Antarctic Science 17 (3), 377–384 (2005) © Antarctic Science Ltd Printed in the UK DOI: 10.1017/S0954102005002816

DNA sequencing and genetic diversity of the 18S–26S nuclear ribosomal internal transcribed spacers (ITS) in nine Antarctic moss species

M.L. SKOTNICKI[1]*, A.M. MACKENZIE[1], M.A. CLEMENTS[2] and P.M. SELKIRK[3]

[1]*Genomic Interactions Group, Research School of Biological Sciences, Institute of Advanced Studies, Australian National University, Canberra, ACT 2601, Australia*
[2]*Centre for Plant Biodiversity, CSIRO Plant Industry, Canberra, ACT 2601, Australia*
[3]*Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia*
**skotnicki@rsbs.anu.edu.au*

Abstract: We have sequenced the 18S–26S nuclear ribosomal DNA ITS region from the genome of nine different moss species from the Ross Sea region of Antarctica. This relatively quick and simple technique enables these species to be readily distinguished, facilitating their taxonomic identification. Only a single moss shoot is required, and for identification of these bryophytes it is only necessary to determine a few hundred nucleotides of the DNA sequence in a single sequencing reaction. Several previously unidentified Antarctic moss specimens were readily characterized by comparison with ITS sequences of known moss species. The relationships between species and locations previously detected by the RAPD (Random Amplified Polymorphic DNA) technique were confirmed by DNA sequencing, demonstrating that the two techniques can be complementary for molecular analysis of the ecology of mosses in Antarctica.

Received 15 June 2004, accepted 15 May 2005

Key words: bryophyte, ITS sequence, molecular taxonomy, molecular ecology, moss dispersal, Victoria Land

Introduction

The origins, colonisation history and dispersal of moss colonies in Antarctica and the sub-Antarctic islands are of interest to bryologists, ecologists and geneticists alike. The flora of continental Antarctica includes only 15 species of mosses (Lewis Smith 1984). These plants, which grow at around 75° to 78°S - with extremes of drought, cold and wind, as well as light - have some remarkable characteristics which make them ideally suited for genetic studies of dispersal and mutagenesis in living plants.

However, their phenotypic plasticity under the extremely harsh climatic conditions and the lack of fertile material often makes identification difficult, if not impossible. Therefore, we have used molecular genetic techniques to assist their taxonomic characterization.

Previously, we have reported the utility of the RAPD (Random Amplified Polymorphic DNA) technique (Rafalski & Tingey 1993) as an aid to taxonomic identification of Antarctic mosses (Skotnicki *et al*. 2000). This technique has proven very useful, for example enabling identification of protonemal growth of a single species near the summit of Mount Erebus as *Campylopus pyriformis*, some 20 years after its collection (Skotnicki *et al*. 2001). We have also used the RAPD technique extensively to analyse relationships within and among different populations of individual moss species in Antarctica (Skotnicki *et al*. 1998a, 1998b, 1999, 2000). It has proven to be a very versatile and effective method, requiring no other genetic information about the organisms, minimal amounts of material, and having the additional advantages of being quick, simple and inexpensive.

The RAPD technique does not, however, allow comparison of DNA sequence information necessary for understanding how moss genomes mutate and evolve, and for detailed comparisons of moss colonies and their dispersal. We have therefore started to investigate the DNA sequences of individual moss loci as an alternative to the RAPD technique for such purposes (Skotnicki *et al.* 2004). We report here the use of DNA sequencing of the nuclear ribosomal ITS region (Baldwin 1992) for taxonomic characterisation of nine moss species from the Ross Sea region of Antarctica.

Materials and methods

Sample collection

Small samples of a few shoots of several moss species were collected at a number of sites in continental Antarctica. These mosses included seven species from cold ground in coastal Victoria Land and nearby islands, and from Wilkes Land: *Bryum argenteum* Hedw., *Bryum pseudotriquetrum* (Hedw.) Gaertn., Meyer et Scherb, *Bryoerythrophyllum* recurvirostre (Hedw.) Chen, *Ceratodon purpureus* (Hedw.) Brid, *Hennediella heimii* (Hedw.) Zand., *Sarconeurum glaciale* (C. Muell.) Card. & Bryhn), and *Schistidium antarctici* (Card.) Savicz. et Smirn., and two species found

https://doi.org/10.1017/S0954102005002816 Published online by Cambridge University Press

Table I. Antarctic moss specimens utilised in this study, with their species, site of collection, Genbank accession number for ITS sequence data, and length of ITS region sequenced (see Genbank details for complete details).

Species	Collection site	Location	Collection no.	Genbank Accession no.	Length of ITS
Bryoerythrophyllum recurvirostre	Granite Harbour, S. Victoria Land	77°00'S, 162°30'E	T576	AY613334	711
Bryum argenteum	Beaufort Island, Ross Sea (BI)	77°00'S, 167°00'E	15f	AY611431	913
	Canada Glacier, Taylor Valley, S. Victoria Land (CG)	77°35'S, 163°15'E	P171	AY611432	903
	Edmonson Point, N. Victoria Land (EP)	74°20'S, 164°30'E	Epa	AY611430	913
	Cape Royds, Ross Island (CR)	77°35'S, 166°10'E	C474	AY611433	919
	Cape Chocolate, S. Victoria Land (CC)	77°57'S, 164°30'E	CC26	AY611429	913
	Granite Harbour, S. Victoria Land (GH)	77°00'S, 162°30'E	C363	AY611434	913
Bryum pseudotriquetrum	Canada Glacier, Taylor Valley, S. Victoria Land	77°35'S, 163°15'E	P159	AY611427	981
	Granite Harbour, S. Victoria Land	77°00'S, 162°30'E	CCC5	AY611426	981
	Crater Cirque, N. Victoria Land (unknown)	72°40'S, 169°10'E	RB1	AY611428	960
Campylopus pyriformis	Mount Erebus, Ross Island (protonema only)	77°30'S, 167°10'E	ME1	AY613328	865
	Mount Melbourne, N. Victoria land	74°21'S, 164°42'E	T845	AY613329	825
Ceratodon purpureus	Granite Harbour, S. Victoria Land	77°00'S, 162°30'E	CC76	AY156590	740
	Edmonson Point, N. Victoria Land	74°20'S, 164°30'E	Epa	AY156591	736
	Crater Cirque, N. Victoria Land (unknown)	72°40'S, 169°10'E	RB2	AY613336	736
	Casey Station, Wilkes Land (unknown)	66°17'S, 110°31'E	SR2	AY156592	706
Hennediella heimii	Beaufort Island, Ross Sea	77°00'S, 167°00'E	ADT #20336	AY613330	727
	Cape Chocolate, S. Victoria Land	77°57'S, 164°30'E	CC16	AY613331	727
Pohlia nutans	Mount Rittmann, N. Victoria Land	73°28'S, 165°37'E	K2	AF479318	874
	King George Island	62°S, 58°W	Convey 218	AF479319	916
Sarconeurum glaciale	Scott Base, Ross Island	77°50'S, 166°45'E	P522	AY613332	775
	Edmonson Point, N. Victoria Land	74°20'S, 164°30'E	ADT #09766	AY613333	775
Schistidium antarctici	SSSI 16, Wilkes Land	66°17'S, 110°31'E	SR8	AY613335	669

