. argenteum Aus               ---------- ------C--- ---------- ----------
B. pseudotriquetrum Aus        ---------- T--------- ----A----- --TGA-----
B. pseudotriquetrum Ant        ---------- T--------- ----A----- --TGA-----
B. capillare NZ NI             ---------A T--------- ----A----- --TGA-----
B. cf. pseudotriquetrum NZ SI  -C-ATC---- T-----C--- ----A----- --T-A-G---
 #361                          ........................................
                               ATG:::AAAT ATTTTCTAAG TCCC:CAAGA CTGAGGACCC


B. subrotundifolium Ant GV     ---------- ---------- ---------- C---------
B. subrotundifolium AntCH1     ---------- ---------- ---------- C---------
B. subrotundifolium AntCH2     ---------- ---------- ---------- C---------
B. subrotundifolium Aus        ---------- ---------- ---------- ----------
B. argenteum NZ SI             ---------- ---------- ---------- ----------
B. subrotundifolium SubAI      ---------- ---------- ---------- ----------
B. argenteum NZ NI             ---------- ---------- ---------- ----------
B. argenteum Aus               ---------- ---------- ---------- ----------
B. pseudotriquetrum Aus        ---A------ ----G----- T-G-C--A-- --CTC----G
B. pseudotriquetrum Ant        ---A------ ----G----- T-G-C--A-- --CCC----G
B. capillare NZ NI             ---------- ----G----- T-G-C--A-C --CCCTTGGG
B. cf. pseudotriquetrum NZ SI  ---------- ---------- T-G-CC-A-- ----------
 #401                          ........................................
                               TTA:TTTTGA CCCGAGAGTT CGAG:TC:CT TC::::::::


                                                                  5.8S ->
B. subrotundifolium Ant GV     ---------- ---------- ---G------ ------T---
B. subrotundifolium AntCH1     ---------- ---------- ---G------ ------T---
B. subrotundifolium AntCH2     ---------- ---------- ---G------ ------T---
B. subrotundifolium Aus        ---------- ---------- ---------- ----------
B. argenteum NZ SI             ---------- ---------- ---------- ----------
B. subrotundifolium SubAI      ---------- ---------- ---------- ----------
B. argenteum NZ NI             ---------- ---------- --------A- ----------
B. argenteum Aus               ---------- ---------- --------A- ----------
B. pseudotriquetrum Aus        GGAGT----- --GA------ ---------G --T-------
B. pseudotriquetrum Ant        GGAGT----- --GA------ ---------: --T--AT---
B. capillare NZ NI             GGAGT----- --GA------ ---------: --T--:----
B. cf. pseudotriquetrum NZ SI  -GAGT----- -C-A------ ---------: :::::AA---
 #441                          ........................................
                               ::::GGGGCT CGAGCACGAG TTGAATCT:A AAACTT:AAC
```

```
B. subrotundifolium Ant GV     ---------- ---------- ---------- ----------
B. subrotundifolium AntCH1     ---------- ---------- ---------- ----------
B. subrotundifolium AntCH2     ---------- ---------- ---------- ----------
B. subrotundifolium Aus        ---------- ---------- ---------- ----------
B. argenteum NZ SI             ---------- ---------- ---------- ----------
B. subrotundifolium SubAI      ---------- ---------- ---------- ----------
B. argenteum NZ NI             ---------- ---------- ---------- ----------
B. argenteum Aus               ---------- ---------- ---------- ----------
B. pseudotriquetrum Aus        ---------- ---------- ---------- ----------
B. pseudotriquetrum Ant        ---------- ---------- ---------- ----------
B. capillare NZ NI             ---------- ---------- ---------- ----------
B. cf. pseudotriquetrum NZ SI  ---------- ---------- ---------- ----------
 #481                          ........................................
                               TTAGAACAAC TCTCAGCAAC GGATATCTTG GCTCTTGCAA


B. subrotundifolium Ant GV     ---------- ---------- ---------- ----------
B. subrotundifolium AntCH1     ---------- ---------- ---------- ----------
B. subrotundifolium AntCH2     ---------- ---------- ---------- ----------
B. subrotundifolium Aus        ---------- ---------- ---------- ----------
B. argenteum NZ SI             ---------- ---------- ---------- ----------
B. subrotundifolium SubAI      ---------- ---------- ---------- ----------
B. argenteum NZ NI             ---------- ---------- ---------- ----------
B. argenteum Aus               ---------- ---------- ---------- ----------
B. pseudotriquetrum Aus        ---------- ---------- ---------- ----------
B. pseudotriquetrum Ant        ---------- ---------- ---------- ----------
B. capillare NZ NI             ---------- ---------- ---------- ----------
B. cf. pseudotriquetrum NZ SI  ---------- ---------- ---------- ----------
 #521                          ........................................
                               CGATGAAGAA CGCAGCGAAA TGCGATACGT AGTGTGAATT


B. subrotundifolium Ant GV     ---------- ---------- ---------- ----------
B. subrotundifolium AntCH1     ---------- ---------- ---------- ----------
B. subrotundifolium AntCH2     ---------- ---------- ---------- ----------
B. subrotundifolium Aus        ---------- ---------- ---------- ----------
B. argenteum NZ SI             ---------- ---------- ---------- ----------
B. subrotundifolium SubAI      ---------- ---------- ---------- ----------
B. argenteum NZ NI             ---------- ---------- ---------- ----------
B. argenteum Aus               ---------- ---------- ---------- ----------
B. pseudotriquetrum Aus        ---------- ---------- ---------- ----------
B. pseudotriquetrum Ant        ---------- ---------- ---------- ----------
B. capillare NZ NI             ---------- ---------- ---------- ----------
B. cf. pseudotriquetrum NZ SI  ---------- ---------- ---------- ----------
 #561                          ........................................
                               GCAGAATTCC GCGAATCATC GAGTTTTTGA ACGCAAGTTG


                                                              ITS 2 ->
B. subrotundifolium Ant GV     ---------- ---------- ---------- ----------
B. subrotundifolium AntCH1     ---------- ---------- ---------- ----------
B. subrotundifolium AntCH2     ---------- ---------- ---------- ----------
B. subrotundifolium Aus        ---------- ---------- ---------- ----------
B. argenteum NZ SI             ---------- ---------- ---------- -----N----
B. subrotundifolium SubAI      ---------- ---------- ---------A -----T----
B. argenteum NZ NI             ---------- ---------- ---------- ----------
B. argenteum Aus               ---------- ---------- ---------- -----N----
B. pseudotriquetrum Aus        ---------- ------A--- ----------T ----------
B. pseudotriquetrum Ant        ---------- ------A--- ----------T ----------
B. capillare NZ NI             ---------- ------A--- ---------- ----------
B. cf. pseudotriquetrum NZ SI  ---------- ------A--- ----------T ----------
 #601                          ........................................
                               CGCCCGAGGC TTGTCCGAGG GCATTTCCGC TAGAGCGTCA
```

```
B. subrotundifolium Ant GV     ---------- --------:: :A-T--CT-- ----------
B. subrotundifolium AntCH1     ---------- --------:: :A-T--CT-- ----------
B. subrotundifolium AntCH2     ---------- --------:: :A-T--CT-- ----------
B. subrotundifolium Aus        ---------- --CC----CA CA-T--CT-- ----------
B. argenteum NZ SI             ---------- --CC----CA CA-T--CT-- ----------
B. subrotundifolium SubAI      ---------- --C------- ----:A---G CGG-------
B. argenteum NZ NI             ---------- ---------- ----:G---G CGG-------
B. argenteum Aus               ---------- ---------- ----:G---G CGG-------
B. pseudotriquetrum Aus        ---------- ---------- -----C--GT ACTTCTGTAC
B. pseudotriquetrum Ant        ---------- ---------- -----:--GT ACTTTTGTAC
B. capillare NZ NI             ---------- -------G-- ----::--GT ACTT------
B. cf. pseudotriquetrum NZ SI  ---------- ---------- ---::---GT ----------
 #641                          ..........................................
                               CCGCGCCCCC CC:ACCACAC TCCCCTAATA ::::::::::


B. subrotundifolium Ant GV     ---------- --C------- ---------- -------:--
B. subrotundifolium AntCH1     ---------- --C------- ---------- -------:--
B. subrotundifolium AntCH2     ---------- --C------- ---------- -------:--
B. subrotundifolium Aus        ---------- --G------- ---------- ----------
B. argenteum NZ SI             ---------- --G------- ---------- ----------
B. subrotundifolium SubAI      ---------- ---------- ---------- ----------
B. argenteum NZ NI             ---------- ---------- ---------- ----------
B. argenteum Aus               ---------- ---------- ---------- ----------
B. pseudotriquetrum Aus        CTGTGCGCC- ---------- ---------- -----AGTGC
B. pseudotriquetrum Ant        CTGTGCGCC- ---------- ---------- -----AGTGC
B. capillare NZ NI             ------GA-- ---------- ---------- -----AGTG-
B. cf. pseudotriquetrum NZ SI  ---------- ---------- ---------- -----AGTG-
 #681                          ..........................................
                               ::::::::GA GTTTGAGTGG AACTGGCCGT CCCGGCACA:


B. subrotundifolium Ant GV     ---------- ---------- ---------- ----------
B. subrotundifolium AntCH1     ---------- ---------- ---------- ----------
B. subrotundifolium AntCH2     ---------- ---------- ---------- ----------
B. subrotundifolium Aus        ---------- ---------- ------A--- ----------
B. argenteum NZ SI             ---------- ---------- ------A--- ----------
B. subrotundifolium SubAI      ---------- ---------- ---------- -----C----
B. argenteum NZ NI             ---------- ---------- ---------- ----------
B. argenteum Aus               ---------- ---------- ---------- ----------
B. pseudotriquetrum Aus        TTAAGGTAC- ---------- ---------- ----------
B. pseudotriquetrum Ant        TTAAGGTAC- ---------- ---------- ----------
B. capillare NZ NI             TTAAG-TAC- ---------- ---------- ----------
B. cf. pseudotriquetrum NZ SI  TTAAG-TGC- ---------- ------C--- ----------
 #721                          ..........................................
                               :::::::::T CGGGTCGGCT GAAATGGAGG GAATTTGGCC


B. subrotundifolium Ant GV     ---------- ---------- ---------- -------:--
B. subrotundifolium AntCH1     ---------- ---------- ---------- -------:--
B. subrotundifolium AntCH2     ---------- ---------- ---------- -------:--
B. subrotundifolium Aus        ---------- ---------- -------C-- -------:--
B. argenteum NZ SI             ---------- ---------- -------C-- -------A--
B. subrotundifolium SubAI      ----C----- ---------- ---------- -------A--
B. argenteum NZ NI             ---------- ---------- C--------- -------A--
B. argenteum Aus               ---------- ---------- C--------- -------A--
B. pseudotriquetrum Aus        ---------- ---------- ---------- ---T---TAC
B. pseudotriquetrum Ant        ---------- ---------- ---------- ---T---TAC
B. capillare NZ NI             ---------- ---------- ---------- -------TTC
B. cf. pseudotriquetrum NZ SI  ---------- ---------- ---------- ---T-TATTC
 #761                          ..........................................
                               GCCGTGGCAT CACTTGCCCC :GCGATC:AG GTGCGATWCT
```

```
                                                                    268 ->
B. subrotundifolium Ant GV     ---------: :--------- ---------- ----------
B. subrotundifolium AntCH1     ---------: :--------- ---------- ----------
B. subrotundifolium AntCH2     ---------: :--------- ---------- ----------
B. subrotundifolium Aus        ---------: :--------- ---------- ----------
B. argenteum NZ SI             ---------T T--------- ---------- ----------
B. subrotundifolium SubAI      ---------T T--------- ---------- ----------
B. argenteum NZ NI             ---------T T--------- ---------- ----------
B. argenteum Aus               ---------T T--------- ---------- ----------
B. pseudotriquetrum Aus        GA-CTCCCAC GG--CTT--- AGTGTCGTGG GACTTGGGTC
B. pseudotriquetrum Ant        GA-CTCCCAC GG--CTT--- AGTGTCGTGG GACTTGGGTC
B. capillare NZ NI             GA-CTCCCAC GG--CTT--- AGTGTCGTGG GACTTGG
B. cf. pseudotriquetrum NZ SI  GA-CTCCCAC GG--C-TCCA AGTGTCGTGG GACTTGGGTC
 #801                          ........................................
                               CGAAAGAGTY K:CA:::::: :::::::::: :::::::;;;


B. subrotundifolium Ant GV     ---------- ---------- ---------- ----------
B. subrotundifolium AntCH1     ---------- ---------- ---------- ----------
B. subrotundifolium AntCH2     ---------- ---------- ---------- ----------
B. subrotundifolium Aus        ---------- ---------- ---------- ----------
B. argenteum NZ SI             ---------- ---------- ---------- ----------
B. subrotundifolium SubAI      ---------- ---------- ---------- ----------
B. argenteum NZ NI             ---------- ---------- ---------- ----------
B. argenteum Aus               ---------- ---------- ---------- ----------
B. pseudotriquetrum Aus        ---------- ---------- G--------- --------::
B. pseudotriquetrum Ant        ---------- ---------- G--------- --------::
B. cf. pseudotriquetrum NZ SI  ---------- ---------C G--------- --------::
 #841                          ........................................
                               TTCCAAGTGC GGGGCTCCC: AGTGAGCCCG AGTGCGTGCC


B. subrotundifolium Ant GV     ---------- ---------- ---------- ----------
B. subrotundifolium AntCH1     ---------- ---------- ---------- ----------
B. subrotundifolium AntCH2     ---------- ---------- ---------- ----------
B. subrotundifolium Aus        ---------C --A------- ---------- ----------
B. argenteum NZ SI             ---------- ---------- ---------- ----------
B. subrotundifolium SubAI      ---------- ---------- ---------- ----------
B. argenteum NZ NI             ---------- ---------- ---------- ----------
B. argenteum Aus               ---------- ---------- ---------- ----------
B. pseudotriquetrum Aus        :--------- ---------- ---------- ----------
B. pseudotriquetrum Ant        :--------- ---------- ---------- ----------
B. cf. pseudotriquetrum NZ SI  :--------- ---------- ---------- ----------
 #881                          ........................................
                               CCGAGTTCCA CAGCAGG
```

Table 2.7. Sequence lengths (bp) of the ITS sequences and 5.8S ribosomal genes lengths in the 12 specimens included in this study.

Taxon (Locality)	**ITS 1**	**5.8S**	**ITS 2**
Bryum subrotundifolium (Cape Hallett, Antarctica 1)	436	95	146
B. subrotundifolium (Cape Hallett, Antarctica 2)	436	95	146
B. subrotundifolium (Garwood Valley, Antarctica)	436	95	146
B. subrotundifolium (Iles Crozét, Subantarctic island)	444	95	156
B. subrotundifolium (Mt. Buffalo, Australia)	444	95	152
B. argenteum (South Is., New Zealand)	445	95	155
B. argenteum (North Is., New Zealand)	450	95	156
B. argenteum (Canberra, Australia)	450	95	156
B. capillare (North Is., New Zealand)	445	95	189
B. cf. *pseudotriquetrum* (South Is., New Zealand)	430	95	185
B. pseudotriquetrum (Cape Hallett, Antarctica)	448	95	204
B. pseudotriquetrum (Mt. Buffalo, Australia)	448	95	205
Mean ITS sequence lengths:	**443**	**95**	**166**

2.3.2 Phylogenetic Relationships

Of 897 characters, 149 (16.6%) were variable with 115 (12.8%) of these being parsimony informative. Two equally parsimonious trees of 188 steps (CI = 0.910, RI = 0.939, RC = 0.854; Figures 2.7 and 2.8) were retained after a branch and bound search of these data. Bootstrap (BS) values are shown on the second branch and bound tree, which had identical topology to the bootstrap majority-rule consensus tree. These trees had a homoplasy index (HI) of 0.090, indicating minimal homoplasy within these data. Homoplasy, when present, reduces the resolution in parsimony-based analyses. The majority-rule consensus tree (Figure 2.9) and the strict consensus tree had identical topologies with 100% support for all the clades in the majority-rule consensus tree. Topology in these cladograms mirrored that of the phylogram generated for Bootstrap 50% majority-rule consensus tree.

