Molecular support for Pleistocene persistence of the continental Antarctic moss *Bryum argenteum*

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Abstract: We examined sequence variation of ITS and *phy*2 for *Bryum argenteum* from Antarctica, sub-Antarctic, New Zealand and Australia to understand better taxonomic delimitations and resolve relationships between these geographic regions. *Bryum argenteum* has been recorded as two species, *B. argenteum* and *B. subrotundifolium*, in all four regions with the latter now referred to as *B. argenteum* var. *muticum*. We found disagreement between taxon delimitations (based on morphology) and molecular markers. All continental Antarctic specimens consistently formed a monophyletic sister group that consisted of both morphologically identified *B. argenteum* varieties, separate to all non-Antarctic specimens (also consisting of both varieties). We suggest, contrary to previous records, that all continental Antarctic (Victoria Land) populations are referable to *B. argenteum* var. *muticum*, while sub-Antarctic, Australian and New Zealand populations included here are *B. argenteum* var. *argenteum*. Additionally, since there was less genetic diversity within Victoria Land, Antarctica, than observed between non-Antarctic samples, we suggest that this is, in part, due to a potentially lower rate of DNA substitution and isolation in northern and southern refugia within Victoria Land since the Pleistocene.

Introduction

The relationships of the Antarctic biota and the degree to which they are isolated from other Southern Hemisphere landmasses is of interest for biogeographic questions examining the origins of these taxa (dispersal, both recent or ancient, versus vicariance) (e.g. Muñoz *et al.* 2004, Sanmartín & Ronquist 2004, Stevens *et al.* 2006, Convey & Stevens 2007) as well as for identifying introduced organisms (e.g. Frénot *et al.* 2005). Critical to any molecular examination, however, is accurate taxonomic identification, and although several papers on moss distribution and taxonomic affinities have been published (e.g. Cardot 1907, Ochi 1979, Seppelt & Kanda 1986, Seppelt & Green 1998, Ochyra *et al.* 2008), the taxonomic status of some of these species remains unsatisfactory (e.g. Seppelt & Green 1998, Seppelt *et al.* 1998, Spence & Ramsay 2002). This is largely because, in the Ross Sea region, diagnostic capsules have only been observed or collected for a single Antarctic species, *Hennediella heimii* (Hedw.) Zand. (Seppelt *et al.* 1998). Without sporophytic features, particularly peristome characteristics, some species may be very difficult to identify unequivocally (Ochi 1979, Seppelt *et al.* 1998). This problem is exacerbated by the morphological plasticity induced by the harsh Antarctic environment (Nakanishi 1979).

The taxonomic identification of Southern Hemisphere populations of the 'silver' *Bryum* complex, especially the morphologically similar taxa *B. argenteum* Brid.

(e.g. Ochyra *et al.* 2008, Seppelt *et al.* 2010) and *B. subrotundifolium* Jaeg. (e.g. Seppelt & Green 1998, Green *et al.* 2005) have been confused, highlighting the particular difficulties with taxonomic identification of these mosses (see also Adams *et al.* 2006). Some small environmental forms of *B. pseudotriquetrum* (Hedw.) P.Gaertn., B.Mey. & Scherb., are also remarkably similar in morphological characteristics to *B. argenteum* (Ochyra *et al.* 2008). Spence & Ramsay (2002) transferred *B. subrotundifolium* to the genus *Anomobryum*. However, Ochyra *et al.* (2008) refuted this and considered this taxon as a synonym of *B. argenteum* var. *muticum*. A key distinguishing feature between the two distinct varieties of *B. argenteum* is the retention of chloroplasts in all leaf cells in var. *muticum* (previously *B. subrotundifolium*), giving the plants a yellow/green colouration, while in var. *argenteum* the distal half to one third of the leaf becomes achlorophyllose, giving the plants a silver/green colouration (see Ochyra *et al.* 2008). Thorough morphological comparisons of vegetative specimens (Seppelt & Kanda 1986, Seppelt & Green 1998), have previously recorded *B. a.* var. *muticum* and *B. a.* var. *argenteum* from continental Antarctica, sub-Antarctica, New Zealand and Australia and the recent treatment by Ochyra *et al.* (2008) describes an almost entirely overlapping distribution between the two varieties. Clearly, the taxonomy within this group remains confused.

