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Phylogeny of Three East Antarctic Mosses

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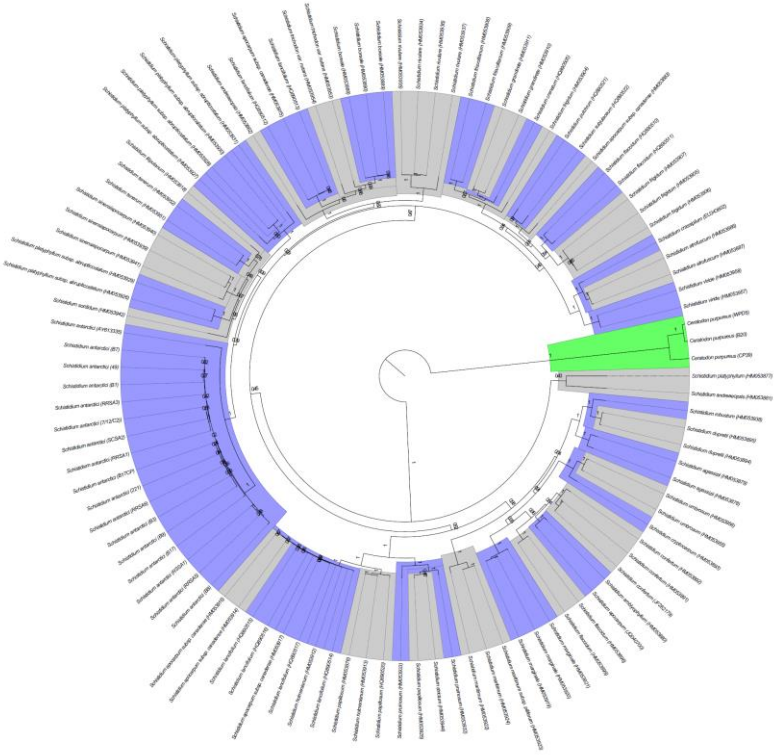

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LIST OF ABBREVIATIONS

ACC	Antarctic circumpolar current
AIC	Akaike information criterion
AICc	Akaike information criterion corrected
ANARE	Australian national Antarctic research expeditions
ASPA	Antarctic specially protected area
BASH	British Antarctic survey herbarium
BEAST	Bayesian evolutionary analysis and sampling trees
BEUTi	Bayesian evolutionary analysis utility
BIC	Bayesian information criterion
DMSO	Dimethyl sulfoxide
dNTPs	deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
GTR	General time reversible
HIMIE	Heard and Macquarie Island expedition
HKY	Hasegawa, Kishino and Yano
LGM	Last glacial maximum
MCMC	Markov-chain-monte-carlo
MEGA	Molecular evolution and genetic analysis
MRCA	Most recent common ancestor
MYA	Million years ago
NYBGH	New York botanic gardens herbarium
PCMEGA	Prince Charles Mountains Expedition: Germany and Australia
PP	Posterior Probability
TN93	Tamura and Nei 1993
tRNA	Transfer ribonucleic acid
RAPD	Randomly amplified polymorphic deoxyribonucleic acid

Common Abbreviation

For simplicity, common abbreviations have not been given in full within the body of the thesis.

bp	Base pairs
DNA	Deoxyribonucleic acid
dH_2O	Distilled water
GPS	Global positioning system
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase A	Ribonuclease A

Gene Abbreviations

For simplicity gene abbreviations only mentioned once in text have not been given in full within the body of the thesis.

5.8S	5.8S ribosomal RNA
18S	18S ribosomal RNA
23S	23S ribosomal RNA
atp	Adenosine triphosphatase
COI	Cytochrome oxidase one
ITS	Internal transcribed spacer
ITS1	Internal transcribed spacer 1
ITS2	Internal transcribed spacer 2
matK	Megakaryocyte-associated tyrosine kinase
nad	Nicotinamide adenine dinucleotide
psb	Photosystem B
rbcL	Ribulose-bisphosphate carboxylase large subunit
rpo	Ribonucleic acid polymerase
rps4	Ribosomal protein subunit 4
trn	Transfer ribonucleic acid

ABSTRACT

Mosses are the key floral component of Antarctic terrestrial ecosystems and provide a model system for the *in vivo* study of freeze tolerance and ultraviolet-B radiation damage in plants. Furthermore, in the Windmill Islands region of East Antarctica, these plants form part of a long term biodiversity study, using mosses as a proxy for the effects of climate change on Antarctic terrestrial ecosystems. However, morphological similarities between Antarctic moss species may make biodiversity measurements error prone. Furthermore, the species status and phylogenetic relationships of Windmill Islands mosses have not been examined using molecular techniques.

To improve the current knowledge of Windmill Islands mosses, phylogenies were constructed for the three most common moss species: *Bryum pseudotriquetrum*, *Ceratodon purpureus* and *Schistidium antarctici*. Phylogenies were constructed based on the chloroplast ribosomal protein subunit 4 (*rps*4) and nuclear non-coding internal transcribed spacer (ITS) genes. Analyses were carried out separately on each species using a Bayesian Markov-chain-monte-carlo (MCMC) analysis and, where sufficient multilocus data was collected, a coalescent-based species delimitation was performed. Additionally, the ability of *rps*4 and ITS to be used for the identification of the three mosses described above, was assessed empirically.

Bryum pseudotriquetrum was the only species for which sufficient multilocus data were obtained to perform both Bayesian MCMC analysis and coalescent-based species delimitation. In *B. pseudotriquetrum*, these analyses indicated cryptic species in the Bunger Hills, Windmill Islands and Prince Charles Mountain regions of Antarctica, when compared to the Northern Hemisphere holotype. For the remaining two species sufficient multilocus

data were not obtained to perform both analyses. Instead Bayesian MCMC analyses were carried out on *C. purpureus rps*4 data and *S. antarctici* ITS data. Analyses indicated that *C. purpureus* populations from East Antarctica are most closely related to populations from Australia and Heard Island, and that populations from the Antarctic Peninsula are most closely related to populations from the Northern Hemisphere. In *S. antarctici* analysis incorporating ITS data from all other *Schistidium* species, revealed that *S. antarctici* is a distinct species, separate from all other *Schistidium* species incorporated into the analysis. Furthermore, it was revealed that the most closely related taxa to *S. antarctici* are cold climate Northern Hemisphere species. This study established that either *rps*4 or ITS could be used to accurately identify *C. purpureus* and *S. antarctici*. However, insufficient variation in the *rps*4 gene indicated that it could not be used alone to unambiguously identify *B. pseudotriquetrum*.

This study has elucidated cryptic species, origins and taxonomic groupings for three species of Antarctic moss: *B. pseudotriquetrum, C. purpureus*, and *S. antarctici*, respectively. In addition it has been shown that ITS and *rps*4 can provide an effective tool for phylogenetic reconstructions and identification of Windmill Islands mosses.

1 INTRODUCTION

1.1 Antarctica

At Earth's southernmost pole, Antarctica is one of the most isolated continents on the planet, containing approximately 70% of the world's fresh water and covering 14 million km^2 (Fox *et al.*, 1994). Antarctica is a neutral continent containing no permanent human population and inhabited by approximately 4,000 researchers from 30 countries, who occupy 40 permanent Antarctic bases (Australian Antarctic Division, 2013). Australia currently operates three Antarctic stations; Mawson (est. 1954), Davis (est. 1957), and Casey (est. 1988), (Australian Antarctic Division, 2013). These stations are located below latitude 60°S and between longitudes 160°E and 45°E, in an area known as the Australian Antarctic Territory (Australian Antarctic Division, 2013) (Figure 1).

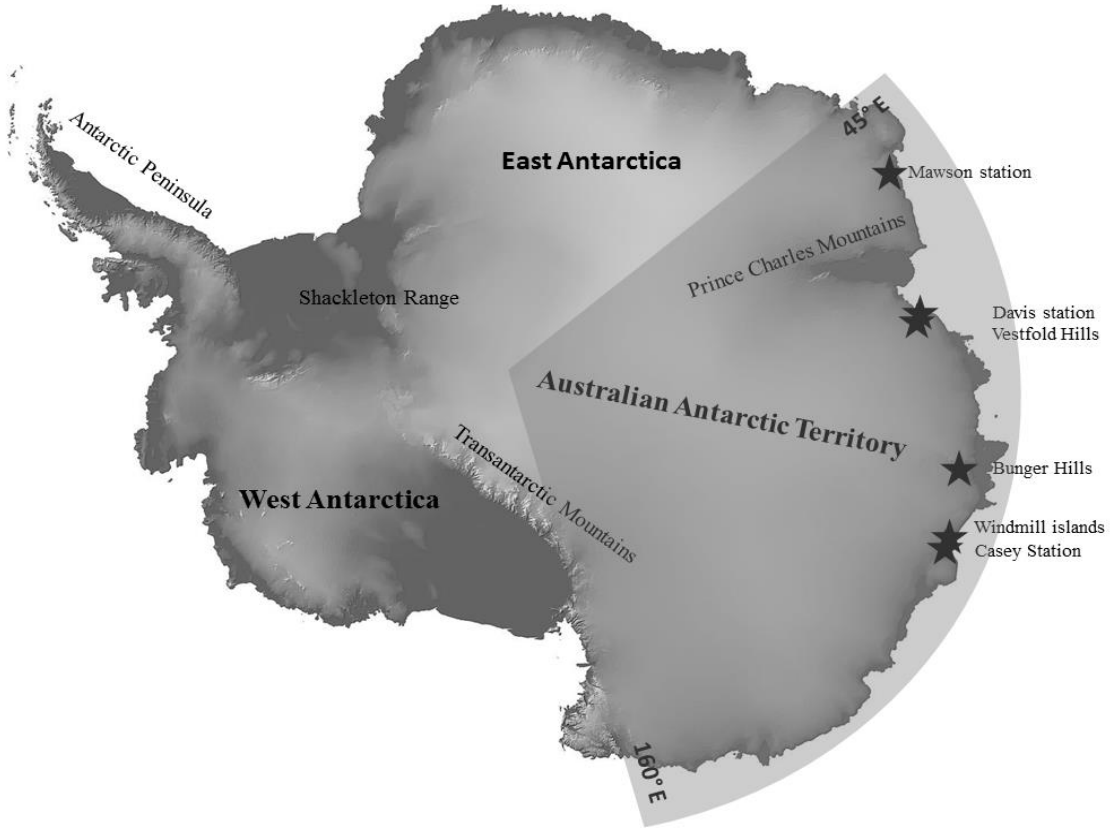

Figure 1. Location of the Australian Antarctic Territory (shaded), Australian Antarctic Stations and key geographical landmarks (stars) (modified from National Aeronautics and Space Administration, 2002).

In 1958, twelve nations (Argentina, Australia, Belgium, Chile, France, Japan, New Zealand, Norway, South Africa, United Kingdom, United States and the USSR) active in Antarctica during the International Geophysical Year signed the Antarctic treaty, which stipulates that Antarctica is to be "…a natural reserve, devoted to peace and science" (Hanessian, 1960). Since this period Antarctica has provided largely pristine and unique environments which are studied by scientists from Australia and around the world.

1.1.1 Geobiological history

The geobiological history of Antarctica is characterised by its separation from the super continent Gondwana during the late Mesozoic, circa 100 million years ago (MYA). During this period, Gondwana experienced a tropical climate (Francis *et al.*, 2002), with flora originally dominated by bryophytes, gymnosperms (non-flowering plants; 419-120 MYA) and later angiosperms (flowering plants; circa 120 MYA–present) (McLoughlin, 2001). Following the subsequent breakup of Gondwana, the flora and fauna diversified (McLoughlin, 2001) and the Antarctic land portion moved south over the Southern Hemisphere (Convey *et al.*, 2008b). Fossil evidence from this period suggests that the terrestrial flora and marine fauna survived this movement (Clarke *et al.*, 1989; Francis and Poole, 2002). The movement of Antarctica southwards did not lead to its direct separation from other continental land-masses; instead land bridges connected the Antarctic Peninsula to South America (Drake Passage) and East Antarctica to Australia (Tasman gateway). The presence of these land bridges allowed for the continuous movement of flora and fauna onto the Antarctic continent, forming two isolated groups of organisms separated by the Transantarctic Mountains (McLoughlin, 2001) (Figure 1). Circa 40 MYA, the opening of both the Drake Passage and Tasman Gateway lead to the establishment of the Antarctic circumpolar current (ACC), which effectively insulated Antarctica from the surrounding

oceans (Convey *et al.*, 2008a). Thermal isolation by the ACC led to the cooling of Antarctica and, when combined with shifts in the earth's orbital parameters and decreasing atmospheric CO_2 levels, led to the glaciation of Antarctica and global cooling (DeConto *et al.*, 2003; Dahms *et al.*, 2012). During this period of global cooling, known as the last glacial maximum (LGM), Antarctica was covered in a permanent ice sheet, which is considered to have caused the extinction of complex terrestrial flora and fauna between 12 and 14 MYA (Ashworth *et al.*, 2007; Lewis *et al.*, 2007).

1.1.2 Persistence of ancient Antarctic life

Additional Antarctic fossil evidence suggests extinction events that extend from the Miocene (23-5 MYA) to the early-Pleistocene (5-2.5 MYA) (Convey *et al.*, 2008a). However there is a growing body of evidence suggesting that isolated relic populations (refugial) may have survived the glaciation of Antarctica. These relic populations may not be represented in the fossil record due to low population densities or rare fossilization. Nevertheless, such species would have survived through the LGM in small isolated communities, restricted to ice-free mountains and rocky outcrops known as nunataks (Convey *et al.*, 2008). Evidence from ^{10}Be and ^{26}Al isotope ratios have shown that areas within the Shackleton Range (Fogwill *et al.*, 2004), the Antarctic Peninsula (Bentley *et al.*, 2006) and Prince Charles Mountains (Fink *et al.*, 2006) have remained ice-free during the late Pleistocene and may have provided ice-free areas capable of supporting terrestrial life. Presently terrestrial Antarctic fauna consists of 15 species of springtails (Collembola), 25 species of mites (Acarina) (Hogg *et al.*, 2002), 14 species of nematodes (Nemathelminthes) (Wharton, 2003) and eight or more species of water bears (Tardigrades) (McInnes *et al.*, 1998). Terrestrial flora is limited to 24 species of mosses (Bryophyta), 1 species of liverwort (Marchantiophyta), 92 species of lichens (Ascomycota and Basidiomycota) and numerous species of algae (reviewed in Bramley-

Alves *et al.,* in press). Evidence relating to the persistence of these Antarctic terrestrial organisms is conflicting. Biogeographical studies suggest that some endemic organisms may be refugial, while others, such as tardigrades, mosses and liverworts, appear to show a continental distribution, suggesting wide dispersal or recent colonisation (Convey *et al.*, 2008b).

Evidence of refugial Antarctic life can be seen in the distribution of modern Antarctic species. This was first reported for the distribution of the nematode *Panagrolaimus magnivulvatus*, which is found only in soils of isolated nunataks (Sohlenius *et al.*, 1996). This same isolation was later observed in endemic mites of the genus *Maudheimia*, which are also present on nunataks throughout the Antarctic continent (Marshall *et al.*, 2000). Survival through periods of Antarctic glaciation was corroborated by phylogenetic evidence suggesting post-Gondwanan speciation (Marshall and Coetzee, 2000). Furthermore, a study of mite biogeography by Pugh and Convey (2000) revealed that maritime and mainland endemic species may have descended from Mesozoic (252-66 MYA) ancestors from the Antarctic Peninsula. Within East Antarctica two previously unknown populations of copepods were discovered with one species suggested to have existed since before the LGM, due to its large distribution and endemic status (Bayly *et al.*, 2003). While on the Antarctic Peninsula a higher diversity of endemic soil microbes (Lawley *et al.*, 2004) and nematodes (Maslen *et al.*, 2006) seems to suggest a recent glacial refuge. In recent years, molecular evidence for refugial populations of springtails living in the Transantarctic Mountains has provided evidence that these springtails have been present for up to one million years (Stevens *et al.*, 2003). This study was later corroborated by evidence of a deep divergence (Stevens *et al.*, 2006) and limited shared haplotypes (adjacent alleles with shared inheritance) (Stevens *et al.*, 2007a) in springtails found within the Transantarctic Mountains. Molecular

studies of cyanobacterial populations near the Transantarctic Mountains also appear to show higher levels of endemic species than previously thought (Taton *et al.*, 2006a; Taton *et al.*, 2006b), suggesting that the Transantarctic Mountains may have provided small ice-free oases during glacial periods.

The evidence of refugial invertebrate populations on mainland Antarctica is present in both molecular and biogeographic studies. However, the same strong argument cannot be made for Antarctic flora. This is in part due to their wide range of dispersal (Longton, 1988), poor representation in the fossil record (Miller, 1982), evidence of anthropogenic introduction of spores (Clarke *et al.*, 2009) and difficulty in species identification (Ochi, 1979). The difficulty in identifying refugial populations of Antarctic flora may be partly due to speciation without morphological variation (cryptic speciation), which may confound the use of morphology to discover deep divergences. Molecular studies of Antarctic mosses suggest differing scenarios in different species. In the cosmopolitan moss *Ceratodon purpureus,* a global study by McDaniel & Shaw (2005) has shown that a single Antarctic population of *C. purpureus* is closely related to Australasian *C. purpureus* populations. The high genetic similarity between Australasian and Antarctic *C. purpureus* populations suggests the recent and possibly continuous re-colonisation of the Antarctic mainland. This is contrasted by low levels of variability in Antarctic populations of the moss *Bryum argenteum*, when compared to populations of the same species from non-Antarctic locations (Simon *et al.*, 2010). Overall, evidence seems to suggest a combination of both coastal and wind driven introduction of Antarctic flora and possible refugial populations restricted to isolated rocky nunataks and mountain regions. However, the anthropogenic introduction of genetic diversity may limit the identification of such refugial colonies to relatively pristine areas.

1.2 Bryophytes lifecycles

Antarctic terrestrial flora consists primarily of bryophytes, which are a group of non-vascular terrestrial plants that includes liverworts (Marchantiophyta), hornworts (Anthrocerophyta) and mosses (Bryophyta). Bryophytes are found on every continent on Earth, but form the dominant floral components in extremes of cold, in Antarctica, the unique bryophyte lifecycle allows for bryophyte (but not vascular plant) survival. The bryophyte lifecycle consists of a dominant haploid stage, (known as the gametophyte) and a diploid stage (known as the sporophyte). The gametophyte is a photosynthetic leafy or thallus (leaf-less) structure, which functions to support both the male (antheridium) and female (archegonium) reproductive organs, and is attached to its substrate through a hair like structure known as a rhizoid. Bryophytes can reproduce sexually or asexually, both by fragmentation and dispersal by wind, water or animals. Sexual reproduction occurs when sperm is transported from the antheridium of one gametophyte to the archegonium of another, by either water or invertebrates (Cronberg *et al.*, 2006; Rosenstiel *et al.*, 2012). The fertilisation process gives rise to a sporophyte. The sporophyte grows attached to the gametophyte by a structure known as a foot and is dependent on the parent gametophyte for both water and nutrients. The sporophyte structure consists of a protective capsule that produces diploid spores. These spores are transported by wind, water or animals to new locations. Upon exposure to the appropriate environmental conditions, they germinate to give rise to the next generation (Figure 2).

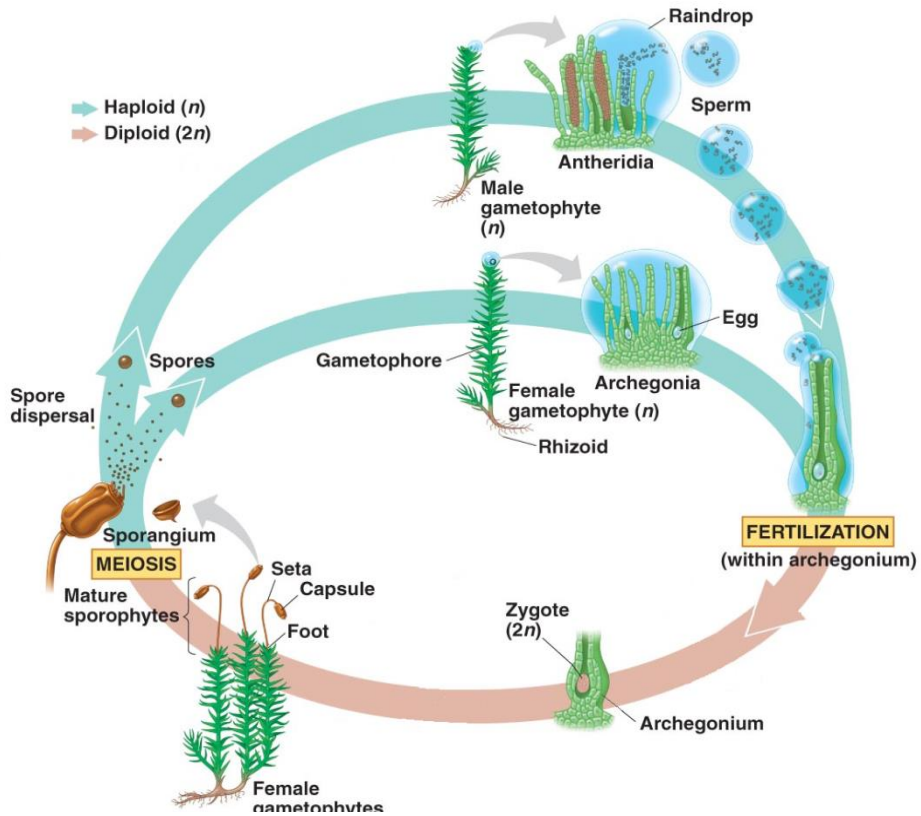

Figure 2. Typical bryophyte lifecycle, where *n* refers to haploid life stages (blue) and 2*n* refers to diploid life stages (pink). Morphological features represent those of a typical moss. (modified from Brotherton *et al.*, 2008).

The lifecycle of Bryophytes allows for wide dispersal, while the rhizoid allows for attachment to substrate poor surfaces. Additionally, the non-vascular nature of bryophytes allows for complete desiccation and freezing. It is these features which have allowed bryophytes to dominate the Antarctic environment.

1.2.1 Moss identification

Of the Antarctic bryophytes, mosses are the most abundant. Mosses are of particular importance, as they grow fast enough (unlike lichens) and are permanent enough (unlike algae) to serve as good model systems for the *in vivo* studies of high ultraviolet-B radiation and freeze tolerance in plants (Takács *et al.*, 1999; Newsham *et al.*, 2002; Dunn *et al.*, 2006; Clarke *et al.*, 2008; Newsham *et al.*, 2009; Turnbull *et al.*, 2009). As mentioned previously, Antarctica hosts only a single species of liverwort (*Cephaloziella varians*), which differs

from mosses both in its macro appearance and the presence of a multicellular rhizoid and unique sporophyte structure.