only on geothermally heated ground: *Campylopus pyriformis* (Schultz) Brid. and *Pohlia nutans* (Hedw.) Lindb. Representative specimens included here are detailed in Table I, where Genbank accession numbers for deposited sequences are also given. In addition, Genbank ITS sequences of *Takakia lepidozioides* Hatt. et H. Inouee (Accession AJ251927) and *Physcomitrella patens* (Hedw.) B.S.G. (Accession X98013) were included in phylogenetic analyses as outgroup species.

Pohlia nutans was collected from geothermally heated ground on volcanic Mount Rittmann (73°28'S, 165°37'E) in northern Victoria Land (Bargagli *et al*. 1996). One other sample of *P. nutans* was obtained from the British Antarctic Survey Herbarium (Convey 218), collected 20 years ago on King George Island (62°S, 58°W) near the Antarctic Peninsula, and stored dry at room temperature. No difference was observed between old dry samples such as this and freshly frozen samples; both yielded genomic DNA suitable for amplification of the desired ITS region.

A few additional unidentified moss specimens were collected in the vicinity of Casey Station (66°17'S 110°31'E) and from Crater Cirque (72°40'S, 169°10'E) in northern Victoria Land, and listed as unknown specimens in Fig. 1.

Small moss samples were air-dried at room temperature, and stored frozen at -20°C until used for DNA isolation. Voucher specimens are held at the University of Waikato in Hamilton, New Zealand and the Australian National University in Canberra, Australia.

DNA isolation, amplification and sequencing

DNA was isolated from single 3–5 mm shoots as described previously (Skotnicki *et al*. 1998a). Both frozen and dry herbarium samples were treated in the same way. Briefly, a shoot was ground in CTAB (cetyl trimethyl ammonium bromide) extraction buffer, extracted with chloroform/ isoamyl alcohol, treated with Rnase A, and concentrated by precipitation with isopropanol. After rinsing the DNA pellets in 70% ethanol, they were resuspended in 15 µl sterile water and stored at 4°C until used for sequencing reactions.

The complete ITS region of the 18S–26S nrDNA was amplified in a polymerase chain reaction (PCR) using the primers ITS1 and ITS4 (or ITS4 and ITS5) designed by White *et al*. (1990). DNA was amplified in a 50 µl reaction