We focused our study on the taxa *B. a.* var. *argenteum* and *B. a.* var. *muticum*. We adopted a molecular systematic

Table I. Moss samples from continental Antarctica (Victoria Land), sub-Antarctic (Îles Crozet), Australia and New Zealand used in this study. Sample ID numbers relate to those used in Figs 1 & 2, collection localities, species identifications and GenBank accessions with sequence lengths obtained for each specimen.

Sample ID	Location	Species[2]	GenBank	
			ITS[3]	*phy*2[3]
SH56	Mount Buffalo, Australia (36°47'S, 146°03'E)	*B. argenteum* var. *argenteum*	GU907057 927 bp	GU907084 694 bp
SH48	Îles Crozet, sub-Antarctica (46°27'S, 52°00'E)	*B. argenteum* var. *argenteum*	GU907058 933 bp	GU907083 696 bp
SH43	Christchurch, New Zealand (43°5'S, 172°7'E)	*B. argenteum* var. *argenteum*	GU907056 927 bp	GU907082 694 bp
SH68	Mount McKay, Australia (36°52'S, 147°15'E)	*B. argenteum* var. *argenteum*	GU907061 921 bp	
SH16	Hamilton, New Zealand (35°53'S, 175°28'E)	*B. argenteum* var. *argenteum*	GU907060 928 bp	GU907078 703 bp
A099	Huntley, New Zealand (37°34'S, 175°09'E)	*B. argenteum* var. *argenteum*	GU907062 928 bp	
SH42	Canberra, Australia (35°18'S, 149°08'E)	*B. argenteum* var. *argenteum*	GU907059 938 bp	GU907081 702 bp
SH34	Garwood Valley, Antarctica (78°03'S, 164°10'E)	*B. argenteum* var. *muticum*	GU907072 934 bp	GU907080 712 bp
RH8/2	Cape Bird, Antarctica (77°13'S, 166°26'E)	*B. argenteum* var. *muticum*	GU907070 934 bp	GU907077 712 bp
C474[1]	Cape Royds, Antarctica (77°35'S, 166°10'E)	*B. argenteum* var. *argenteum*	AY611433 934 bp	
MG20/10	Miers Valley, Antarctica (78°05'S, 164°45'E)	*B. argenteum* var. *muticum*	GU907071 934 bp	GU907076 712 bp
P171[1]	Canada Glacier, Antarctica (77°35'S, 163°15'E)	*B. argenteum* var. *argenteum*	AY611432 928 bp	
SH21	Cape Hallet, Antarctica (72°18'S, 170°18'E)	*B. argenteum* var. *muticum*	GU907066 928 bp	
SH27	Cape Hallet, Antarctica (72°18'S, 170°18'E)	*B. argenteum* var. *muticum*	GU907068 928 bp	
SH17	Cape Hallet, Antarctica (72°18'S, 170°18'E)	*B. argenteum* var. *muticum*	GU907067 928 bp	GU907079 712 bp
SH47	Marble Point, Antarctica (76°26'S, 163°50'E)	*B. argenteum* var. *muticum*	GU907069 928 bp	
SH61	Mount Hotham, Australia (36°59'S, 147°09'E)	*B. argenteum* var. *muticum*		GU907085 694 bp
15F[1]	Beaufort Island, Antarctica (76°55'S, 166°54'E)	*B. argenteum* var. *argenteum*	AY611431 928 bp	
20333	Beaufort Island, Antarctica (76°55'S, 166°54'E)	*B. argenteum* var. *argenteum*	GU907063 928 bp	GU907075 712 bp
BIE1	Beaufort Island, Antarctica (76°55'S, 166°54'E)	*B. argenteum* var. *muticum*	GU907064 928 bp	
EP[1]	Edmonson Point, Antarctica (74°20'S, 164°30'E)	*B. argenteum* var. *argenteum*	AY611430 928 bp	
C363[1]	Granite Harbour, Antarctica (77°00'S, 162°30'E)	*B. argenteum* var. *argenteum*	AY611434 928 bp	
CC26[1]	Cape Chocolate, Antarctica (77°57'S, 164°30'E)	*B. argenteum* var. *argenteum*	AY611429 928 bp	
20317	Granite Harbour, Antarctica (77°00'S, 162°30'E)	*B. argenteum* var. *argenteum*	GU907065 928 bp	
CCC5[1]	Granite Harbour, Antarctica (77°00'S, 162°30'E)	*B. pseudotriquetrum*	AY611426 996 bp	
P159[1]	Canada Glacier, Antarctica (77°35'S, 162°15'E)	*B. pseudotriquetrum*	AY611427 996 bp	
A137	Casey, Antarctica (66°15'S, 110°33'E)	*B. pseudotriquetrum*	GU907073 992 bp	
SH69	Mount Buffalo, Australia (36°47'S, 146°03'E)	*B. pseudotriquetrum*	GU907074 994 bp	GU907086 694 bp