In temperate regions, sporophytes provide the main diagnostic feature for identifying bryophytes, and in mosses allow for species level identification (Shaw and Renzaglia, 2004). Sporophytes are essentially spore capsules suspended above moss gametophytes by a long stalk known as a seta. In particular, it is the capsule that contains the diagnostic morphological features. These include the calyptra, (a protective sheath covering the capsule), the operculum (a plug-like structure sealing the capsule) and the peristome teeth (finger-like projections extending from the capsule) (Figure 3).

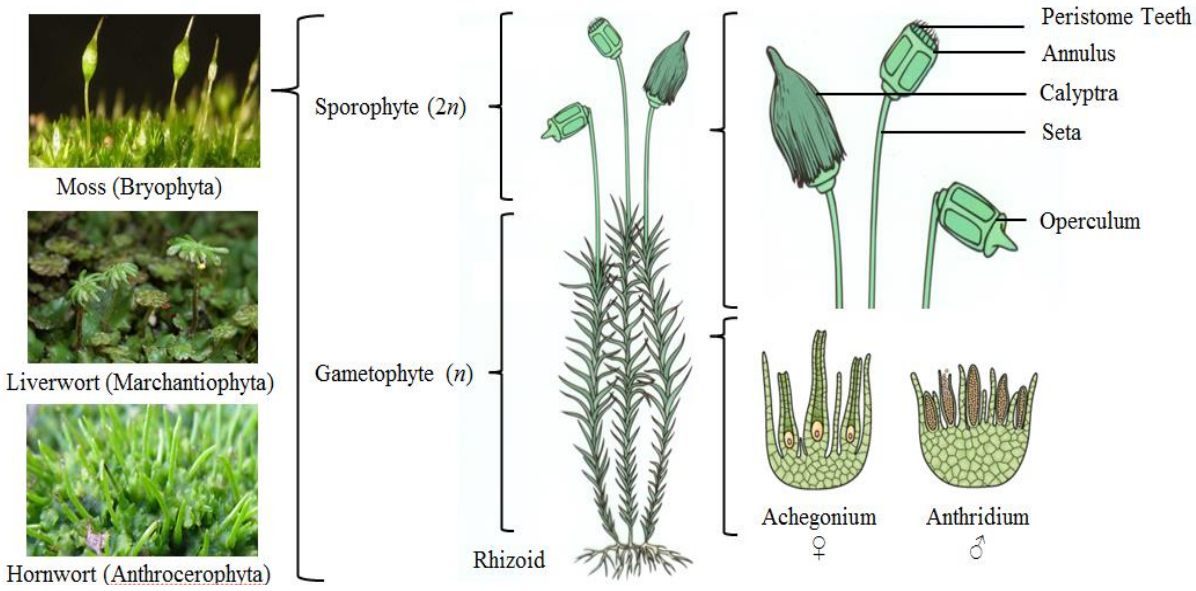

Figure 3. General morphological features of the three bryophyte lineages (left), and a typical moss gametophyte and sporophyte (right), with *n* representing the haploid life stage and 2*n* representing the diploid life stage (modified from Hamel, 2013).

The morphological variability in sporophytes has been used to identify the 12,700 moss species currently known to science (Crosby *et al.*, 1999). The use of sporophytes for the identification of moss species has been carried out since the 1800's (Hedwig, 1801), although it is still problematic. In general, mosses are globally distributed and share highly conserved

morphologies (Shaw *et al.*, 2005). Furthermore, many species rarely produce sporophytes. This is particularly evident in cold climates, where mosses rely primarily upon vegetative reproduction, making species identification challenging (Convey *et al.*, 1993; Smith *et al.*, 2002).

1.2.2 Antarctic mosses and identification

Antarctic mosses present a prime example of challenging morphological identification. Sporophytes have only been recorded in 10% of continental Antarctic mosses, many of which produce sporophytes rarely and only within the more temperate Antarctic Peninsula (Convey and Smith, 1993; Smith and Convey, 2002). This limited sporophyte production in Antarctic mosses is due to a number of factors, including: possible imbalance in sex ratios, large degrees of isolation between sexes and most importantly, environmental conditions, which may limit gametophyte and sporophyte maturation and hinder sperm transport (Smith and Convey, 2002). The lack of sporophyte production initially led to an explosion in moss species during Antarctic exploration in the 19th century, based primarily on the assumption that isolated populations must represent different species (Shaw, 2001). However, in recent years many of these species have been re-classified based upon leaf and cell morphology and assumed to be morphological variants of more globally distributed moss species (Ochyra *et al.*, 2008).

1.2.2.1 East Antarctic mosses

Along the East Antarctic coast, mosses are found in small rocky and protected oases, where they commonly grow in nutrient rich strata, deposited by ancient penguin colonies (Emslie *et al.*, 2005). These oases occur in a number of locations, but the most commonly observed are the Vestfold Hills, Bunger Hills and Windmill Islands. In particular, the Windmill Islands are composed of nine ice bound islands and five small peninsulas spread over a distance of 40

km (Figure 4). Located on the Bailey Peninsula within the Windmill Islands is Casey Station (a permanent Australian research station), which provides a base of operation for research on the surrounding peninsulas. Less than a kilometre from Casey Station is the Antarctic specially protected area 135 (ASPA135), an area protected from human impact under the Antarctic Treaty (Hanessian, 1960). Within ASPA135, terrestrial flora are studied as part of a long term biodiversity study, monitoring the effects of climate change on Antarctic terrestrial ecosystems (Australian Antarctic Division, 2013).

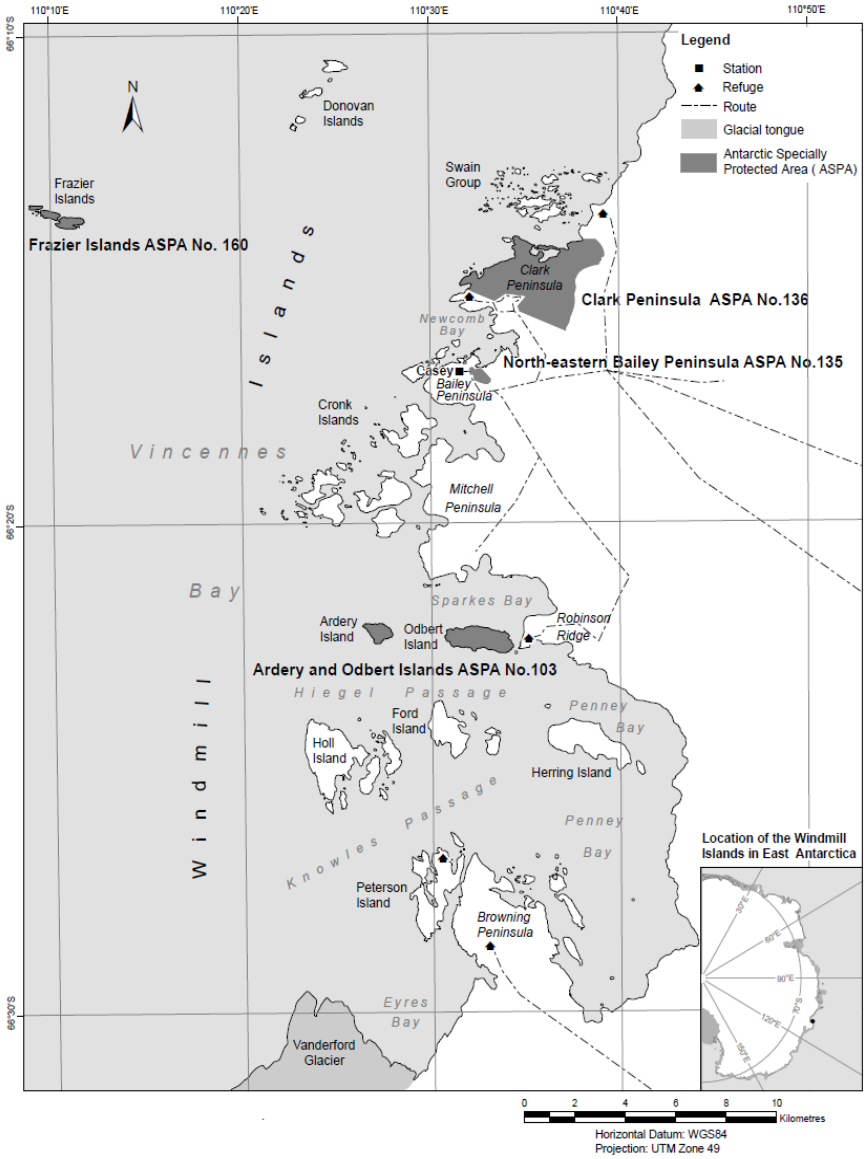

Figure 4. Map of the Windmill Islands region, showing the location of the five ice-bound peninsulas, Casey Station and ASPA135. Inset, location of the Windmill Islands with respect to the East Antarctic coast. (Australian Antarctic Data Centre, 2013).

Within the Windmill Islands region of Antarctica, the most abundant moss species are: *Bryum pseudotriquetrum*, *Ceratodon purpureus* and *Schistidium antarctici* (formerly *Grimmia antarctici*), each of which are also found in abundance along the East Antarctic coast (Ochyra *et al.*, 2008). In the ASPA135 long term biodiversity study, these species are identified exclusively by leaf and cell morphologies, which have been shown to be highly variable in response to water and ultraviolet irradiance (Robinson *et al.*, 2000; Robinson *et al.*, 2005). With respect to the Windmill Islands mosses, the species *B. pseudotriquetrum* and *C. purpureus* are globally distributed and morphologically variable. However, both species are morphologically distinct from one another, limiting confusion. On the other hand, the Antarctic endemic species *S. antarctici* is morphologically very similar to *C. purpureus* and, as a result, is commonly misidentified.

Genetic studies on the three Windmill Islands moss species have occurred in recent years. Initially these studies focused on population genetics using allozymes (enzyme variants) (Melick *et al.*, 1994) and later randomly amplified polymorphic DNA (RAPD) (Skotnicki *et al.*, 1998a; Skotnicki *et al.*, 1998c; Skotnicki *et al.*, 1999). However, these studies produced vastly conflicting results, with allozymes identifying populations as clonal and RAPDs suggesting high levels of variability. Later studies identified fungal contamination that inflated the measurement of genetic variability using RAPDs (Stevens *et al.*, 2007b), while a microsatellite study of *C. purpureus* confirmed that populations were largely but not exclusively clonal (Clarke *et al.*, 2009). Studies addressing the identification of Antarctic mosses have focused on the use of the nuclear non-coding internal transcribed spacer (ITS) region (Skotnicki *et al.*, 2005) and a combination of the ITS and the chloroplast ribosomal protein subunit 4 (*rps*4). These studies identified mosses based on sequence data from morphologically similar herbarium specimens. They showed, through phylogenetic analysis,

that ITS and *rps*4 can successfully distinguish the moss species *B. pseudotriquetrum, S. antarctici, Coscinodon lawianus* and *C. purpureus* (Skotnicki *et al.*, 2012). However, no study has yet examined the population origins or phylogenetic relationships of these same species between Antarctic and non-Antarctic locations.

In particular, unpublished phylogenetic work (Kato pers. comm.) suggests that *B. pseudotriquetrum* may actually be a cryptic Northern and Southern Hemisphere species. Cryptic species have been identified in the Antarctic moss species *Bryum argenteum* (Simon *et al.*, 2010) and the trans-Antarctic moss *Pyrrhobryum mnioides* (McDaniel *et al.*, 2003). With regards to *C. purpureus*, a global study of the taxa identified separate Northern and Southern Hemisphere populations, but included only a single replicate from East Antarctica (McDaniel and Shaw, 2005). Furthermore, a microsatellite study showed increased genetic variability with proximity to Antarctic research stations, suggesting the human introduction of spores (Clarke *et al.*, 2009). Finally, with reference to the Antarctic endemic species *S. antarctici*, no molecular studies of the genus *Schistidium* have incorporated *S. antarctici* samples. As such, its species status and placement within the *Schistidium* genus is not known.

The origins, species status and phylogenetic relationships of Windmill Islands mosses to those from pan Antarctic and non-Antarctic locations have not yet been comprehensively studied. Therefore, prediction of the effects of climate change, based upon Windmill Islands mosses, cannot be confidently applied to other Antarctic location. As such, phylogenetic analyses of the three most common Windmill Islands mosses can elucidate how representative Windmill Islands mosses are compared to the same species from other locations, and improve the overall understanding of the species.

1.3 Molecular markers

In order to carry out phylogenetic analyses and species delimitation (the use of multilocus data to establish the boundaries of species) on the Antarctic moss species: *B. pseudotriquetrum, C. purpureus* and *S. antarctici* the appropriate molecular markers must be selected. In the case of East Antarctic mosses, this relates to the delimitation of haploid and essentially clonal populations. Due to these requirements, molecular markers containing both high and low levels of variability are required. Markers with high levels of variability are useful for delimiting closely related or clonal taxa, while markers with low levels of variability are useful for separating distantly related populations (as in the case of refugial populations). The number of molecular markers used must also be taken into consideration. There is no definitive number of markers required for a phylogenetic study. However, the majority of bryophyte phylogenetic studies have utilised 2-3 markers (Stech *et al.,* 2010), making 2-3 markers both comparable and efficient in the time required to sequence multiple loci from each sample. Furthermore, the molecular markers used should be obtained from multiple cellular compartments in order to account for the different evolutionary histories of nuclear and plastid genomes. In relation to land plants, a number of molecular markers have been used: the *atpF–atpH* spacer, *matK, rbcL, rpoB, rpoC1*, the *psbK–psbI* spacer and *trnH–psbA*. However, these molecular markers have not been particularly successful in bryophytes, often only able to discriminate taxa at the genus level (Hassel *et al.,* 2013). A range of molecular markers have been used in genetic studies of Antarctic mosses, including allozymes (Melick *et al.,* 1994), microsatellites (Clarke *et al.,* 2008; Clarke *et al.,* 2009), RAPDs (Skotnicki *et al.,* 1998a; Skotnicki *et al.,* 1998c; Skotnicki *et al.,* 1999), single locus nuclear markers (Skotnicki *et al.,* 2005) and multilocus markers (Skotnicki *et al.,* 2012). Of these techniques, allozymes and microsatellites are not appropriate for the construction of a

phylogeny. However, the use of multilocus markers has proven useful for the phylogenetic analysis of Antarctic moss species (Skotnicki *et al.*, 2012).

For the selection of these markers, a number of factors must be taken into account including comparability, sensitivity and efficiency (Sunnucks, 2000). In the case of comparability, the molecular markers that are chosen must be comparable with markers used in previous studies. In bryophyte molecular studies, the most commonly used markers are: the nuclear ribosomal genes *18S-ITS1-5.8S-ITS2-28S* (collectively known as the ITS region), the mitochondrial nicotinamide adenine dinucleotide (nad) gene, of which the subunit 5 is most commonly used (*nad*5), and the chloroplast transfer ribonucleic acid (tRNA) cluster, which consists of the genes *TrnF-TrnL-TrnT-rps*4-*TrnS*, of which *rps*4 is the most used bryophyte molecular marker (Stech and Quandt, 2010). From this collection of markers, the nuclear marker ITS and chloroplast marker *rps*4 have proven to be the most comparable and efficient in terms of easy amplification (Stech and Quandt, 2010) and prior use in Antarctic mosses (Skotnicki *et al.*, 2005; Skotnicki *et al.*, 2012). The ITS region provides a highly variable marker for the study of population variability in essentially clonal populations, while the less variable marker *rps*4 also satisfies the requirement for markers from multiple cellular compartments. As such, the molecular markers ITS and *rps*4 provide an effective combination from multiple cellular compartments for the identification of Antarctic moss species and the construction of phylogenies.

1.3.1 Internal transcribed spacer

The ITS region is an approximately 1,000 bp stretch of nuclear ribosomal RNA (nrRNA) coding genes, which can exist as copies with up to 3,000 repeats (Rogers *et al.*, 1987). These copies are homogenised by a process known as concerted evolution (Stech and Quandt,

2010), resulting in an easily amplified region with a high copy number, whilst overcoming the issue of paralogs associated with other nuclear markers. In terms of use, the complete ITS region is one of the most commonly used bryophyte nuclear markers and is almost exclusively amplified using the universal primers designed by White, *et al.,* (1990). These primers, amplify the complete sequences of *ITS1-5.8S-ITS2* and include fragments of both the flanking *18S* and *26S* rRNA genes. However, the modified amplification primer ITSHP5 has also been utilised to amplify the same region (Simon *et al.*, 2010). The amplification of the ITS region exclusively involves the amplification of the complete ITS region, including fragments of the *18S* and *26S* genes. However, the analysis of this marker rarely utilises the complete ITS region, due to the presence of large length variations in *ITS1* between species. To overcome this issue, often only the ITS2 region is analysed, with the information contained within this region sufficient for species identification and also for phylogenetic analyses in Antarctic mosses (Skotnicki *et al.*, 2005). This makes ITS a useful nuclear marker for the construction of phylogenies between closely related populations and for species delimitation.

1.3.2 Ribosomal protein subunit 4

The marker *rps*4 is a fast-evolving protein coding gene found within the chloroplast transfer RNA (tRNA) region. This region consists of four tRNA genes and *rps*4 (*TrnF-TrnL-TrnT-rps*4-*TrnS*). The marker *rps*4 is one of the most commonly used bryophyte molecular markers, as it provides higher levels of information over the commonly employed plant chloroplast marker *rbcL* (O'Brien *et al.*, 2007; Quandt *et al.*, 2007). In addition, due to the high abundance of chloroplast DNA in total plant DNA, its amplification is relatively easy (Souza-Chies *et al.*, 1997). The use of *rps*4 for molecular studies was established by the development of universal primers by Taberlet *et al.*, (1991) and their first use in a

phylogenetic study of grasses (Nadot *et al.*, 1994). In mosses, *rps*4 has primarily been employed as a single locus marker for the delimitation of moss genera and more recently for the delimitation of moss species when used as part of a multilocus approach (Magombo, 2003; Pedersen *et al.*, 2007; Skotnicki *et al.*, 2012). The use of *rps*4 for the study of Antarctic mosses is relatively new, with the first study published by Skotnicki *et al.*, (2012). This study used *rps*4 and the ITS region in order to identify moss species from the Prince Charles Mountains and in doing so provided sequence data for the two common East Antarctic species *B. pseudotriquetrum* and *S. antarctici*. Although *rps*4 is not appropriate for species level delimitation or barcoding (Stech and Quandt, 2010), this study demonstrated that *rps*4 when combined with the ITS region can be effectively used to identify Antarctic mosses, making *rps*4 a useful and comparable marker.

1.4 Phylogenetic methods and species delimitation

In order to use the molecular markers ITS and *rps*4 to identify origins, refugial populations and cryptic species, a method of phylogenetic analysis that can incorporate multiple markers and delimit species in an unbiased manner is needed. The most common methods of phylogenetic analyses are Maximum Likelihood, and Bayesian Methods. Maximum Likelihood methods of analysis use statistical measures of likelihood based upon prior assumptions in order to calculate the most likely relationship between taxa. To overcome the subjectivities (use of prior assumptions) associated with Likelihood methods, Bayesian methods of analysis can be used. In Bayesian analysis, prior assumptions are established computationally based upon the data. In order to establish these priors in a manner currently computationally possible with data sets greater than a few sequences, a Markov-chain-monte-carlo (MCMC) method is employed (Joseph, 2004). This method involves changing each parameter in a starting prior and establishing the probability of this new prior based upon the

data. If the new prior is more probable than the previous, then the prior is accepted and the process is repeated. The number of times this process is repeated is dependent upon the time required for the MCMC algorithm to reach an equilibrium (known as the burn-in time), in which the probabilities of newly generated priors are not improving; at this point the prior with the highest probability is selected and used for further analysis. This method is known as a Bayesian MCMC analysis, and can be carried out using the software packages MrBayes (Huelsenbeck et al., 2001; Ronquist et al., 2003) and Bayesian evolutionary analysis and sampling trees (BEAST) (Drummond et al., 2007).

As well as requiring a phylogenetic analysis method which is as unbiased as possible, a method that can take into account multiple loci is needed. This is generally achieved by concatenating sequence data. However concatenation can often give biased results, as the locus containing the highest level of variation often determines the resulting phylogenetic trees. As such, a method which analyses each loci separately, but incorporates them into a single tree is required. This can be achieved using coalescent theory; coalescent theory works by constructing a multispecies coalescent, which is essentially a species tree inferred from the individual gene trees of each locus. Coalescent theory has recently been incorporated into the modified version of BEAST known as *BEAST allowing for the incorporation of multilocus data without concatenation (Heled *et al.*, 2010).

Finally, in order to identify cryptic species, a testable species definition is required. In general, a species is considered an independently evolving population of organisms with no (allopatric) or little (parapatric) gene flow, and has diverged sufficiently enough to be distinct from its most recent common ancestor (MRCA) (Fujita *et al.*, 2012). In order to then identify allopatric and parapatric species with significant divergence from the MRCA proxies are

used. Until recently this has been achieved through the use of morphology (known as the morphological species concept), or through the inability of one organism to produce fertile offspring with another (known as the biological species concept). Currently, there are 26 different species concepts (Wilkins, 2002), with many researchers opting for an integrative taxonomy, whereby different species concepts are applied under different circumstances (Mishler *et al.*, 1987; Will *et al.*, 2005; Fujita *et al.*, 2012). However, none of these species concepts can adequately delimit species in all circumstances, with most criteria for species delimitation exhibiting much subjectivity. Since the development of techniques such as PCR and DNA sequencing, molecular techniques have become one of the primary methods of delimiting species.

In particular, the use of DNA barcoding has been one of the primary methods for species identification. DNA barcoding was first proposed by Hebert *et al.*, (2003) who proposed the use of the cytochrome oxidase I (*COI*) gene as a barcode for the delimitation of species. The use of *COI* is advantageous as it is a highly conserved, short (648 bp) region of mitochondrial DNA found within all eukaryotes, making it easily amplified by universal primers. Nevertheless, the use of *COI* for DNA barcoding has been subject to much criticism relating to its ability to effectively separate species. In order to effectively delimit species using a DNA barcode, the within species divergence must be less than the between species divergence (Moritz *et al.*, 2004). However, empirical studies have reported that this is not always the case (Waugh, 2007). Confounding this are the differing degrees of *COI* variability between species, such that the required variability for two populations to be considered separate species will depend on the species being examined. In the case of plants, *COI* has been found to evolve too slowly to be useful for the delimitation of plant species (Barcode of Life Database, 2012) and as a result the genes *matK* and *rbcL* have been generally accepted

as DNA barcodes in plants (Hollingsworth *et al.,* 2009; Barcode of Life Database, 2012). However, *matK* and *rbcL* do not overcome many of the issues associated with DNA barcoding, nor do they allow for re-construction of evolutionary histories (Will *et al.*, 2005), which can be used to quantify divergence from the MRCA and to identify deep divergences characteristic of refugial populations.