https://doi.org/10.1017/S0954102005002816 Published online by Cambridge University Press

```
                  1                                                                                                    90
Bryum arg BI      GTCACCGCGC CCCCCCACCA CACTATGTTA G--------- ---------- ---------- -AGTCTGAGT GGAACTGGCC GTCCCGG---
Bryum arg CG      GTCACCGCGC CCCCCCACCA CACTCTCTTA G--------- ---------- ---------- -AGTCTGAGT GGAACTGGCC GTCCCGG---
Bryum pseud CG    GTCACCGCGC CCCCCCACCA CACTCCCC-A AGTAC---TT -----TTGTA CCTGT-GCGC CAGTTTGAGT GGAACTGGCC GTCCCGGAGT
Bryum pseud GH    GTCACCGCGC CCCCCCACCA CACTCCCC-A AGTAC---TT -----TTGTA CCTGT-GCGC CAGTTTGAGT GGAACTGGCC GTCCCGGAGT
Unknown 2         GTCACCGCGC CCCCCCACCA CACTCCCCCA AGTAC---TT -----CTGTA CTTGT-GCGC CAGTTTGGGT GGAACTGGCC GTCCCGGAGT
Pohlia MR         GTCACCGCGC CCCCCCCACC GGGTCCC-AA A--------- ---------- ---------C CGGCCCGAGT GAAACTGGCC GTCCCAGCGA
Pohlia MM         GTCACCGCGC CCCCCC-ACC GGGTCCC-AA A--------- ---------- ---------C CGGCCCGAGT GAAACTGGCC GTCCCAGCGA
Campylopus ME     GTCACCGCGA CACCCACCAA TTTTCCCTCT CGCCCGGATT -----TTTCG GGCGG-GACA AAAATCGAGT GGAACTGGCC ATCCGAATG-
Campylopus MM     GTCACCGCGA CACCCACCAA TTT-CCCTTC CGCCCGAATC -----TTTCG GCCGG-GACA AAAATCGAGT GGAACTGGCC ATCCGAATGA
Ceratodon GH      GTCACCGCGA CACCCACC-- ----AACCCA CTCC---A-- ------AGTG GG-------- ----CTGAGT GAAACTGGCC ATCCGAATGG
Ceratodon EP      GTCACCGCGA CACCCACC-- ----AACCCA CTCC---A-- ------AGTG GG-------- ----CTGAGT GAAACTGGCC ATCCGAATGG
Unknown 1         GTC-CCGCGA CACCCACC-- ----AACCCA CTCC---A-- ------AGTG GG-------- ----CTGAGT GAAACTGGCC ATCCGAATGG
Unknown 3         GTCACCGCGA CACCCACC-- ----AACCCA CTCC---A-- ------AGTG GG-------- ----CTGAGT GAAACTGGCC ATCCGAATGG
Hennediella BI    GTCACCGCGA CACCCACCCG ACAAACACTA CTCCT--CC- -----TCGGA GTAG---CGC TCGTTGGAGT GAAAGTGGCC ATCCGAATGA
Hennediella CC    GTCACCGCGA CACCCACCCG ACAAACACTA CTCCT--CC- -----TCGGA GTAG---CGC TCGTTGGAGT GAAAGTGGCC ATCCGAATGA
S. glaciale SB    GTCACCGCGA CACCCACC-- ----AAACCA CTCCT--AT- -----TTTTG GA------GT --GTTTGAGT GAAAGTGGCC ATCCGAATGG
S. glaciale EP    GTCACCGCGA CACCCACC-- ----AAACCA CTCCT--AT- -----TTTTG GA------GT --GTTTGAGT GAAAGTGGCC ATCCGAATGG
Bryoerythro GH    GTCACCGCGA CACCCACC-- ----AAACCA CACC------ -----AAGTG G--------- ---TTCGAGT GAAACTGGCC ATCCGAA---
Schistidium WL    GTCACCGCGA CACCCACCGA CCCCCCACCC CCCCTCCTTC AAAAAGAGGG GTTCTTGGGC CGGGCCGAGT GGAACTGGCC GTCCCAGAAT

                  91                                                                                                  180
Bryum arg BI      ---------- ---------- ---------- --------CA A----TCGGG TCGGCTGAAA TGGAGGGA-A TTTGGC--CG CCGTGGCATC
Bryum arg CG      ---------- ---------- ---------- --------CA A----TCGGG TCGGCTGAAA TGGAGGGA-A TTTGGC--CG CCGTGGCATC
Bryum pseud CG    GCT------- ---------- ---------- ---TAAGGTA C----TCGGG TCGGCTGAAA TGGAGGGA-A TTTGGC--CG CCGTGGCATC
Bryum pseud GH    GCT------- ---------- ---------- ---TAAGGTA C----TCGGG TCGGCTGAAA TGGAGGGA-A TTTGGC--CG CCGTGGCATC
Unknown 2         GCT------- ---------- ---------- ----AAGGTA C----TCGGG TCGGCTGAAA TGGAGGGA-A TTTGGC--CG CCGTGGCATC
Pohlia MR         GGC-AC---G CC-------- --------GC GAGCGTGCTG G----CTGGG TCGGCTGAAA TAGAGGGA-A ACTCGTATCG CCGTGGCGAC
Pohlia MM         GGC-AC---G CC-------- --------GC GAGCGTGCTG G----CTGGG TCGGCTGAAA TAGAGGGA-A ACTCGTATCG CCGTGGCGAC
Campylopus ME     ---------- ---------- ---------- ---TGAATCA TTACATTGGG TTGGCTGAAA TGGAGGGA-A CTTATC--CG GTTTAGGGCC
Campylopus MM     A--------- ---------- ---------T GAATGAATCA TCACATTGGG TTGGCTCAAA TGGAGGGA-A CTTATC--CG GTTTAGGGTC
Ceratodon GH      GGCCAC---- CCGGGACTGA --AAATGTCC TCGC--GGTG GCAGATTGGG TTGGCTGAAA TGGAGGGA-A TTTGGC--CG CCGTGGTGAC
Ceratodon EP      GGCCAC---- CCGGGACTGA --AAATGTCC TCGC--GGTG GCAGATTGGG TTGGCTGAAA TGGAGGGA-A TTTGGC--CG CCGTGGTGAC
Unknown 1         GGCCAC---- CCGGGACTGA --AAATGTCC TCGC--GGTG GCAGATTGGG TTGGCTGAAA TGGAGGGA-A TTTGGC--CG CCGTGGTGAC
Unknown 3         GGCCAC---- CCGGGACTGA --GAATGTCC TCGT--GGTG GCAGATTGGG TTGGCTGAAA TGGAGGGA-A TTTGGC--CG CCGTGGTGAC
Hennediella BI    GGCCGT---- ---------- --------AC TCGTACGGCA A--GATTGGG TTGGCTGAAA TGTAGGGA-A TTTGGC--CG CCGTGGTAGA
Hennediella CC    GGCCGT---- ---------- --------AC TCGTACGGCA A--GATTGGG TTGGCTGAAA TGTAGGGA-A TTTGGC--CG CCGTGGTAGA
S. glaciale SB    GGCCGCCGCG CCTTTCCTTT TTTGGG--GG GCGCGCGGCG GCAGATTGGG TTGGCTGAAA TGGAGGGA-A TTTGGC--CG CCGTGGTGAC
S. glaciale EP    GGCCGCCGCG CCTTTCCTTT TTTGGG--GG GCGCGCGGCG GCAGATTGGG TTGGCTGAAA TGGAGGGA-A TTTGGC--CG CCGTGGTGAC
Bryoerythro GH    ---------- ------CCT- --------GT GAAAACAGAG G----TTGGG TTGGCTGAAA TAGAGGGGGA TTTGGC--CG CCGTGTTGTC
Schistidium WL    TTTTCC---- -----GC--- --CGCGAAGC GG-CAGGGGA G--GACTGGG TCGGCTGAAA TGGATGGA-A TCC-GCCCCG CCGTGGCGAA

                  181                                                                                                 270
Bryum arg BI      AC-TTGCCCC GCGATCAGGT GCGA-TCTCG AA-------- ------AGAG TCAT------ ------T-CC AA---GTGCG -GGGCTCCCA
Bryum arg CG      AC-TTGCCCC GCGATCAGGT GCGA-TCTCG AA-------- ------AGAG TCAT------ ------T-CC AA---GTGCG -GGGCTCCCA
Bryum pseud CG    AC-TTGCCCC GCGATCAGGT GTGA-TTACG AACTCCCACG GCACTTAGTG TCGTGGGACT TGGGTCTTCC AA---GTGCG -GGGCTCCCG
Bryum pseud GH    AC-TTGCCCC GCGATCAGGT GTGA-TTACG AACTCCCACG GCACTTAGTG TCGTGGGACT TGGGTCTTCC AA---GTGCG -GGGCTCCCG
Unknown 2         AC-TTGCCCC GCGATCAGGT GTGA-TTACG AACTCCCACG GCACTTAGTG TCGTGGGACT TGGGTCTTCC AA---GTGCG -GGGCTCCCG
Pohlia MR         AT-TCGCCCC GCGATCAGGT GC---TCACA TC-------- ------GTGT TCGT------ --------CA AA---GTGCG -GGGTTGCAG
Pohlia MM         AT-TCGCCCC GCGATCAGGT GC---TCACA TC-------- ------GTGT TCGT------ --------CA AA---GTGCG -GGGTTGCAG
Campylopus ME     CCCCAAACCC GCGATTAGGT GATC-CAAGT ---------- -----AAACC TTG------- ---GAAATCA AA--TGTGCG -CGGTTTGGG
Campylopus MM     C--TAAACCC GCGATTAGGT GATC-CAAGT TGT------- ----TAAACC TTG------- ---GAAATCA AA--TGTGCG -CGGTTTCGG
Ceratodon GH      AC---AGCCC GCGATCAGGT GATC-CA--- ----CCCGGG AGTAAAATCC CAGTTGGT-- ------CTCA AA--CGTGCG CGCG---CTG
Ceratodon EP      AC---AGCCC GCGATCAGGT GATC-CA--- ----CCCGGG AGTAAAATCC CAGTTGGT-- ------CTCA AA--CGTGCG CGCG---CTG
Unknown 1         AC---AGCCC GCGATCAGGT GATC-CA--- ----CCCGGG AGTAAAATCC CAGTTGGT-- ------CTCA AA--CGTGCG CGCG---CTG
Unknown 3         AC---AGCCC GCGATCAGGT GATC-CA--- ----CCCGGG AGTAAAATCC CAGTTGGT-- ------CTCA AA--CGTGCG CGCG---CTG
Hennediella BI    AC---AGCCC GCGATCAGGT GATC-CAG-- ---------- -GTGCAAACC TGG------- ----AACTCA AA--CGTGCG CGGG---CTC
Hennediella CC    AC---AGCCC GCGATCAGGT GATC-CAG-- ---------- -GTGCAAACC TGG------- ----AACTCA AA--CGTGCG CGGG---CTC
S. glaciale SB    AC---AGCCC GCGATCAGGT GGTC-CAG-- GTC-CT---- --TAGGGACC CGG------- ----AACTCA AA--CGTGCG TCGGC-CGTG
S. glaciale EP    AC---AGCCC GCGATCAGGT GGTC-CAG-- GTC-CT---- --TAGGGACC CGG------- ----AACTCA AA--CGTGCG TCGGC-CGTG
Bryoerythro GH    AC---AGCCA GCGATCAGGT GATC-CAA-- GT-------- GGTAAAAGAC TTG------- ---GAACTCA AA--CGTGCG CTGGGACCTT
Schistidium WL    AC---AGCCC GCGATCAGGT GATCATCCCT CCCCCTCCTC CCCGAAAAGG GAGGAGGGGG AGGGAG--GG AGTGTGTTGG TTGG---CTG
```