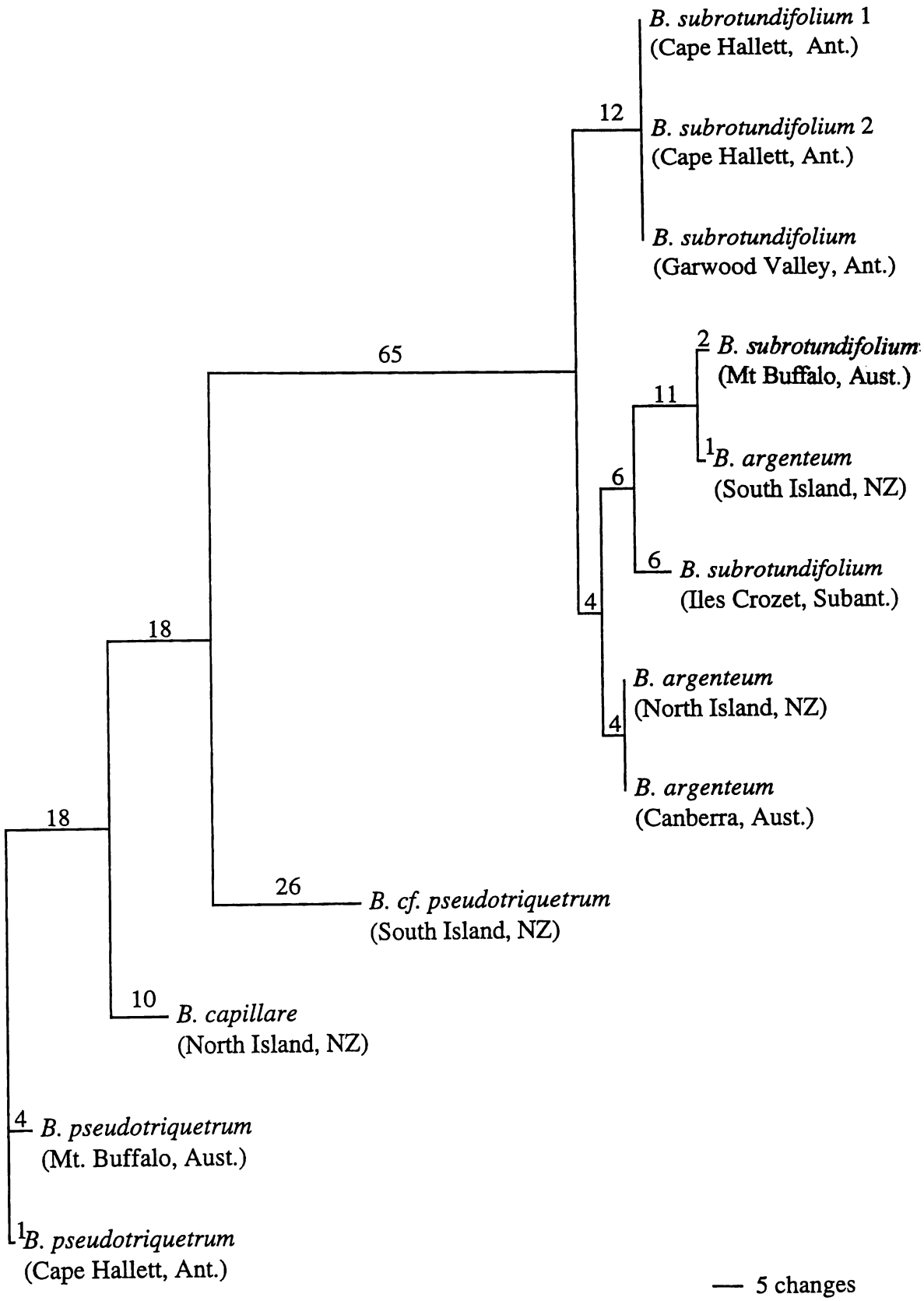


Figure 2.7: First of two phylograms of 188 steps showing branch lengths (above branches) generated from a branch and bound search of the aligned data in Figure 2.6. Abbreviations: Ant. - Antarctica, Aust. Australia, NZ - New Zealand, Subant. - SubAntarctic islands.

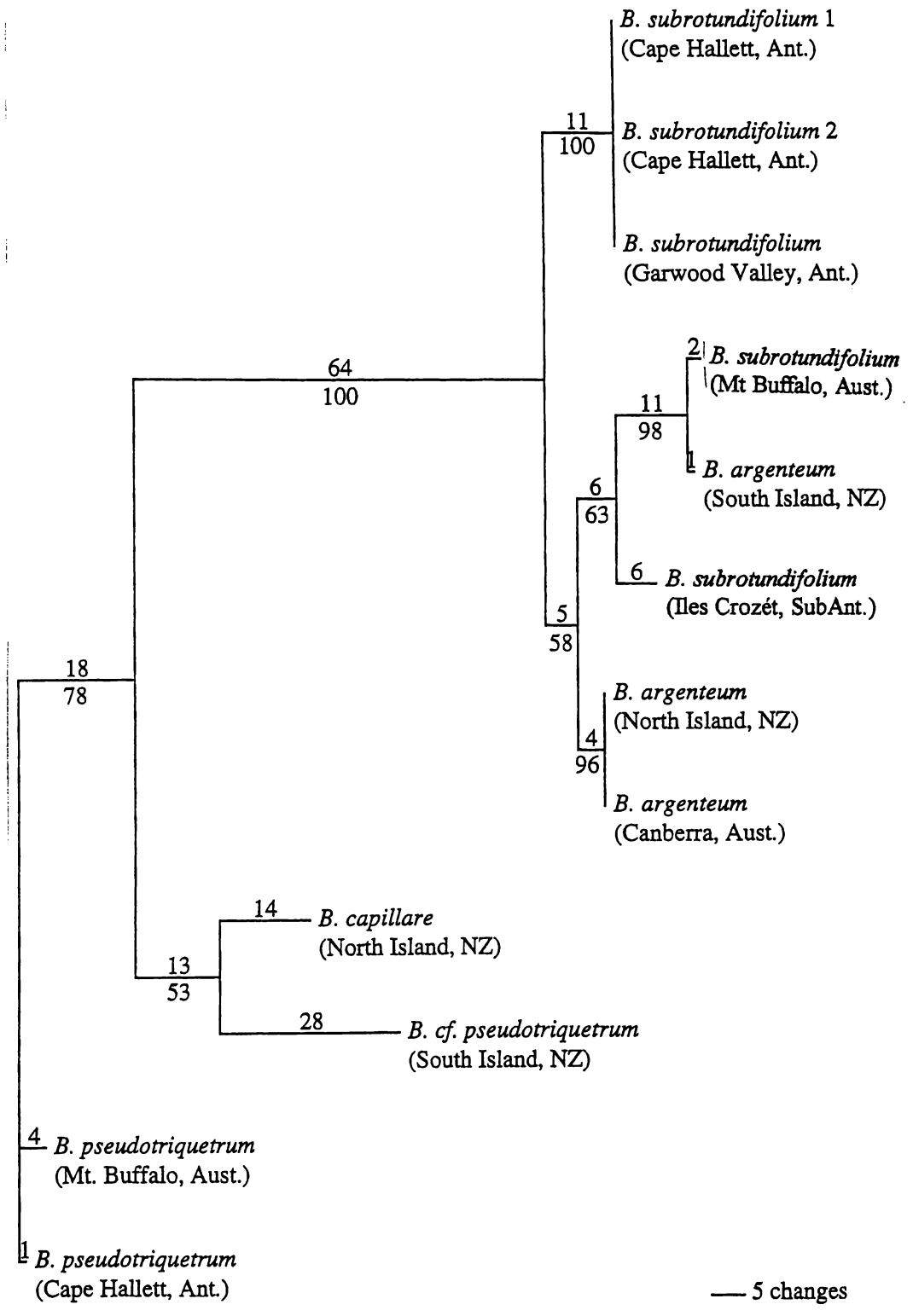


Figure 2.8. Second of two phylograms of 188 steps showing branch lengths (above branches) and bootstrap support values (beneath branches) generated from a branch and bound search of the aligned data in Figure 2.6. Abbreviations as in Figure 2.7.

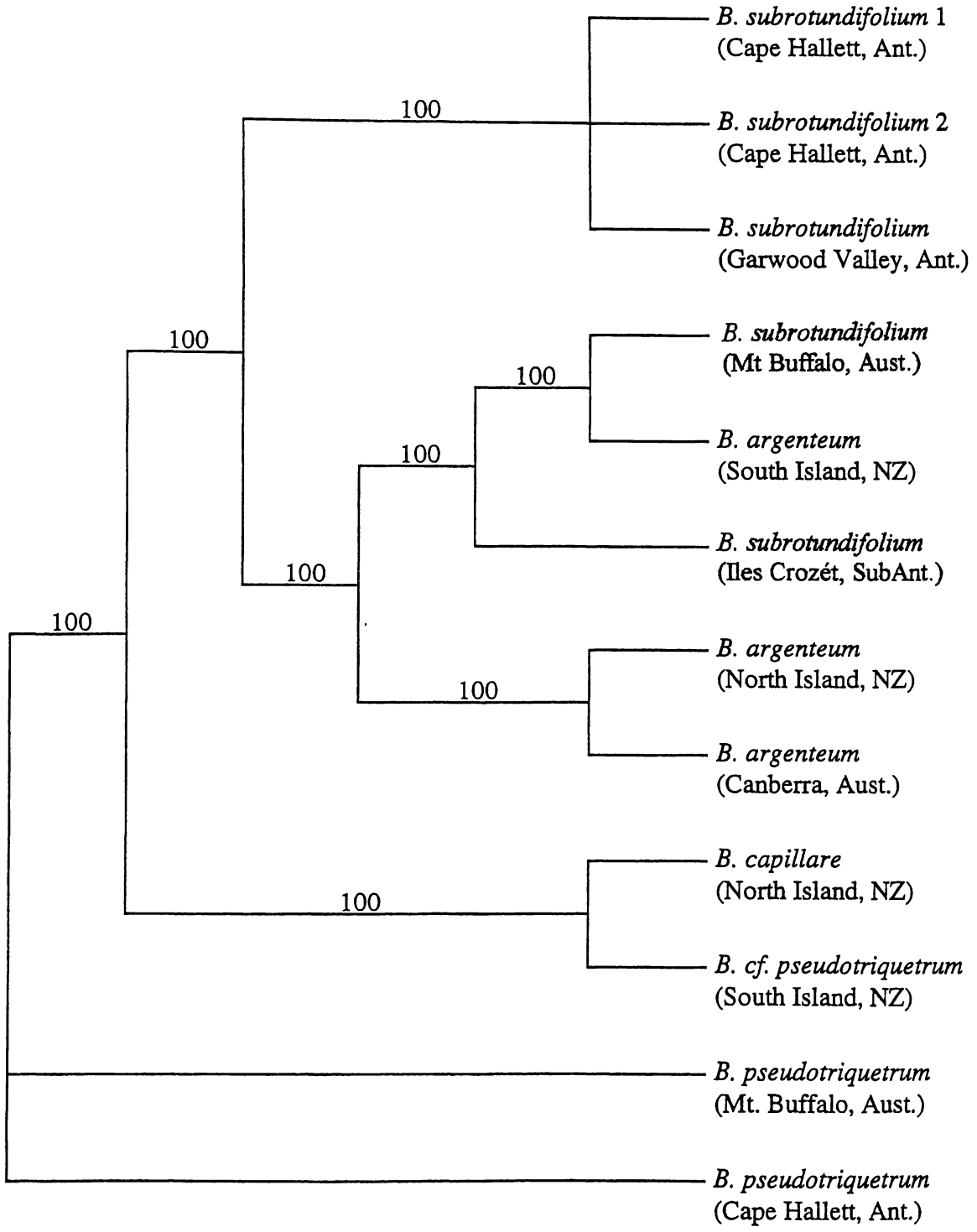


Figure 2.9. Majority-rule consensus cladogram generated from the aligned sequence data in Figure 2.6. Abbreviations as in Figure 2.7.

Two main clades are resolved from these data, comprised of one large clade (100% BS), containing the three *B. argenteum* specimens and the five *B. subrotundifolium* specimens, and one small clade (53% BS), comprised of *B. capillare* and *B. cf. pseudotriquetrum*. The three Antarctic *B. subrotundifolium* specimens are genetically identical and form a clade (100% BS) within the larger *B. argenteum/B. subrotundifolium* clade (58% BS). The *B. subrotundifolium* from Australia is sister to *B. argenteum* from the South Island of New Zealand (98% BS), with this group sister to *B. subrotundifolium* from Iles Crozét (63% BS). The sister group to this clade contains *B. argenteum* from the North Island of New Zealand and *B. argenteum* from Australia (96% BS) with these specimens also genetically identical to each other. The silver *Bryum* species, *B. capillare* forms a clade with *B. cf. pseudotriquetrum* (53% BS) which forms a distantly related (96 steps) sister group to the *B. argenteum/B. subrotundifolium* clade. The *B. cf. pseudotriquetrum* is more closely related to *B. capillare* (42 steps) than it is to the outgroup *B. pseudotriquetrum* specimens (59 steps) from Antarctica and Australia.

2.4 DISCUSSION

2.4.1 PCR Amplification Products

In several of the Antarctic samples two PCR products were observed 1000-1100bp and approximately 550bp in length. The second band was attributed to fungal symbionts associated with Antarctic mosses. The implications of this symbiosis on RAPD analyses of Antarctic moss populations is investigated further in Chapter Three.

2.4.2 Sequence Characteristics

ITS 1 and ITS 2 sequences are each generally less than 300bp each in angiosperms (Baldwin, 1992; Baldwin, 1993; Wojciechowski et al., 1993; Baldwin et al., 1995; Sang et al., 1995; Downie and Katz-Downie, 1996; Gielly et al., 1996; Liston et al., 1996; Manos, 1997; Kelly, 1998, Kornkven et al., 1998; Ainouche and Bayer, 1999; Alice and Campbell, 1999; Li et al., 1999; Noyes and Rieseberg, 1999; Starr et al., 1999; Vargas et al., 1999). The ITS 1 region is similar to this in length in the moss family Brachytheciaceae at 321bp, while the ITS 2 region was only half as long at

159bp. In the *Bryum* species, however, the ITS 1 region is considerably longer (approximately 150bp) with an average length of 443bp. ITS 2 in the *Bryum* species included in this study is closer in length to that in the Brachytheciaceae with an average length of 166bp. This is only half as long as the ITS 2 region in angiosperm species.