The solution to identifying cryptic species is the use of coalescent-based species delimitation, which is carried out in the software BP&P (Rannala *et al.,* 2003). Similar to analyses that incorporate coalescent theory, coalescent-based species delimitation incorporates multilocus data, considering each locus separately. However, unlike the coalescent-based analyses carried out in *BEAST, coalescent-based species delimitation relies on a guide tree, which in this case is generated through Bayesian MCMC analysis in *BEAST. Once a guide tree is specified, BP&P works to identify the probability of speciation at each node in the tree by statistically quantifying whether branches represent independently evolving allopatric or parapatric populations.

In summary, this study will investigate evidence of refugial populations, origins and cryptic species in the three common Windmill Islands mosses *B. pseudotriquetrum, C. purpureus* and *S. antarctici*. This will be achieved using the molecular markers ITS and *rps*4 and Bayesian MCMC analyses in *BEAST and coalescent-based species delimitation in the software BP&P. Furthermore, this study will empirically assess the ability of ITS and *rps*4 to identify the species mentioned above, in an attempt to improve future Windmill Islands moss biodiversity measurements.

2 METHODS

2.1 Plant material and sample distributions

The plant material used in this investigation was sourced from both recent Antarctic expeditions and herbarium collections and was combined with publicly available sequence data; these are described in greater detail below.

2.1.1 Fresh plant material

Fresh plant material was collected between 2004 and 2013 from three East Antarctic locations (Windmill Islands; Figure 4, Bunger Hills; Figure 5 and Vestfold Hills; **Error! Reference source not found.**), one sub-Antarctic location (Heard Island; Figure 7) and from the University of Wollongong campus (UOW). Within each location several samples were collected from multiple sub-sites (Table 1).

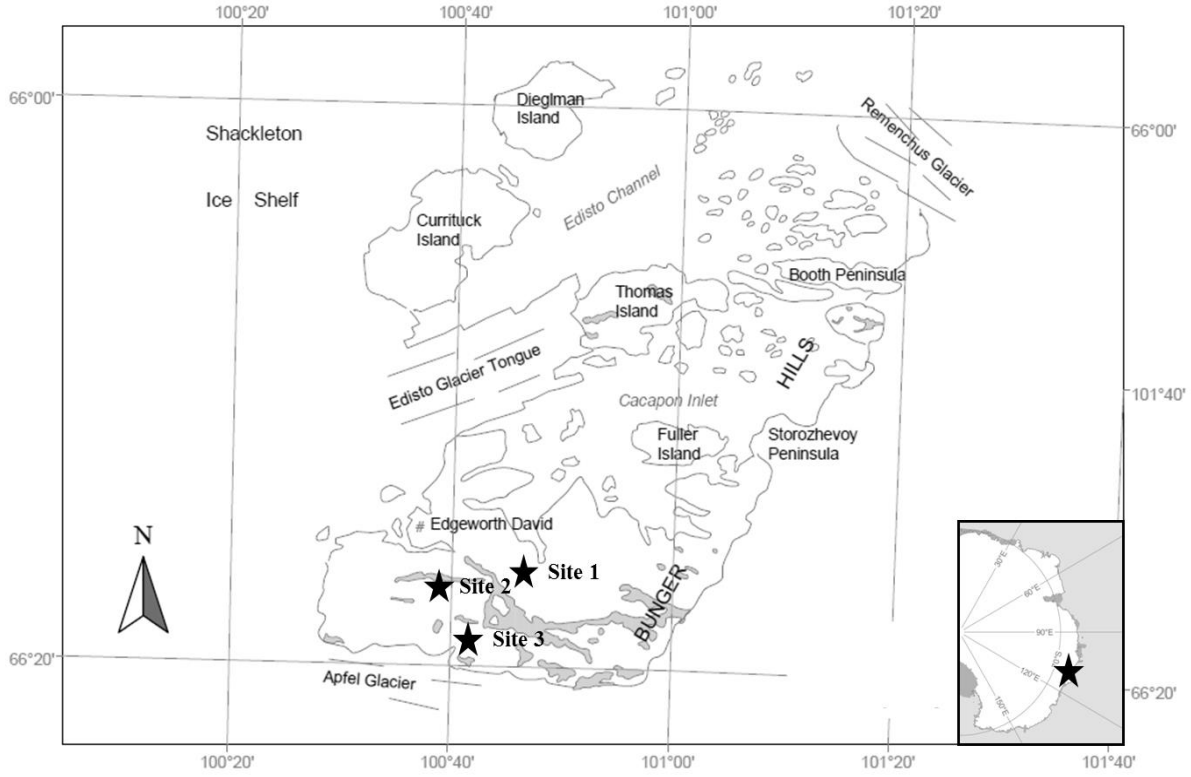

Figure 5. The Bunger Hills, showing the location of moss collection sites (stars). Inset, location of the Bunger Hills with respect to the East Antarctic coast. (Modified from the Australian Antarctic Data Centre, 2013).

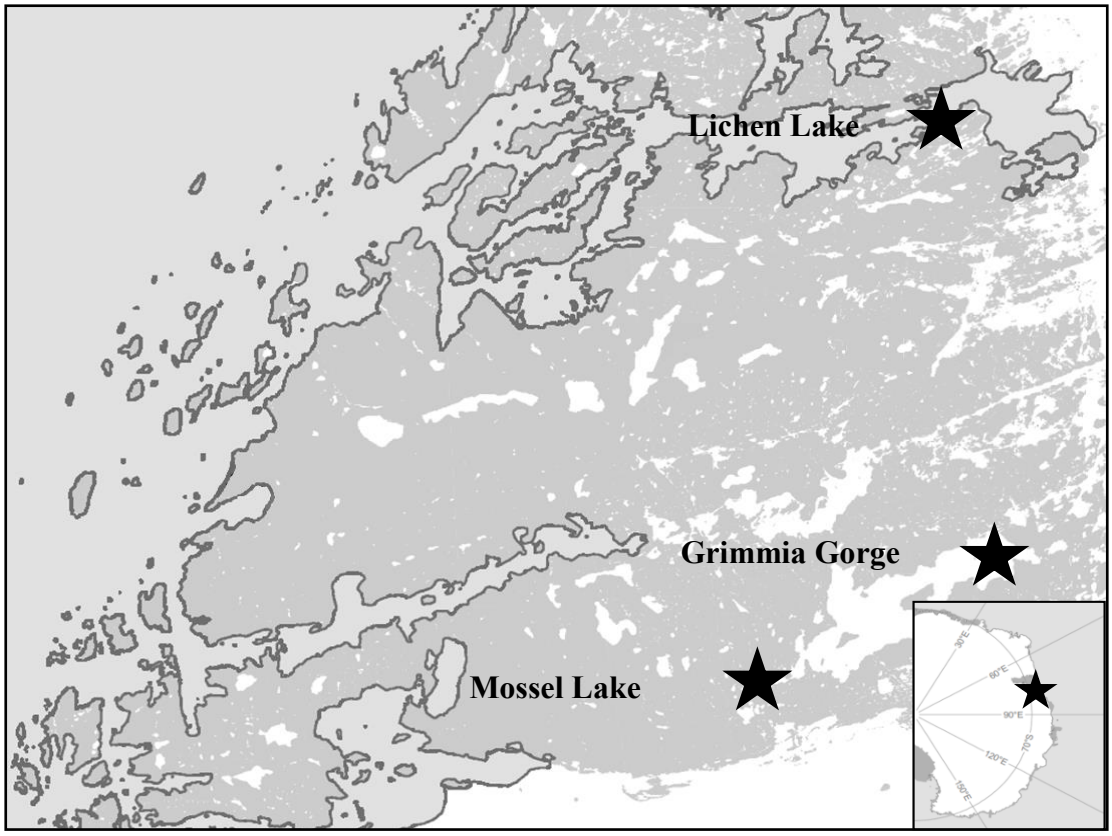

Figure 6. The Vestfold Hills, showing the location of moss collection sites (stars). Inset, location of the Vestfold Hills with respect to the East Antarctic coast. (Modified from the Australian Antarctic Data Centre, 2013).

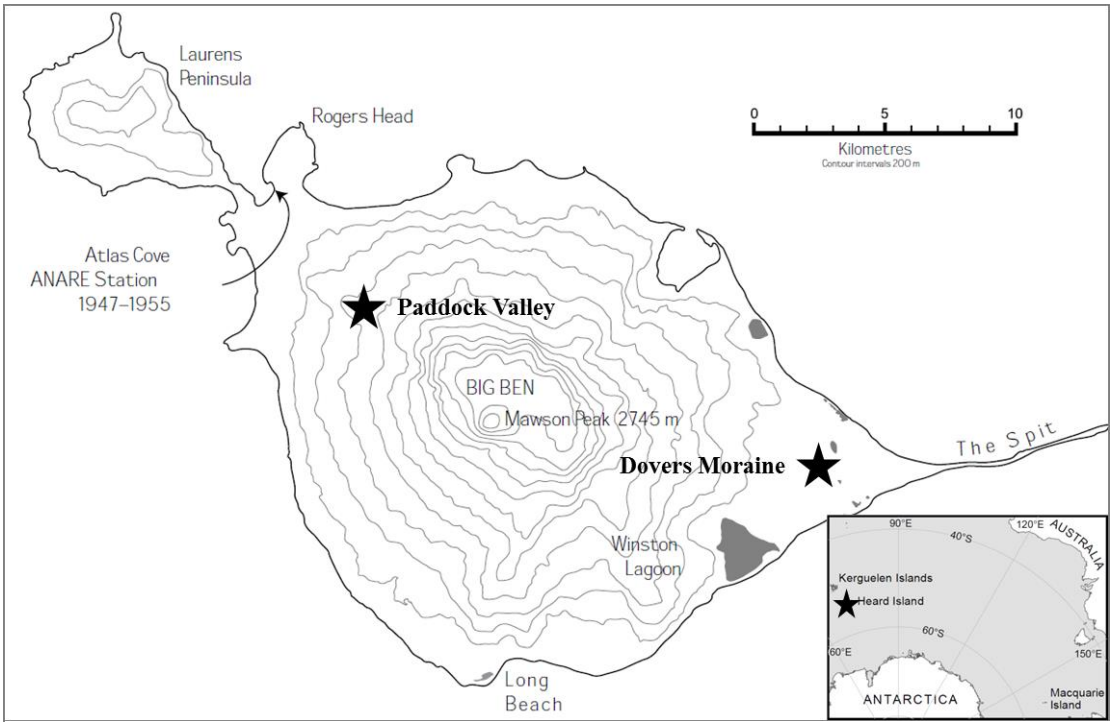

Figure 7. Heard Island, showing the location of moss collections sites (stars). Inset, location of Heard Island with respect to Antarctica and Australia. (Modified from the Australian Antarctic Data Centre, 2013).

Table 1. Fresh samples: Collection locality, with the hemisphere of collection given in bold and underlined, followed by the collection localities in bold and the name of collection sites within each locality (sub-sites) given in italics. Where sub-sites are ambiguously named (no geographical features were present) GPS (latitude & longitude) positions are given. Collection source and year refer to the expedition on which samples were collected, while the collector is given in brackets (full names found in acknowledgments).

Collection locality	**Collection Source and Year**	**Separation of sub-sites**
Southern Hemisphere		
Heard Island	HIMIE 2004 (Turnbull)	15 km
Dovers Moraine		
Paddock Valley		
East Antarctica		
Windmill Islands**,** Budd coast	ANARE 2005 & 2012 (Robinson, Bramley-Alves & Nydahl)	7 Km
Bailey Peninsula		
Browning Peninsula	ANARE 2005 (Robinson & Clarke)	
Clark Peninsula		
Mitchell Peninsula		
Robinson Ridge		
Bunger Hills Knox coast		4 km
Site 1 (-66.29° 100.66°)		
Site 2 (-66.28° 100.78°)		
Site 3(-66.28° 100.68°)		
Vestfold Hills, Ingrid Christian Coast		14 km
Grimmia Gorge		
Lichen Lake		
Mossel Lake		
Australia		
New South Wales		70 km
University of Wollongong	2013 (Author)	
Macquarie University	2005 (Clark)	
Victoria		
Mt Beauty		
Canberra		
Australian National University		

ANARE: Australian national Antarctic research expedition
HIMIE: Heard Island and Macquarie Islands Expedition

Plant material was collected as either individual gametophytes or whole moss clumps. Individual gametophytes were collected using tweezers to sample a small number of photosynthetically active gametophytes, which were placed into sterile 1.5 mL Eppendorf tubes. Whole moss clumps were collected using a small trowel and placed into sterile plastic containers. Samples collected between 2005 and 2013 were collected for genetic and carbon isotope measurements and so were air-dried (until a stable mass was obtained) and stored at -20°C (Windmills Islands, Bunger Hills, Vestfold Hills and UOW). Samples collected in 2004 were collected for measurements of photosynthetic and photo-protective pigments and as such, were frozen in liquid nitrogen and stored at -20°C (Heard Island). Following collection, samples were transported to the University of Wollongong and stored at -20°C prior to analysis. For further details see Clark *et.al* (2008 and 2009).

2.1.2 Herbarium specimens

Herbarium specimens were sourced from the British Antarctic survey herbarium (BASH) and the New York botanic gardens herbarium (NYBGH) (Table 2). In general, herbarium moss samples were air-dried following collection and stored in paper envelopes maintained in temperature (25°C) and humidity (45%) controlled conditions. Following transport to the University of Wollongong, these samples were transferred into sterile Eppendorf tubes and stored at -20°C prior to analysis.

Table 2. Herbarium Samples: Collection locality, with the hemisphere of collection given in bold and underlined, followed by the collection localities in bold and the name of collection sites within each locality (sub-sites) given in italics. Collection source and year refer to the herbarium and year of sample collection, respectively. Morphological identification (ID) refers to the species identification as determined by the source.

Collection locality	Collection Source and Year	Morphological ID
Northern Hemisphere		
Europe		
Conicus, Gottlan, Hovgard, Sweden	DUKE 1966	*C. purpureus*
Africa		
Ain Zahalta Bornmuller, Saudi Arabia	DUKE 1910	*C. purpureus*
Ifrane, Morocco	DUKE 1969	*C. purpureus*
North America		
Lake Hazen, Ellesmere Is, Canada	NYBGH 1967	*C. purpureus*
Mauna Kea, Hawaii	RBGE 2005	*C. purpureus*
New York, America	NYBGH 2005	*C. purpureus*
Asia		
Sindure, Nepal	RBGE 1989	*C. purpureus*
Zara/Sivas Province, Turkey	RBGE 1960	*C. purpureus*
Southern Hemisphere		
Africa		
Lichenya, Malawi	RBGE 1991	*C. purpureus*
South America		
Provincia Sauta Cruz , Argentina	BASH 1978	*C. purpureus*
East Antarctic		
Browning Peninsula, Budd Coast	BASH 1985	*C. purpureus*
Central Basin, Victoria Land	BASH 1995	*C. purpureus*
Crater Cirque, Costal Victoria land	BASH 1991	*C. purpureus*
Davis station, Vestfold Hills	BASH 1905	*B. pseudotriquetrum*
Grimmia Gorge, Vestfold Hills	BASH 1905	*B. pseudotriquetrum*
Ice Axe Peak, Dronning Maud	BASH 1988	*C. purpureus*
Southern Danielle Peninsula, Victoria Land	BASH 1996	*C. purpureus*
West Anchorage Is	BASH 1995	*C. purpureus*
West Antarctic		
Deception Is, South Shetland Is	BASH 2002	*C. purpureus*
Deception Is, South Shetland Is	BASH 1994	*C. purpureus*
Charcot Is, Antarctic Peninsula	BASH 1999	*C. purpureus*
Curville Is, Antarctic Peninsula	BASH 1993	*C. purpureus*
Moe Is, South Orkney Is	BASH 1985	*C. purpureus*
North Leonie Is, Antarctic Peninsula	BASH 1995	*C. purpureus*
Visokai Is, South Sandwich Is	BASH 1997	*C. purpureus*

BASH: British Antarctic survey herbarium
DUKE: Duke University Cryptogamic Herbarium
NYBGH: New York botanic gardens herbarium
RBGE: Royal Botanic Garden Edinburgh

2.1.3 Morphological identification

All fresh and herbarium samples were morphologically identified using the taxonomic keys in the Illustrated Moss Flora of Antarctica (Ochyra *et al.*, 2008). *B. pseudotriquetrum* was identified by the presence of large rhomboid cells and an ovate leaf shape (Figure 8A). *C. purpureus* (Figure 8B) and *S. antarctici* (Figure 8C) are morphologically very similar, but are distinguished by the presence of sinuous cells in *S. antarctici*. In addition to the three Antarctic mosses listed above, the Antarctic moss *Bryoerythrophyllum recurvirostrum* (Figure 8D), the Antarctic liverwort *Cephaloziella varians* (Figure 8E) and numerous unidentified algae (Figure 8F) were found in samples. In a number of cases, identification proved difficult and micrographs of samples were sent to Joy Williams (UOW) and Alison Downing (Maquarie University) for identification.

All fresh and herbarium samples were morphologically identified with the aid of a Leica MS5 dissecting microscope. Where cell structure was required for morphological identification, moss leaves were removed and photographed under an Olympus BHA transmission microscope (Olympus, Australia) fitted with a DCM510 microscope camera (ScopeTek, Australia). During the morphological identification process, samples were separated into individual gametophytes and cleaned by removing moribund (appearing dead) and fungal material, before being transferred to a sterile 1.5 mL Eppendorf tube. In order to reduce contamination, microscope slides were cleaned with 70% ethanol, while tweezers (used to remove moss leaves) were flame sterilised between samples.

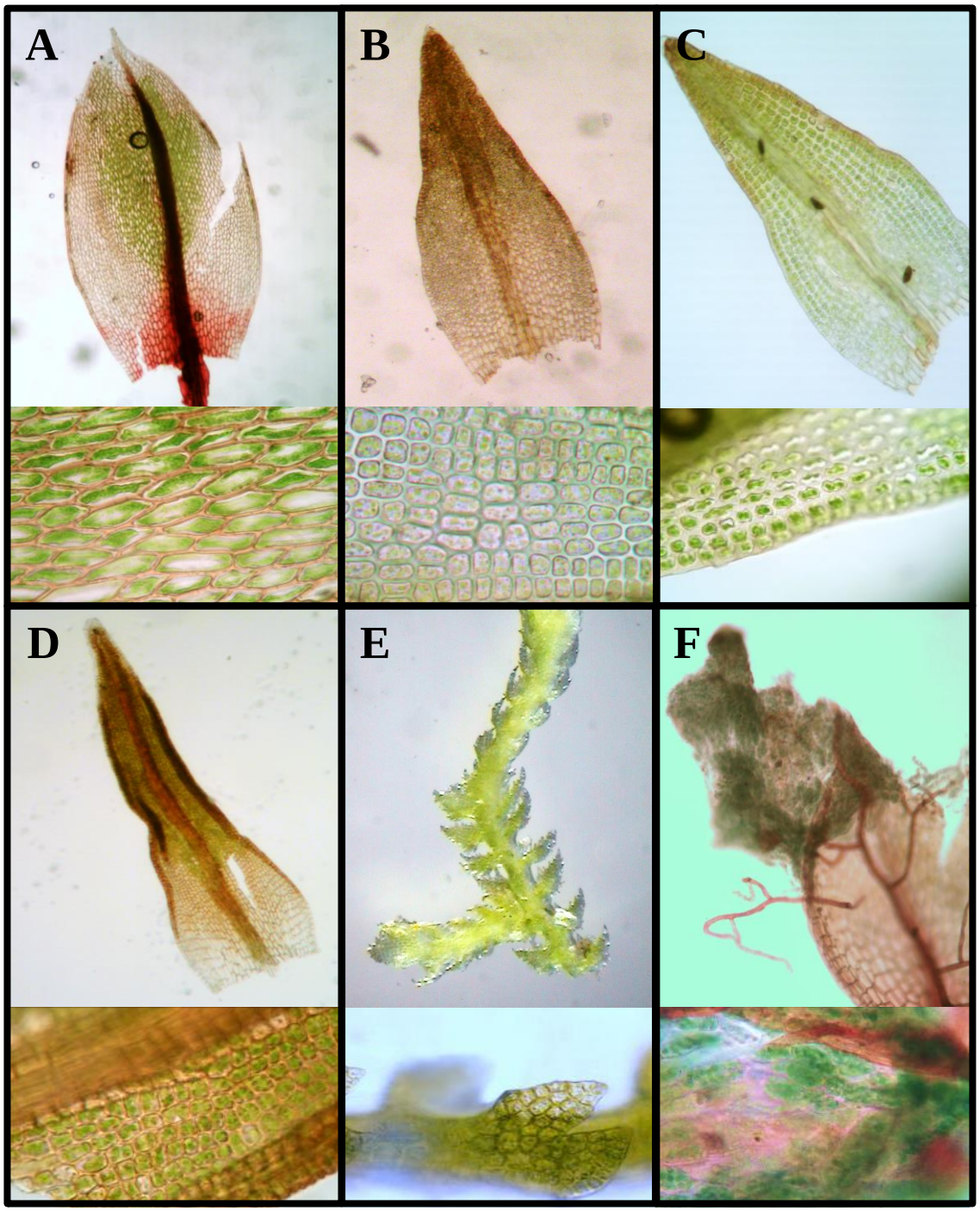

Figure 8. Micrographs of A) *B. pseudotriquetrum*, B) *C. purpureus*, C) *S. antarctici*, D) *B. recurvirostrum*, E) *C. varians* and F) algae. Upper most images in each pair are at 10X magnification while lower images illustrate cell structure when photographed at 40X magnification.

2.1.4 Geographic sampling of Bryum pseudotriquetrum

Samples of *B. pseudotriquetrum* were identified from three sub-sites in the Windmill Islands and all three sites in the Bunger Hills. In order to increase the geographic sampling range, *B. pseudotriquetrum* data was supplemented with *rps*4 and ITS data from Northern Hemisphere locations (n = 2) and the Antarctic Peninsula (n = 1) provided by Kengo Kato (Kengo *et al.*, 2013) and from two locations in the Prince Charles Mountains (Skotnicki *et al.*, 2012) (Figure 9 and

Table 3).