Fig. 1. Comparison of partial nucleotide sequences of the conserved nuclear ribosomal DNA ITS1–5.8S–ITS2 spacer region from nineteen mosses from Antarctica (two examples of most species, plus three unidentified samples): *Bryum argenteum*, *Bryum pseudotriquetrum*, *Bryoerythrophyllum recurvirostre*, *Campylopus pyriformis*, *Ceratodon purpureus*, *Pohlia nutans*, *Hennediella heimii*, *Sarconeurum glaciale* and *Schistidium antarctici.* This analysis was obtained using CLUSTAL-W, by comparing approximately 1000 nucleotides of the ITS1–5.8S–ITS2 region. Only a partial but representative alignment, of 270 nucleotides of the ITS2 region, is shown here for reasons of clarity. The first six nucleotides shown are the 3' end of the 5.8S region, followed by 263 nucleotides of ITS2.

volume containing 5 pmol of each primer, 1 µl moss genomic DNA and 45 µl PCR SUPERMIX High Fidelity (GIBCOBRL). The thermocycling regime was 1 cycle of 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 8 min. Amplification products were checked by

https://doi.org/10.1017/S0954102005002816 Published online by Cambridge University Press

agarose gel electrophoresis (Sambrook *et al*. 1989). Specific amplification products were excised from an agarose gel and purified using the QIAquick Gel Extraction Kit (QIAGEN).

10–50 ng of the purified double stranded PCR product was sequenced from both ends and the centre using 3.2 pmol of either ITS1, ITS4 or ITS2 primers (Rosenthal & Charnock-Jones 1992, White *et al*. 1990, Tracy & Mulcahy 1991) and the ABI Prism Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems). The cycling regime was 30 cycles of 94°C for 30 s, 50°C for 15 s and 60°C for 4 min. Sequencing reactions were purified by ethanol precipitation and analysed on an ABI Prism 377 DNA Sequencer. The complete sequence of the ITS1–5.8S–ITS2 region was determined for each sample using Chromas software (Technelysium Pty Ltd) to edit and assemble the sequencing chromatograms.

Sequence alignment and analysis

Moss ITS gene sequences were first aligned using the CLUSTAL-W program (Thompson *et al*. 1994) via the Australian National Genomic Information Service (ANGIS), with multiple alignment parameters set at the default values: a gap opening penalty of 10 (range 1–100), a gap extension penalty of 5 (range 0.10–100) and gap separation penalty of 8 (range 1–50). Sequences were then further manually aligned using BioEdit version 5.0.9 (Hall 1999). The aligned files were exported through MacClade (Maddison & Maddison 1992) for analysis using PAUP version 4.0b4a (Swofford 1998).

The complete ITS sequences were initially used to produce an alignment and cladogram, which clustered the different mosses well enough to identify the species taxonomically. However, alignment of only the ITS2 region also produced a cladogram which gave excellent separation of the specimens into species groups. The most parsimonious trees were determined and bootstrap analyses were done to test support for the resulting clades (Felsenstein 1985).

ITS sequences have been deposited with Genbank; accession numbers and ITS sequence lengths are given in Table I for the nine Antarctic moss species.