ITS 2 regions are thought to be more highly conserved than ITS 1 in angiosperms (Hershkovitz and Lewis, 1996; Liston et al., 1996) and the length polymorphisms in the ITS 1 region from *Bryum* species suggest that this generalisation also applies to bryophyte species. The ITS 1 region 150bp longer in the *Bryum* species than it is in the Brachtheciaceae which suggests that this region is especially variable in this taxon. ITS 2 region was up to 59bp longer in the *B. pseudotriquetrum* and *B. capillare* specimens than it was in the *B. argenteum* and *B. subrotundifolium* specimens. Several insertions of up to 26bp were made to align the shorter sequences with *B. pseudotriquetrum* and *B. capillare*. This suggests that length polymorphisms are prevalent in ITS 2 as well as ITS 1. Insertions such as these in non-coding DNA of mosses may explain the high levels of genetic variation observed in these plants (Giannasi, 1978; Cummins and Wyatt, 1981; Wyatt et al., 1989a; Wyatt et al., 1989b; Derda and Wyatt, 1990; Stoneburner et al., 1991; Adam et al., 1997; Skotnicki et al., 1997, 1998a, b, c; Derda and Wyatt, 1999a; Derda and Wyatt, 1999b). Strict selection controls on coding regions through the haploid-dominant gametophyte is thought to inhibit morphological and physiological evolution. Non-coding regions are not effected by natural selection and could, therefore, mutate freely.

2.4.3 Phylogenetic Relationships

The *B. subrotundifolium* specimens, which were hypothesised to comprise a single clade, instead comprise a well supported clade containing *B. subrotundifolium* and *B. argenteum*. The Antarctic *B. subrotundifolium* populations comprise a smaller clade within the this clade. No variation was detected between *B. subrotundifolium* populations from Cape Hallett and the Garwood Valley, in Antarctica. This contrary to the levels of variation detected in isozyme analyses for Antarctic *B. subrotundifolium* populations from Cape Bird, Lake Fryxell, Cape Royds, the

Garwood Valley, the Taylor Valley and Granite Harbour (Adam et al., 1997), which detected variation (35%and above) between most of these sites. Within population variation was not detected in the moss populations at these sites using isozymes. In contrast to this, RAPD data reveal exceptionally high levels of variation within sites such that shoots from a single clump are considered genetically distinct (Adam et al., 1997; Skotnicki et al. 1997; 1998a). Fungal symbionts identified in this research may provide an alternative explanation for the high levels of variation detected using this technique (Chapter Three).

Populations within the large *B. subrotundifolium/B. argenteum* clade exhibit considerable between site variation. The variation separating the Antarctic *B. subrotundifolium* populations from the remaining populations (16 steps) is less than the variation separating the *B. subrotundifolium* populations from Australia and Iles Crozét (19 steps). The Australian *B. subrotundifolium* specimen, which was collected from the same population as the specimen originally identified by Spence (unpub. data), is more closely related to the *B. argenteum* populations from Australia and New Zealand (3-23 steps) than it is to the Antarctic *B. subrotundifolium* (35 steps). The same can be said for the population from Iles Crozét (16-18 steps cf. 28 steps), which was conclusively identified as *B. subrotundifolium* (R. Ochyra, pers. comm.).

The position of *B. cf. pseudotriquetrum* in the second main clade with the silver *Bryum* species *B. capillare* suggests that the identity of this specimen is incorrect. *Bryum cf. pseudotriquetrum* was sterile, exhibiting *B. pseudotriquetrum*-like gametophytic characters, but with leaves less decurrent than are generally observed in *B. pseudotriquetrum* (A. Fife, pers. comm.). This is a typical example of the inadequacy of gametophytic characters in taxonomic identification. It also illustrates the usefulness of molecular data in identifying ambiguous, sterile specimens, although this is can be expensive and requires existing genetic information from the taxon in question.

The positions of *B. argenteum, B. capillare* and *B. pseudotriquetrum* in the phylograms suggest that genetic distance between congeners within *Bryum* is large.

The *B. argenteum* clade is separated from *B. capillare*, the other silver *Bryum* taxon by 91 steps, while non silver species *B. pseudotriquetrum* in the outgroup is separated from *B. capillare* by 46-49 steps.

These data suggest that *B. subrotundifolium* is conspecific with *B. argenteum* and that the Antarctic populations are an ecotypic variant of *B. argenteum*. This is concordant with RAPD studies by Skotnicki et al. (1998a), which revealed little genetic differentiation (19%) between Antarctic populations of *B. argenteum s.l.* and Australian and New Zealand populations of *B. argenteum s.s.* Somewhat less variation (7%) differentiated the New Zealand and Australian populations, while the majority of the variation was observed within populations.

These data raise the question of the status of *B. subrotundifolium* as a taxonomic unit. The implication that this species is conspecific with *B. argenteum* based on molecular data suggests that *B. subrotundifolium* be void. The morphological differences distinguishing these taxa are minimal, based on leaf colour, leaf shape and the transient occurrence of chloroplasts in hyaline lamina cells after incubation without light. It is conceivable that this latter feature is an adaptation of the Antarctic material in response to the absence of sunlight over the winter months. A reappraisal of *B. subrotundifolium* is required with further consideration given to the morphological features thought to differentiate this taxon from *B. argenteum.*

2.4.4 Biogeography

Biogeographic relationships as estimated in the hypothesis tree are not clearly reflected in this phylogenetic analysis. The Antarctic populations are represented in a differentiated within the tree. However, the Australian and New Zealand populations are interspersed and it is not possible conclusive statement about the biogeographic relationships within the Southern Hemisphere. These results are similar to those obtained using RAPDs which also resolved the Australian and New Zealand populations as interspersed within each other. The inclusion of further specimens from the Subantarctic islands between Australia and New Zealand may help to clarify the relationships between the Antarctic moss populations in Victoria Land and the

surrounding landmasses.

2.4.5 Dispersal

The apparent lack of differentiation between the Australian and New Zealand populations suggests that suggests that either dispersal is occurring between these two land masses or that little genetic divergence exists between these populations. Some support has been generated for this latter postulate in recent studies using isozymes. These studies revealed little genetic differentiation between bryophyte populations from Europe and North America (Odrzykoski and Szweykowski, 1991; Wyatt et al., 1993) compared to the intercontinental differentiation among angiosperm species. Nei's (1972) genetic identity values (I) for intercontinentally disjunct species of the moss species *Rhizomnium magnifolium* and *R. punctatum* in Europe and North America were >0.92 (Wyatt et al., 1993). Similar values (>0.92) were found in the North American and European populations of the liverwort *Conocephalum conicum* (Odrzykoski and Szweykowski, 1991). These values are much lower in angiosperms (e.g. I = 0.43, Parks and Wendel, 1990; and I = 0.26-0.51, Hoey and Parks, 1991), which are generally observed to exhibit strong intercontinental disjunctions (Crawford et al., 1992). Further analysis of other specimens from Australian and New Zealand localities are required to ascertain whether the lack of apparent divergence between Australian and New Zealand populations of *B. argenteum* is due to gene flow via long distance dispersal or not.

2.5 CONCLUSIONS

There appears to be less variation between moss populations in Antarctica than there is between populations in Australia and New Zealand. The Antarctic *B. subrotundifolium* appears to be an ecotypic variant of cosmopolitan *B. argenteum* rather than a separate species. These data suggest that *B. subrotundifolium* is conspecific with *B. argenteum*. A revision of *B. subrotundifolium* is required to determine if this taxon is valid or if it represents a morphological variant of cosmopolitan *B. argentum*.

The inclusion of further specimens from other silver *Bryum* taxa is required for future

research to determine whether or not the silver *Bryum* species form a monophyletic assemblage. This will also enable the genetic distances between a greater range of *Bryum* species to be investigated, which will further our understanding of the relationships the Antarctic moss populations have with cosmopolitan *B. argenteum*.

CHAPTER THREE

FUNGAL HITCHHIKERS: A POSSIBLE EXPLANATION FOR HIGH LEVELS OF GENETIC VARIATION AMONG ANTARCTIC BRYOPHYTES

3.1 INTRODUCTION

3.1.1 Random Amplified Polymorphic DNA (RAPD) Analyses

Random amplified polymorphic DNA (RAPD) analysis uses primers of arbitrary sequence (10bp long) to randomly amplify DNA. No prior knowledge of the DNA sequence for a sample is required for this technique. Differences in banding patterns for a sample reflect the presence and absence of complementary binding primer sites in the genomic DNA for that individual. These differences in the RAPD profile of an individual are detected by the presence or absence of scorable bands on an agarose gel.

Reproducibility in RAPDs is generally poor, with this technique favoured for speed, convenience and simplicity rather than reliability (Jones et al., 1997; Parker et al., 1998). Two RAPD studies, comparing levels of variation in population of Antarctic *Bryum subrotundifolium* Jaeg. with those in populations of *B. argenteum* Hedw. from New Zealand and Australia, report contrasting results. The first study (Skotnicki et al., 1997) reports less variation in the Antarctic populations than in the temperate populations, while the second study (Skotnicki et al., 1998a) reports the opposite relationship with more variation reported in the Antarctic populations than in the temperate populations. Faint irreproducible bands were reported in the RAPD profiles of the Antarctic *B. subrotundifolium* in the first study. This coincides with the reports of higher levels of variation in the Antarctic populations and may explain the discrepancy between these two studies.

DNA contamination has the potential to be a significant problem in RAPDs (Jones et al., 1997; Black IV, 1996) as the random primers used in this technique do not discriminate between DNA from the organism of interest and contaminating DNA.

This is becomes less of a problem in large organisms where the proportion of contaminating DNA is small in comparison to the amount target DNA. However, RAPD studies in Antarctic mosses have used DNA extracted from single shoots a few millimetres in length rendering the samples very susceptible to contamination from parasites, pathogens and phoretic organisms, which make up a larger proportion of the total DNA extracted (Black VI, 1996). These DNA extractions yield small, inconsistent quantities of DNA which can also create ambiguities in RAPD data as reaction products vary with DNA quality and concentration (Black IV, 1996).

3.1.2 Hypervariability in Antarctic Mosses Detected by RAPDs

RAPD analyses have been used to investigate Antarctic populations of *B. argenteum s.l.*, *B. pseudotriquetrum* Gaertn., Meyer et Scherb., and *Ceratodon purpureus* (Hedw.) Brid. (Adam et al., 1997; Skotnicki et al., 1997, 1998a, b, c). These analyses have detected very high levels of genetic variation within a single plant, such that shoots within a moss clump give a unique profile. For example, six moss shoots from a clump of *B. pseudotriquetrum* sampled at 1cm intervals and analysed with five primers gave RAPD profiles which differed from each other by 4 to 22 bands (Skotnicki et al., 1998c). This level of variation is unusually high given that Antarctic mosses are presumed to reproduce primarily by asexual reproduction (Seppelt et al., 1998; but see Filson and Willis, 1975; Kanda and Ochi, 1986; Seppelt et al., 1992; Covey and Lewis Smith, 1993). Mutations induced by high levels of ultraviolet light have been proposed as an explanation for this variation (Skotnicki et al., 1998a, b, c). Somatic mutations induced by this mechanism would be expressed immediately in the haploid-dominant gametophyte, rather than persisting as a masked recessive allele, as they can diploid organisms, which would increase levels of genetic variation.

Adam et al. (1997) used RAPDs and isozyme electrophoresis in a parallel study to assess levels of variation in Antarctic *B. argenteum s.l.* populations from Cape Bird, Cape Royds, Garwood Valley, Lake Fryxell and Granite Harbour. RAPDs revealed genetic differences at two levels in these populations: between sites and between shoots in a clump. The isozyme revealed much less variation with four out of 16

isozyme loci studied exhibiting polymorphisms. Two alleles were found for each polymorphic locus. No within-site variation was detected using this technique with several of the study sites showing no between-site variation. The increased levels of variation detected with RAPDs was attributed to the higher sensitivity of RAPDs which generally reveal more genetic variation than isozymes (Liu and Furnier, 1993; Parker et al., 1998).

3.1.3 Antarctic Fungal Symbionts

Fungal species have been recognised as a integral part the Antarctic biota since the first microbiological investigations in 1960 (Flint and Stout, 1960; di Menna, 1960). They are ubiquitous and have been isolated from a wide variety of microhabitats such as avian detritus, soils, mosses, wood and lakes (Del Frate and Caretta, 1990; Baublis et al., 1991; Onofri et al., 1992; Azmi and Seppelt, 1997). *Phoma, Chrysosporium, Geomyces,* and *Thelebolus* are the most common fungal genera in Antarctica and are generally associated with plants and animals (Del Frate and Caretta, 1990). Fungal species associated with Antarctic mosses are thought to be saprophytic (Del Frate and Caretta, 1990), although, pathogenic fungi have been observed infecting *Hennediella heimii* (Hedw.) Zander. (Greenfield, 1983). Pathogenic fungi are responsible for concentric rings of infection, caused by radial spreading of the fungal hyphae, observed in maritime Antarctic moss species (Longton, 1973; Fenton, 1983). Such fungal hyphal rings are also common on mosses from the Granite Harbour region and on Beaufort Island, southern Victoria Land (S. Hunger pers. obs.; R. Seppelt, pers. comm.).