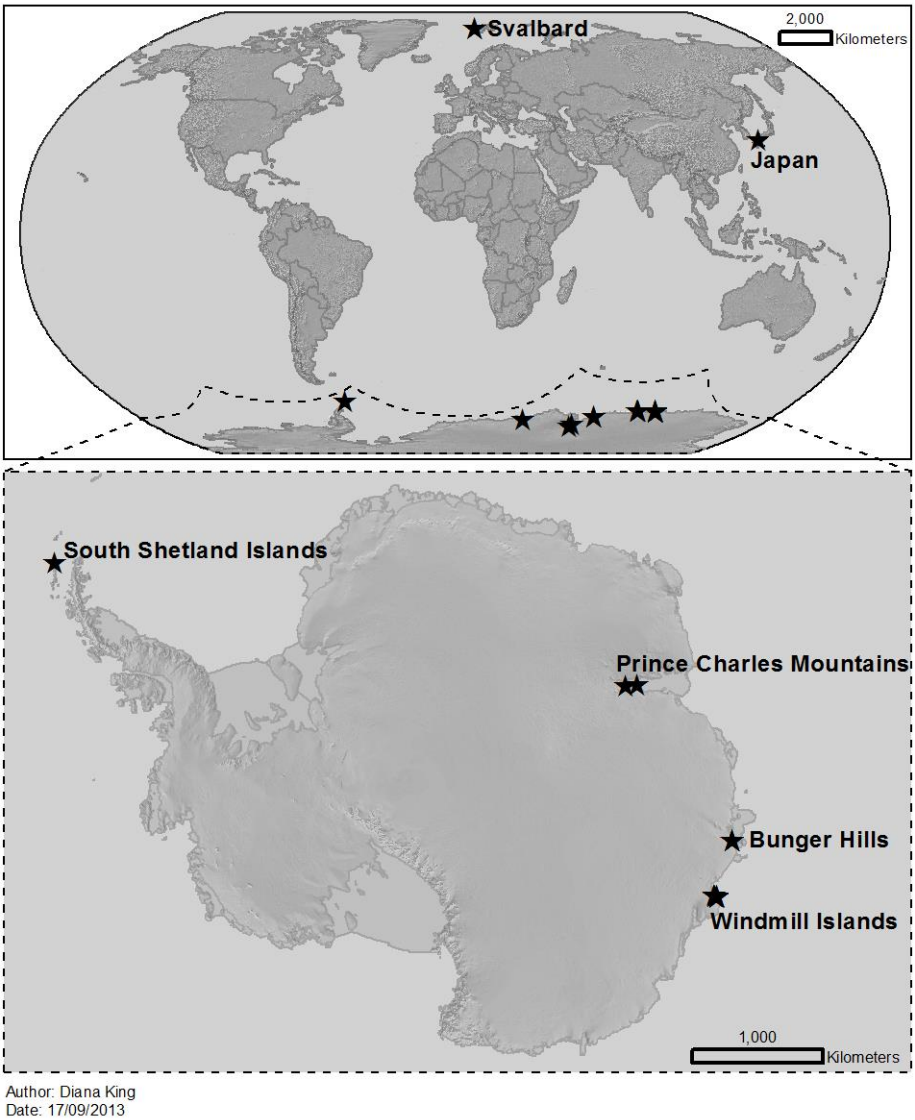

Figure 9. Map showing the locations of samples used for analysis (Stars). Top; Location of samples in relation to the world; Bottom, location of samples with respect to the Antarctic continent.

Table 3. *Bryum pseudotriquetrum* samples used in analyses; Collection locality, with the hemisphere of collection given in bold and underlined, followed by the collection localities in bold and the name of collection sites within each locality (sub-sites) given in italics. Sample identification (ID) or GenBank Accession numbers are given for each sample. Where sample ID's are followed by '*' samples were collected from the same moss clump.

Collection locality	GPS Position (Latitude & Longitude)	ID/Accession Number	Collection Source and Year
Northern Hemisphere			
Hiroshima prefecture, Japan	78.99°, 12.66°	TY-14438	(Kengo *et al.*, 2013)
Svalbard islands, Ny-Ålesund, Norway	34.40°, 132.46°	F03042	(Kengo *et al.*, 2013)
Southern Hemisphere			
East Antarctic			
Bailey Peninsula, Windmill Is	-66.28° 110.54°	B4*	ANARE 2005
		B5*	
		B6*	
	-66.45° 110.51°	94	
	-66.28° 110.54°	170	
Clark Peninsula, Windmill Is	-66.25° 110.59°	SCBP4	
	-66.24° 110.59°	SCBP3	
Robinson Ridge, Windmill Is	-66.37° 110.59°	RRBP6	
	-66.37° 110.58°	RRBP4	
	-66.36° 110.59°	RRBP	
Bunger Hills, Site 1	-66.29° 100.66°	B17	
Bunger Hills, Site 2	-66.29° 100.78°	B18	
Bunger Hills, Site 3	-66.28° 100.69°	B19	
	-66.28° 100.69°	B20	
	-66.28° 100.69°	B23	
	-66.28° 100.69°	CP10	
Prince Charles Mts,	-72.01° 68.82°	JQ040696 (ITS), JQ040701 (rps4)	(Skotnicki *et al.*, 2012)
Prince Charles Mts,	-72.78° 68.04°	JQ040697 (ITS), JQ040702 (rps4)	(Skotnicki *et al.*, 2012)
West Antarctic			
South Shetland Islands	-62.00° -58.00°	TY26709	(Kengo *et al.*, 2013)

ANARE: Australian national Antarctic research expedition

PCMEGA: Prince Charles Mountains Expedition: Germany and Australia

2.1.5 Geographic sampling of Ceratodon purpureus

Samples of *C. purpureus* were identified from two sub-sites in the Windmill Islands and two sub-sites on Heard Island. A further three samples of *C. purpureus* were also collected from a single moss clump from the University of Wollongong. In order to increase geographic sampling, herbarium specimens and all GenBank *C. purpureus* sequences were incorporated into the sampling. (Figure 10 and Table 4).

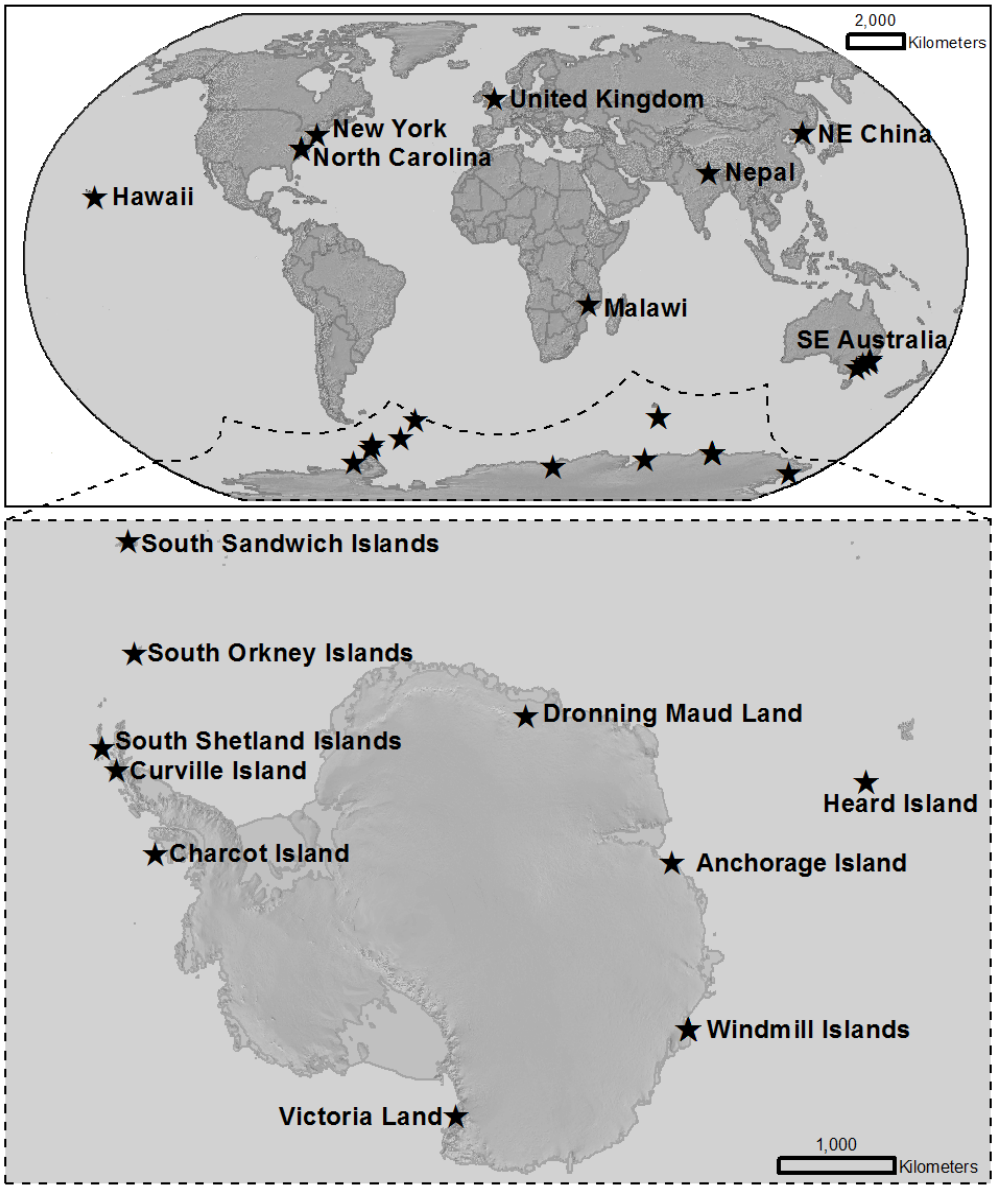

Figure 10. Map showing the location of *C. purpureus* samples used for analysis. Top; location of samples in relation to the world. Bottom; location of samples with respect to the Antarctic continent.

Table 4. *Ceratodon purpureus* samples used in analyses; Collection locality, with the hemisphere of collection given in bold and underlined, followed by the collection localities in bold and the name of collection sites within each locality (sub-sites) given in italics. Sample identification (ID) or GenBank Accession numbers are given for each sample. Where sample ID's are followed by '*' samples were collected from the same moss clump.

Collection Locality	GPS Position (Latitude & Longitude)	ID/Accession Number	Collection source and year
Northern Hemisphere			
Europe			
Berkshire Reading, United Kingdom	51.45° -0.97°	AJ554004	(Hedderson *et al.*, 2004)
North America			
Duke University, Durham, North Carolina	36.00° -78.94°	AY908123	(Shaw *et al.*, 2005)
		AY908122	
		AY908121	
		AF435271	(Farge *et al.*, 2002)
Mauna Kea, Hawaii	19.82° -155.47°	CP8	BASH 2005
New York, America	40.86° -73.88°	CP11	NYBGH 2005
Asia			
Changbai, North East China	41.42° 128.20°	FJ572605	(Liu *et al.*, 2010)
		FJ572589	
Sindure, Nepal	28.17° 84.30°	CP17	RBGE 1998
Southern Hemisphere			
Heard Island			
Paddock Valley, Heard Island	-53.08° 73.50°	CP39	HIMIE 2004
Dovers Moraine, Heard Island	-53.08° 73.50°	CP44	
Africa			
Lichenya, Malawi	-15.55° 35.83°	CP13	RBGE 1991
Australia			
Australian National University	-35.28° 149.12°	CP16	
Mt Beauty, Victoria	-36.74° 147.17°	CP5	2005 (Clark)
Macquarie University, Sydney	-33.77° 151.11°	CP7	
University of Wollongong	-34.40° 150.86°	B10*	2013 (Author)
		B11*	
		B12*	

Table 4. (Continued)

East Antarctica			
Bailey Peninsula, Windmill Is	-66.28° 110.54°	CPMP	ANARE 2005
	-66.33° 110.47°	CP6	
	-66.28° 110.54°	76	
	-66.16° 110.32°	B20	
	-66.28° 110.54°	166CP	
Clark Peninsula, Windmill Is	-66.25° 110.57°	219	
	-66.25° 110.55°	WPD5	
	-66.25° 110.56°	WPD8	
Central Basin, Victoria Land	-74.33° 165.13°	CP20	BASH 1995
Ice Axe Peak, West Dronning Maud	-71.47° 31.08°	CP21	BASH 1988
West Anchorage Is	-68.56° 77.93°	CP34	BASH 1995
West Antarctica			
Deception Is, South Shetland Islands	-62.98° -60.66°	CP23	BASH 2002
Moe Is, South Orkney Is	-60.74° -45.74°	CP24	BASH 1985
Deception Is, South Shetland Is	-62.97° 60.50°	CP25	BASH 1994
Curville Is, Antarctic Peninsula	-64.71° -62.68°	CP28	BASH 1993
Visokai Is, South Sandwitch Islands	-54.27° -36.49°	CP30	BASH 1997
Charcot Is, Antarctic Peninsula	-69.75° -75.25°	CP32	BASH 1999

ANARE: Australian national Antarctic research expedition

BASH: British Antarctic survey herbarium

HIMIE: Heard Island and Macquarie Islands Expedition

NYBGH: New York botanic gardens herbarium

RBGE: Royal Botanic Garden Edinburgh

2.1.6 Geographic sampling of Schistidium antarctici

Samples of *S. antarctici* were identified from three sub-sites in the Windmill Islands and two sub-sites from the Bunger Hills (Figure 11 and

Table 5). Due to the Antarctic endemic status of *S. antarctici*, samples were not available from Heard Island, Wollongong or other non-Antarctic locations. As for *B. pseudotriquetrum* and *C. purpureus* all GenBank sequences (ITS and *rps*4) were incorporated into analyses (List available in appendix, Table 8 & Table 9).

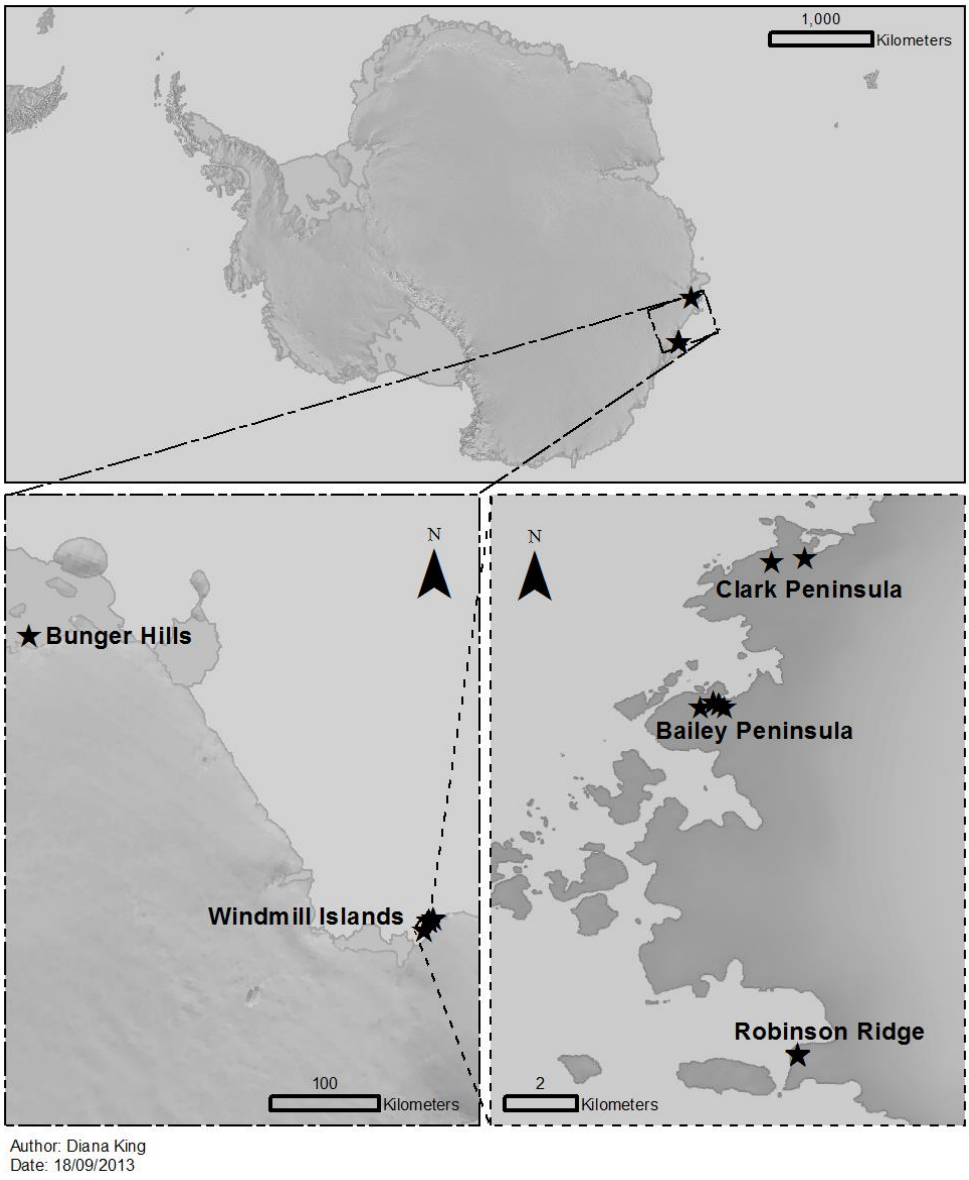

Figure 11. Map showing the location of *S. antarctici* samples used for analysis. Top; location of the Bunger Hills and Windmill Islands within Antarctica. Bottom left; location of the Bunger Hills and Windmill Islands in relation to the East Antarctic coast. Bottom right; location of samples collected from within the Windmill Islands.

Table 5. *Schistidium antarctici* samples used in analyses; Collection locality, with the hemisphere of collection given in bold and underlined, followed by the collection localities in bold and the name of collection sites within each locality (sub-sites) given in italics. Sample identification codes (ID) are given for each sample. Where sample ID's are followed by '*' samples were collected from the same moss clump.

Collection Locality	**GPS Position (Latitude & Longitude)**	**ID**	**Collection year and Source**
Southern Hemisphere			
East Antarctica			
Bailey Peninsula, Windmill Is	-66.28° 110.54°	B1*	ANARE 2005

		B2*	
		B3*	
	-66.28° 110.53°	B7	
	-66.28° 110.53°	B8	
	-66.28° 110.53°	B9	
	-66.28° 110.53°	B14	
	-66.28° 110.53°	B15	
	-66.28° 110.53°	B16	
	-66.28° 110.53°	B17	
	-66.28° 110.52°	RSSA1	ANARE 2012
	-66.28° 110.54°	7/12C2	ANARE 2005
Robinson Ridge, Windmill Is	-66.37° 110.59°	RRSA6	
	-66.36° 110.58°	RRSA5	
	-66.37° 110.58°	RRSA3	
	-66.36° 110.59°	49	
Clark Peninsula, Windmill Is	-66.25° 110.59°	SCSA2	ANARE 2012
	-66.25° 110.57°	221	ANARE 2005
	-66.28° 110.54°	17/12B3	
	-66.28° 110.53°	200SA	
Bunger Hills, site 1	-66.30° 100.67°	B17CP	
Bunger Hills, site 3	-66.29° 100.66°	B22	

ANARE: Australian national Antarctic research expedition

2.2 DNA extraction

Total genomic DNA was extracted from single moss gametophytes (<1 mg dry weight) using the Plant DNeasy Mini Kit (QIAGEN, Australia), with the following changes: AP1 (lysis) buffer and RNase A were added to samples prior to tissue disruption; plant tissue was disrupted using a TissueLyser (QIAGEN, Australia) (four minutes at 30 Hz); and DNA was eluted in 50 μL of AE (elution) buffer. DNA concentration and fragment size was determined

using agarose gel electrophoresis. Extracted DNA (5 µL) was mixed with 2 µL of 6X loading dye (Sigma-Aldrich, Australia) and 5 µL of dH_2O then electrophoresed (100 V for 45 min) in Tris/Borate/ Ethylenediaminetetraacetic acid (EDTA) buffer (1 mM Tris, 45 mM boric acid and 1 mM EDTA [pH 8]) on a 1% agarose gel. DNA was stained for 30 min using ethidium bromide (1 µg/mL) followed by a 5 min de-stain in dH_2O. Comparisons of fragment size and DNA concentration were made against 5 µL (2 µg) of λ/HindIII marker (Sigma-Aldrich, Australia), electrophoresed under identical conditions.

2.3 PCR amplification and gel excision

All PCR reactions were carried out in a total volume of 20 µL. Each 20 µL reaction mixture contained one unit of hot start polymerase (IMMOLASE or MyTaq HS; Bioline, Australia), 1-10 ng of template DNA or 1 µL of dH_2O (negative control), 60 nM of each primer (Sigma, Australia), 3.75 mM $MgCl_2$, 580 µM of deoxynucleotide triphosphates (dNTPs) and 1X ImmoBuffer (Bioline, Australia). Amplifications were carried out using a Corbett Research gradient PalmCycler™ II (Corbett Research, Australia). Following amplification, 20 µL of each PCR product was mixed with 4 µL of 6X loading dye and electrophoresed (as describe in section 2.2) on a 1% agarose gel, to separate the various PCR products. Gels were stained with ethidium bromide as described above and PCR products visualised using a UV transilluminator. Bands of interest were excised from the gel and purified using a Wizard SV Gel and PCR clean up kit (Promega, USA) with modifications, whereby DNA was eluted in 15 µL of nuclease free water (Promega, USA) that had been preheated to 65°C.

2.3.1 PCR of DNA from fresh material

For the amplification of *rps*4 and ITS from DNA extracted from fresh material, the hot start DNA polymerase IMMOLASE was used. In order to activate the hot start polymerase, an initial activation step for 10 min at 95°C was carried out. This was followed by an initial

denaturation step for 30 s at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s and elongation for 2 min at 72°C. Amplification of ITS was carried out utilizing the primer combination GGAAGGAGAAGTCGTAACAAGG (ITSHP5) (Simon *et al.*, 2010) and TCCTCCGCTTATTGATATGC (ITS4) (White *et al.*, 1990). Amplification and sequencing of *rps*4 utilized the primer combination ATGTCCCGTTATCGAGGACCT (RPS5) and TACCGAGGGTTCGAATC (TrnS) (Souza-Chies *et al.*, 1997). Following amplification, PCR products were separated, visualised, excised and purified as described above.