[1] Sequences from Skotnicki *et al.* (2005)

[2] Species names based on original taxonomic identification, such that *Bryum argenteum* var. *argenteum* = specimens originally identified as *Bryum argenteum*, and *Bryum argenteum* var. *muticum* = specimens originally identified as *Bryum subrotundifolium*.

[3] Sequence lengths for fragments prior to addition of gaps for accurate alignments; not all specimens were run with *phy*2 given the lower resolution compared to ITS

approach to unravel the taxonomic and biogeographic issues of these mosses and to ask the question: Do the Antarctic populations represent a genetically distinct taxon (suggesting isolation) or are they similar (mixed) to non-Antarctic populations (suggesting recent gene flow)? We utilized sequence variation of two nuclear markers, ribosomal (nr) internal transcribed spacer (ITS) regions and a fragment of the phytochrome 2 (phy2) gene; this approach avoids problems associated with both single gene phylogenies and environmentally driven morphological plasticity.

Materials and methods

Plant material

Specimens for DNA analyses were collected from Antarctica, New Zealand, and Australia (Table I). The samples were placed in paper envelopes and air dried as soon as possible after collection, transferred to sealed plastic bags and stored at -74°C until DNA extraction. Voucher specimens are deposited in the University of Waikato herbarium (WAIK), and the Australian Antarctic Division herbarium (ADT - now located in HO).

Molecular analyses

Total genomic DNA was extracted from 100–500 mg of clean, dried, herbarium or frozen moss material using a CTAB extraction technique (Rogers & Bendich 1985). PCR amplification and sequencing of the ITS region from the moss samples utilized the primer combination ITSHP5 [5'-GGAAGGAGAAGTCGTAACAAGG-3'] (LMS, Smithsonian Institute) and ITS4 [5'-TCCTCCGCTTATTGATATGC-3'] (White et al. 1990). Amplification and sequencing of a portion of the phy2 gene employed the primers; phy2(forward) [5'-GGCATGGAAATGATGTGTTG-3'] and phy2(reverse) [5'-CATCACTGTACCCATCTCG-3'] (McDaniel & Shaw 2005). PCR was performed in 0.5 ml thin-walled microcentrifuge tubes using 1–10 ng of template DNA, 1 X PCR buffer (Roche), 1.5 mM $MgCl_2$, 0.5 μM of each primer, 0.25 mM each of dATP, dTTP, dCTP, dGTP (Boehringer Mannheim) and 1 unit Taq DNA polymerase (Roche) in a total volume of 20 μl. Amplification was performed using a Biometra T1 thermocycler (Whatman Biometra) with the following protocol: 5 m at 95°C, followed by 29 cycles of 30 s at 95°C, 30 s at 50°C and 45 s at 72°C. PCR products were electrophoresed on a 1.0% agarose (SeaKem LE) minigel and visualized with ethidium bromide. Where multiple amplification products were generated in a single reaction, products c. 1 kb were excised individually and purified using a Perfectprep gel cleanup kit (Eppendorf).