Results

ITS sequencing of Antarctic mosses

The ITS region was sequenced from over 30 different specimens of Antarctic mosses from a range of locations, although results for only 19 of the samples are presented here since some proved to have identical ITS sequences. Three of the specimens included here had not been identified by morphological methods, and several had only been provisionally identified by traditional methods. By analysis of the ITS sequences, the specimens fell into nine distinct species. At least one, and usually several isolates of

```
                   1                                                                                          90
B. argenteum EP    TATTTTGACC CGAGAGTTCG AGTCCTCCGG GGCTCGAGCA CGAGTTGGAT CTAAAACTTT AACTTAGAAC AACTCTCAGC AACGGATATC
B. argenteum BI    TATTTTGACC CGAGAGTCCG AGTCCTCCGG GGCTCGAGCA CGAGTTGGAT CTAAAACTTT AACTTAGAAC AACTCTCAGC AACGGATATC
B. argenteum CC    TATTTTGACC CGAGAGTTCG AGTCCTCCGG GGCTCGAGCA CGAGTTGGAT CTAAAACTTT AACTTAGAAC AACTCTCAGC AACGGATATC
B. argenteum CG    TATTTTGACC CGAGAGTTCG AGTCCTCCGG GGCTCGAGCA CGAGTTGGAT CTAAAACTTT AACTTAGAAC AACTCTCAGC AACGGATATC
B. argenteum CR    TATTTTGACC CGAGAGTTCG AGTCCTCCGG GGCTCGAGCA CGAGTTGGAT CTAAAACTTT AACTTAGAAC AACTCTCAGC AACGGATATC
B. argenteum GH    TATTTTGACC CGAGAGTTCG AGTCCTCCGG GGCTCGAGCA CGAGTTGGAT CTAAAACTTT AACTTAGAAC AACTCTCAGC AACGGATATC
Consensus          ********** ******* ** ********** ********** ********** ********** ********** ********** **********

                   91                                                                                        180
B. argenteum EP    TTGGCTCTTG CAACGATGAA GAACGCATCG AAATGCTATA CGTAGTGTCA ATTGCAGAAT TCNNANAATN ATCGAGTTTT TGANCGCAAG
B. argenteum BI    TTGGCTCTTG CAACGATGAA GAACGCAGCG AAATGCGATA CGTAGTGTGA ATTGCAGAAT TCCGNGAATC ATCGAGTTTT TGAACGCAAG
B. argenteum CC    TTGGCTCTTG CAACGATGAA GAACGCAGCG AAATGCGATA CGTAGTGTGA ATTGCAGAAT TCCGCGAATC ATCGAGTTTT TGAACGCAAG
B. argenteum CG    TTGGCTCTTG CAACGATGAA GAACGCAGCG AAATGCGATA CGTAGTGTGA ATTGCAGAAT TCCGCGAATC ATCGAGTTTT TGAACGCAAG
B. argenteum CR    TTGGCTCTTG CAACGATGAA GAACGCAGCG AAATGCGATA CGTAGTGTGA ATTGCAGAAT TCCGCGAATC ATCGAGTTTT TGAACGCAAG
B. argenteum GH    TTGGCTCTTG CAACGATGAA GAACGCANCG AAATGCNATA CGTAGTGTGA ATTGCAGAAT TCCGNGAATC ATCGAGTTTT TGAACGCAAG
Consensus          ********** ********** ******* ** ****** *** ******** * ********** **    ***  ********** *** ******

                   181                                                                                       270
B. argenteum EP    TTGNGCCTGA GGCTNGTNCA AGGGCATTNC AGCTACAGCG TCACCGCGCC CCCCCACTAC AGT------A TGTTAGAGTC TGAGTGGAAC
B. argenteum BI    TTGCGCCTGA GGCTTGTCCN AGGGCATTTC NGCTAGAGCG TCACCGCGCC CCCCCACCAC ACT------A TGTTAGAGTC TGAGTGGAAC
B. argenteum CC    TTGCGCCCGA GGCTTGTCCG AGGGCATTTC CGCTAGAGCG TCACCGCGCC CCCCCACCAC AGT------C TGTTAGAGTC TGAGTGGAAC
B. argenteum CG    TTGCGCCCGA GGCTTGTCCG AGGGCATTTC CGCTAGAGCG TCACCGCGCC CCCCCACCAC ACT------C TCTTAGAGTC TGAGTGGAAC
B. argenteum CR    TTGCGCCCGA GGCTTGTCCG AGGGCATTTC CGCTAGAGCG TCACCGCGCC CCCCCACCAC AACTCCCGAA TGCGGAGTTT TGAGTGGAAC
B. argenteum GH    TTGNGCCTGA GGCTNGTCCN AGGGCATTNC NGCTAGAGCG TCACCGCGCC CCCCCACNAC ANT------A TGTTAGAGTC TGAGTGGAAC
Consensus          *** *** ** **** ** *  ******** *  **** **** ********** ******* ** *          *       *  **********
```

Fig. 2. Comparison of 270 bp of the conserved nuclear ribosomal RNA ITS1–5.8S–ITS2 nucleotide sequences of six different Antarctic isolates of *Bryum argenteum*, obtained from Edmonson Point (EP), Beaufort Island (BI), Cape Chocolate (CC), Canada Glacier (CG), Cape Royds (CR) and Granite Harbour (GH). The first 470 bp were identical for all six isolates (except for a single nucleotide difference in the BI sample), as were the last 250 bp, so only the middle 270 bp of ITS2 with nucleotide variability is shown in the alignment, for clarity. The consensus shows where all six specimens had identical nucleotide sequences.

https://doi.org/10.1017/S0954102005002816 Published online by Cambridge University Press

each of the nine species were analysed, though results for only two isolates of each species are included here for clarity; other specimens tested for the same range of species gave similar results to those included here.

The region sequenced included the 3' terminal nucleotides of the 18S locus, all of the ITS1 region, all of the 5.8S locus, and all of the ITS2 locus. In all but a few samples, approximately 700–1000 nucleotides were analysed depending on the length of the ITS region amplified with primers ITS4 and ITS5. Sequencing with primers ITS2, ITS4 and ITS5 enabled the full sequence to be constructed, and the lengths are included in Table I. Some of the results are summarized in Fig. 1, which shows an alignment constructed with CLUSTAL-W using only 270 nucleotides from the ITS2 sequences. Alignments using the full ITS sequences gave similar results, but the small representative segment is shown in Fig. 1 for clarity. The 5.8S region was much more highly conserved, showing few nucleotide differences between the genera and none within species. The ITS1 region showed a little variation, but gave a similar species-dependent characteristic alignment.

Figure 1 clearly demonstrates that even a small portion of the ITS region can be used to distinguish between nine of the main moss species which have been reported from the Ross Sea region of Antarctica (Lewis Smith 1984). Moreover, Fig. 1 also demonstrates that some genetic variability can occur within this relatively conserved sequence, visible in some of the moss species where multiple specimens were analysed.

ITS variability within Bryum argenteum

By comparison of a range of different samples of individual species, it was possible to assess the extent of within-species nucleotide variation in the ITS region. One example of such variation is shown in Fig. 2, where six sequences are aligned for different isolates of *Bryum argenteum* from a range of sampling sites in Victoria Land, Antarctica. The specimen from Beaufort Island was obtained from a large continuous turf of *B. argenteum* (Seppelt *et al.* 1999), whereas other specimens were from small isolated colonies; this did not appear to affect the results.

For the first 550 bp, and the last 250 bp of the complete ITS1–5.8S–ITS2 alignment for these six specimens, no nucleotide differences were detected (data not shown; see Genbank submissions). Only within a 270 bp segment of the ITS2 region were any differences detected, and this segment with nucleotide variation is shown in Fig. 2. Several samples, but particularly the isolate from Edmonson Point, showed polymorphism at a few nucleotide positions in the ITS2 sequence. This is indicated by "N" in the alignment shown in Fig. 2, usually representing at least two alternative nucleotides, which could not be established with certainty from the ABI sequencing curves despite repetition of experiments.