2.3.2 PCR of DNA from herbarium material

DNA extracted from herbarium plant material often contains higher concentrations of PCR inhibitors and shorter fragments of DNA, which can make amplification of genes difficult (Savolainen *et al.*, 1995). For the amplification of *rps*4 and ITS from DNA extracted from herbarium specimens (9-100 years old), the hot start DNA polymerase MyTaq HS was used. In preliminary trials, amplification with MyTaq HS led to higher yields of amplicon, when compared to the polymerases IMMOLASE and Biotaq (Bioline, Australia). In order to amplify *rps*4 and ITS, a modified PCR protocol was used: MyTaq HS was activated by an initial activation step for 1 min at 95°C, this was followed by an initial denaturation step for 15 s at 95°C, followed by 35 cycles of denaturation at 95°C for 15 s, primer annealing at 60°C for 15 s and elongation for 30 s at 72°C.

The amplification of *rps*4 from herbarium material often produced amplicons too weak for gel excision and sequencing. To concentrate these weak PCR products for sequencing, *rps*4 was amplified in replicate tubes (n=5) and PCR products pooled. One hundred μL of this pooled PCR product was mixed with 20 μL of 6X loading dye and electrophoresed as

described in section 2.2. Amplicons were then excised and purified as described in section 2.3. Amplification of the complete ITS region (1000 bp) from herbarium material was often not possible using the primer combination ITS4 and ITSHP5. Instead, the smaller ITS1 region of ITS was amplified using the primer combination ITSHP5 (Simon *et al.*, 2010) and GCTGCGTTCTTCATCGATGC (ITS2). Amplified products were then visualised and bands of interest excised and purified as described above.

2.4 Sequencing of amplified DNA

Sequencing reactions were carried out using the Perkin-Elmer DNA sequencing kit (Perkin-Elmer, USA) and standard sequencing conditions (95°C for 30 s and 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 2 min) for *rps*4. Sequencing of the complete ITS region used the standard sequencing protocol described above, but with the following modifications; due to the length of the complete ITS region, the internal primers GCATCGATGAAGAACGCAGC (ITS3) and ITS2 (White *et al.*, 1990) were also used to initiate sequencing reactions; and 5% dimethyl sulfoxide (DMSO) was added to each sequencing reaction. Following the sequencing reaction, DNA was precipitated using 2 μL of EDTA (125 mM), 2 μL of sodium acetate (1 M), 20 μL of sequencing product and 50 μL of ethanol (95%). The mixture was vortexted and incubated at room temperature (15 min) before being centrifuged (13,000 *g* for 15 minutes). The supernatant was then removed and the DNA pellet washed with cold (-20°C) 70% ethanol. The mixture was then centrifuged (13,000 *g* for 15 minutes), the residual 70% ethanol removed and the DNA pellet allowed to air dry. Precipitated DNA was sequenced by Margaret Phillips (UOW sequencing facility) using a 3130XL genetic analyser (Applied Biosystems, Australia). All sequencing chromatograms were checked in ChromasPro V1.7.5 (Technelysium Pty Ltd., Australia) and consensus sequences generated in BioEdit V7.1.9 (Hall, 1999) using the Cap-Contig Assembly program. All generated sequences were identified using GenBank BLASTn

searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) based upon the GenBank nucleotide collection.

2.5 Phylogenetic analyses of DNA sequence data

Phylogenetic analyses were carried out on the complete *rps*4 gene and the complete ITS region. Sequences were aligned using the CLUSTAL-W algorithm (Thompson *et al.*, 1994) within Molecular evolution and genetic analysis 5 (MEGA5) (Tamura *et al.*, 2011). In cases where CLUSTAL-W produced poor alignments (in which the length of the alignment was much longer than any individual sequence), the alignment tool MAFFT (http://mafft.cbrc.jp/alignment/server/) was used and alignments corrected manually. Due to differing evolutionary pressures on gene regions and therefore differing rates of evolution, analysis is improved by partitioning alignments into different gene regions and allowing for multiple nucleotide substitution models (Brandley *et al.*, 2005). Ribosomal protein subunit 4 data was partitioned into 1st, 2nd and 3rd codon partitions, while ITS was partitioned into ITS1, 5.8S and ITS2 regions. Partitioned alignments were then imported into the program partition finder V1.1.1 (Lanfear *et al.*, 2012), which searches partitioned alignments and identifies the most appropriate nucleotide substitution model for each partitioned region. Partition finder was run using all nucleotide substitution models found in the program BEAST (Drummond *et al.*, 2007a). The Akaike information criterion (AIC), Akaike information criterion corrected (AICc) and Bayesian information criterion (BIC) were used to identify the most appropriate model for each partition. In most cases all criteria identified the same model for each partition. Where criteria were in disagreement, the model supported by two criteria was selected. Once nucleotide substitution models had been established for each partition, alignments were imported into Bayesian evolutionary analysis utility V1.7.5 (BEAUTi) (Rambaut *et al.*, 2007), which allows for the specification of nucleotide substitution models, clock models, tree priors and chain lengths prior to Bayesian MCMC analysis in the program

BEAST V1.7.5 (Drummond and Rambaut, 2007a). In circumstances where *rps*4 and ITS data were available for each sample, the modified version of BEAST (*BEAST; Heled and Drummond, 2010) was used. The program *BEAST allows for the incorporation of coalescent theory for the construction of a species tree from multilocus data. Following analysis in BEAST or *BEAST, stationarity of each parameter was assessed using the software Tracer V1.5 (Drummond *et al.*, 2007b). Stationarity was considered reached if the Effective Sample Size (ESS) of each parameter had a score ≥100. The burn-in time (the time required for parameters to reach stationarity) was taken as the first 10% of each logged parameter. Once stationarity had been confirmed, a consensus tree was generated using TreeAnnotator V1.7.5 and the tree viewed in the software program Figtree V1.4.0. In cases where *BEAST was used, the software BP&P V1.1.1 (Rannala and Yang, 2003) was then used to delimit separate species (Figure 12).

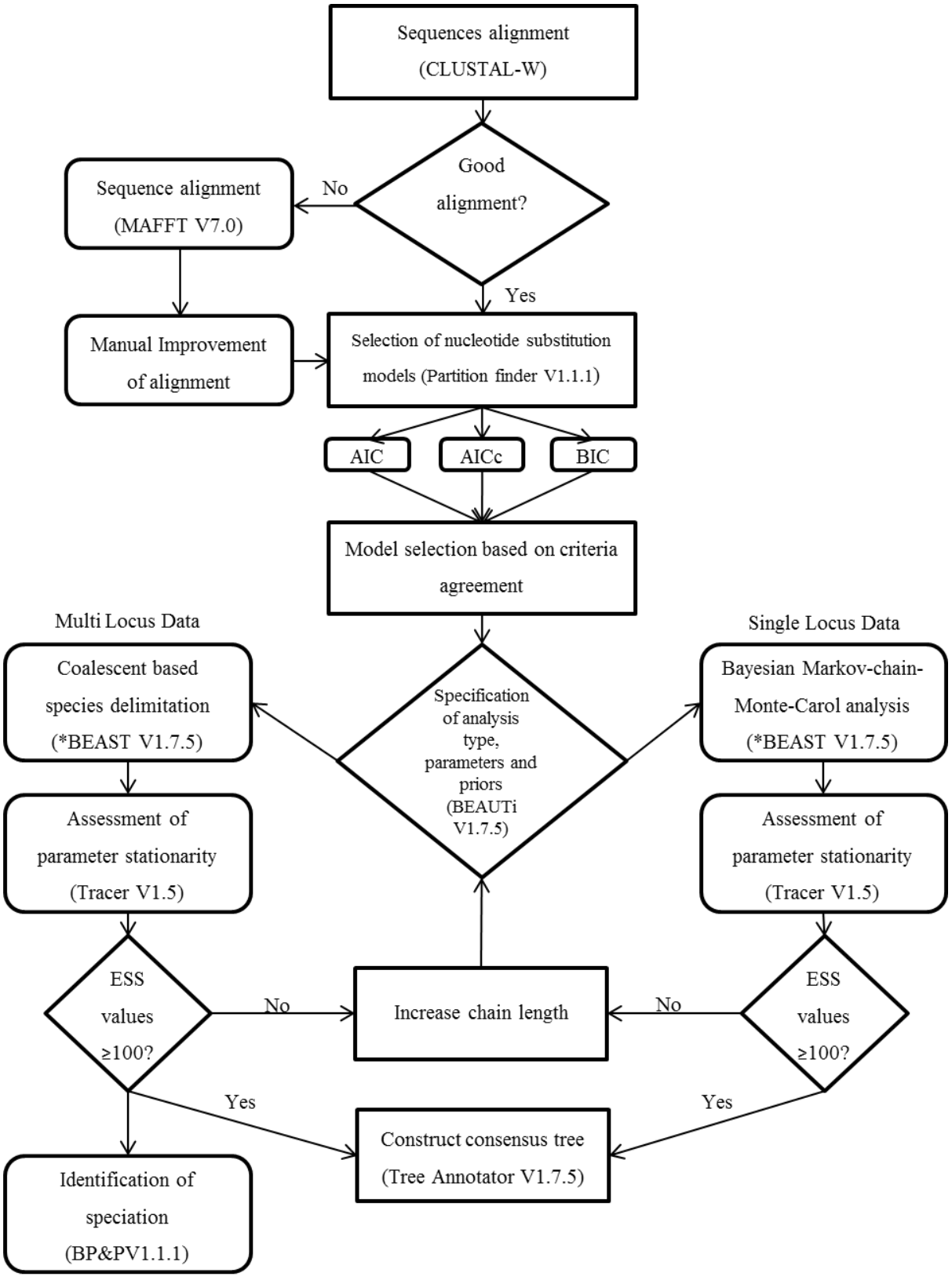

Figure 12. Flow diagram illustrating the phylogenetic analysis process.

The type of analysis carried out on each dataset depends upon the data available, particularly if the data are multi locus or single locus (Table 6). For the analysis of *B. pseudotriquetrum*, both *rps*4 and ITS data were available for each specimen. As such, coalescent-based species delimitation could be carried out. For analysis of data from both *C. purpureus* and *S. antarctici*, sufficient data was only obtained from one locus. Therefore, only Bayesian MCMC analyses could be carried out. In *C. purpureus*, difficulty obtaining ITS data from herbarium specimens meant that only sufficient *rps*4 data had been obtained. In *S. antarctici*, herbarium specimens were not available. In this case, GenBank data were used. However, no paired data sets were publicly available and analysis was thus carried out separately on ITS and *rps*4 data sets.

Table 6. Parameters and priors used for each analysis, where GTR refers to a general time reversible model (Tavaré, 1986), HKY refers to a Hasegawa-Kishino-Yano model (Hasegawa *et al.*, 1985) and TN93 refers to a Tamura-Nei 93 model (Tamura *et al.*, 1993). Models that incorporated a gamma distribution or invariant sites are denoted by +G (Gamma) and +I (Invariant).

Parameters	***B. pseudotriquetrum***	***C. purpureus***	***S. antarctici* (*rps*4)**	***S. antarctici* (*ITS*)**
Analysis	*BEAST	BEAST	BEAST	BEAST
Data	ITS and *rps*4	*rps*4	*rps*4	ITS
Substitution model	GTR+G	HKY	HKY	GTR+I+G (ITS1) TN93+I+G (5.8S & ITS2)
Chain length	100,000,000	100,000,00	100,000,00	100,000,000
Parameters logged	10,000	1,000	10,000	1,000
Base frequency	Estimated	Estimated	Estimated	Estimated
Clock model	Strict	Strict	Logarithmic	Logarithmic
Tree prior	Yule Process	Coalescent: Constant size	Yule Process	Yule Process

3 RESULTS

The three common East Antarctic moss species: *B. pseudotriquetrum*, *C. purpureus* and *S. antarctici* form part of a long term biodiversity study in the Windmill Islands and serve as indicators for the effects of climate change on Antarctic terrestrial ecosystems. However, the identification of moss species from the Windmill Islands is difficult. Furthermore, it is not known whether Windmill Islands mosses are representative of the same species from other locations. As such, a molecular basis of identification and phylogeny is highly desirable. In this section, results obtained during the optimisation of PCR and the phylogenetic analyses of the three species mentioned above are detailed. Furthermore, the ability of the molecular markers ITS and *rps*4 to identify *B. pseudotriquetrum*, *C. purpureus* and *S. antarctici* are empirically assessed.

3.1 PCR amplification and optimisation

The molecular markers ITS and *rps*4 were selected due to their location in distinct genomic compartments and differing levels of phylogenetic resolution. Although protocols for the amplification of these two markers from mosses have been published (McDaniel and Shaw, 2005; Skotnicki *et al.*, 2012), these protocols required considerable optimisation in order to produce reliable sequence data.

3.1.1 Internal transcribed spacer

The amplification of ITS consistently produced multiple strong amplicons, ranging from 600 to 1,200 bp in size and varying in number between species, samples and annealing temperatures (Figure 13).

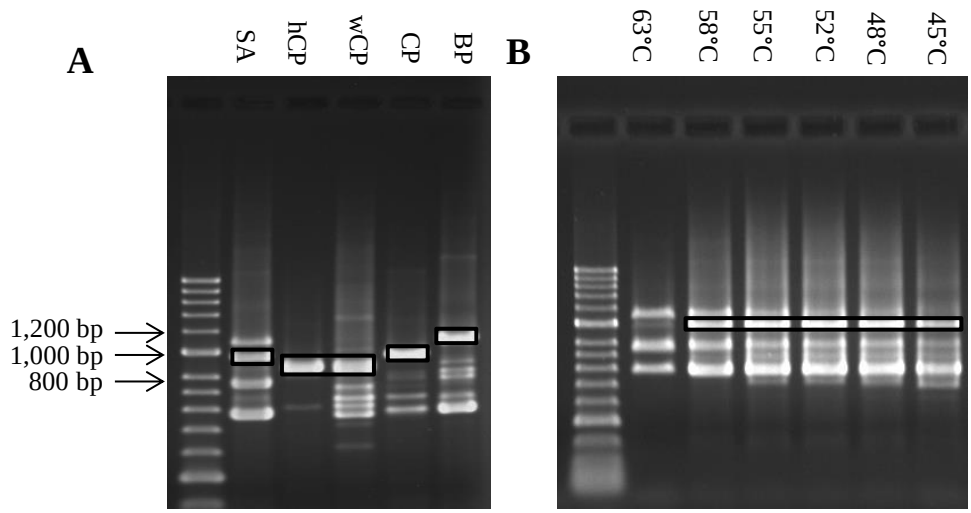

Figure 13. A) Internal transcribed spacer region amplified from 1-10 ng of DNA extracted from single gametophytes of *S. antarctici* (SA), herbarium *C .purpureus* (20 years old) (hCP), Wollongong *C. purpureus* (wCP), Antarctic *C. purpureus* (CP) and Antarctic *B. pseudotriquetrum* (BP). B) Annealing temperature gradient of ITS amplified from 1-10 ng of DNA extracted from a gametophyte of *S. antarctici*. Amplicon sizes were determined via comparison to a Hyperladder II marker, electrophoresed under identical conditions. Black boxes indicate the location of moss ITS amplicons.

To identify the multiple amplicons, the ITS region was amplified from the moss species *B. pseudotriquetrum*, *C. purpureus* and *S. antarctici*. Amplicons were separated by gel electrophoresis and individually excised and sequenced. Sequencing of some amplicons proved difficult, due to the presence of strong secondary structures, evident through a sharp signal drop in sequencing chromatograms. This was particularly evident in amplicons of 1,000 bp in size, in *C. purpureus* and *S. antarctici*. Once sequenced, amplicons were identified by BLASTn searches and revealed the presence of the moss ITS band at 1,000 bp in size (*C. purpureus* and *S. antarctici*) and 1,200 bp in size (*B. pseudotriquetrum*). Amplicons larger than 1,200 bp were identified as green algal ITS genes, while amplicons below 1,000 bp in size were identified as fungal ITS genes (Table 7 and Figure 14).

Table 7. Contaminant genes identified following gel excision and sequencing of the ITS region. Genes were identified by BLASTn searches, with E value (probability of a chance match), Identity score (Identity) and accession number of the top BLAST match given. The region of Antarctica is given in bold and underlined, followed by the collection locality in bold and sub-sites in italics. The collection site represents the collection locality of the moss sample from which the genes were isolated. Species refers to the identity of the closest matched sequence available in GenBank.

Moss collection site	GPS Position (Latitude & Longitude)	Species	Identity	Score	E value	BLASTn Accession number
East Antarctica						
Bunger Hills Knox coast						
Site 1	-66.29° 100.66°	*B. pseudotriquetrum*	*Ulothrix*[A]	96%	0	DQ821516.1
Site 1	-66.29° 100.66°	*B. pseudotriquetrum*	*Chloromonas*[A]	97%	0	AB734112.1
Site 1	-66.29° 100.66°	*B. pseudotriquetrum*	*Chloromonas*[A]	98%	0	AB734112.1
Site 3	-66.28° 100.68°	*B. pseudotriquetrum*	*Geomyces*[F]	96%	0	FJ977924.1
Windmill Islands, Budd coast						
Bailey Peninsula, ASPA135	-66.28° 110.54°	*C. purpureus*	*Ascomycetes*[F]	97%	0	HQ211827.1
	-66.28° 110.53°	*B. pseudotriquetrum*	*Neocystis mucosa*[A]	86%	7E-76	JQ920366.1
	-66.28° 110.53°	*B. pseudotriquetrum*	*Trebouxiophyceae*[A]	96%	0	FJ554399.1
	-66.28° 110.53°	*B. pseudotriquetrum*	*Phaeosphaeria*[F]	97%	0	KC965394.1
	-66.28° 110.53°	*S. antarctici*	*Phialocephala virens*[F]	97%	2E-85	KC456683.1
	-66.28° 110.53°	*B. pseudotriquetrum*	*Pseudendocloniopsis botryoides*[A]	98%	0	FR865755.1
Clark Peninsula	-66.25° 110.57°	*S. antarctici*	*Sphaerocystis*[A]	98%	0	HQ404871.1
	-66.25° 110.55°	*C. purpureus*	*Chlorosarcinopsis*[A]	98%	0	HQ246437.1
Robinson's Ridge	-66.36° 110.58°	*S. antarctici*	*Trebouxia impressa*[A]	92%	0	AJ249570.1
	-66.37° 110.58°	*S. antarctici*	*Physcia adscendens*[F]	97%	0	FR799268

[A]Algal genes
[F]Fungal genes

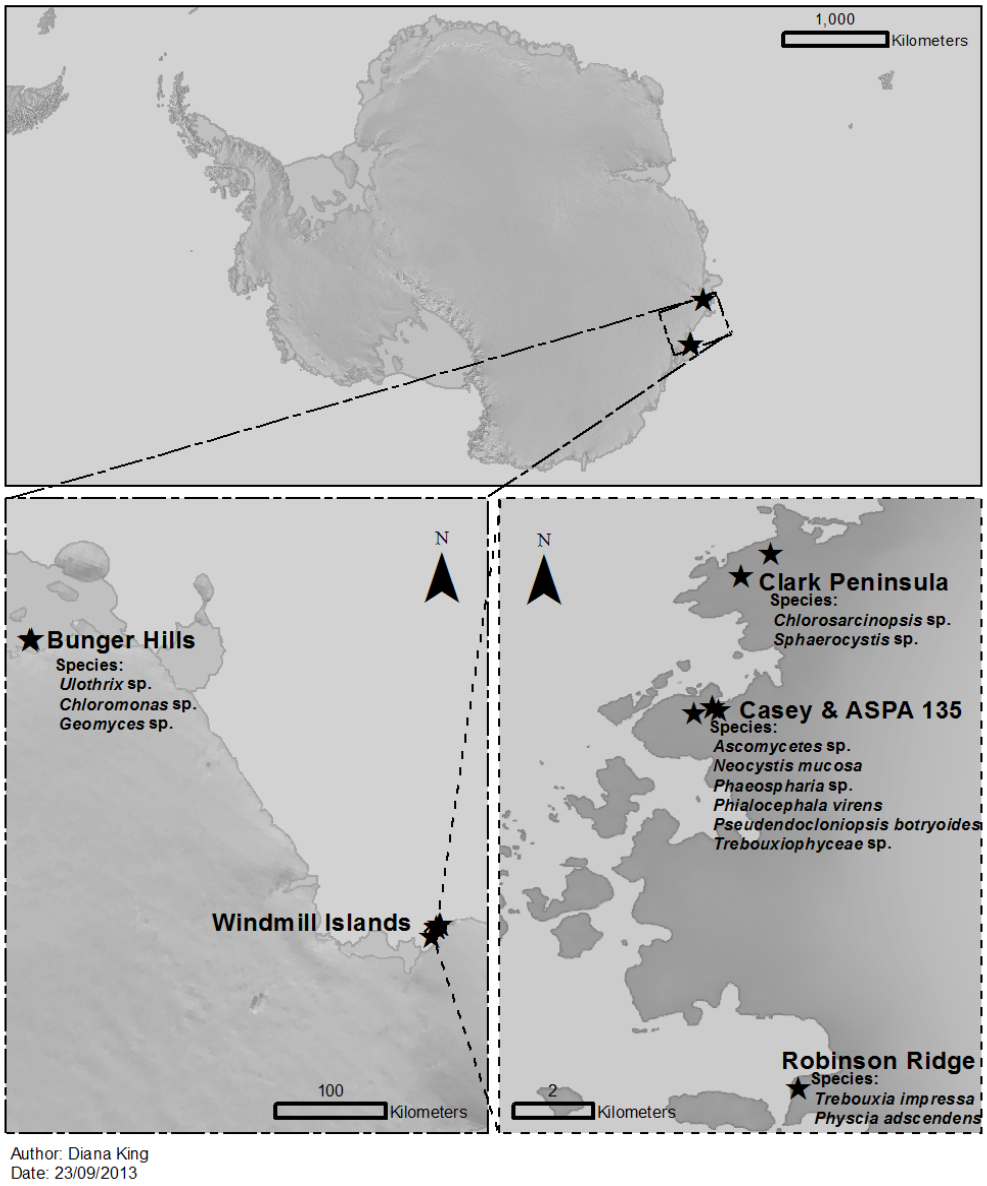

Figure 14. Map showing the location of fungal and algal ITS genes isolated from moss samples of *B. pseudotriquetrum*, *C. purpureus* and *S. antarctici* from the Windmill Islands and Bunger Hills. Collection site of moss from which samples were isolated is marked by a star, with the gene identity given below. Top; location of the Bunger Hills and Windmill Islands in relation to Antarctica. Bottom left; location of the Bunger Hills and Windmill Islands in relation to the East Antarctic coast. Bottom right; location of samples collected from within the Windmill Islands.