Purified PCR products were quantified with a Nanodrop spectrophotometer (NanoDrop Technologies). Forward and reverse sequences were generated using BigDye[TM] Terminator
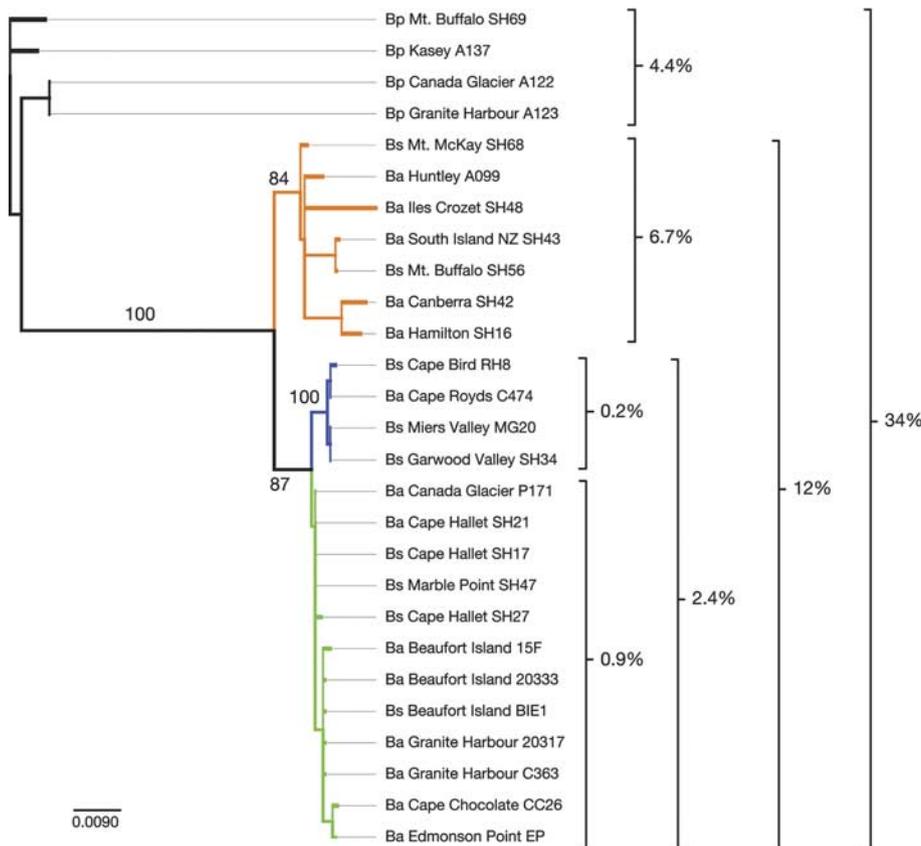

Fig. 1. Neighbor Joining tree derived from a 1128 bp ITS alignment (including gaps). The tree is rooted using Bryum pseudotriquetrum samples as an outgroup. Clades are colour coded; orange for non-Antarctic specimens, blue for southern Antarctic specimens, and green for northern Antarctic specimens. Percentage consensus support values from 100 bootstrap replicates are indicated adjacent to relevant branches. Specimen labels include a morphologically based taxonomic identifier (Bp = Bryum pseudotriquetrum, Ba = Bryum argenteum, Bs = Bryum argenteum var. muticum (previously Bryum subrotundifolium), followed by sample location and sample identification code (see Table I). A scale bar is given, in number of substitutions per site. The percentage of variable sites in subsets of the data is indicated highlighting the low variability in the Antarctic samples.
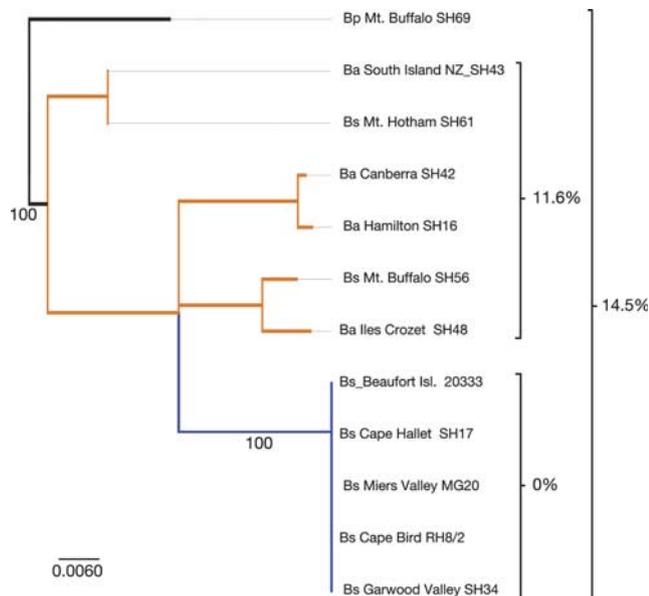
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Fig. 2. Neighbor Joining tree derived from a 715 bp *phy*2 alignment (including gaps). The tree is rooted using *Bryum pseudotriquetrum* as an outgroup. Clades are colour coded; orange for non-Antarctic specimens, blue for Antarctic specimens. Percentage consensus support values from 100 bootstrap replicates are indicated adjacent to relevant branches. Specimen labels include a morphologically based taxonomic identifier (Bp = *Bryum pseudotriquetrum*, Ba = *Bryum argenteum*, Bs = *Bryum argenteum* var. *muticum* (previously *Bryum subrotundifolium*), followed by sample location and sample identification code (see Table I). Scale bar indicates number of substitutions per site. The percentage of variable sites in subsets of the data is indicated.