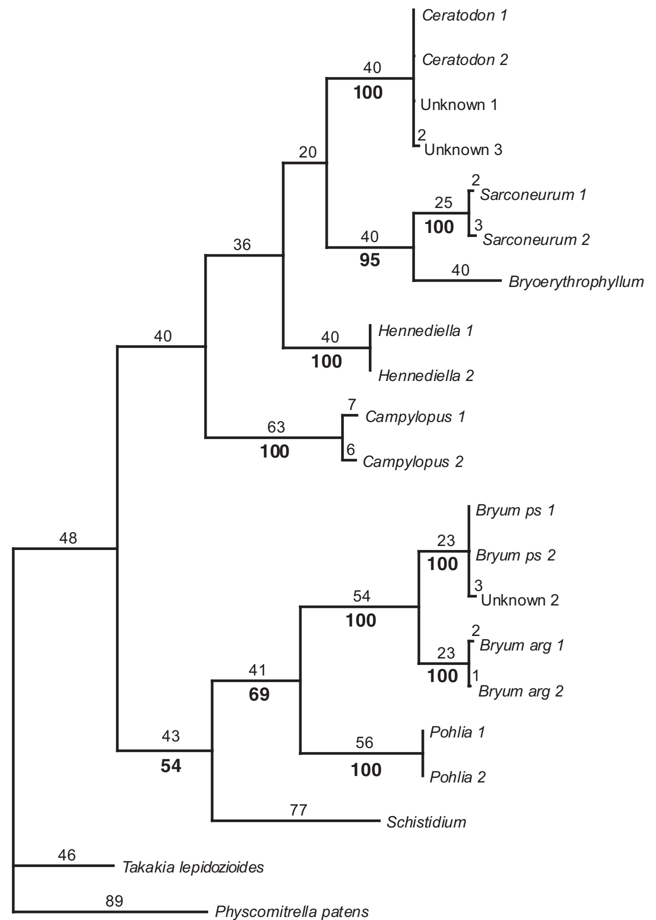

Fig. 3. Cladogram produced from a PAUP alignment of the ITS2 sequence for nine different species of moss found in the Ross Sea region of Antarctica. The nineteen samples are the same as in Fig. 1, including *Takakia lepidozioides* and *Physcomitrella patens* as outgroup species. Numbers above the lines show the number of changes in the nucleotide sequences; numbers below the lines represent the bootstrap support for those branches.

Samples of *B. argenteum* collected from geographically close sites were genetically similar. However the most remote sample collected, at Edmonson Point some 300 km north of the others, exhibited more differences within the ITS2 region (as well as more mixed nucleotide sequences) than did the samples from sites closer together. Similar results were obtained for other *B. argenteum* isolates (unpublished data); all samples collected from more distant locations showed higher levels of sequence differences than did colonies collected from nearby sites.

Variability between isolates of other Antarctic moss taxa

Initially, sequences of the complete ITS region of the nine Antarctic moss species, and the unidentified mosses, were analysed together with the outgroup species *Takakia lepidozioides* and *Physcomitrella patens*. However, genetic

https://doi.org/10.1017/S0954102005002816 Published online by Cambridge University Press

variability within the ITS1 sequence caused the alignments to be unreliable, and therefore only the ITS2 sequence was used for subsequent analyses. The new alignment contained 589 nucleotides of the 5.8S and ITS2 regions, of which 295 were potentially parsimony-informative. PAUP analysis of this alignment produced eight equally nearly identical parsimonious trees; tree length = 870; consistency index (CI) = 0.6989; retention index (RI) = 0.7815; and rescaled consistency index (RC) = 0.5461. One cladogram is shown in Fig. 3; only two samples of each moss species are included for clarity, but similar results were obtained for up to six other samples tested from each species. The cladogram shows the clustering and distinction of the different species, as well as characterization of three samples of previously unknown identity - two samples of *Ceratodon purpureus* (one from Casey and another from Crater Cirque), and one specimen of *Bryum pseudotriquetrum* from Casey.

The cladogram in Fig. 3 also shows that there can be genetic variation between different isolates of the same species, even within this relatively conserved multicopy region. Although only a limited number of moss samples is included in this cladogram, many other samples showed similar levels of genetic variation.

Utility of ITS for identification of unknown mosses

Figure 3 also includes three mosses which had not previously been identified by morphological means, mainly because of phenotypic plasticity. These three samples were readily identified by comparison of either the ITS1 or the ITS2 sequences obtained, as shown for ITS2 in Fig. 3. One species from Casey and one from Crater Cirque were both characterized as *Ceratodon purpureus*, another from near Casey was identified as *Bryum pseudotriquetrum*.

However, comparison of a much smaller region of the sequence was sufficient for identification of these species, and for distinction of the dominant moss species of the Ross Sea region. Figure 1 also includes the three unknown samples in an alignment of only 270 nucleotides within the ITS2 region, and these mosses are again identified as *Ceratodon purpureus* and *Bryum pseudotriquetrum.* This result demonstrates that this small, readily amplifed and relatively conserved section of the genome is sufficient for differentiation and characterization within the nine Antarctic moss species included here.

Discussion

This report demonstrates the utility of DNA sequencing of the ITS region for genetic analysis of nine species of Antarctic mosses, enabling taxonomic identification based on only a partial nucleotide sequence of the conserved nuclear ribosomal 18S–26S DNA ITS region. The results shown here are typical of many we have already obtained for most of these nine Antarctic moss species, confirming that for these plants, as for mosses from temperate regions, this part of the genome can be used for taxonomic purposes, and potentially for phylogenetic analysis in future, with incorporation of additional data (Shaw & Schneider 1995, Bopp & Capesius 1996, Patterson *et al*. 1998, Chiang & Schaal 1999, Shaw 2001, Vanderpoorten *et al*. 2001, Shaw *et al*. 2003). However, a major difference between Antarctic and temperate mosses is their lack of sexual reproduction in the extreme climate of Antarctica (Seppelt *et al.* 1992), meaning that any changes in nucleotide sequences in Antarctic mosses have most likely arisen either by immigration or by mutation *in situ.*

There is currently some uncertainty as to the precise identity of the Antarctic material of certain *Bryum* species, with the name *Bryum argenteum sensu lato* being used here until further taxonomic research is completed (Skotnicki *et al*. 1998b, 2000, J. Spence, personal communication 2001). The results presented here demonstrate that the six Antarctic isolates analysed all have very similar ITS sequences, with just a little variation seen in ITS2, and with some mixed populations of this region in three of the six isolates. The genetically mixed populations, found at Granite Harbour, Edmonson Point and Beaufort Island, probably represent mutation happening within the moss, with the multicopy nature of the ITS region allowing amplification and maintenance of more than one nucleotide sequence within individual moss shoots.