In order to identify if the algal and fungal genes were a product of laboratory contamination, ITS was amplified from a sterile cultured gametophyte of *C. purpureus*. Amplification resulted in one strong ITS band at 1,000 bp, showing that the presence of algal and fungal genes in Antarctic samples was not due to laboratory contamination.

3.1.2 Ribosomal protein subunit 4

Amplification of *rps*4 consistently produced a single strong amplicon of 700 bp in size in all moss species examined (Figure 15A). However, sequencing consistently resulted in noisy chromatograms (chromatograms containing multiple bases at each nucleotide position). Optimisation of both PCR annealing (Figure 15B) and sequencing annealing temperatures, failed to decrease the noise in subsequent data. Alternatively, gel excision of the PCR amplicon proved effective and resulted in consistently high quality data.

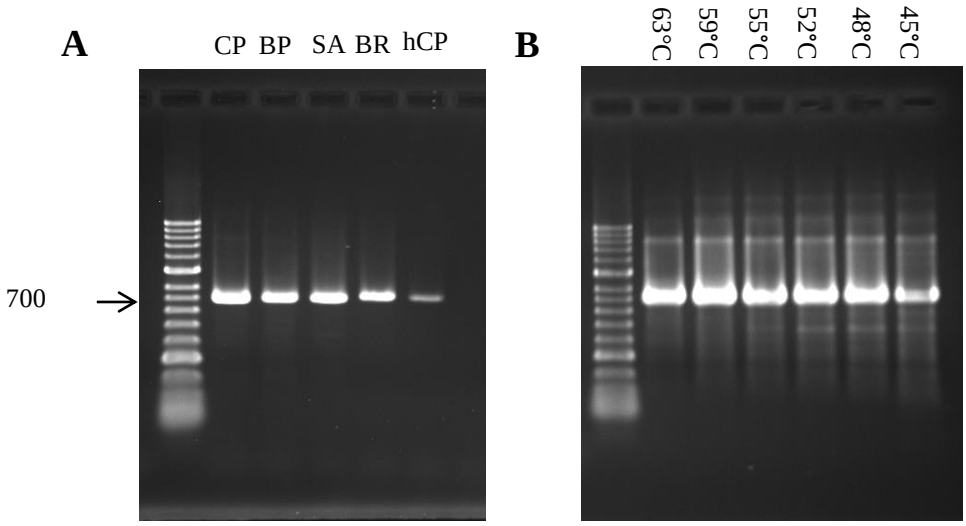

Figure 15. Gel electrophoresis showing A) *rps*4 amplified from 1-10 ng of DNA extracted from single gametophytes of Antarctic *C. purpureus* (CP), *B.* pseudotriquetrum (BP), *S. antarctici* (SA), *B. recurvirostrum* (BR) and a herbarium specimen (20 years old) of *C. purpureus* (hCP). B) Annealing temperature gradient of *rps*4 amplified from 1-10 ng of DNA extracted from a gametophyte of *S. antarctici*. Amplicon sizes were determined via comparison to Hyperladder II marker, electrophoresed under identical conditions.

3.2 Bryum pseudotriquetrum

Recent evidence has suggested that Antarctic *B. pseudotriquetrum* may be a distinct species to *B. pseudotriquetrum* populations from the Northern Hemisphere (Kengo *et al.*, 2013). Furthermore, whether Antarctic *B. pseudotriquetrum* populations represent a single or multiple species has not been investigated. In order to address these issues, coalescent-based species delimitation was carried out on samples of *B. pseudotriquetrum*. Moreover, the ability of ITS and *rps*4 to identify Antarctic *B. pseudotriquetrum* samples was assessed.

3.2.1 Phylogeny of Bryum pseudotriquetrum

Phylogenetic analysis of *B. pseudotriquetrum* sequence data involved the use of only those samples from which both *rps*4 and ITS had been sequenced and in all cases incorporated a sample of *B. argenteum* collected from the University of Wollongong (UOW) as an out-group.

The delimitation of species using analytical approaches that incorporate the coalescent has only recently become possible (Fujita *et al.*, 2012). Nevertheless, all current approaches are computationally intensive. Ideally, analytical inference of the phylogeny and species delimitation should be carried out at the same time, but this is not computationally feasible at present. Instead, the phylogeny must first be determined, species limits proposed, and then species limits tested using coalescent approaches. For these reasons an initial phylogeny was constructed through *BEAST. Once this initial phylogeny had been established, species limits were proposed. Samples from the Bunger Hills (Bunger Hills, n = 6) and Windmills Islands (Windmill Islands, n = 10) were proposed as two separate groups based on sample location. The single samples from Japan and Norway were proposed as a single group (Japan and Norway, n = 2), whilst the two samples from the Prince Charles Mountains were grouped

with a single sample from the South Shetland Islands (Prince Charles Mountains, n = 3). A second *BEAST analysis, defining species limits as described above, was carried out (Figure 16). Results from this tree revealed strong posterior probabilities (PP) (the probability of the tree node given the data) for Prince Charles Mountain samples forming a sister clade to samples from the Windmill Islands, Bunger Hills and Northern Hemisphere (PP = 0.8927). Lower support was found for the separation of Northern Hemisphere samples from samples collected from the Windmill Islands and Bunger Hills (PP = 0.4979). While the highest support was found for distinguishing samples from the Windmill Islands and Bunger Hills (PP = 0.955).

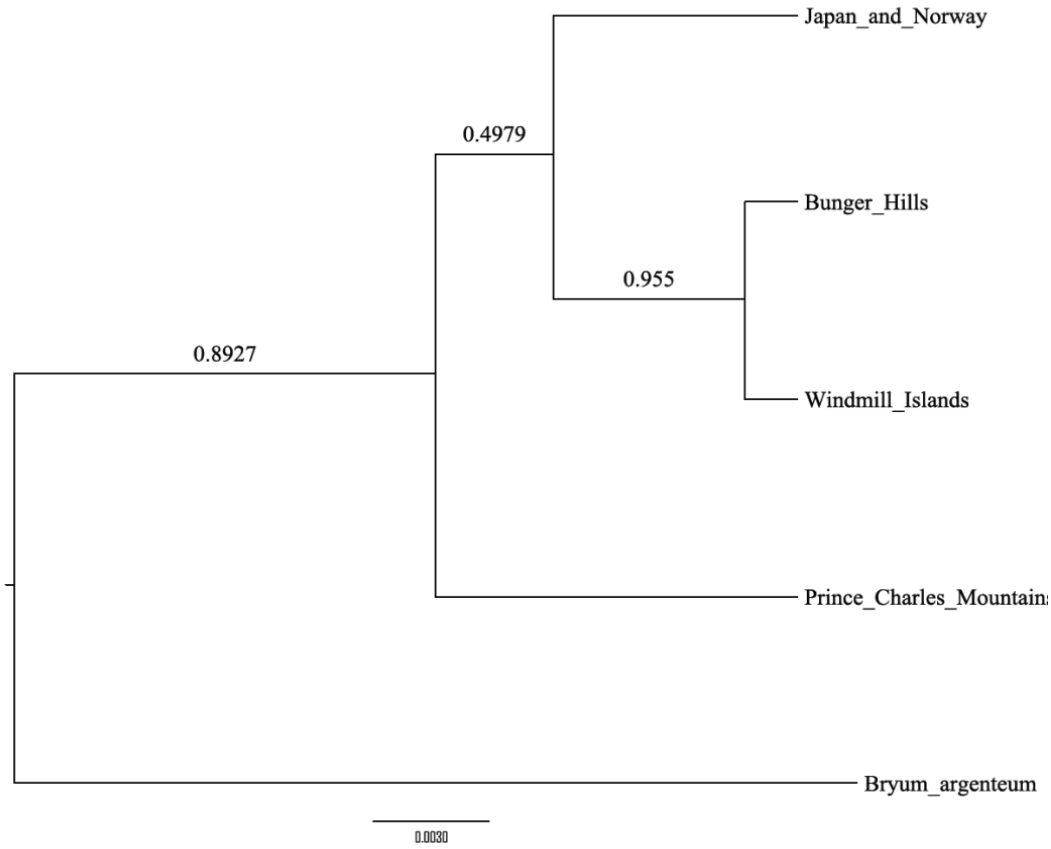

Figure 16. *BEAST tree generated using *rps*4 and ITS sequence data from samples of *B. pseudotriquetrum* collected from Japan and Norway (TY-14438 and F03042) (n = 2), Bunger Hills (B17, B18, B19, B20, B23 and CP10) (n = 6), Prince Charles Mountains (JQ040696 & JQ040701, JQ040697 & JQ040702 and TY26709) (n = 3) and Windmill Islands (B4, B5, B6, RRBP6, RRBP4, RRBP, SCBP4, SCBP3, 94 and 170) (n = 10). Tree was rooted using a single sample of *B. argenteum* collected from the University of Wollongong. Scale bar represents evolutionary change in branches, while values on branches are posterior probabilities for each respective node.

Following analysis in *BEAST, Species limits were tested using BP&P, which calculates the probability of speciation at each node in the tree (Figure 17). Posterior probabilities suggested that speciation events separated populations from the Bunger Hills (n = 6) and Windmill Islands (n = 10) (PP = 0.97975), Windmill Islands and Bunger Hills from Northern Hemisphere populations (n = 2) (PP = 1.00000) and Prince Charles Mountain populations (n = 3) from all other populations (PP = 1.00000).

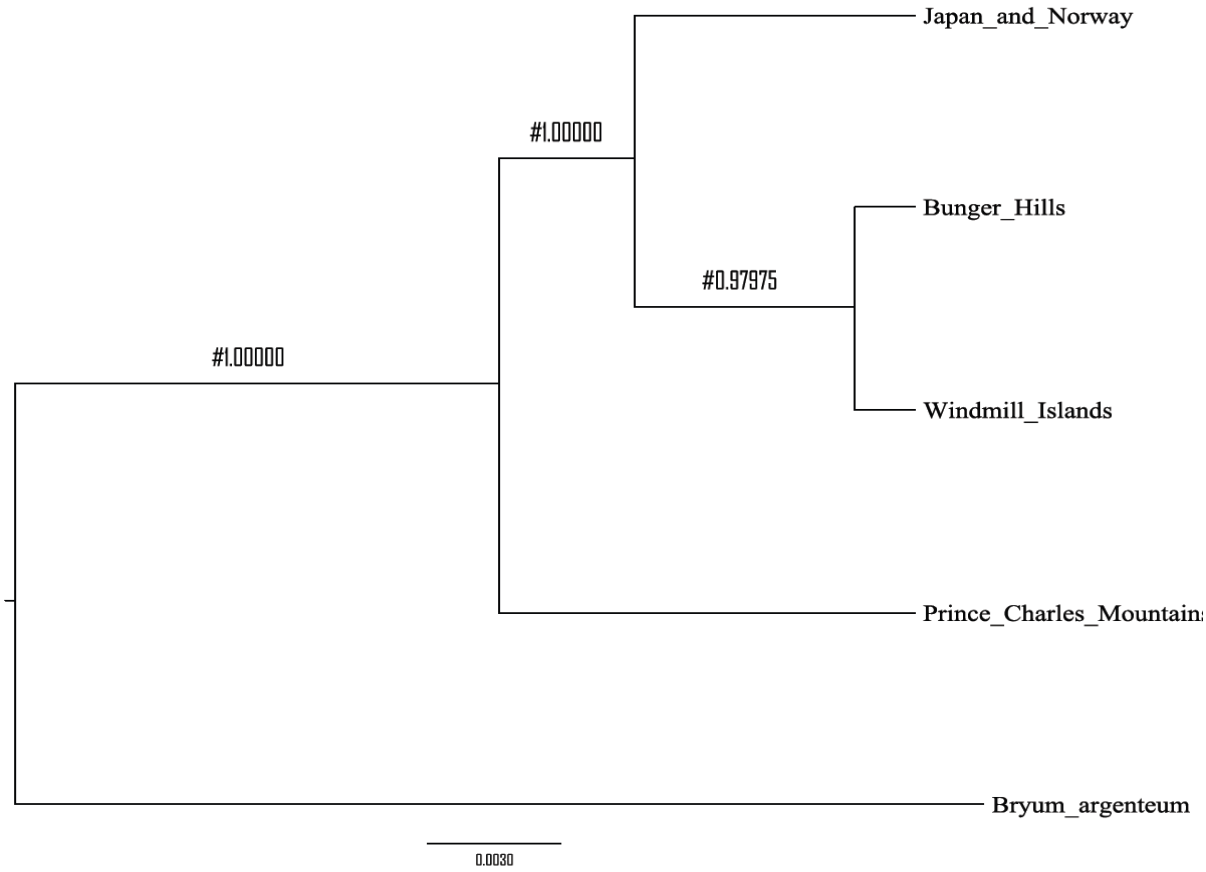

Figure 17. Tree generated via *BEAST using *rps*4 and ITS sequence data from samples of *B. pseudotriquetrum* collected from the Japan and Norway (TY-14438 and F03042) (n=2), Bunger Hills (B17, B18, B19, B20, B23 and CP10) (n=6), Prince Charles Mountains (JQ040696 & JQ040701, JQ040697 & JQ040702 and TY26709) (n=3) and Windmill Islands (B4, B5, B6, RRBP6, RRBP4, RRBP, SCBP4, SCBP3, 94 and 170) (n=10). Tree was rooted using a single sample of *B. argenteum* collected from the University of Wollongong and used as a guide tree for analysis in BP&P. Values represent probabilities of speciation at each node, with values greater than 0.95 considered significant and scale bar representing evolutionary change in branches.

3.2.2 Ability of ITS and rps4 to identify Bryum pseudotriquetrum

Samples morphologically identified as *B. pseudotriquetrum* were consistently identified as *B. pseudotriquetrum* based upon BLASTn matches using the complete ITS sequence. Furthermore, in cases where only partial sequence data was obtained, 500 bp from either ITS1 or ITS2 consistently identified samples as *B. pseudotriquetrum*. In contrast, the ability of *rps*4 to identify samples of *B. pseudotriquetrum* was poor. Samples from all locations were consistently identified as the moss species *Bryum lisae*. Furthermore, a single sample from Robinson Ridge in the Windmill Islands (RRBP6) was identified as *Bryum elegans.*

3.3 *Ceratodon purpureus*

A recent phylogenetic study by McDaniel and Shaw (2005) has identified separate Southern and Northern Hemisphere populations of *C. purpureus*. However, this study only included a single isolate from Antarctica. As such, the origins of Antarctic *C. purpureus* populations are poorly known. Furthermore, the identification of *C. purpureus* is difficult, and samples are often misidentified as *S. antarctici*. To address these issues, Bayesian MCMC analysis of Antarctic and herbarium samples of *C. purpureus* was performed. Additionally, the ability of ITS and *rps*4 to identify Antarctic *C. purpureus* populations was assessed.

3.3.1 Phylogeny of Ceratodon purpureus

Ceratodon purpureus sequence data was analysed using only a single locus, *rps*4, as ITS sequence data proved difficult to obtain from many herbarium specimens. Bayesian MCMC analysis was carried out in BEAST (Figure 18). In order to root the tree, three *B. recurvirostrum* samples originally identified as *C. purpureus* from the Vestfold Hills were incorporated into the analysis.

Results from this analysis revealed five distinct clades. All samples collected from East Antarctica (n = 8) formed a well-supported clade (PP = 0.98) incorporating samples from Heard Island (n = 2) and Australia (n = 6), with the exception of a single sample from Anchorage Island. Samples collected from Northern Hemisphere locations formed a poorly supported clade (PP < 0.35) incorporating samples from the Antarctic Peninsula, surrounding islands and a single sample from South Africa. Although support for this clade was low, a highly supported sub-clade (PP = 0.99) was resolved incorporating all samples from the Antarctic Peninsula and a single sample from Anchorage Island. A single sample from North Carolina and Nepal formed a separate clade (PP = 0.99), basal to both the Australia and East Antarctic clade and the Northern Hemisphere and Antarctic Peninsula clade. A single sample from Moe Island, located off the coast of the Antarctic Peninsula, formed a well-supported branch (PP = 1.0) basal to all other groups in the tree, with the exception of the out-group.

3.3.2 Ability of ITS and rps4 to identify Ceratodon purpureus

Samples morphologically identified as *C. purpureus* were consistently identified as *C. purpureus* in BLASTn searches using either ITS or *rps*4. Furthermore, the use of either ITS or *rps*4 effectively identified samples of *B. recurvirostrum* misidentified as *C. purpureus*. In both cases the accuracy of sequence data was confirmed by the incorporation of the misidentified samples into phylogenetic analyses as an out-group. As with *B. pseudotriquetrum*, in cases where only partial sequence data was obtained, 500 bp from ITS1 or ITS2 proved sufficient for identifying samples. In contrast, when full length *rps*4 data could not be obtained, samples were often incorrectly identified as uncultured streptophytes.

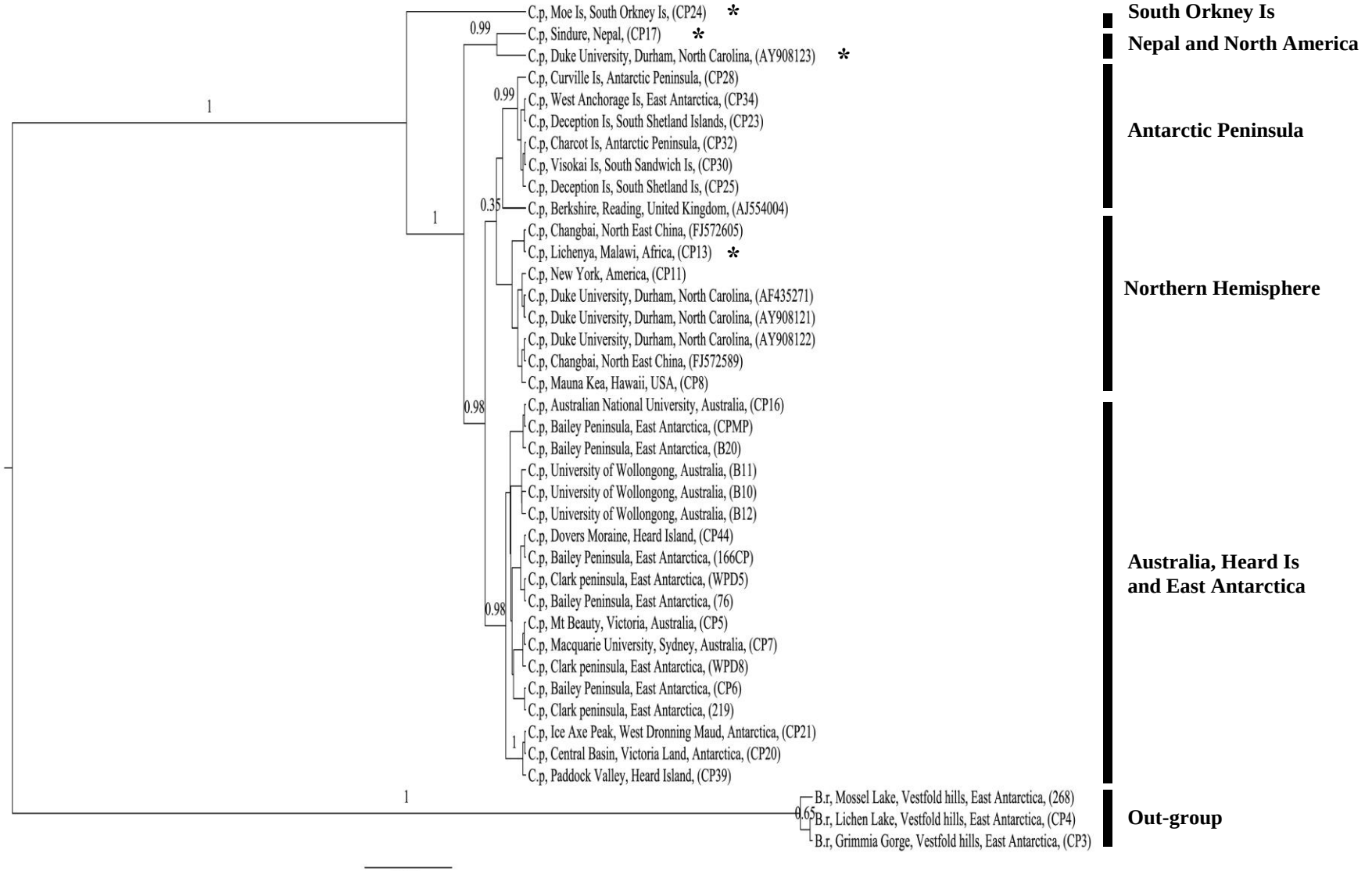

Figure 18. Tree generated via Bayesian MCMC analysis of *rps*4 sequence data originating from samples of *C. purpureus* (C.p) and rooted using three samples of *B. recurvirostrum* (B.r). Outliers highlighted by *. The collection locality of each sample is listed followed by the sample identification code or GenBank Accession number. Distinct clades are given on the right-hand side, values present on branches represent posterior probabilities of support for each node (values below 0.35 are not shown for clarity) and scale bar represents evolutionary divergence in branches.

3.4 Schistidium antarctici

Schistidium antarctici is one of a few mosses native to Antarctica and along with *B. pseudotriquetrum* and *C. purpureus* is one of the most common East Antarctic mosses (Ochyra *et al.*, 2008). However, like Antarctic *B. pseudotriquetrum* and *C. purpureus*, *S. antarctici* has never been examined using a molecular approach. As such, the presence of cryptic species of *S. antarctici* has not been investigated. Similarly, nothing is known of the origins of *S. antarctici*. To address these issues, Bayesian MCMC analysis was carried out on samples of *S. antarctici* and all available *Schistidium* sequences in GenBank. Additionally, the ability of ITS and *rps*4 to identify *S. antarctici* samples was assessed.

3.4.1 Phylogeny of Schistidium antarctici

In total, 108 *Schistidium* ITS sequences and 20 *Schistidium rps*4 sequences were available on GenBank and incorporated into the analysis of ITS and *rps*4 respectively (full list available in appendix, Table 8 & Table 9).

3.4.1.1 Analysis of ITS

Initial alignment of the ITS region using CLUSTAL-W proved difficult due to the presence of large gaps in the ITS1 region. Alignments produced using CLUSTAL-W contained many ambiguous gaps and aligned regions. In order to improve this, the alignment tool MAFFT was used, resulting in an alignment containing fewer gaps and ambiguous regions, and requiring little manual improvement. BEAST analysis resulted in a tree containing high support for nodes close to the branch tips, but with decreasing support towards the tree base (Figure 19).