Multiple bands were consistently amplified in polymerase chain reaction (PCR) products of the internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA (nrDNA) from Antarctic moss specimens. Inspection of the moss specimens at 6.3X magnification revealed the presence of fungal hyphae among the moss shoots (Figure 3.1). Hyphae were generally associated with green shoot tips of the moss. Leaf apices of the shoots in the Granite Harbour samples were white and appeared to be damaged. Both of these observations are concordant with the observation by Seppelt and Kanda (1986) of fungi associated with damaged leaf apices in the upper

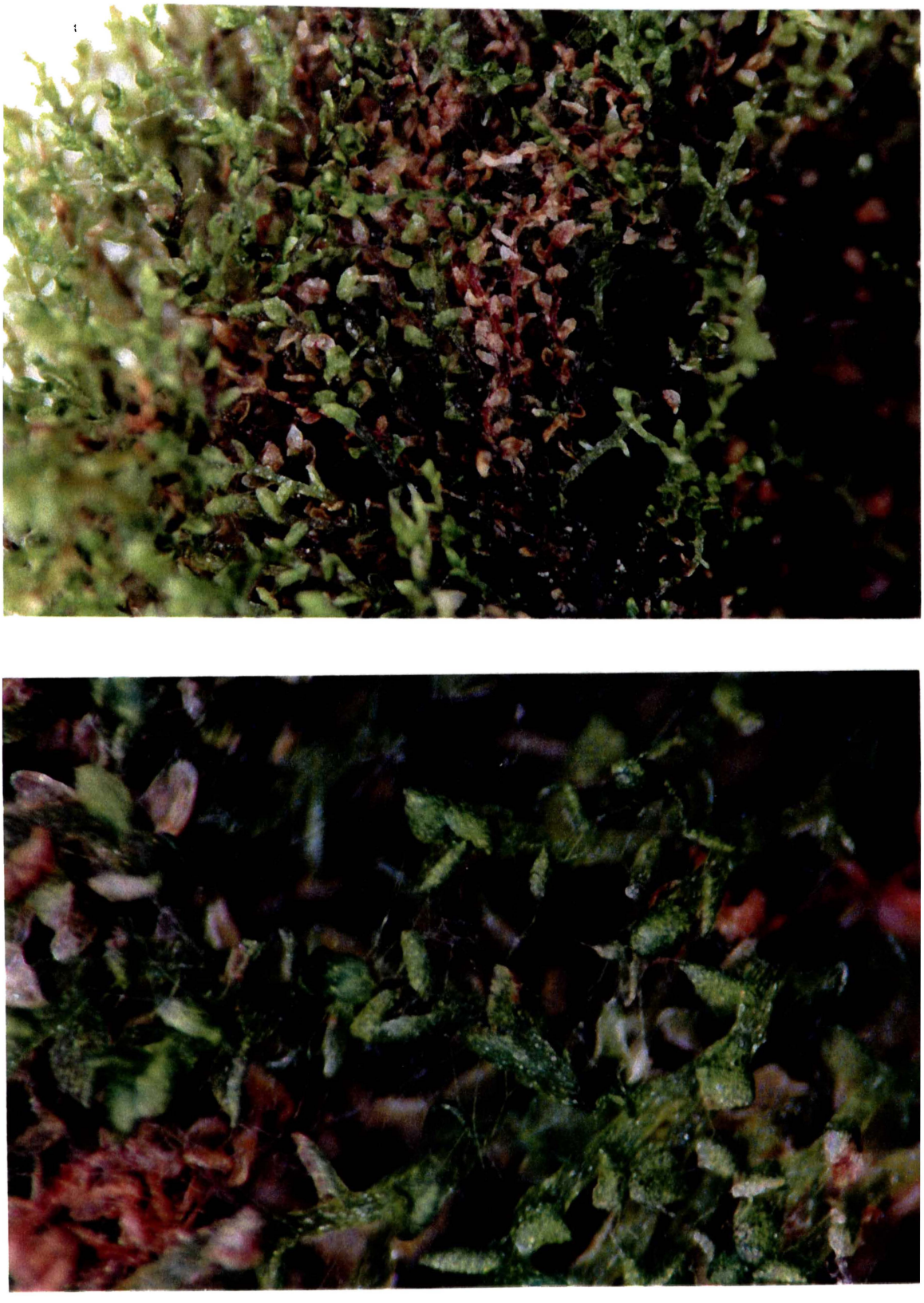


Figure 3.1. Fungal hyphae among the foliage of Antarctic *B. subrotundifolium* at 3.0X (top) and 6.3X (bottom) magnification. This specimen had been rehydrated, in a sealed petri dish and left for several weeks to develop protonemal growth.

stems of mosses. Mycological researchers (Onofri et al., 1992) have isolated 11 fungal species from a single Antarctic moss sample indicating the potential for fungi and other phoretic organisms to contaminate moss DNA.

3.1.4 Objective

The objective of this research was to determine if fungal symbionts associated with Antarctic moss samples could explain the high levels of genetic variation detected by RAPD analysis. Fungal specimens were cultured and DNA extracted so that the RAPD profiles produced by these organisms could be compared to the RAPD profiles of contaminated moss samples. This will enable any bands in the moss profile contributed by fungal contaminants to be identified.

3.2 MATERIALS AND METHODS

3.2.1 Plant Material

Specimens for DNA analyses and fungal culture included in this study are listed in (Table 3.1). Samples were placed in paper envelopes and dried as soon as possible after collection, then transferred to zip-lock plastic bags and stored at -74°C until DNA extraction. Voucher specimens were deposited at the University of Waikato herbarium (WAIK).

3.2.2 DNA Sequencing and Analysis

Amplification products were sequenced at the Waikato University DNA Sequencing Facility as described in Chapter Two. Electropherograms of the ITS sequences were edited using Sequencher version 3.0 (Genecodes). A Basic BLAST searched though Genbank (http://www.ncbi.nlm.nih.gov/) was performed to identify organisms with high genetic identity to the ITS sequence from the secondary band.

Table 3.1. List of specimens included in this study. Specimens in which PCR bands were observed are indicated as are specimens from which fungal cultures were grown. Herbarium specimens are indicated with an asterisk (*).

Taxon	Multiple PCR bands	Fungal Cultures	Location	Accession Number
Bryum argenteum Hedw.			Australia:	
	No		Canberra (35°18'S, 149°08'E)	SH42
			New Zealand:	
	No		Hamilton (35°53'S, 175°28'E)	SH16
	No		Christchurch (43°5'S, 172°7'E)	SH43
B. capillare Hedw.			New Zealand:	
	Yes		Hamilton (35°53'S, 175°28'E)	SH38
B. pseudotriquetrum (Hedw.)			Antarctica:	
Gaertn., Meyer et Scherb.	Yes	Yes	Cape Hallett (72°18'S, 170°18'E)	SH23-1
			Australia:	
	Yes		Mt. Buffalo (36°47'S, 146°03'E)	SH69
			New Zealand:	
	No		Canterbury (43°09'S, 171°40'E)	CHR515077*
B. subrotundifolium Jaeg.			Antarctica:	
	Yes	Yes	Cape Hallett 1 & 2	SH21 &
	Yes	Yes	(72°18'S, 170°18'E)	SH27-1
	No	Yes	Garwood Valley (78°03'S, 164°10'E)	SH34
	Yes	Yes	Granite Harbour (77°00'S, 162°34'E)	KMG2
			Subantarctica:	
	No		Iles Crozet (46°27'S, 52°00'E)	SH48*
			Australia:	
	Yes		Mt. Buffalo (36°47'S, 146°03'E),	SH56-1
Ceratodon purpureus (Hedw.)			Antarctica:	
Brid.	Yes	Yes	Cape Hallett 1 & 2 (72°18'S, 170°18'E)	SH24
		Yes	Granite Harbour (77°00'S, 162°34'E)	KM5
Hennediella heimii (Hedw.)			Antarctica:	
Zander	No	Yes	Garwood Valley (78°03'S, 164°10'E)	SH35

Figure 3.2. Secondary ITS bands amplified in Antarctic *B. subrotundifolium* samples. Lane 1: 100 base pair DNA ladder (Life Technologies Inc.), bright bands are at 1200bp (top) and 600bp (middle). Lanes 2, 3 and 4: Antarctic *B. subrotundifolium* samples from Cape Hallett (accession number SH21), Garwood Valley, (accession number SH34), and Granite Harbour (accession number KMG2), respectively. ITS bands are 1000-1100bp long while the contaminant band is approximately 550bp long.

3.2.3 Fungal Cultivation and Microscopy

Moss specimens from Cape Hallett, Granite Harbour and the Garwood Valley were examined at 6.3X magnification with a stereo microscope (Olympus, 5Z60). One healthy shoot from frozen and dried specimens was depressed into separate petri dishes containing malt extract agar (MEA: 1.5% malt extract, 0.2% yeast extract, 0.9% agar) and incubated at 15°C for six days. Colony morphology was examined (6.3X magnification) and colony diameter measured to estimate the extent of fungal contamination in different localities. Bacterial species were also present in some of the cultures, but these exhibited limited growth in comparison to the fungal colonies and were soon out-competed by the fungi. Fungal cultures were incubated for a further three months at room temperature to allow the cultures to mature at which time colony morphology was examined again. Pycnidia from fruiting cultures were mounted in a drop of water on a microscope slide and a coverslip depressed onto the sample to expel the spores. The slides were then examined under 100X magnification for spore morphology.

3.2.4 DNA Extraction

Liquid cultures (5mL aliquots of malt extract media: 1.5% malt extract, 0.2% yeast

extract in 25mL universal bottles) were inoculated with fungal hyphae from the fungal culture obtained from SH21 in the petri dishes (see above) using sterile forceps. These cultures were incubated for four days at 15°C shaking at 300rpm. Single fungal colonies from liquid cultures were placed into a 1.5mL microfuge tube and centrifuged at 18,200g for 10 minutes. The supernatant was removed and the pellet completely dried in a HetoVac CT110 for 30 minutes. DNA was extracted from the prepared fungal pellets using the technique described by Rogers and Bendich (1985; Appendix II).

3.2.5 RAPDs

RAPD analysis was performed on Antarctic *B. subrotundifolium* specimens, SH27-1 and SH21 and fungal colonies cultured from SH21. Fungal DNA from SH21 was also added to a DNA sample from SH27-1 (1:1 w/w) to obtain RAPD profiles for artificially contaminated sample. This made it possible to compare the bands produced in the artificially contaminated sample with bands from a contaminant with a known profile.

RAPD reactions based on those described by Skotnicki et al. (1998a, b, c), were prepared in a 10μL volume containing 1X PCR buffer (Boehringer Mannheim), dATP, dTTP, dCTP and dGTP at 0.025mM each, 0.3mM primer OPA03 or OPA13 (Operon Technologies Inc.), 0.5 Units *Taq* DNA polymerase (Boehringer Mannheim) and 5-10ng of template DNA. The amplification protocol was as follows: 1 cycle of 3 minutes at 94°C, 2 minutes at 40°C and 3 minutes at 72°C; 43 cycles of 10 seconds at 94°C, 10 seconds at 40°C and 50 seconds at 72°C; with a final extension period for 3 minutes at 72°C. Ramp times were set at 1°C/second. Reactions were electrophoresed on a 1.5% agarose gel at 5V/cm for 2.5 hours.

3.3 RESULTS

3.3.1 Molecular Identification

A DNA fragment (approximately 550bp in length) was amplified from Antarctic *B. subrotundifolium* DNA (SH21 and KMG2) in conjunction with the moss DNA fragment (1000-1100bp; Figure 3.3). This fragment did not amplify in the Garwood

Valley sample (SH34). Basic BLAST search results from the DNA sequence of the contaminant fragment indicated 97% sequence homology between the secondary ITS band and the partial sequence 18S ribosomal RNA gene of *Ampelomyces humuli*, *A. quercinus* and *P. glomerata*.

3.3.2 Fungal Cultures

After the six day incubation period at 15°C fungal colonies consisted of an extensive white mycelium, covered in a grey-green dust-like substance, with a faint red pigment diffusing into the media at the base of the colony. These observations are characteristic of *Phoma herbarum* Westend. (Rai and Rajak, 1993). The two samples from the Garwood Valley yielded much less prolific fungal growth than samples from Cape Hallett and Granite Harbour (Table 3.2).

3.3.3 Morphological Identification

Only one fungal species, common to all moss samples (including the Granite Harbour specimen), was observed on the malt extract agar after extended incubation at room temperature. Other smaller bacterial and fungal colonies observed after the initial incubation period were obliterated by the rapid, extensive growth of the predominant fungal species. This specimen was characterised as *Phoma* sp. based on the morphological description for this genus (Rai and Rajak, 1993) and thought to be *P. herbarum* Westend. (D. M°New, pers. comm.). Colonies changed from green (15°C) to brown after incubation at room temperature with the development of an extensive mycelial mat which permeated the media (Figure 3.3). Black, globose to subglobose, flask-like, pseudoparenchymatous pycnidia were also observed, but did not form until two months after the initial incubation period. Pycnidia are not usually formed on nutrient-rich media (D. M°New, pers. comm.) and were induced on MEA, which is a nutrient-rich media, only after nutrient levels had been sufficiently depleted by the extensive fungal growth.

Phoma herbarum can be differentiated from *Phoma glomerata* (Corda) Wollenw. & Hochapf. by the absence of chlamydospores and dictyochlamydospores (Rai and Rajak, 1993). This feature could not be confirmed from microscopic examination of

spores from these cultures (D. McNew, pers. comm.), preventing a positive species-level identification.

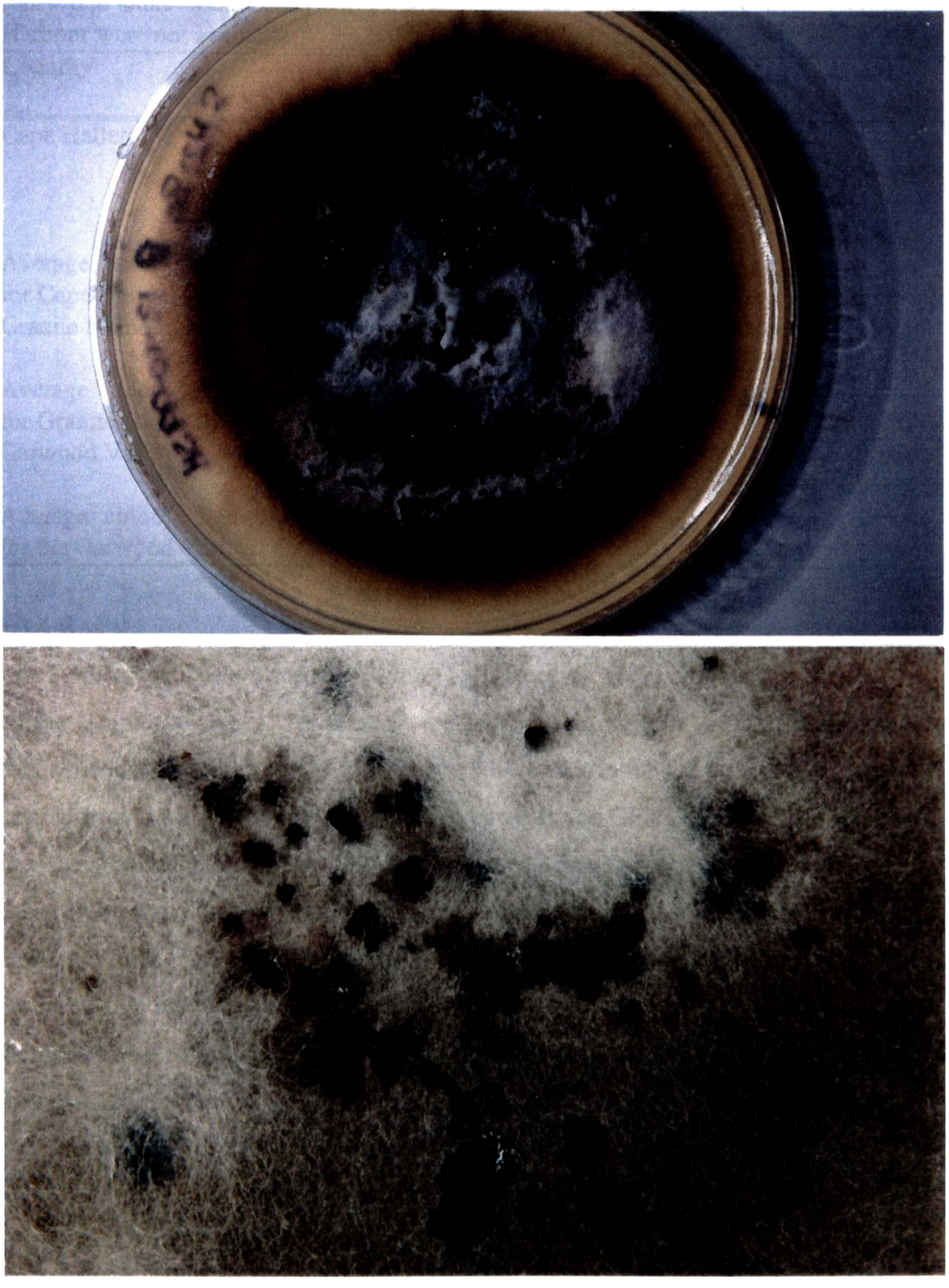


Figure 3.3. Colony morphology of *Phoma* sp. grown on MEA isolated from Antarctic moss specimens (top). Pycnidia (6.3X magnification) formed after prolonged growth (three months) at room temperature (bottom).