The ITS2 sequences obtained were not sufficiently different to distinguish cryptic species within the *B. argenteum* samples, and the six Antarctic sequences showed no differences within ITS1 or the 5.8S loci. The only nucleotide differences observed in the Antarctic *B. argenteum* samples occurred within the ITS2 locus, where sequences varied by 1–2% maximum. These Antarctic specimens also varied from two Australian and New Zealand *B. argenteum* samples analysed by up to 8% within the ITS region. However, the Antarctic *B. argenteum* sequences differed by approximately 25–30% from sequences of three *B. pseudotriquetrum* isolates from Victoria Land.

It is interesting to note that Longton & Hedderson (2000) found that samples of *B. argenteum* from the Ross Sea region were most closely related to specimens from the Arctic and formed a monophyletic group with these, rather than with *B. argenteum* specimens from elsewhere. Thus, until many more *B. argenteum* samples are analysed, it is not possible to distinguish another possibly cryptic species within the Antarctic *B. argenteum* populations.

DNA sequencing of the ITS region, as well as other genetic loci, in temperate and Antarctic mosses has clearly shown that these haploid plants can maintain higher levels of genetic variation even in conserved multicopy loci than might have been expected (Skotnicki *et al.* 2004, Shaw *et al*. 2003). In some temperate species, it has been

https://doi.org/10.1017/S0954102005002816 Published online by Cambridge University Press

proposed that this variation can lead to "cryptic speciation" (Shaw 2001). The few moss species which can colonize and survive the rigors of continental Antarctica grow in probably the harshest environment on earth (Walton 1990), and are also subjected to increased levels of UV irradiation (and thus potential mutation) due to the annual expansion of the ozone hole (Farman *et al*. 1985, Clilverd *et al*. 2003).

The results presented here confirm that levels of ITS sequence variation are also significant in Antarctic moss species. Overall, samples from sites very close together were much more likely to have identical sequences, and the further apart samples were collected then the more likely they were to exhibit greater differences within the nucleotide sequences. For example, the *Hennediella* specimens shown in Fig. 3 were collected from sites within 100 km of each other and had identical ITS2 sequences, whereas the specimens of *Sarconeurum* were collected from sites separated by several hundred kilometres and had nucleotide differences within their ITS sequences. This was not always the case, and specimens of *Ceratodon* separated by hundreds or thousands of kilometres were found to sometimes have identical ITS sequences.

As these mosses appear to reproduce solely by vegetative means (Seppelt *et al*. 1992), such genetic variation appears most probably caused by mutation (Skotnicki *et al*. 2004), and could be involved in evolution of new species (cryptic or otherwise, over time) perhaps more adapted to specifically withstand the extremely harsh climate of the region. We are also currently sequencing several more genetic loci from these Antarctic mosses, including the *trn*L-F, *rps*4 and *rbc*L chloroplast genes (De Luna *et al*. 2000, Pedersen *et al*. 2003, Virtanen *et al*. 2003), to assist with determining the extent of genetic variability, origins and evolution of moss species in this isolated and extreme location. However, the ITS region appears to be excellent for analysis of Antarctic mosses, enabling identification and analysis of some genetic variability between populations.

In Antarctica, the climatic extremes often lead to phenotypic plasticity of moss species, making identification on purely morphological grounds difficult and sometimes impossible. One such example is the single moss protonemal specimen collected from Mount Erebus, which could not be differentiated into the morphologically recognisable vegetative form (Broady 1984). This specimen could therefore not be identified for nearly 20 years, until molecular genetics was used to demonstrate that it was closely related to *Campylopus pyriformis* on Mt Melbourne some 300 km to the north (Broady *et al*. 1987, Skotnicki *et al*. 2001). Now, its identification has been further confirmed by use of the ITS sequence, showing that this specimen of *C. pyriformis* is very closely related to the Mount Melbourne population of that species (Fig. 3).

Many other samples of moss have less extreme phenotypic plasticity, and we have demonstrated here that DNA sequencing is a fast, simple and minimally damaging technique for characterisation. Three unidentified moss samples were included in the results presented here, but we have also used the same method with previously unidentified or uncertain examples of *Bryum argenteum*, *Hennediella heimii*, *Pohlia nutans* (Bargagli *et al*. 2004), and *Sarconeurum glaciale.* While the RAPD technique can be used for such characterization of Antarctic mosses (Skotnicki *et al*. 2000, 2002), this method is sometimes difficult to reproduce reliably without very careful work. We have confirmed that RAPD results are mirrored by DNA sequencing results, some of which are presented here. Thus DNA sequencing of the ITS region offers an alternative and simple technique, which can easily be combined with traditional morphological methods to provide definitive taxonomic identification of Antarctic mosses. For population studies, the two methods have proven complementary, with RAPDs facilitating the screening of large numbers of samples for overall population genetic analysis, and DNA sequencing enabling more detailed comparison of a few moss samples.

Acknowledgements

We thank R. Bargagli for providing specimens from Mount Rittmann and Crater Cirque in Victoria Land, and R.I. Lewis Smith and H. Peat for British Antarctic Survey Herbarium specimens. This work was supported by an Australian Antarctic Science Research Grant and the Hermon Slade Foundation. We also thank ANARE, Antarctica New Zealand and the Italian Antarctic Research Programme for logistical support to collect moss specimens under permit from various regions in Antarctica. We thank Lars Hedenas and an unnamed referee for their critical comments.

References

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

Bargagli, R., Broady, P.A. & Walton, D.W.H. 1996. Preliminary investigation of the thermal biosystem of Mount Rittmann fumaroles (northern Victoria Land, Antarctica). *Antarctic Science*, **8**, 121–126.

Bargagli, R., Skotnicki, M.L., Marri, L., Pepi, M., Mackenzie, A. & Agnorelli, C. 2004. New record of moss and thermophilic bacteria species and physico-chemical properties of geothermal soils on the northwest slope of Mt. Melbourne (Antarctica). *Polar Biology*, **27**, 423–431.

Bopp, M. & Capesius, I. 1996. New aspects of bryophyte taxonomy provided by a molecular approach. *Botanica Acta*, **109**, 368–372.

Broady, P.A. 1984. Taxonomic and ecological investigations of algae on steam-warmed soil on Mt Erebus, Ross Island, Antarctica. *Phycologia*, **23**, 257–271.

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

https://doi.org/10.1017/S0954102005002816 Published online by Cambridge University Press

Chiang, T.Y. & Schaal, B.A. 1999. Phylogeography of North American populations of the moss species *Hylocomium splendens* based on the nucleotide sequence of internal transcribed spacer 2 of nuclear ribosomal DNA. *Molecular Ecology*, **8**, 1037–1042.

Clilverd, M.A., Clarke, E., Rishbeth, H., Clark, T.D.G. & Ulich, T. 2003. Solar cycle: solar activity levels in 2100. *Astronomy and Geophysics*, **44**, 20–22.