chemistry (Perkin-Elmer Applied Biosystems) for all sequences. This ensured sequence information was obtained for the entire ITS region, including the 5.8S ribosomal gene. The products of these reactions were then separated on a capillary ABI3730 genetic analyser (Applied Biosystems Inc) at the Allan Wilson Centre Genome Service, Massey University. All sequences generated here were verified using GenBank BLASTn searches. Sequences were aligned and edited using Sequencher (Gene Codes ver. 4.2). Trees were built in Geneious ver. 4.7 (Drummond *et al.* 2009) from these alignments.

Results

ITS

ITS sequence data included the ITS1 and ITS2 regions, the 5.8S rDNA gene and a flanking region of the 18S rDNA gene. Sequenced fragments ranged from 921 to 938 bp in length for *B. a.* var. *argenteum* and *B. a.* var. *muticum* samples, and from 992 to 996 bp for the outgroup *B. pseudotriquetum* samples (Table I). The majority of the genetic variability is clustered in

the ITS regions, and the introduction of sizeable gaps was required to align sequences from all three taxa.

The phylogenetic reconstruction from the ITS alignments consistently recovered a clear separation of Antarctic *Bryum* samples from Australasian and sub-Antarctic samples (Fig. 1). Additionally, within the Antarctic group there is a discrete, well-supported grouping of samples from the most southern and Ross Island localities. Closer inspection of the sequence variability underlying the tree structure reveals a considerable difference in the sequence divergence observed between the samples from continental Antarctic and the non-Antarctic samples. Across all the *B. a.* var. *argenteum* and *B. a.* var. *muticum* samples the variability is 12%, with an average similarity of 96% between any two samples. However, within the non-Antarctic samples 6.7% of sites are variable, while only 2.4% are variable in the Antarctic sample set. Furthermore, the majority of the variability seen within the Antarctic sample set is accounted for by differences between samples from Ross Island and southern McMurdo Dry Valley sites (Cape Bird, Cape Royds, Garwood Valley, Miers Valley) compared to the continental samples from lower (more northern) latitudes. Sequence variability in the southern sites is only 0.2%, and for the samples from lower latitudes in continental Antarctica is 0.9%.

phy2

Alignments of *phy*2 were 715 bp in length, including gaps (Table I). The resulting phylogenetic reconstruction supports the Antarctic/non-Antarctic split seen for ITS (Fig. 2). There is no division between southern (including Ross Island) and northern Antarctic samples. The variability across the *B. a.* var. *argenteum* and *B. a.* var. *muticum phy*2 sequences is 12.9%. However, *B. a.* var. *muticum* samples from continental Antarctica have identical *phy*2 sequences.