Table 3.2. Fungal colony diameter (mm) after a six day incubation period at 15°C seeded from single shoots taken from dried and frozen Antarctic moss specimens (only one plate was seeded for each sample). NB: Dried samples from Granite Harbour were not available.

Locality	Taxon	Accession Number	Colony Diameter	
			Frozen	Dried
Cape Hallett	*B. subrotundifolium*	SH21	25mm	18mm
	B. subrotundifolium	SH27-1	23mm	17mm
	B. pseudotriquetrum	SH23-1	16mm	16mm
	C. purpureus	SH24	35mm	15mm
Average colony diameter for Cape Hallett:			25mm	17mm
Granite Harbour	*B. subrotundifolium*	KMG2	20mm	-
	C. purpureus	KM5	25mm	-
Average colony diameter for Granite Harbour:			23mm	-
Garwood Valley	*B. subrotundifolium*	SH34	15mm	2mm
	H. heimii	SH35	2mm	0mm
Average colony diameter for the Garwood Valley:			7mm	1mm

3.3.4 RAPDs

The *B. subrotundifolium* specimen (SH21) produced the most polymorphic bands of all the samples analysed. Ten bands were produced using primer OPA13 and nine bands produced with primer OPA03 (Figure 3.4). Bands in the OPA03 profile for this sample were more poorly resolved than the bands in the OPA13 profile. The second *B. subrotundifolium* specimen (SH27-1) produced less polymorphic bands with only one scorable band present in the OPA13 profile and two scorable bands present in the OPA03 profile. The SH27-1 specimen artificially contaminated with fungal DNA produced five scorable bands with the OPA13 primer compared to one produced in the non-contaminated sample. Five bands for each primer were produced from the fungal DNA. At least two of these bands were present in each of the SH21 profiles. The bands from the fungi profiles were not observed in the non-contaminated SH27-1 sample and only one band from the fungi profile was observed in the artificially contaminated SH27-1 sample.

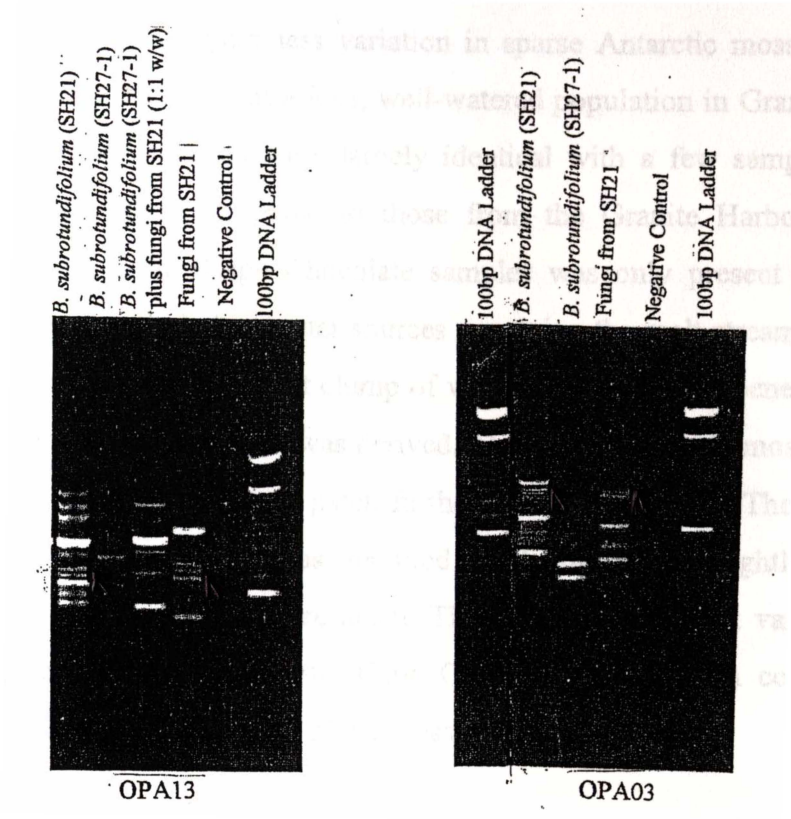


Figure 3.4. RAPD profiles of Antarctic *B. subrotundifolium* and the associated fungal species amplified using OPA13 (lanes 1-4) and OPA03 (lanes 8-10). Shared bands are indicated by black arrows. Two bands in the RAPD profiles of the fungi (lanes 4 & 10) also appeared in the RAPD profiles of the *B. subrotundifolium* specimen from which the fungi was isolated (SH21; lane 1 & 8). Bright bands in the 100bp DNA ladders (Life Technologies) are 2000bp (top), 1200bp (middle) and 600bp (bottom) in length.

3.4 DISCUSSION

Contamination in the Antarctic mosses appeared to be less prolific in samples from the Garwood Valley (an extremely dry and harsh locality) than they were in those from Cape Hallett (a sheltered site, abundant free water present) based on the size of fungal colonies produced in these regions. This observation is supported by the absence of a secondary band product in the PCR amplification of the ITS region in the Garwood Valley specimen. Mycological studies also follow this trend with up to 58 colony forming units (CFU) detected beneath a moss turve in the Taylor Valley (Baublis et al., 1991) and approximately 75,000 yeast cells per gram of moss isolated from specimens in the McMurdo Sound Region (di Menna, 1960). These observations suggest that the extent of fungal contamination is related to the amount of free-water available in the area.

Skotnicki et al. (1998c) report less variation in sparse Antarctic moss populations from Cape Chocolate than from a lush, well-watered population in Granite Harbour. The Cape Chocolate samples were largely identical with a few samples showing minimal variation when compared to those from the Granite Harbour site. The variation detected in the Cape Chocolate samples was only present in the moss clumps growing directly below water sources supplying the melt-stream from which these samples were taken. The first clump of variable samples was beneath the snow bank from which the melt-stream was derived. The second variable moss clump was growing alongside a second snow patch further along the channel. The melt-stream between these snow patches was reported as being only "slightly damp" in midsummer when the samples were taken. These data suggest that variation in the moss population growing along the Cape Chocolate melt-stream correlates with increased free-water in the vicinity of the moss populations.

Preliminary results from this research using RAPDs suggest that fungal contaminants can artificially bias levels of genetic variation dectected using this technique. At least two bands out of the 9 to 10 bands in each of the RAPD profiles of the *B. subrotundifolium* specimen (SH21) are evident in the RAPD profile of the fungi isolated from that specimen. The artificially contaminated SH27-1 sample yielded four more bands than the non-contaminated sample. However, only one of these was common to the fungal DNA profile. The lack of scorable bands in the non-contaminated *B. subrotundifolium* specimen (SH27-1) suggests amplification problems in this sample. This may have been due to variation in the concentrations of DNA in the reactions and will be investigated further in future studies.

Microsatellite DNA markers are currently being developed as a alternative technique to investigate population-level genetics in Antarctic moss populations (Chapter Four). This technique is also PCR based, however it is much less sensitive to contamination. Regions of the genome containing polymorphic microsatellite DNA is targeted by specific primers developed for the target species and will only amplify DNA from that species and close relatives of it. Microsatellite DNA markers are expensive and time-comsuming to develop. However, once developed they are more

reliable than RAPDs, easy to use and can detect variation at different levels within a population depending on the amount of polymorphism at a locus.

The use of PCR of a known region as a positive control to detect contamination in DNA samples has potential. For example, several DNA samples may be screened for contamination then analysed with RAPDs to gain an estimate of the expected levels of variation for that organism from DNA samples which are known to be contamination-free. Screening can then be performed intermittently throughout the remaining samples and in samples which contain unusually high levels of variation. This will ensure that high levels of variation detected within samples is unequivocally due to variation within the organism of interest.

The ITS region is particularly useful in this regard as it is highly polymorphic and ranges in length between distantly related taxa. This enables ITS products of different taxa produced by PCR to be detected by electrophoresis on an agarose gel. This region is universal, with primer binding sites situated within the ribosomal genes, which are highly conserved in all organisms. It also exists in high copy numbers throughout the genome facilitating amplification. Sequencing this region and performing a BLAST search through Genbank (http://www.ncbi.nlm.nih.gov/) enables organisms with high genetic identity to the contaminant to be identified if required. However, this does not always result in a positive identification of the organism of interest and is essential that morphological data is considered in conjunction with molecular data in identifying unknown taxa.

3.5 CONCLUSIONS

These data suggest that contaminating fungal DNA may contribute to the levels of variation detected by RAPDs. And that high levels of variation reported in moss populations correlates strongly with favourable fungal habitats. Future research into the levels of variation in Antarctic mosses will use microsatellite DNA markers and enable the current reported levels of genetic variation in Antarctic moss species to be re-evaluated without concerns of contaminants.

CHAPTER FOUR

DEVELOPMENT OF MICROSATELLITE DNA MARKERS IN THE COSMOSPOLITAN MOSS SPECIES *BRYUM ARGENTEUM* HEDW.

4.1 INTRODUCTION

4.1.1 Biodiversity in Continental Antarctica

Fifteen species of *Bryum* have been described in continental Antarctica since the first botanical collections were made in 1820 (Literary Gazette and Journal of Belles Lettres, 10 Nov, 1821, pp712-713). These fifteen species have now been subsumed into only two species: *Bryum subrotundifolium* Jaeg. and *Bryum pseudotriquetrum* (Hedw.) Gaertn., Meyer et Scherb. (Ochi, 1979; Seppelt and Kanda, 1986). This huge reduction in the floristic inventory of Antarctica could be masking the true levels of biodiversity, especially given the morphological variation that exists within these species. Genetic studies aimed at the population-level will enable us to map levels of biodiversity in the Antarctic bryoflora. By understanding and monitoring the levels of genetic variation present in moss populations we will be better prepared to report and maintain the current levels of biodiversity in Antarctica.

4.1.2 Population Genetics

Antarctic mosses provide an interesting study group from a population genetics perspective. Sexual reproductive structures are reported to occur in only 10% of the moss species in Continental Antarctica (Convey and Lewis Smith 1993; e.g. Seppelt et al., 1992; Kanda and Ochi, 1986). New populations are established by wind and avian dispersal of asexual propagules (Rudolf, 1970). This, coupled with a haploid-dominant vegetative form suggests little opportunity for the introduction of genetic variation. Molecular evidence, however, suggests otherwise.

4.1.3 Genetic Variation of Mosses

Genetic studies using isozymes and RAPDs have detected levels of variation comparable to those found in angiosperms (Table 4.1; Hamrick, 1979; Cummins and

Wyatt, 1981; Vries et al., 1983; Bramen, 1986; Hofman, 1988 Wyatt et al., 1989a; Wyatt et al., 1989b; Derda and Wyatt, 1990; Stoneburner et al., 1991; Derda and Wyatt, 1999a; Derda and Wyatt, 1999b). This genetic variation is not reflected in phenotypic differences suggesting that bryophyte species may evolve at a physiological level (Wyatt, 1985; Dewey, 1989) or with morphological evolution so subtle it is undetectable (Wyatt, 1985).

Table 4.1: Genetic variability levels in bryophytes and angiosperms determined by isozyme analysis (Adapted from Wyatt et al., 1989b). Abbreviations: P, percentage of polymorphic loci per population; A, mean number of alleles per locus per population; H_s, mean expected heterozygosity per locus per population.

Taxon	A	P	H_s	Reference
Mosses:				
Plagiomnium cilliare	1.40	31.1	0.079	Wyatt et al., 1989a
P. ellipticum	1.80	50.0	0.123	R. Wyatt et al., (*unpub. data*)
P. insigne	1.17	16.7	0.065	R. Wyatt et al., (*unpub. data*)
Mean values for *Plagiomnium*	1.46	32.6	0.089	
Plagiothecium curvifolium	1.90	48.0	0.190	Hofman, 1988
P. denticulatum	1.70	37.0	0.170	Hofman, 1988
P. ruthei	1.60	35.0	0.160	Hofman, 1988
P. latebricola	1.30	25.0	0.110	Hofman, 1988
P. nemorale	1.70	39.0	0.160	Hofman, 1988
P. undulatum	1.20	16.0	0.090	Hofman, 1988
Mean values from *Plagiothecium*	1.56	33.3	0.150	
Racopilum. capense	1.29	29.0	0.069	Bramen, 1986
R. convolutaceum	1.43	25.0	0.102	Bramen, 1986
R. cuspidigerum	1.37	24.3	0.093	Vries et al., 1983
R. cuspidigerum	1.71	50.0	0.242	Bramen, 1986
R. intermedium	1.43	43.0	0.093	Bramen, 1986
R. robustum	1.29	29.0	0.127	Bramen, 1986
R. spectabile	1.93	52.3	0.198	Vries et al., 1983
R. spectabile	1.62	45.0	0.168	Bramen, 1986
R. strumiferum	1.57	56.0	0.180	Bramen, 1986
R. tomentosum	1.62	43.0	0.174	Bramen, 1986
Mean values for *Racopilum*	1.53	35.4	0.145	
Range of mean values:	1.46-1.56	32.6-35.4	0.089-0.150	
Vascular Plants:				
Range of mean values:	1.35-2.56	22.0-75.3	0.079-0.354	Hamrick, 1979

Randomly amplified polymorphic DNA analysis (RAPDs; Adam et al., 1997; Skotnicki et al., 1997, 1998a, b, c) detected exceptionally high levels of genetic variation in Antarctic moss species. In a study comparing populations of Australian and New Zealand *B. argenteum* and Antarctic *B. argenteum s.l.* (Skotnicki et al., 1998a), 75% of the variation detected occurred within these populations, with genetic differences detected between adjacent moss shoots. The identification of fungal symbionts in Antarctic moss species provide an alternative explanation for these levels of variation (see Chapter Three), which have not been detected using more reliable techniques such as isozymes (Adam et al., 1997) and ITS sequences (see Chapter Two).

4.1.4 Microsatellite DNA Markers

Microsatellite DNA consists of tandem repeats of nucleotides (2 - 6 nucleotides in length; Queller et al., 1993) rarely longer than 60 repeats (Goldstein and Pollock, 1997). These regions are estimated to occur every 50 kb in plant genomes (Morgante and Olivieri, 1993). They can be amplified from milligram amounts of sample using the polymerase chain reaction (PCR) once primers which flank repeat sequences of interest have been designed (Gupta et al., 1996).

The discrete, codominant mode of inheritance demonstrated by these markers makes them ideal for studying population-level genetics and phylogenetic relationships between populations (Schlotterer and Pemberton, 1994). They are highly polymorphic, varying in length between individuals within a species or a population (Gupta et al., 1996). Their polymorphic character is attributed to slippage of DNA polymerase during replication (Levinson and Gutman, 1987). Loss of a single repeat unit is the most frequently observed form of polymorphism, although larger changes also occur (Weber and Wong, 1993; Amos and Rubinzstein, 1996). Goldstein and Pollock (1997) postulate unequal crossing over during recombination as feasible mechanism for mutations based on similar observations in minisatellite DNA (repeats of 10-60 nucleotides; Jefferys et al., 1988).

Microsatellite DNA markers are largely species-specific, although they can often be

used in closely related species (Strassmann et al., 1996; e.g.: Arévalo et al., 1994; Bhebhe et al., 1994). The specificity of the primers used in microsatellite DNA analysis reduces the chance of contaminants from unrelated organisms influencing the results. These markers are expensive and time consuming to develop, however, they are more reliable than RAPDs and can be used to analyse large numbers of population-level DNA samples using PCR once developed.