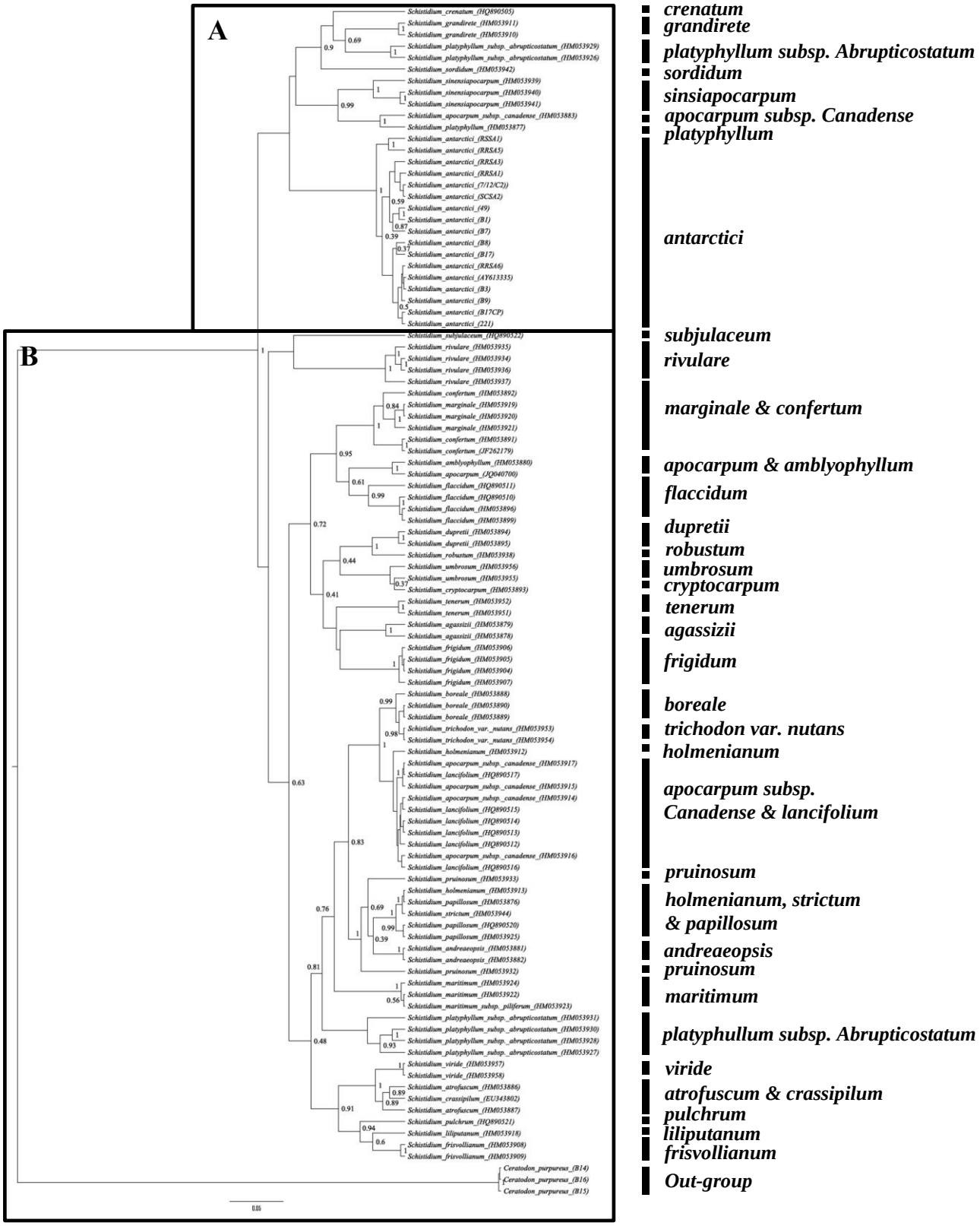

Figure 19. Tree generated via Bayesian MCMC analysis of ITS sequence data originating from samples of *S. antarctici*, and congeneric *Schistidium* sequences available on GenBank and rooted using ITS sequence data from *C. purpureus* (out-group). The species name for each sample is listed followed by the corresponding GenBank accession number or sample identification code. Species groups are listed (far right). Values present on branches represent posterior probabilities of support for each node (values below 0.35 are not shown for clarity) and scale bar representing evolutionary changes in branches.

In general two large clades were resolved. The clade in panel A (Figure 20) contained all *S. antarctici* samples and a group containing the sister taxa *Schistidium crenatum* (n = 1), *Schistidium grandirete* (n = 2), *Schistidium sordidum* (n = 1), *Schistidium platyphyllum* (n = 1), *Schistidium sinensiapocarpum* (n = 3), *Schistidium platyphyllum subsp. Abrupticostatum* (n = 6) and *Schistidium apocarpum subsp. Canadense* (n = 6) (Figure 19, Section A). However, support for the separation of *S. antarctici* from its sister clade is low (PP < 0.35). Within the *S. antarctici* sister clade, well supported grouping of the species: *S. grandirete* (PP = 1), *S. platyphyllum subsp. Abrupticostatum* (PP = 1), *S. sordidum* (PP = 0.9) and *S. sinensiapocarpum* (PP = 1) were resolved. With regards to *S. antarctici* all samples formed a single monophyletic group. Two samples within the *S. antarctici* clade, originating from Robinson Ridge (RRSA1 & RSSA5), were resolved as more divergent to all others (PP = 1). Samples of *S. antarctici* originated from three East Antarctic locations, although, no geographical structure was resolved between samples and the collection localities. Based on branch lengths, *S. antarctici* samples have undergone rapid evolution, with few divergences. In contrast, species within the sister taxa have experienced rapid evolution, following multiple divergences.

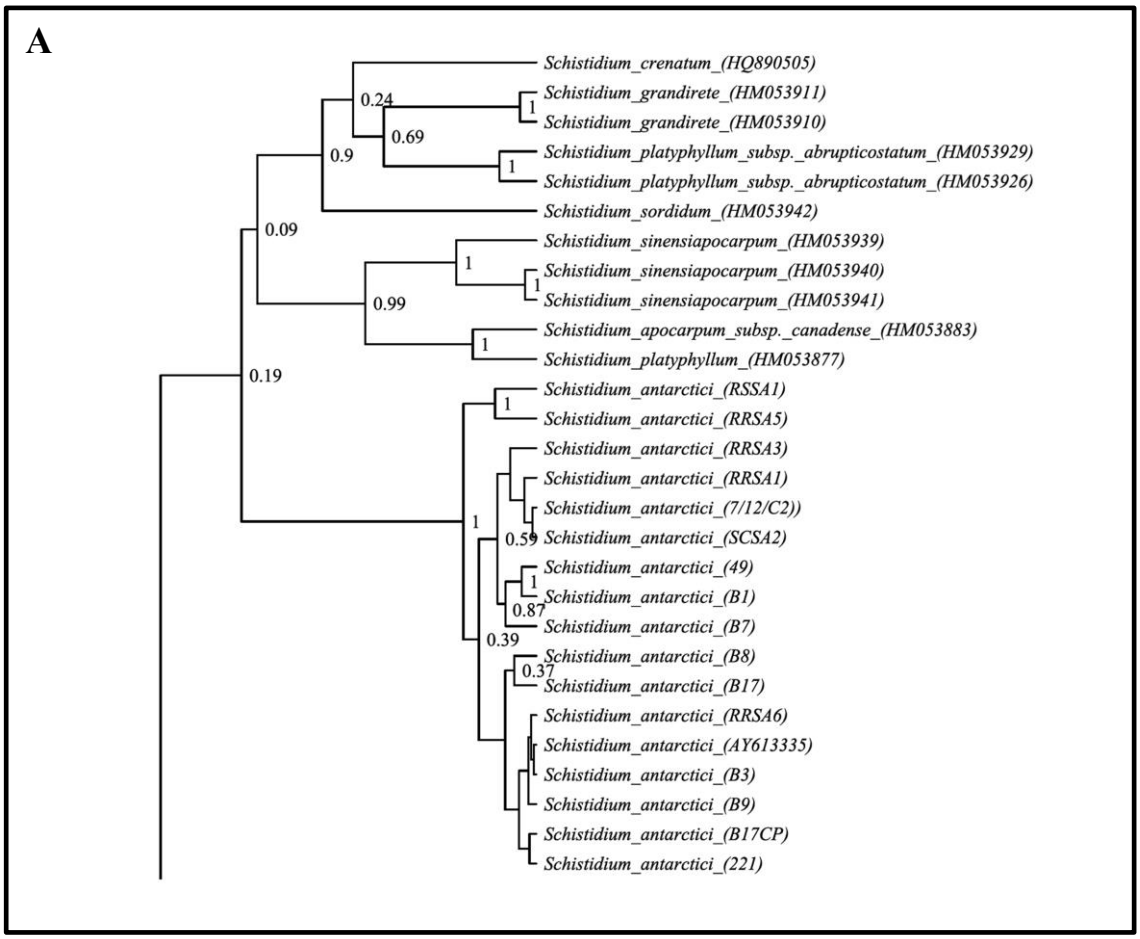

Figure 20. Section A of Figure 19 generated via Bayesian MCMC analysis of ITS sequence data originating from samples of *S. antarctici* and congeneric *Schistidium* sequences available in GenBank and rooted using ITS sequence data from *C. purpureus.* The species name of each sample is listed followed by the corresponding GenBank accession number or sample identification code. Values present on branches represent posterior probabilities of support for each node. Scale bar represents evolutionary change in branches

The large clade resolved in section B contains all other *Schistidium* taxa forming three distinct but poorly supported sub-clades. Well supported grouping were resolved for all species, with the exception of *Schistidium apocarpum* (n = 1) *Schistidium confertum* (n = 3), *S. apocarpum subsp. Canadense* (n = 5) and *Schistidium holmenianum* (n = 2) which were resolved as paraphyletic groups. Furthermore, of note is the paraphyly resolved for *S. platyphyllum subsp, Abrupticostatum* which formed a grouping of two samples in section A and four samples in section B.

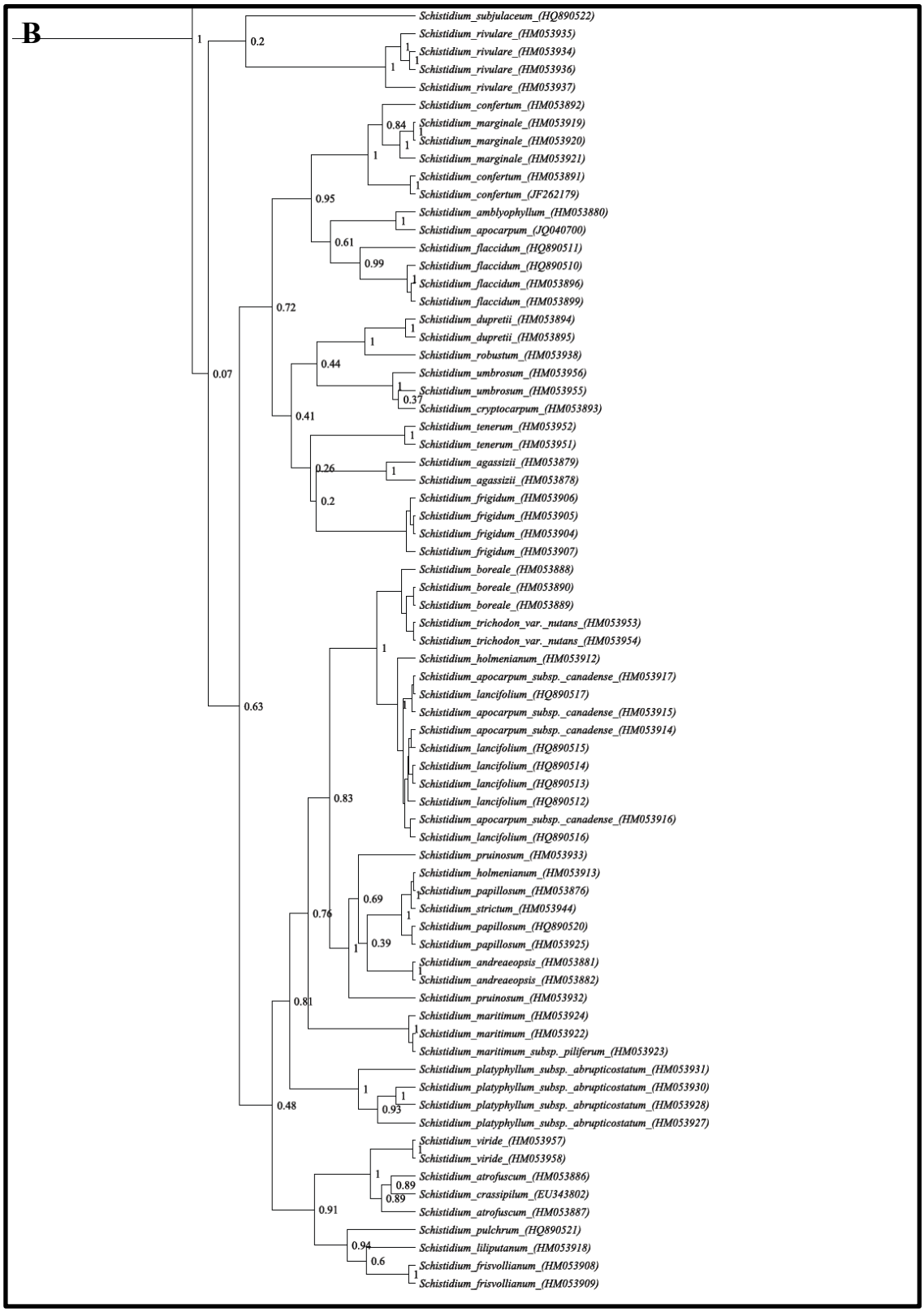

Figure 21. Section B of Figure 20Figure 19 generated via Bayesian MCMC analysis of ITS sequence data originating from samples of *S. antarctici* and congeneric *Schistidium* sequences available in GenBank and rooted using ITS sequence data from *C. purpureus.* The species name of each sample is listed followed by the corresponding GenBank accession number or sample identification code. Values present on branches represent posterior probabilities of support for each node. Scale bar represents evolutionary change in branches.

3.4.1.2 Analysis of rps4

Bayesian MCMC analysis of the *rps*4 locus (Figure 22) incorporated all *Schistidium rps*4 sequence data available in GenBank (n = 18). Alignment of *rps*4 was carried out in CLUSTAL-W, resulting in no ambiguous regions or gaps.

Phylogenetic analysis of the *rps*4 locus resulted in a tree containing high support for nodes closer to the tree root, but with decreasing support toward the tree tips. Two well-supported (PP = 1) clades were resolved. The first of these clades contained all *S. antarctici* samples as a single monophyletic group, with the exception of a single sample from the isolated Prince Charles Mountains (JQ040761) (Skotnicki *et al.*, 2012). Although inter-clade support was low within the *S. antarctici* clade, six samples (Bu22, RSSA1, B15, 17/12C2, B8 & RRSA6) appear to be more divergent than all other *S. antarctici* samples. Furthermore, as resolved in the ITS phylogeny, no *S. antarctici* samples formed any groupings based upon the geographical collection localities.

The second clade resolved contained all other taxa and the single outlier *S. antarctici* sample. As in the *S. antarctici* clade, inter-clade support was low. As such, no well supported grouping of any taxa exist. Although inter-clade support was low, the placement of the outlier *S. antarctici* sample outside of the *S. antarctici* clade was well supported. Additionally, when the two clades were compared, it can be seen that more genetic divergence has occurred within the *S. antarctici* clade than within the clade formed by all other taxa.

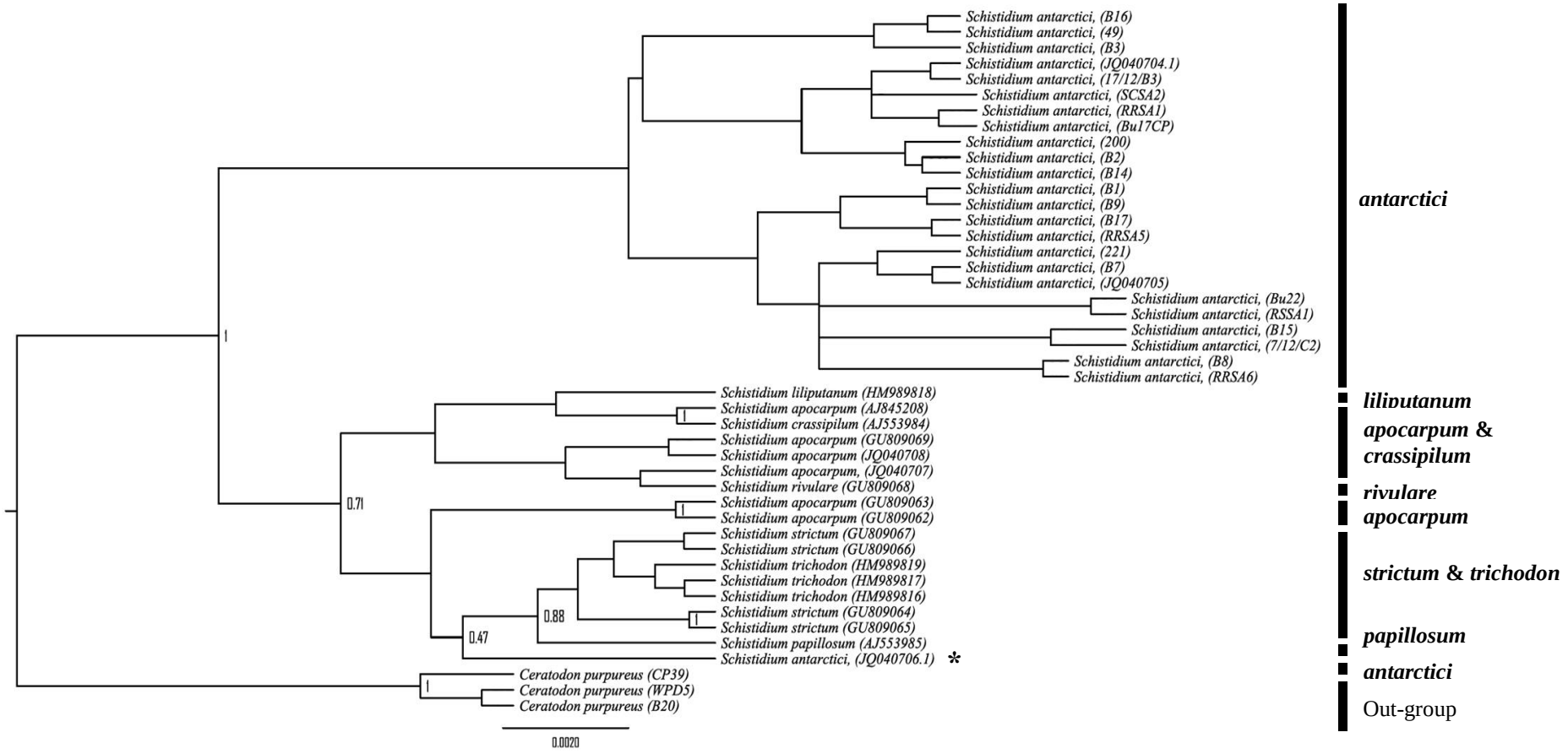

Figure 22. Tree generated via Bayesian MCMC analysis of *rps*4 sequence data originating from samples of *S. antarctici*, and congeneric *Schistidium* sequences available on GenBank and rooted using *rps*4 sequence data from *C. purpureus.* The species name for each sample is listed followed by the corresponding GenBank accession number or sample identification code. Species groups are given on the right-hand side and outliers highlighted by *. Values present on branches represent posterior probabilities of support for each node (values below 0.35 are not shown for clarity) and scale bar represents evolutionary divergence in branches.

3.4.2 Ability of ITS and rps4 to identify Schistidium antarctici

As with *C. purpureus*, BLASTn searches using complete *rps*4 and ITS sequences proved effective for the identification of samples morphologically identified as *S. antarctici*. Furthermore, a number of samples initially identified as *S. antarctici* were confirmed as *C. purpureus* based upon sequence data from ITS and *rps*4. To confirm the reliability of sequence matches, these samples were incorporated into phylogenetic analyses as out-groups. Moreover, as in *B. pseudotriquetrum* and *C. purpureus*, 500 bp of either ITS1 or ITS2 proved effective in identifying samples. Once again, incomplete *rps*4 data resulted in misidentification of samples as uncultured streptophytes.

4 DISCUSSION

This study has identified cryptic species of the moss *B. pseudotriquetrum* from locations in the Bunger Hills, Prince Charles Mountains, Windmill Islands and the Northern Hemisphere. Moreover, it demonstrated that populations of *C. purpureus* from the Windmill Islands are very closely related to populations of the same species from Australia and Heard Island. Populations of the Antarctic moss *S. antarctici* were shown to form a single monophyletic group, with the exception of a single sample analysed using the *rps*4 locus. Lastly, the PCR amplification and sequencing of the ITS region revealed the presence of a large diversity of algae and fungi closely associated with the moss gametophytes.

4.1 PCR and contaminant genes

Amplifications of ITS and *rps*4 were carried out using published protocols and produced amplicons consistent with published amplicon sizes (McDaniel and Shaw, 2005; Skotnicki *et al.*, 2005; Simon *et al.*, 2010; Skotnicki *et al.*, 2012). However, issues associated with poor quality data were present when investigating both loci. Sequencing of the ITS region was hindered by the presence of strong secondary structures in the species *C. purpureus* and *S. antarctici*. Secondary structures form between complementary bases in single stranded DNA. These secondary structures cause polymerases to stall, hindering the incorporation of dNTPs and causing sharp signal drops in sequencing chromatograms. As secondary structures do not form in all single stranded DNA copies, they can give rise to multiple amplicons in PCR reactions. These multiple amplicons would consist of short ITS fragment (up till the secondary structure) and complete ITS amplicons. However, no evidence of short ITS fragments were identified, as they were likely too small in size. To allow for sequencing of the ITS region, 5% DMSO, which disrupts secondary structure formation, was added to all sequencing reactions. Once high quality data could be obtained for the ITS region, the

identification of multiple ITS amplicons revealed a wide diversity of contaminant fungi and algae, which likely resulted in the poor sequence data obtained from *rps*4. Being a chloroplast gene, *rps*4 is only present in plants (Stech and Quandt, 2010). As such, it is likely that gel excision improved sequence data by removing weakly co-amplified algal genes. In order to sequence ITS, gel excision was used to excise the moss amplicons, using amplicon size as a guide. Therefore, the identification of contaminant genes (following the initial investigation of ITS amplicons) was biased towards contaminants with ITS regions between 1,000 and 1,200 bp in size. Nevertheless, a large diversity of foreign genes were still identified. Algal genes were more commonly isolated from *B. pseudotriquetrum*, while fungal genes were more commonly isolated from *C. purpureus* and *S. antarctici*. It is likely that this is a result of the growth habit of *B. pseudotriquetrum*, which is commonly found in wetter locations (Lockhart *et al.*, 2012). Futhermore, the loose growth habit of *B. pseudotriquetrum* may allow for light to penetrate moss clumps, aiding algal photosynthesis (Wasley *et al.*, 2006).