Discussion

Bryum argenteum var. *argenteum* is recorded (based on morphology) as having a cosmopolitan distribution including (but not confined to) the Maritime Antarctic and Antarctic Peninsula south to Alexander Island, Edward VII Peninsula, and in Victoria Land south to about 80°S. *Bryum argenteum* var. *muticum* has an almost identical range, but excludes Edward VII Peninsula (Ochyra *et al.* 2008). Both ITS and *phy*2 sequence data show a clear separation of Antarctic and non-Antarctic samples. Furthermore, taxonomic identification of the specimens based on morphological characters that separated *B. a.* var. *argenteum* from *B. a.* var. *muticum* in our specimens from Antarctica, Australia and New Zealand, is not supported by either molecular marker used here.

The ITS phylogeny (Fig. 1) illustrates a close sister relationship of the Antarctic and non-Antarctic samples, while the *phy*2 data (Fig. 2) indicates that the Antarctic

samples (suspected to all be *B. a.* var. *muticum*) form a clade nested within the *B. a.* var. *argenteum* lineage. In either case, the data support a close relationship of all these *Bryum* specimens, with clear separation between continental Antarctic populations and populations from lower (more northern) latitudes.

The continental Antarctic populations are characterized by low genetic variability, compared to samples from lower latitudes, indicative of a lower DNA substitution rate and a probable persistence in refugia, at least since the Holocene and likely to have been isolated in Antarctica since the Pleistocene. This result can be partly explained due to a lack of sexual reproduction (and hence longer generation time) in these mosses in the Antarctic environment (Seppelt *et al.* 1992) in conjunction with isolation in refugia. Populations restricted to a mode of vegetative reproduction will accumulate fewer substitutions than those able to reproduce sexually. This is concordant with an explanation for the same phenomenon observed in the Antarctic moss *Ceratodon purpureus* (Hedw.) Brid. (Skotnicki *et al.* 2000).

Levels of genetic variability in the ITS sequence data are consistent with those reported by Skotnicki *et al.* (2005) and Stevens *et al.* (2007). However, with a larger sample size we have identified a split in the Antarctic populations that separate the most southern localities from those at lower (more northern) latitudes in Victoria Land. This is similar to the genetic breaks identified for other Antarctic organisms in the same region and which have been used to infer the presence of ancient (Holocene–Pleistocene) glacial refugia (e.g. Stevens & Hogg 2003, McGaughran *et al.* 2010). The genetic variability seen in the ITS sequences of the Antarctic specimens results largely from the comparison between northern and southern samples, with very little variation evident within each group (see Fig. 1). When sample size of the northern and southern populations is taken into account, the genetic variation observed is similar (0.1–0.4%), indicating similar constraints in both regions. It is possible that these two lineages represent separate colonization events from discrete refugia within Victoria Land.

There are currently very few molecular studies on Antarctic bryophytes leading to an assumption of recent dispersal and colonization in Antarctica. Here, we suggest the level of molecular divergence shows a clear signal of isolation across the Holocene and most probably into the Pleistocene for *B. a.* var. *muticum* and it is likely that this variety is endemic to Victoria Land, continental Antarctica. It is also apparent that *B. a.* var. *argenteum* does not occur in Victoria Land and is restricted to the non-Antarctic localities included in the present study. These restricted (and non-overlapping) distributions for both varieties are in contrast to previous morphological work that suggested largely overlapping cosmopolitan distributions (see Ochyra *et al.* 2008). Morphologically, the extreme variants of both varieties are readily identifiable (Ochyra *et al.* 2008). However, they are connected by a continuous range of morphological variants. The molecular evidence we have presented unequivocally refutes the separation of the two taxa in Victoria Land. To clarify this taxonomic dilemma it is essential for future work to examine the 'silver' *Bryum* group in a worldwide context, only then can we ultimately assign the Antarctic taxon to *B. a.* var. *muticum*, another taxon (not studied here) or be considered as potentially an Antarctic endemic taxon. A promising avenue of further study will also be a molecular clock analysis of this group with much wider geographic sampling along with other Antarctic and non-Antarctic (e.g. South American) bryophytes to examine biogeographic hypotheses (e.g. Convey & Stevens 2007, Convey *et al.* 2008, 2009). This will allow the hypothesis of a slower rate of molecular evolution, refugial populations and persistence in Antarctica to be directly tested, and allow the timing of colonization events to be examined at regional and continental-wide scales.

Acknowledgements

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