4.1.5 Study group

Bryum argenteum Hedw. is cosmopolitan moss species. This species is taxonomically complex, exhibiting morphological variation in different geographical regions (Seppelt and Kanda, 1986). The wide range of this species makes it ideal for studying population genetics in bryophytes from Antarctica and temperate regions. Another cosmopolitan species *Ceratodon purpureus* (Hedw.) Brid., which also occurs in Antarctica (Burley and Pritchard, 1990; Seppelet and Hancok, 1991), was used as a positive control for this research.

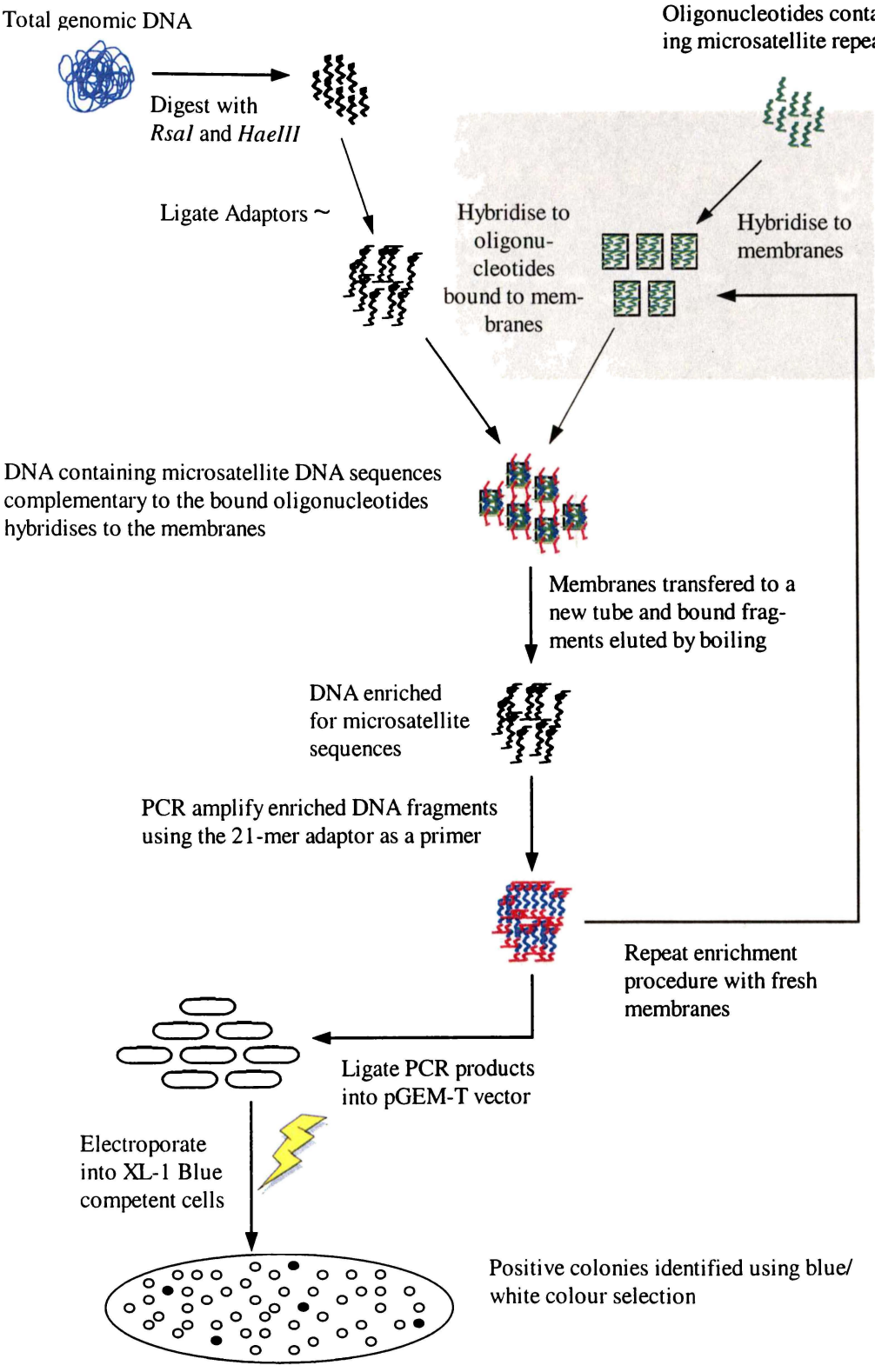
4.1.6 Objectives

The aim of this research was to develop microsatellite DNA markers for future fine-scale population-genetics studies of Antarctic mosses using New Zealand specimens of *B. argenteum* and *C. purpureus*. Specifically, **1)** to design primers which could be used to amplify highly polymorphic microsatellite DNA regions in Antarctic moss populations, **2)** to test these primers across a range of species in the genus *Bryum*, including the Continental Antarctic species *Bryum subrotundifolium* Jaeg. and *B. pseudotriquetrum* (Hedw.) Gaertn., Meyer et Scherb. and, if they work, **3)** to assess the amount of genetic variation in Antarctic populations of *C. purpureus* and *B. subrotundifolium*.

4.2 MATERIALS AND METHODS

Microsatellite DNA marker development was based on the techniques described by Karagyozov et al. (1993) and Edwards et al. (1996). These techniques are reported to yield a 50-fold enrichment of DNA containing microsatellite repeats (Karagyozov et al., 1993). A higher proportion of clones containing different types of microsatellite

repeats have also been reported with the technique described by Edwards et al. (1996). The schematic diagram in Figure 4.1 illustrates the main steps in the protocol.



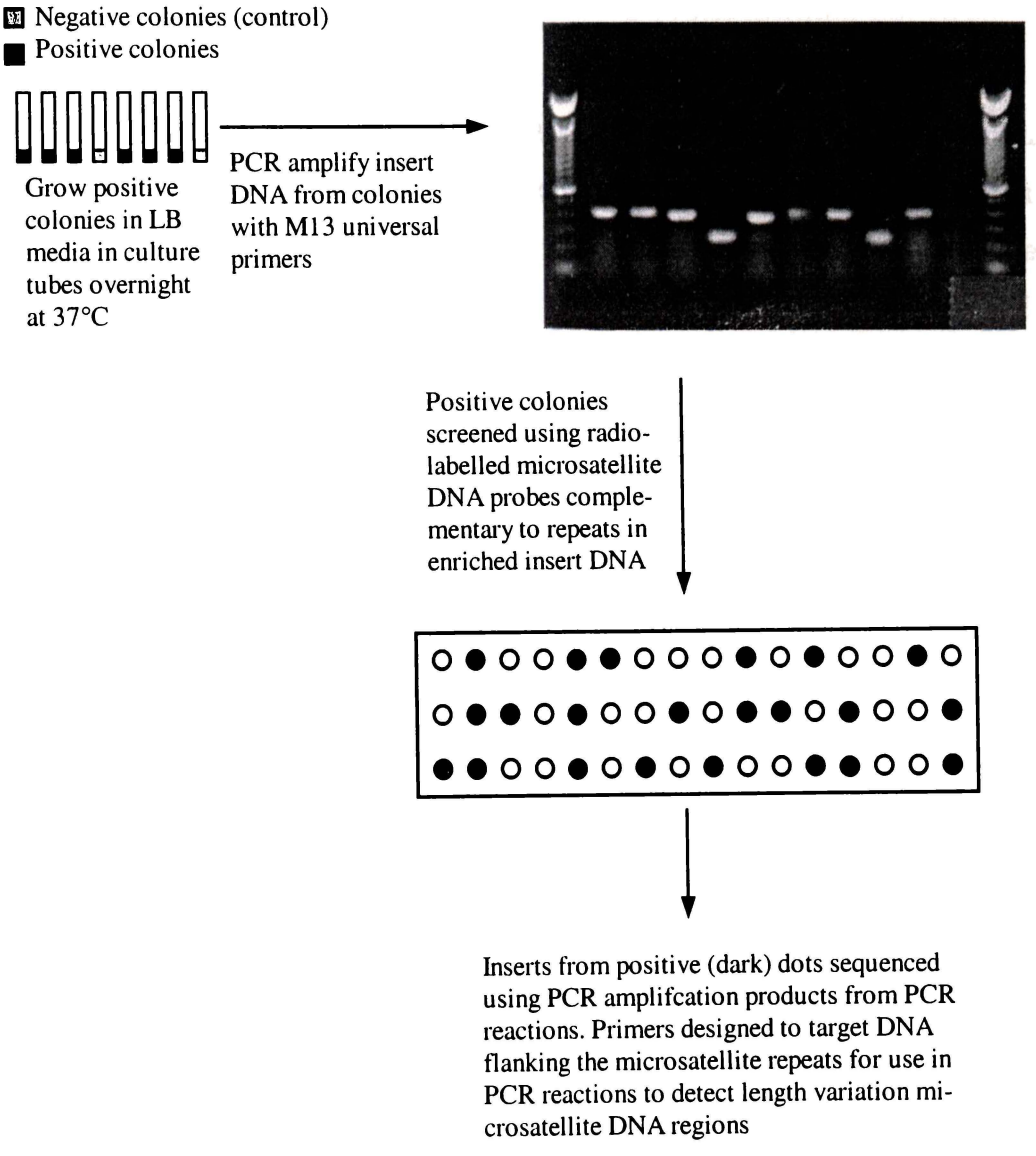


Figure 4.1. Schematic diagram of DNA preparation and enrichment for microsatellite repeats based on techniques described by Karagyozov et al. (1993) and Edwards et al. (1996).

4.2.1 Plant Material

Fresh specimens of *Ceratodon purpureus* (Hedw.) Brid. and *Bryum argenteum* Hedw. were collected from Hamilton, New Zealand (35°53'S, 175°28'E; Appendix I). Specimens were inspected for contaminating material then dried in paper envelopes. Duplicates of each specimen were stored at -74°C and voucher specimens lodged into WAIK.

Total genomic DNA was extracted from 100mg of dried, green, leaf material from both species using the CTAB extraction technique described by Rogers and Bendich (1985; Appendix II). DNA samples were screened for contaminants by amplification of a the internal transcribed spacer (ITS) sequences in nuclear ribosomal DNA (nrDNA; Chapter Two) prior to use in microsatellite DNA marker development. This region is present in all organisms and is flanked by highly conserved ribosomal genes to which primers that amplify this region are targeted. Contaminating DNA in a sample is indicated by the presence of more than one band when polymerase chain reaction (PCR) amplification products are electrophoresed on an agarose gel.

4.2.2 Membrane Preparation

Seven oligonucleotides ($(GA)_7$, $(GT)_7$, $(CAA)_6$, $(CAT)_6$, $(GAA)_6$, $(GATA)_{10}$ and $(CATA)_{10}$) (Life Technologies) containing microsatellite repeats were hybridised to fifty 0.5cm^2 sections of Hybond® N^+ (Amersham) membrane. Each oligonucleotide was suspended in TE buffer [10mM Tris (pH 8.0), 1mM EDTA (pH 8.0)] at a concentration of 1μg/μL. 20μg of the di- and trinucleotide repeats ($(GA)_7$, $(GT)_7$, $(CAA)_6$, $(CAT)_6$ and $(GAA)_6$) and 40μg of the tetranucleotide repeats ($(GATA)_{10}$ and $(CATA)_{10}$) were combined in 4mL of 3 x standard saline citrate (SSC) [450mM NaCl, 45mM sodium citrate (pH 7.0)]. 80μl of this solution was spotted on to the nitrocellulose membrane squares and dried at 37°C for three hours. The oligonucleotides were then cross-linked to the membranes by baking at 80°C for two hours. Unbound oligonucleotides were washed from the membranes at 45°C in 10mL of hybridisation buffer [50% formamide, 3 x SSC, 25mM sodium phosphate (pH 7.0), 0.5% sodium dodecyl sulphate (SDS)] for two days and shaken several times over this period. The membranes were then rinsed in distilled water, followed by

boiling for 10 minutes in 1% SDS, and stored at -20°C until required.

4.2.3 DNA Preparation

One microgram of DNA from each species was digested at 37°C for one hour with *RsaI* and *HaeIII* in separate reactions to give two sets of digested DNA from each species [1μg DNA, 20U enzyme, 1X restriction buffer (Boehringer Mannheim), distilled water to 40μL]. Each digest was electrophoresed on a 1% agarose minigel to confirm digest success (Figure 4.2). Complementary adaptors (21-mer: 5'-CTC TTG CTT GAA TTC GGA CTA-3' and 5'-end phosphorylated 25-mer: 5'-pTAG TCC GAA TTC AAG CAA GAG CAC A-3'; Karagyozov et al., 1993) were ligated to 15 μl of each digest [100ng 21-mer, 100ng 25-mer, 2U T4 DNA ligase (Boehringer Mannheim), 1 x ligation buffer (Boehringer Mannheim), distilled water to 40 μL]. The reactions were incubated at 37°C for five minutes then left at room temperature for 16 hours.

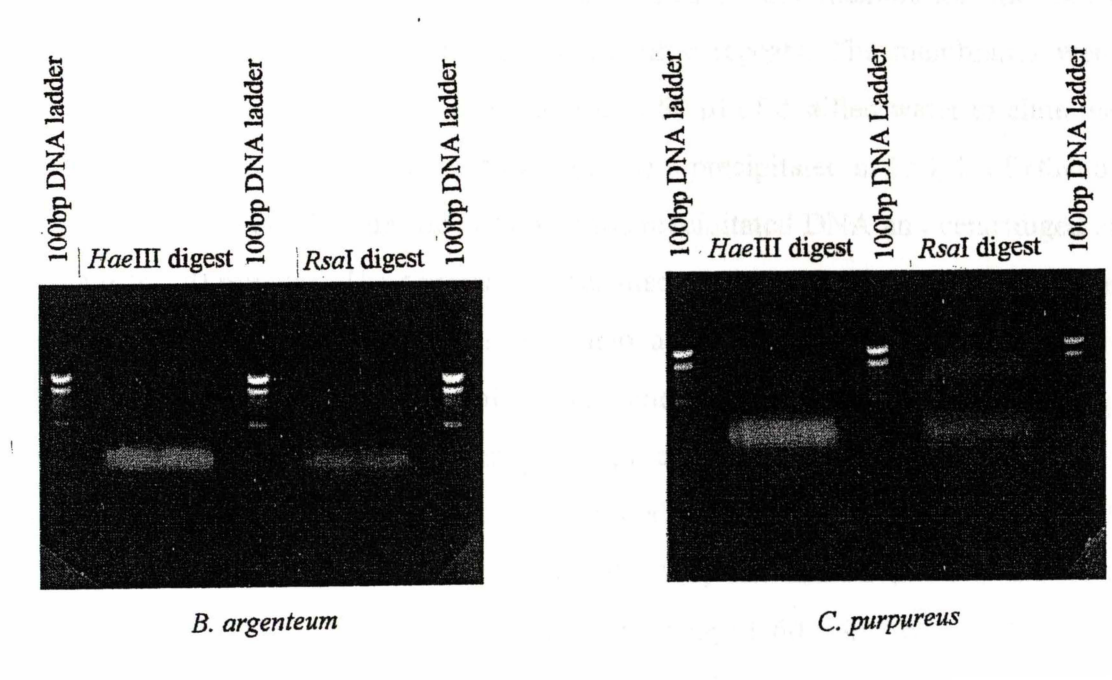


Figure 4.2. Total genomic DNA from *B. argenteum* and *C. purpureus* digested with *RsaI* and *HaeIII*.