De Luna, E., Buck, W.R., Akiyama, H., Arikawa, T., Tsubota, H., González, D., Newton, A.E. & Shaw, A.J. 2000. Ordinal phylogeny within the hypnobryalean pleurocarpous mosses Inferred from cladistic analyzes of three chloroplast DNA sequence data sets: *trn*L-F, *rps*4, and *rbc*L. *The Bryologist*, **103**, 242–256.

Farman, J.C., Gardiner, B.G. & Shanklin, J.D. 1985. Large losses of total ozone in Antarctica reveal seasonal ClOx/Nox interaction. *Nature*, **315**, 207–210.

Felsenstein, J. 1985. Confidence limits on phylogenesis: an approach using the bootstrap. *Evolution*, **39**, 783–791.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95–98.

Lewis Smith, R.I. 1984. Terrestrial plant biology of the sub-Antarctic and Antarctic. *In* Laws, R.M., *ed. Antarctic ecology*. London: Academic Press, 61–162.

Longton, R.E. & Hedderson, T. 2000. What are rare species and why conserve them? *Lindbergia* **25**, 53–61.

Maddison, W.P. & Maddison, D.R. 1992. *MacClade: analysis of phylogeny and character evolution* (version 3.0) Sunderland, MA: Sinauer Associates.

Patterson, E., Boles, S.B. & Shaw, A.J. 1998. Nuclear ribosomal DNA variation in *Leucobryum glaucum* and *L. albidum* (Leucobryaceae): a preliminary investigation. *The Bryologist*, **101**, 272–277.

Pedersen, N., Cox, C.J. & Hedenäs, L. 2003. Phylogeny of the moss family Bryaceae inferred from chloroplast DNA sequences and morphology. *Systematic Botany* **28**, 471–482.

Rafalski, J.A. & Tingey, S.V. 1993. Genetic diagnostics in plant breeding: RAPDs, microsatellites and machines. *Trends in Genetics*, **9**, 275–280.

Rosenthal A. & Charnock-Jones, D.S. 1992. New protocols for DNA sequencing with dye terminators. *DNA Sequence*, **3**, 61–64.

Sambrook, J., Fritsch, E.F. & Maniatis, T. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. New York: Cold Spring Harbor Press, 1500 pp.

Seppelt, R.D., Green, T.G.A. & Skotnicki, M.L. 1999. Notes on the flora, vertebrate fauna and biological significance of Beaufort Island, Ross Sea, Antarctica. *Polarforschung*, **66**, 53–59.

Seppelt, R.D., Green, T.G.A., Schwartz, A.J. & Frost, A. 1992. Extreme southern locations for moss sporophytes in Antarctica. *Antarctic Science*, **4**, 37–39.

Shaw, A.J. 2001. Biogeographic patterns and cryptic speciation in bryophytes. *Journal of Biogeography*, **28**, 253–261.

Shaw, A.J. & Schneider, R.E. 1995. Genetic biogeography of the rare "copper moss," *Mielichhoferia elongata* (Bryaceae). *American Journal of Botany*, **82**, 8–17.

Shaw, A.J., Cox, C.J., Goffinet, B., Buck, W.R. & Boles, S.B. 2003. Phylogenetic evidence of a rapid variation of pleurocarpous mosses (Bryophyta). *Evolution*, **57**, 2226–2241.

Skotnicki, M.L., Bargagli, R. & Ninham, J.A. 2002. Genetic diversity in the moss *Pohlia nutans* on geothermal ground of Mount Rittmann, Victoria Land, Antarctica. *Polar Biology*, **25**, 771–777.

Skotnicki, M.L., Mackenzie, A.M. & Selkirk, P.M. 2004. Mosses surviving on the edge: origins, genetic diversity and mutation in Antarctica. Molecular systematics of Bryophytes. *Monographs in Systematic Botany from the Missouri Botanical Garden*, **98**, 388–403.

Skotnicki, M.L., Ninham, J.A. & Selkirk, P.M. 1998b. Genetic diversity in the moss *Bryum argenteum* in Australia, New Zealand and Antarctica. *The Bryologist*, **101**, 412–421.

Skotnicki, M.L., Ninham, J.A. & Selkirk, P.M. 1999. Genetic diversity and dispersal of the moss *Sarconeurum glaciale* on Ross Island, East Antarctica. *Molecular Ecology*, **8**, 753–762.

Skotnicki, M.L., Ninham, J.A. & Selkirk, P.M. 2000. Genetic diversity, mutagenesis and dispersal of antarctic mosses - a review of progress with molecular studies. *Antarctic Science*, **12**, 363–373.

Skotnicki, M.L., Selkirk, P.M. & Beard, C. 1998a. RAPD profiling of genetic diversity in Antarctic populations of the moss *Ceratodon purpureus*. *Polar Biology*, **19**, 172–176.

Skotnicki, M.L., Selkirk, P.M., Broady, P., Adam, K.D. & Ninham, J.A. 2001. Dispersal of the moss *Campylopus pyriformis* on geothermal ground near the summits of Mount Erebus and Mount Melbourne, Victoria Land, Antarctica. *Antarctic Science*, **13**, 280–285.

Swofford, D.L. 1998. *PAUP*: Phylogenetic Analysis Using Parsimony and other methods*. Sunderland, MA: Sinauer Associates.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673–4680.

Tracy, T.E. & Mulcahy, L.S. 1991. A simple method for direct automated sequencing of PCR fragments. *BioTechniques*, **11**, 68–75.

Vanderpoorten, A., Shaw, A.J. & Goffinet, B. 2001. Testing controversial alignments in *Amblystegium* and related genera (Amblystegiaceae: Bryopsida). Evidence from rDNA ITS sequences. *Systematic Botany*, **26**, 470–479.

Virtanen, V. 2003. Phylogeny of the Bartramiaceae (Bryopsida) based on morphology and on *rbc*L, *rps*4, and *trn*L-*trn*F sequence data. *The Bryologist*, **106**, 280–296.

Walton, D.W.H. 1990. Colonization of terrestrial habitats - organisms, opportunities and occurrence. *In* Kerry, K.R. & Hempel, G., *eds. Antarctic ecosystems, ecological change and conservation*. Berlin: Springer, 51–60.

White, T.J., Bruns, T., Lee, S. & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* Innis, M., Gelfand, D., Sninsky, J. & White, T., *eds. PCR Protocols: a guide to methods and applications*. San Diego, CA: Academic Press, 315–322.

https://doi.org/10.1017/S0954102005002816 Published online by Cambridge University Press