4.2.4 Enrichment for Microsatellite DNA

Fragments with the 21-mer and 25-mer adapters ligated to each end were amplified using PCR [20μM 21-mer, 50ng ligated *Rsa*I digestion fragments, 50ng ligated *Hae*III digestion fragments, 200μM deoxyribonucleotide triphosphates (dNTPs; Boehringer Mannheim), 1X J buffer (Appendix III), 1U *Taq* DNA polymerase (Boehringer Mannheim)]. The reaction was amplified for 30 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 30 seconds at 72°C in an Eppendorf Mastercycler Gradient thermal cycler.

20μg of the PCR fragments were denatured by heating at 95°C for ten minutes, followed by the addition of 600μL of hybridisation solution [50% formamide, 3 x SSC, 0.1 x Church and Gilbert solution (Appendix III)] and 10μg of the 21-mer to prevent concatamerisation of the complementary ends of the fragments (Karagyozov et al., 1993). Two of the nitrocellulose membranes with oligonucleotides cross-linked to their surface were added to this solution and incubated shaking at 37°C for 24 hours to hybridise between oligonucleotides fixed to the membranes and DNA fragments containing complementary microsatellite repeats. The membranes were then transferred to two fresh tubes and boiled in 50 μl of distilled water to elute the bound DNA fragments. The eluted fragments were precipitated in 150μL of ethanol with 5μl of linear acrylamide added to bind the precipitated DNA and centrifuged at 17,000*g* for 20 minutes. The supernatant was discarded and the pellet washed twice with 300μL of 70% ethanol, dried in the Hetovac CT110 and then resuspended in 25μL of TE buffer. One microlitre of the resupended fragments was amplified using the protocol described above. The enrichment procedure above was repeated with the final products being reamplified using a modified PCR protocol with a longer final extension period [25 cycles of one minute at 94°C, one minute at 55°C and two minutes at 72°C followed by a final extension cycle of 60 minutes at 72°C]. The enriched, amplified fragments were then purified using the QIAquick PCR Purification Kit (Qiagen) and electrophoresed on a 1.5% agarose gel (SeaKem LE, FMC Bioproducts).

4.2.5 Microsatellite DNA Library

Purified fragments were ligated into the pGEM®-T Easy Vector using the pGEM®-T Easy Vector System (Promega) with overnight incubation at 4°C [10ng fragments, 50ng pGEM®-T Easy Vector, 1 X Rapid Ligation Buffer, 3U T4 DNA ligase, made up to 10μL with distilled water]. Plasmids were transformed into freshly prepared XL-1 Blue electroporation competent cells (Stratagene: Appendix IV) using a Gene Pulser™ with pulse controller (BioRad). Five microlitres of ligation reaction was added to 50μL of cells on ice and incubated for at least 30 seconds before electroporating in a 0.1cm electroporation cuvette (BioRad) at 1.8kV, 200Ω and 2μF. 945μL of warm (37°C) SOC (Appendix III) was added to the cells immediately after electroporation. The cells were then transferred to 4mL of SOC at 37°C and incubated for 60 minutes at 37°C shaking at 225rpm. One hundred microlitres of this culture was spread onto warm (37°C) LB plates (Appendix III) containing 0.1M glucose, 0.1mg/mL ampicillin and 0.05mg/mL tetracycline and incubated for 16+ hours at 37°C. The plates had been spread with 2mg of X-Gal and 0.8mg of IPTG 30 minutes prior to plating for colour selection of positive colonies.

Positive (entirely white or white with blue centre) colonies were selected from the plates and cultured in liquid media for PCR amplification and screening for microsatellite repeats. The tip of a sterile toothpick was touched to the centre of a colony and dropped into a culture tube containing 5mL LB broth (containing 0.1mg/mL ampicillin and 0.05mg/mL tetracycline). The cultures were grown at 37°C shaking at 300rpm overnight. 500μL of culture was centrifuged at 17,000*g* for 10 minutes and the supernatant decanted. The pellet was resuspended in 200μL of sterile distilled water and 2μL of this suspension added to 23μL of master mix [1X PCR buffer (Boehringer Mannheim), 1.5mM $MgCl_2$, 2μM each of M13 reverse universal primer (5'-GGA AAC AGC TAT GAC CAT G-3') and M13 forward universal primer (5'-TGT AAA ACG ACG GCC AGT-3'; Life Technologies Inc.), 37.5μM each of dATP, dCTP, dTTP and dGTP (Boehringer Mannheim) and 0.04U/μL *Taq* DNA polymerase (Boehringer Mannheim)] to amplify the insert DNA and amplified as follows: 94°C for $5^1/_2$ minutes; 35 cycles of 94°C for 60 seconds, 50°C for 30 seconds, 72°C for 60 seconds; and a final extension cycle of 72°C for 10 minutes.

Samples containing insert DNA were identified as being longer than 251 base pairs (i.e. the multiple cloning site (MSC) between and including the two M13 universal primers in pGEM-T® is 251 base pairs. Therefore, the amplified MSC in plasmids containing insert DNA will be 251 base pairs plus the length of the insert).

Clones containing insert DNA were screened for microsatellite DNA repeats using [γ^{32}P]ATP labeled oligonucleotide probes. 1M NaOH and 50mM EDTA (pH 8.0) was added to 10μL samples of the amplified insert to a final concentration of 0.4M NaOH and 10mM EDTA and heated to 99°C for 10 minutes then chilled on ice. 2μL of this mixture was spotted on to a piece of Hybond® N^+ membrane (Amersham) labelled with a pencil and left to dry at room temperature for thirty minutes. The DNA was then cross-linked to the membrane by exposure to UV light at 120kJ/cm.

The oligonucleotides used in the initial enrichment for microsatellite repeats were labelled with [γ^{33}P]ATP to screen for microsatellite DNA. 40pmoles of each dinucleotide was incubated with 1X polynucleotide kinase (PNK) buffer, 20U PNK, and 0.5M [γ^{33}P]ATP for one hour at 37°C followed by deactivation of PNK at 68°C for 10 minutes.

The dot blotted membrane was prehybridised in 5-10mL of Church and Gilbert solution rotating, at room temperature, for 30 minutes in a Hybaid Midi Duel 14 Oven. The prehybridisation solution was decanted and replaced with 5-10mL of fresh Church and Gilbert solution, followed by the addition of 5μL of radiolabelled probe and the probe left to hybridise to the membrane overnight at room temperature, rotating in a Hybaid Midi Duel 14 Oven. This solution was decanted into a 50mL Falcon tube and retained at 4°C in a shielding perspex container. The membrane was rinsed in 10-15mL of 3X SSC with 0.1% SDS followed by two 10 minute washes at room temperature in the rotating Hybaid Oven. The Geiger counter was passed over the washed membrane to check for activity before it was put down with Kodak X-ray film in a lead cassette and incubated at room temperature overnight. The film was developed in Kodak developing solution shaking gently for two minutes then drained and transferred to Kodak fixative solution for two minutes before thorough rinsing in running tap water for five minutes and hanging to dry. The X-ray film was handled in

safe (red) light conditions at all stages of development to prevent exposing the film to white light.

PCR products from positive dot blots were sequenced at the Waikato DNA Sequencing Facility using the M13 forward universal primer with BigDye™ Terminator chemistry (Perkin-Elmer Applied Biosystems). The products of these reactions were then separated on a 4.5% polyacrylamide gel using an ABI 377 Automated DNA Sequencer (Perkin-Elmer Applied Biosystems) and the electropherograms examined for microsatellite DNA repeats.

4.3 RESULTS

Initial attempts at ligating the enriched PCR amplified inserts were unsuccessful due to insufficient extension time in the final cycle of PCR. Ligation into the pGEM-T® Easy Vector system relies on the presence of A-tails, added by *Taq* DNA polymerase in the final stage of amplification. These were not being added to the PCR products as the final extension time of the PCR protocol not allowing enough time for *Taq* DNA polymerase to complete the reaction. To address this problem the final extension time was extended from 30 seconds to 60 minutes.

Ligated plasmid was transformed into freshly prepared XL-1 Blue electroporation competent cells with transformation efficiencies of 5 X 10^9 CFU/μg of DNA in the control plasmid (pUC 18) and 2 X 10^6 CFU/μg in the positive pGEM-T control. Efficiencies were lower in the sample transformations at approximately 1 X 10^4 CFU/μg. Positive clones for each species were screened for inserts using PCR (Figure 4.3). The inserts were relatively uniform in size ranging from 100-200bp in length. The amplified bands ranged from approximately 350-450bp in length including the MCS which was 251bp long. All blue colonies contained inserts, including colonies with blue perimeters and white centres. No inserts were detected in the white colonies.

Thirty colonies from each species confirmed as containing inserts detected after screening with PCR were screened for microsatellite repeats with radiolabelled

oligonucleotides. Eleven out of the sixty dot blots screened were positive, five for *B. argenteum* and six for *C. purpureus* (Figure 4.4).

Figure 4.3. PCR amplified MCS from the pGEM-T® Easy Vector in transformed XL-1 Blue electroporation competent cells. Lanes 1 & 12: 100bp DNA Ladders (Life Technologies Inc.). Lanes 2-4: Colonies containing insert DNA from *B. argenteum*. Lane 5: Blue colony control for *B. argenteum* (no insert). Lanes 6-8 & 10: Colonies containing insert DNA from *C. purpureus*. Lane 9: Blue colony control for *C. purpureus* (no insert). Lane 11: Negative control (no DNA).

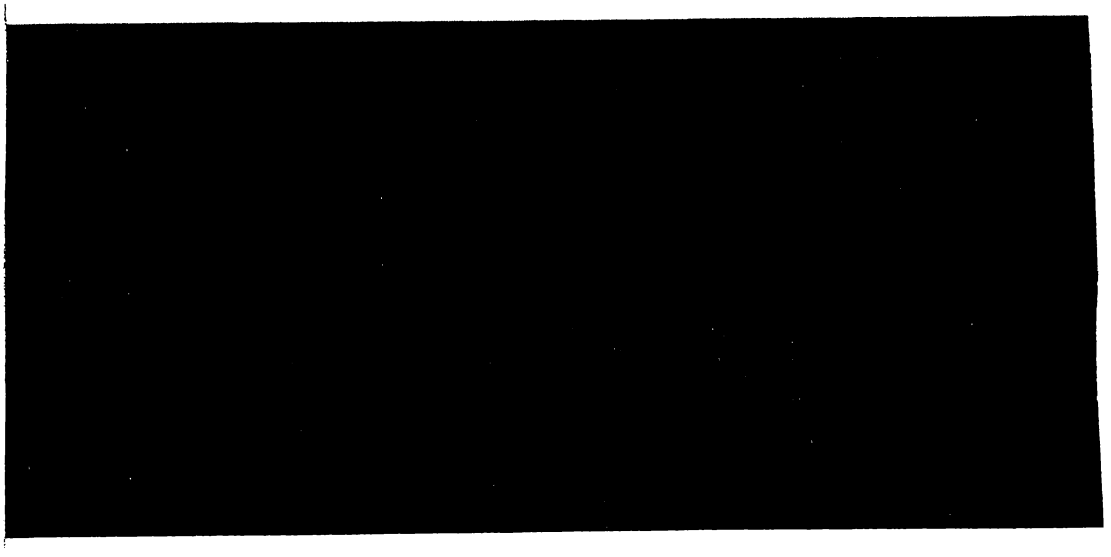


Figure 4.4. Autoradiograph of dot blots screened for microsatellite DNA with [γ^{33}P]ATP-labelled probes (*B. argenteum* (Ba) samples top of strip, *C. purpureus* (Cp) samples below). Positive dot blots are marked with a tick.

Inserts positive for microsatellite DNA as indicated by the dot blots were sequenced directly off the PCR amplified inserts using the M13 forward universal primer. Sequences for the inserts were devoid of microsatellite repeats indicating that the enrichment procedure had failed and that non-specific radiolabelling was occurring during the dot blot hybridisation.

4.4 DISCUSSION

The failure of this protocol to enrich for microsatellite DNA is most likely due to non-specific hybridisation of the digested DNA to the oligonucleotides bound to the Hybond® N^+ membrane (Amersham). PCR was successful after each enrichment procedure indicating hybridisation and elution of the DNA from the membranes had been successful. Optimisation of the hybridisation conditions by increasing hybridisation temperature may have increased the specificity of hybridisation sufficiently to ensure only DNA containing microsatellite repeats complementary to the oligonucleotides were hybridised. Non-specific hybridisation of the radio-labelled oligonucleotide probes was also a problem in the dot blots as none of the positively labelled blots contained microsatellite repeats. Further experimentation to optimise hybridisation temperatures for this protocol are required to eradicate this problem.

Size selection after the initial digest reactions would also be advantageous. A greater size range of products and would have facilitated identification fragments of different lengths in recombinant clones. This is especially important when using PCR based enrichment procedures as it minimises selection of copies of the same fragment in different clones.

Optimisation of the above technique was not pursued due to time restrictions. Instead, a second enrichment protocol (Ostrander et al., 1992) which uses *dut-/ung-* competent cells and helper phage to enrich for single-stranded products containing microsatellite repeats was commenced. Briefly, DNA is digested and size selected (300-700bp) then ligated into a plasmid vector. Ligation success is confirmed by transformation into competent cells with colour selection capacity. Ligation reactions are transformed again into *dut-/ung-* cells and spread on to plates containing

ampicillin to select for cells containing plasmids. 5000-7500 CFU are required and these are resuspended off the plate surface in media containing ampicillin. Aliquots of these cells are used to generate single-stranded DNA with helper phage (Vieira and Messing, 1987). Oligonucleotides containing microsatellite repeats are used in primer extension reactions, converting single-stranded DNA containing microsatellite repeats into double stranded DNA. This DNA is purified and ligated, then transformed into competent cells with colour selection capacity. These cells are screened for microsatellite DNA using radiolabelled probes and positive clones sequenced.

Enrichment using this technique relies on the *dut-/ung-* cells which are deficient in dUTPase (the *dut* gene product (*dut-*)) and uracil-N-glycosylase (the *ung* gene product (*ung-*)). This allows the incorporation of dUTP into DNA as it is not digested by dUTPase or removed by uracil-N-glycosylase. Thus, circular, single-stranded DNA rich in uracil repeats can be isolated with the addition of helper phage (Vieira and Messing, 1987). Primer extension reactions generate a circular, double-stranded product which is selected for upon transformation into an *Escherichia coli* strain as single-stranded DNA is not taken up as efficiently as double-stranded DNA (Kunkel, 1985; Kunkel et al., 1987) and also because of the degradation of DNA rich in uracil by the uracil-N-glycosylase present in the wild-type *E. coli* strain (Lindahl, 1982). The double stranded products, which also contain a uracil-rich strand are repaired with the strand generated by primer extension acting as a template. The resulting colonies are enriched for microsatellite repeats complementary to the primers used in the primer extension reactions. Microsatellite DNA marker development will be pursued using this protocol and will be completed in ongoing research.

4.5 FUTURE RESEARCH

Recent research using RAPDs has been unable to decipher clear patterns of gene flow in Antarctic moss populations due to the extremely high levels of variation being detected with this technique (Adam et al., 1997; Skotnicki et al., 1997, 1998a, b, c). Little is known about the polymorphisms revealed in RAPD analyses making it difficult to trace a specific polymorphism without knowing the genealogy of the

specimen being analysed. Microsatellite DNA polymorphisms are amplified using specific primers, which analyse one locus at a time. This yields much more fine-scale information about the origin of the polymorphisms.

The development of microsatellite DNA markers in cosmopolitan moss species will increase our understanding of gene flow and genetic variation in Antarctic moss species. It is still unknown whether Antarctic moss populations are derivatives of relict populations which arose through vicariance or if they established through long distance dispersal. Detection of unique alleles in distinct populations will enable dispersal ranges of mosses to be investigated and hopefully provide evidence for the colonisation of Antarctica.

CHAPTER FIVE

SUMMARY

The phylogenetic reconstructions presented in this research suggest the current classification of *Bryum subrotundifolium* Jaeg. for the Antarctic silver moss is incorrect and that the Antarctic populations are actually an ecotypic variant of the ubiquitous, cosmopolitan silver *Bryum* species *B. argenteum* Hedw. The Antarctic populations of this moss formed a well supported clade with New Zealand and Australian populations of *B. argenteum*. Australian material collected from populations identified unequivocally as *B. subrotundifolium* were more closely related to populations of *B. argenteum* than they were to the Antarctic material. Moss populations from the Subantarctic islands, also unequivocally identified as *B. subrotundifolium* (R. Ochyra, pers. comm.), were grouped with the *B. argenteum* populations as well.

Species descriptions for these two taxa are very similar, with *B. subrotundifolium* differentiated from *B. argenteum* on the basis of foliage colour, leaf shape and the transient occurrence of chlorophyllose cells in the hyaline leaf apices induced in culture under low light. Foliage colour and leaf shape are often variable within a taxon and are generally not viewed as reliable taxonomic characteristics (Nakanishi, 1979) and it is possible that the induction of chlorophyllose cells in the Antarctic specimens is an adaptation to the extended hours of darkness over the winter period. Reappraisal of the taxonomic status of *B. subrotundifolium* is required with consideration given to these morphological characteristics to decide whether or not all material referred to as *B. subrotundifolium* can be subsumed into *B. argenteum.*

Random amplified polymorphic DNA (RAPD) analyses have revealed exceptionally high levels of variation in Antarctic mosses, such that shoots within a single moss clump are genetically distinct. This is unusual given that reproduction in Antarctic mosses is thought to occur primarily through dispersal of asexual propagules. Multiple bands detected in polymerase chain reaction (PCR) products in Antarctic moss samples indicated the presence of persistent contaminants associated with DNA from these samples. The contaminating bands were sequenced then BLAST searched

through Genbank and were found to be derived from a fungus. An investigation has commenced to determine if fungal symbionts associated with Antarctic mosses could explain the high levels of variation detected in previous studies using random amplified polymorphic DNA (RAPDs) analysis. Preliminary results suggest that the presence of contaminating fungal DNA does artificially bias the amount of genetic variation detected using this technique. The observation that fungal contamination appears to be more of a problem in wetter sites is reflected in increased levels of genetic variation detected in RAPD analyses of moss populations from more moist areas.

Microsatellite DNA markers are currently being developed for use in population-level investigations of Antarctic mosses. This technique is much less sensitive to contamination than RAPDs. The primers used in PCR of microsatellite DNA are targeted for a specific species and close relatives of it, unlike RAPDs which use non-specific, random primers. Microsatellite DNA markers are expensive and time-consuming to develop. However, they are far more reliable than RAPDs, require minimal amounts of DNA, are simple to use once developed and can detect variation at different levels within a population depending on the amount of polymorphism detected at a locus.

Future research will be aimed at intensive population-level analysis of Antarctic moss populations. Questions pertaining to the colonisation of Antarctica largely remain unanswered. It is hoped that the implementation of microsatellite DNA markers will reveal whether Antarctic moss populations are remnants of relict populations or if they have been recently introduced through long distance dispersal. Levels of gene flow within Antarctic moss populations will also be addressed. These mosses are thought to reproduce primarily by asexual means which suggests little opportunity for the introduction of genetic variation within these populations. However, equivocal results using RAPDs has indicated exceptionally high levels of variation. Understanding the levels of genetic variation present in Antarctic moss populations will enable conservation strategies targeted at preserving the biodiveristy of Antarctica to be developed.

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APPENDIX I: Moss specimens collected by Sarah Hunger (SH)

Taxon	Accession Number	Source	Date (D/M/Y)	Location	Notes
Ceratodon purpureus	SH1	wild	19.09.1998	Hamilton, New Zealand	
Ceratodon purpureus	SH2	wild	19.10.1998	Hamilton, New Zealand	Same population as SH1
Sphagnum sp.	SH3	wild	07.11.1998	Karangahake, New Zealand	
Unknown	SH4	wild	07.11.1998	Karangahake, New Zealand	
Sphagnum sp.	SH5	wild	07.11.1998	Karangahake, New Zealand	
Ceratodon purpureus	SH6	wild	07.11.1998	Karangahake, New Zealand	
Unknown	SH7	wild	07.11.1998	Karangahake, New Zealand	
Unknown	SH8	wild	07.11.1998	Karangahake, New Zealand	
Bryum argenteum	SH9	wild	07.11.1998	Karangahake, New Zealand	
Unknown	SH10	wild	07.11.1998	Karangahake, New Zealand	
Sphagnum sp.	SH11	wild	07.11.1998	Karangahake, New Zealand	
Ceratodon purpureus	SH12	wild	07.11.1998	Karangahake, New Zealand	
Ceratodon purpureus	SH13	wild	09.11.1998	Hamilton, New Zealand	Same population as SH1
Ceratodon purpureus	SH14	wild	10.11.1998	Hamilton, New Zealand	Same population as SH1
Ceratodon purpureus	SH15	wild	11.11.1998	Hamilton, New Zealand	Same population as SH1
Bryum argenteum	SH16	wild	11.11.1998	Hamilton, New Zealand	
Bryum argenteum	KMG2	wild	Summer season 1994	Granite Harbour, Antarctica	Collected by Kadmiel Mysek, 1994 - summer season and stored at -20°C. DNA degraded.
Ceratodon purpureus	KM5	wild	Summer season 1994	Granite Harbour, Antarctica	Collected by Kadmiel Mysek, 1994 - summer season and stored at -20°C.
Bryum subrotundifolium	SH17 (1 to 116)	wild	04.01.1999	Cape Hallett, Antarctica	Sub-population level samples
Bryum subrotundifolium	SH18	wild	01.01.1999	Cape Hallett, Antarctica	
Bryum subrotundifolium	SH19 (1 to 13)	wild	04.01.1999	Cape Hallett, Antarctica	Sub-population level samples
Bryum subrotundifolium	SH20 (1 to 3)	wild	06.01.1999	Cape Hallett, Antarctica	Sub-population level samples
Bryum argenteum	SH21	wild	06.01.1999	Cape Hallett, Antarctica	c.f. Antarctic *Bryum subrotundifolium*
Bryum pseudotriquetrum	SH22	wild	06.01.1999	Cape Hallett, Antarctica	
Bryum pseudotriquetrum	SH23 (1 to 10)	wild	04.01.1999	Cape Hallett, Antarctica	Sub-population level samples

Taxon	Accession Number	Source	Date (D/M/Y)	Location	Notes
Ceratodon purpureus	SH24	wild	08.01.1999	Cape Hallett, Antarctica	
Bryum subrotundifolium	SH25	wild	08.01.1999	Cape Hallett, Antarctica	
Bryum subrotundifolium	SH26 (1 to 8)	wild	09.01.1999	Cape Hallett, Antarctica	Sub-population level samples
Bryum subrotundifolium	SH27 (1 to 10)	wild	06.01.1999	Cape Hallett, Antarctica	Sub-population level samples
Bryum argenteum	SH28	wild	09.01.1999	Cape Hallett, Antarctica	c.f. Antarctic *Bryum subrotundifolium*
Sarconeurum glaciale	SH29	wild	15.01.1999	Scott Base, Antarctica	
Bryum subrotundifolium	SH30	wild	15.01.1999	Scott Base, Antarctica	
Bryum subrotundifolium	SH31	wild	16.01.1999	Arrival Heights, Antarctica	
Hennediella heimii (Others)	SH32 (1 to 10)	wild	18.01.1999	Garwood Valley, Antarctica	Sub-population level samples. Mixed colony also including *Bryum subrotundifolium*, *Bryum pseudotriquetrum* and *Nostoc*.
Bryum subrotundifolium	SH33	wild	18.01.1999	Garwood Valley, Antarctica	
Bryum subrotundifolium	SH34	wild	18.01.1999	Garwood Valley, Antarctica	
Hennediella heimii	SH35	wild	18.01.1999	Garwood Valley, Antarctica	
Hennediella heimii (Others)	SH36	wild	18.01.1999	Garwood Valley, Antarctica	Mixed colony also including *Bryum subrotundifolium*, *Bryum pseudotriquetrum* and *Nostoc*.
Bryum sp.	SH37	wild	20.04.1999	Hamilton, New Zealand	
Bryum sp.	SH38	wild	23.04.1999	Hamilton, New Zealand	
Bryum sp.	SH39	wild	25.04.1999	Karangahake, New Zealand	

APPENDIX II: DNA extraction protocol for fresh and frozen moss samples

Adapted from Scott O. Rogers and Arnold J. Bendich (1985).

1) Grind 100mg of tissue with liquid nitrogen in a mortar and pestle. Transfer the ground tissue to a 1.5ml microfuge tube.

2) Add 500µl of 1 X CTAB extraction buffer with 1% PVP (polyvinylpyrrolidone) at 65°C. Mix gently then incubate at 65°C for 3 minutes.

3) Add 500µl chloroform/isoamyl alcohol (24:1) and mix gently but thoroughly. Centrifuge at 12,800*g* for 1 minute in Eppendorf 5140 centrifuge.

4) Transfer the top layer to another microfuge tube and add $^{1}/_{10}$ 10 X CTAB at 65°C and mix gently.

5) Add an equal volume of chloroform/isoamyl alcohol, mix gently but thoroughly, then centrifuge at 12,800*g* for 1 minute in Eppendorf 5140 centrifuge.

6) Transfer the top phase to a new tube and add an equal volume of 1 X CTAB precipitation buffer and mix gently. Centrifuge at 12,800*g* for 1 minute in Eppendorf 5140 centrifuge, decant the supernatant and air dry.

7) Rehydrate the pellet in 50µl of distilled water. Heat at 37°C for 5-10 minutes to facilitate rehydration if necessary.

8) Add 100µl of 95% ethanol at -20°C (Maybe stored at -20°C overnight at this point). Centrifuge at 12,800*g* for 1 minute in Eppendorf 5140 centrifuge then decant the supernatant.

9) Add 300µl of 80% ethanol and resuspend the pellet. Centrifuge at approximately 15,000*g* for 1 minute then decant the supernatant. Dry in Hetovac CT110.

10) Rehydrate the pellet in 100µl of distilled water.

11) Add 1µl of RNase A and incubate at 37°C for 1 hour.

12) Electrophorese for 2-3 hours on a 1.5% agarose gel (SeaKem LE) at 50 volts.

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APPENDIX III: Microsatellite DNA Marker Development Reagents

10 x J Buffer		10mL
450mM	Tris-HCl (pH 8.0)	0.569g
110mM	$(NH_4)_2SO_4$	0.145g
45mM	$MgCl_2.6H_2O$	0.0914g
67mM	2-mercaptoethanol	46.8µL
45µM	EDTA (0.5M)	0.9µL
2.5mM	Spermidine (200mg/mL)	31.5µL

Dissolve in 8mL of distilled water then add concentrated HCl to pH 8.0. Make up to 10mL then filter sterilise though 0.2µm filter.

Church and Gilbert Solution	500mL
sodium dodecyl sulphate (SDS)	35.0g
0.05M EDTA	5mL

Make up to 250mL with distilled water and add 250mL phosphate buffer (89.0g $Na_2HPO_4.2H_2O$ dissolved in 900µL distilled water. Add H_3PO_4 to pH 7.2, make up to 1L then autoclave for 20 minutes).

SOC	250mL
Bactotryptone	5.0g
Bactoyeast	1.25g
NaCl	0.13g

Make up to 250mL with distilled water and autoclave for 20 minutes. Allow to cool then add 0.9g glucose and 625µL 4M $MgCl_2$

LB media	1L
Bactotryptone	10g
Bactoyeast	5g

Add 950 mL distilled water. Adjust pH to 7.0 and make up to 1L. Autoclave for 20 minutes.

APPENDIX IV: Preparation of XL-1 Blue electroporation competent cells

1) Dilute XL-1 Blue cells from glycerol stock (41µL of cells into 1mL of L broth) and spread 50µL onto a 100mm diameter plate of minimal media containing a selective agent. Grow at 37°C overnight until colonies have reached approximately 2-4mm in diameter. Can be stored at 4°C covered in parafilm™ for 3-4 months.

2) Prepare 3 x 10mL glass culture tubes with 3mL of LB + tetracycline (3mL of LB + 12µL tetracycline stock solution). Inoculate each tube with a single colony from the plate above using a sterile toothpick and grow shaking overnight at 37°C.

3) Prepare 900mL of L broth and 100mL of potassium phosphate, autoclave each solution separately, then combine (retain 5mL of this solution for blanking the spectrophotometer).

4) Divide the solution into four 1000mL erylenmeyer flasks and inoculate each portion with 1.5mL of culture from step 2 above (i.e. divide one tube of culture between two erylemeyer flasks - the extra tube was in case of growth failure in one tube). Incubate the cultures shaking at 37°C until the OD_{600} reaches approximately 0.6 (3-5 hours). Check the OD_{600} every 30 minutes after one hour of incubation. Take 0.25mL for each reading. Ensure that the centrifuge and rotor is turned on 30 minutes before the cultures are ready to allow the rotor to equilibrate at 4°C.

5) Transfer the cultures to chilled centrifuge tubes and place on ice for 15 minutes once the desired OD_{600} has been reached. Refill the flasks with distilled water and place on ice as well. **Keep the cells cold from this point onwards.**

6) Transfer the cells to the cooled centrifuge tubes, balance the tubes with the chilled distilled water from the flasks then centrifuge at 4000g (max.) for 10 minutes.

7) Decant the supernatant and refill the centrifuge tubes one third full with the chilled distilled water and resuspend the cells by gentle swirling using a spatula to facilitate resuspension if necessary. Centrifuge the cells again at 4000g (max.) for 10 minutes. Label 20 2mL flat bottomed tubes with the strain of bacteria and date and place in an epi-rack in the -20°C freezer. Place 10% glycerol and wide bore transfer pipette tips on ice.

8) Decant the supernatant and refill the centrifuge tubes one third full with the chilled distilled water and resuspend the cells by gentle swirling using a spatula to facilitate resuspension if necessary. Transfer two of the resuspended solutions into the other two centrifuge tubes (i.e. 1 → 2 and 3 → 4 = 1 + 2 and 3 + 4). Centrifuge the cells again at 4000g (max.) for 10 minutes.

9) Decant the supernatant and add 1.25mL of 10% glycerol to the tubes. Resuspend the solutions by swirling but be sure to keep them as cool as possible.

10) Remove labeled tubes from the -20°C freezer and place on ice. Transfer 175µL of cells to each tube using the chilled wide bore pipette tips.

11) Transfer the cells to a labelled box and store -80°C. Always check competency (by transforming one subaliquot with 10pg of uncut plasmid) with every batch of transformations.

N.B. Should retain competency for six months to two years.