Of the algal and fungal groups identified, no groups were present in both the Bunger Hills and Windmill Islands. In the Bunger Hills, the Genus *Chloromonas* was identified from two separate samples. Interestingly, this genus of alga has not been previously reported in the Bunger Hills, but is present in the Windmill Islands as the species *C. rubroleosa* and is responsible for red snow (Ling *et al.*, 1993; Ling *et al.*, 1998). Additionally, a second red snow-forming algal genus (*Ulthorix)* was identified. Again this genus has previously been recorded in the Windmill Islands, but not in the Bunger Hills (Ling, 1996). A single fungal gene from the genus *Geomyces* was identified in a sample from the Bunger Hills. The genus *Geomyces* consists of freeze tolerant filamentous fungi, commonly found in Arctic permafrost (Rice *et al.*, 2006). The Antarctic distribution of *Geomyces* is commonly associated with bird, seal and human colonies (Marshall, 1998). By contrast, although the

Bunger Hills contain bird colonies, no *Geomyces* species have been reported previously. The Windmill Islands region contained the highest levels of biodiversity. This is expected as the Windmill Islands contain some of the highest levels of biodiversity on the Antarctic continent (Selkirk *et al.*, 1987; Smith, 1988). However, this high level of biodiversity was primarily localised to Baily Peninsula, the site of the current Casey Station. This may be a result of greater sampling within the region or may represent human introduction of fungal and algal diversity, much of which has been shown to originate from European locations (Azmi *et al.*, 1998b; Frenot *et al.*, 2005). The high levels of algal diversity on Baily Peninsula may also be due to the high level of moisture in the ASPA135 site, which is formed from a low lying basin. In contrast, Robinson Ridge, Browning and Mitchell peninsula are drier sites. However, regions of Clark Peninsula are very wet and high levels of algal and fungal diversity may be present in this region, but not represented in this study due to low levels of sampling on Clark Peninsula (Australian Antarctic Division, 2013).

Of the fungal species identified in the Windmill Islands the genera *Ascomycota*, *Trebouxiophyceae*, (*T. impressa*) and *Physcia*, (*P. adscendens*) have been previously identified from this region (Antarctic Treaty Consultative Meetings, 1991). Contrastingly, the algal genera *Neocystis* (*N. mucosa*), *Pseudendocloniopsis* (*P. botryoides*), *Chlorosarcinopsis* and *Sphaerocystis* have not been recorded in Antarctica. Interesting, the known distributions of *N. mucosa*, *P. botryoides* and *Chlorosarcinopsis* are from cold Northern Hemisphere locations (Guiry *et al.*, 2013). Additionally, the genus *Sphaerocystis* is only known from Signy Island, a small island off the coast of the Antarctic Peninsula (Broady, 1976).

The two fungal genera identified, *Phaeospharia* and *Phialocephala* (*Phialocephala virens*) are plant pathogenic fungi. *Phaeosphaeria* belongs to the Asomycota fungal division.

Phaeosphaeria has been isolated from the Antarctic grass *Deschampsia antarctica* found on the sub-Antarctic South Orkney Islands and Antarctic Peninsula (Stchigel *et al.*, 2004). Contrastingly, *P. virens* and its related genus *Phialocephala* have not been recorded in Antarctica but are associated with Northern Hemisphere alpine environments (Stoyke *et al.*, 1991; Hambleton *et al.*, 1997) and contribute to root rot in some plants (Siegfried *et al.*, 1992).

For the identification of moss species, ITS and *rps*4 proved variable. Ribosomal protein subunit 4 and ITS sequence data from the species *B. pseudotriquetrum, C. purpureus,* and *S. antarctici* are present in GenBank. However, the identification of each species using BLASTn search matches proved variable. For the identification of *B. pseudotriquetrum*, *rps*4 proved unreliable, consistently identifying samples as *Bryum lisae* and *Bryum elegans*. This is likely a result of the low variability in *rps*4 between species (Liu *et al.*, 2010). However, it may also indicate that Antarctic *B. pseudotriquetrum* was misidentified, although this is unlikely as *B. lisae* and *B. elegans* have never been recorded in Antarctica. The final possibility is that the Genbank specimens for *B. lisae* and *B. elegans* have been misidentified. A major difficulty in the identification of Antarctic mosses is the delimitation of *C. purpureus, S. antarctici* and *B. recurvirostrum,* which share overlapping distributions and very similar morphologies. Skotnicki *et al.*, (2012) found *rps*4 to be effective in delimiting populations of *C. purpureus* and *S. antarctici*. This is supported by evidence from this study, where in all cases *rps*4 correctly identified samples of these three species. Furthermore, in one case a sample of *C. purpureus* from the BASH (CP3) and two *S. antarctici* samples collected during the 2005 ANARE, were confirmed as *B. recurvirostrum* and *C. purpureus* respectively using *rps*4 sequence data. Moreover, the identification was confirmed by ITS sequence data and the incorporation of samples into phylogenetic analyses (Figure 18 and

Figure 19). For the identification of *B. pseudotriquetrum, C. purpureus, S. antarctici* and *B. recurvirostrum* ITS proved reliable and consistent in all cases. This again is in agreement with Skotnicki *et al*., (2012). However, as mentioned above, sequence data from ITS proved much more difficult to obtain and yielded contaminant genes, whereas no contaminant genes were isolated during the amplification and sequencing of *rps*4. Together these observations suggest the ITS is the most reliable marker for identifying Antarctic mosses. However, within the Windmill Islands, where *B. pseudotriquetrum* is easily identified by morphological means, *rsp*4 is most feasible.

A limitation of this form of species identification is that it is most reliable when databases contain gene information from all genera, and assumes that all species have been identified correctly. However, this is not presently the case. It is likely that the bulk of Antarctic algal and fungal species are not represented in the GenBank database. Nevertheless, in all cases ITS sequences from contaminant taxa resulted in very close matches, suggesting that for the foreign genes identified in this study GenBank proved adequate. Finally, even though a number of studies have examined the distributions of Antarctic fungi and algae in soils (for full review see Broady, 1979; Ruisi *et al.*, 2007), the high diversity and novelty of the algae and fungi identified in this study suggests that much work is still required. Furthermore, it appears that moss turfs, which have been poorly studied, are a rich source of biodiversity yet to be thoroughly examined.

4.2 Bryum pseudotriquetrum

Recent evidence has suggested that what has previously been considered a single cosmopolitan population of the moss *B. pseudotriquetrum* is instead a discrete Antarctic and Northern Hemisphere species (Kengo *et al.*, 2013). Consequently, this study identified four cryptic species from what was considered to be a homogeneous population of *B. pseudotriquetrum*. *Bryum pseudotriquetrum* is considered to be highly variable in its morphology and is found in almost all environments in both the Northern and Southern Hemisphere (Ochyra *et al.*, 2008). It now seems likely that *B. pseudotriquetrum* is instead multiple cryptic species adapted for specific environments.

Coalescent-based species delimitation identified four distinct cryptic species present in the Bunger Hills, Windmill Islands, Prince Charles Mountains and the Northern Hemisphere. Populations from the Bunger Hills were most closely related to populations from the Windmill Islands. This suggests that ancestral populations may have colonised the East Antarctic coast following the de-glaciation of the Bunger Hills 20,000 to 30,000 years ago (Gore *et al.*, 2001) and the subsequent de-glaciation of the Windmill Islands 5,500 years ago (Goodwin, 1993). However, samples from the Vestfold Hills, which became ice free 10,000 to 25,000 years ago (Adamson *et al.*, 1983), would be required to strengthen this hypothesis.

Most closely related to the Windmill Islands and Bunger Hills samples are two *B. pseudotriquetrum* samples originating from the Northern Hemisphere. As suggested by Kato (2013), these samples represent a distinct species from all Antarctic samples. Moreover, the Northern Hemisphere samples, being the first described as *B. pseudotriquetrum*, represent the *B. pseudotriquetrum* (Hedw.) holotype (Gärtner *et al.*, 1802). However, caution must be applied since the two Northern Hemisphere samples are unlikely to capture the full variability

in Northern Hemisphere populations. Moreover, non-Antarctic Southern Hemisphere populations have not been included in this analysis (Ochyra *et al.*, 2008). In terms of origins, samples from the Bunger Hills and Windmill Islands are more closely related to Northern Hemisphere samples than samples from the Prince Charles Mountains. This suggests that *B. pseudotriquetrum* samples from the Windmill Islands and Bunger Hills originated as a result of a colonisation event, while samples in the Prince Charles Mountains might be refugial. However, in order to conclusively establish the origins of these three Antarctic *B. pseudotriquetrum* species, greater sampling is required, both around Antarctica and the Northern and Southern Hemispheres. Furthermore, tree nodes require dating, a process that requires a key event with a known date in order to calibrate evolutionary clocks. In particular, accurate measurements of de-glaciation may provide such information for future studies.

In recent years, cryptic speciation has become more evident in bryophytes (comprehensively reviewed in Shaw, 2001). Exploration during the 19th Century led to an explosion in bryophyte species, based primarily on the assumption that isolated populations must represent different species (Shaw, 2001). This is evident in Antarctic mosses, with *B. pseudotriquetrum* from the Bunger Hills previously known as *Bryum korotkeviczia* (Savicz-Lyubitskaya, 1959; Savicz-Lyubitskaya, 1960), *B. pseudotriquetrum* from the Antarctic Peninsula known as *Ptychostrum pseudotriquetrum* (Ochyra et al., 2008) and *B. pseudotriquetrum* from Madagascar described as *Bryum austroventricosum* (Crosby *et al.*, 1983). In total, *B. pseudotriquetrum* has been described as at least 15 different species, which in recent years have been reclassified as the single species *B. pseudotriquetrum* (Ochyra *et al.*, 2008). However, evidence of high levels of genetic variation in Antarctic *B. pseudotriquetrum* (Skotnicki *et al.*, 1998b), combined with the high levels of morphological plasticity in

varying environments provided early evidence of multiple species within the *B. pseudotriquetrum* species group.

There are many definitions of a species, but in general the morphological and biological species concepts have proven effective for classifying organisms. However, in the case of Antarctic bryophytes, sexual reproduction is rare (Convey and Smith, 1993). Moreover, bryophytes show a large geographical range and little morphological differentiation (Shaw, 2001). As such, molecular techniques are most appropriate. A species is generally considered to be an independently evolving population (Fujita *et al.*, 2012). However, molecular methods other than coalescent-based species delimitation are not capable of identifying independently evolving population, and as such could not be used to delimit cryptic moss species. Although, once a population is establish as being a single species, other methods of species identification prove appropriate. The use of a DNA barcoding approach using *rps*4 and ITS as suggested by Skotnicki *et al.*, (2012) and Skotnicki *et al.*, (2005) proved effective in identifying *C. purpureus, S. antarctici* and *B. recuroviorstrum*. Although, this method correctly identified *B. pseudotriquetrum* populations, it could not identify cryptic species, as cryptic species have likely been misidentified in public databases. This highlights the benefits of coalescent-based species delimitation, as employed in this study, which enable statistical identification of independently evolving populations (Fujita *et al.*, 2012) and as such overcomes many issues associated with other methods (see Chapter 3). In the case of *B. pseudotriquetrum*, this study employed the first use of coalescent-based species delimitation in bryophytes and identified definitive cryptic populations in the Windmill Islands and Bunger Hills and possible cryptic populations in the Northern Hemisphere and Prince Charles Mountains. To confidently confirm the presence of cryptic species in the

Northern Hemisphere and Prince Charles Mountains, greater sampling is required from both the Northern and Southern Hemisphere.

4.3 Ceratodon purpureus

As with *B. pseudotriquetrum*, *C. purpureus* is a morphologically variable cosmopolitan moss species (Ochyra *et al.*, 2008). Similar to *B. pseudotriquetrum*, it was initially classified as a number of different species: *Ceratodon antarcticus*, (Holzinger, 1902) *Ceratodon grossiretis* (Cardot, 1908), *Ceratodon minutifolius* (Cardot, 1911), *Ceratodon validus* (Horikawa *et al.*, 1963) and *Ceratodon kinggeoricus* (Kanda, 1986), all of which are currently considered as *C. purpureus* (Ochyra *et al.*, 2008). However, in contrast to *B. pseudotriquetrum*, no evidence of cryptic species has been reported in *C. purpureus*. Genetic studies of *C. purpureus* have identified discrete Northern and Southern Hemisphere populations (McDaniel and Shaw, 2005) and identified that relatedness in Antarctic *C. purpureus* populations is likely related to geographical separation (Skotnicki *et al.*, 1998c). However, no study has examined the origins of Antarctic populations, until this study.

Bayesian MCMC analysis of *rps*4 sequences identified populations from East Antarctica as most closely related to populations from Australia and Heard Island. This corroborates work by Skotnicki *et al.*, (2004) who identified a close relatedness between East Antarctic *C. purpureus* populations and those found in Australia and on Heard Island. Furthermore, Skotnicki *et al.*, (2004) incorporated samples from Northern Hemisphere populations. However, these results were unfortunately not presented and further comparisons cannot be made. Surprisingly, the analyses carried out in the present study resolved populations of *C. purpureus* from the Antarctic Peninsula as more closely related to populations from the Northern Hemisphere than East Antarctica. This is in contrast to work by McDaniel and

Shaw (2005) who identified discrete Northern and Southern Hemisphere populations, with little movement between hemispheres. Unfortunately, McDaniel and Shaw (2005) only incorporated a single East Antarctic sample and as such the same results were not elucidated.

It would be expected that moss communities that are closer geographically would be more closely related. In addition, evidence suggests that the relatedness of bryophyte communities is strongly correlated with wind patterns (Muñoz *et al.*, 2004). However, the Antarctic continent is isolated from surrounding land masses by the Antarctic circumpolar current and strong circumpolar winds. Furthermore, due to limited sporophyte production in Antarctic mosses, long range dispersal by winds is probably limited. Nevertheless, low levels of spore have been recorded in Victoria Land Antarctica, showing that transfer by wind is possible (Linskens *et al.*, 1993). Aside from low levels of spore transfer by winds, the close relationship of East Antarctic *C. purpureus* to Australian *C. purpureus* might be due to migratory birds, the Tasman gateway acting a land bridge between Australia and Antarctica, or more recently humans. The Tasman Gateway connected Australia to Antarctic and collapsed before the LGM. Therefore if relatedness between Australian and East Antarctic *C. purpureus* is due to the Tasman Gateway, *C. purpureus* populations must be refugial populations which have survived through the LGM. However, no evidence of refugial populations has been found in East Antarctica. On the other hand, evidence exists for the human introduction of *C. purpureus* to Antarctica. Evidence from *C. purpureus* microsatellites shows higher levels of genetic diversity with proximity to Australian research stations (Clarke *et al.*, 2009). Additionally, studies of fungi have found increased fungal diversity in human disturbed sites (Azmi *et al.*, 1998a; Connell *et al.*, 2008). These findings suggest that human exploration and research may be one of the key factors in both the introduction of moss and fungi to Antarctica and increasingly its movement within the

continent. The other possibility is the introduction of spores via migratory birds. Research has shown that bryophytes are transported by birds for nest building (Breil *et al.*, 1976), although no specific research has been carried out examining the introduction of flora and fauna to Antarctica by migratory birds. As such, the impacts of migratory birds are not known, although it seems likely that both migratory birds and Antarctic researchers impact on the introduction of flora and fauna to the Antarctic continent.

The Northern Hemisphere clade that was resolved during phylogenetic analysis grouped all Northern Hemisphere samples with the samples from the Antarctic Peninsula, along with a single Southern Hemisphere sample from Malawi, Africa. This contrasts with the phylogenetic analysis carried out by McDaniel and Shaw (2005), who identified three samples from Capetown, South Africa as grouping strongly with other Southern Hemisphere samples. This distinction is likely a result of the location of the samples, with Malawi located much closer to the equator than Capetown. Within the Northern Hemisphere clade, two poorly supported sub-clades were resolved, containing all Antarctic samples, with the exception of a single sample from the UK and all Northern Hemisphere samples. However, support for these two sub-clades was low and a second locus is required for confirmation. The close relationship of samples from the Antarctic Peninsula with samples from the Northern Hemisphere can be partially explained by the close proximity of South America to the Antarctic Peninsula, which may act as a land bridge for dispersal between the Northern and Southern hemisphere. However, the incorporation of samples from South America in future studies is required to confirm this hypothesis. Contrastingly, refugial and endemic populations of nematodes, microbes and wingless insects that have been identified on the Antarctic Peninsula suggest that the Antarctic Peninsula is geographically isolated (Convey *et al.*, 2008).

The Antarctic Peninsula is one of the most temperate and hospitable locations on the Antarctic continent and contains more human research stations than any other Antarctic location. In particular, the British Antarctic Survey operates seven Antarctic research stations within the Antarctic Peninsula. The single UK sample which grouped with samples from the Antarctic Peninsula was generated as part of a larger study investigating the subclass Dicranidae (Hedderson *et al*., 2004). Its similarity to samples from the Antarctic Peninsula may be due to laboratory contamination during its extraction, or to the introduction of genetic diversity from the UK into the Antarctic Peninsula by the long history of human habitation. No introduced species have been found on the Antarctic Peninsula. However, the human mediated introduction of the North Atlantic crab spider (*Hyas araneus*) around the Antarctic Peninsula shows that it could easily occur (Tavares *et al.*, 2004). It seems most probable that the close relation between Antarctic Peninsula *C. purpureus* and Northern Hemisphere *C. purpureus* is related to the late separation of the Drake Passage, a land Bridge connecting South America to the Antarctic Peninsula. Moreover, the high level of human activity in the Antarctic Peninsula and short distance for migratory birds makes it likely that genetic diversity has been introduced into the region. However, further studies are required to identify this.

Finally, phylogenetic analysis revealed the presence of three highly divergent samples. A single sample from Nepal formed a well-supported clade with a single GenBank sample from North Carolina. Four North Carolina samples were incorporated from a study of moss diversity (Shaw *et al.*, 2005). Its placement may represent laboratory contamination. Contamination of the author amplified and sequenced sample from Nepal can be ruled out as any contamination would result in the sample being grouped into another clade. As such, this

sample may represent a unique isolate. Further to this, a single highly divergent sample was identified from South Orkey Is; whilst this single sample may be from a refugial population, further replicates are required for verification.

The molecular marker *rps*4 provides an effective phylogenetic tool due to its ease of amplification, even from old material. However, *rps*4 is limited by low phylogenetic resolution. Future work will require the incorporation of multiple markers of a higher resolution to identify fine scale relationships, while incorporating more comprehensive sampling. Finally, work remains to be done to specifically identify possible introductions of flora and fauna to the Antarctic continent by bird and human movement.

4.4 Schistidium antarctici

Schistidium antarctici is one of seven moss species found only in Antarctica (Ochyra *et al.*, 2008). However, unlike other native Antarctic species, *S. antarctici* is very common. Furthermore, its morphological similarities with *C. purpureus* can make identification of Windmill Islands specimens difficult. The species status of *S. antarctici* has never been examined using a molecular approach. As such, *S. antarctici* may represent multiple cryptic species or morphological variants of *Schistidium* species found elsewhere. In this section, the species status of *S. antarctici* is examined using all *Schistidium* ITS and *rps*4 data available on GenBank. Moreover, the location of *S. antarctici* within the *Schistidium* genus is discussed.

4.4.1 Internal transcribed spacer

The ITS region contains high levels of phylogenetic information. However, issues associated with its sequencing (as mentioned in section 4.1) and alignment made obtaining data and analyses difficult. Alignment of the ITS region in *B. pseudotriquetrum* and *C. purpureus* proved relatively straightforward, as alignments within a single species often contain few gaps. However, in the case of *S. antarctici* the incorporation of other *Schistidium* species required the insertion of large gaps, resulting in ambiguously aligned regions. To overcome this, often only part of the ITS2 region is analysed, although this requires the exclusion of informative sequence data. Instead, the analysis of RNA secondary structures in the ITS1 and ITS2 regions are used to improve alignments (Milyutina *et al*., 2010). In this study the software MAFFT was used, which incorperates RNA secondary structure to improve alignments.

Following alignment, analysis of the ITS region revealed *S. antarctici* as a single monophyletic group. As paired multilocus data was not obtained nor available for *Schistidium* samples, speciation in each node could not be quantified by coalescent-based species delimitation. Therefore, identification of a species is based upon all samples forming a monophyletic group, with less variation between samples than to other taxa (Moritz and Cicero, 2004). Based upon this criterion, *S. antarctici* appears to be a unique species rather than a morphological variant of other *Schistidium* species. *Schistidium antarctici* samples included in this analysis were sampled from three locations (Prince Charles Mountains, Bunger Hills and Windmill Islands) spread over 2,000 km of the East Antarctic coast. However, unlike *B. pseudotriquetrum*, no evidence of cryptic species was present. Furthermore, no distinct groupings based upon geographical locations were identified. However, samples from West Antarctica were not available for this study, therefore distinct

East and West Antractic populations or possible cryptic species may not have been revealed. More comprehenive sampling and the incorporation of multiple loci are required in future studies to confirm that no cryptic species or geographic structure is present.

Being an Antarctic endemic species, it is likley that *S. antarctici* has been present in Antarctica longer than cosmopolitan species. Due to the slow and consistent growth of Antarctic mosses, evidence of the prescence of *S. antarctici* prior to other moss species can be seen in the abundance of long moss shoots (Robinson Pers. Comm). Additionally the long term prescence of *S. antarctici* may be evidenced in the ITS phylogeny. Branch lengths show that following divergance from its sister taxa, *S. antarctici* underwent rapid evolution, followed by a period of limited change. This may represent the evolution of *S. antarctici* in response to the cooling Antarctic climate, prior to the LGM. However, as in *B. pseudotriquetrum,* key events are required in order to date tree nodes and confirm this hypothesis.

Phylogenetic analysis using ITS revealed that the sister taxa to *S. antarctici* are the *Schistidium* species *S. crenatum, S. grandirete, S. sordidum, S. platyphyllum, S. sinensiapocarpum, S. platyphyllum subsp. Abrupticostatum* and *S. apocarpum subsp. Canadense*. Interestingly, *Schistidium* species most closely related to *S. antarctici* are all restricted to cold climate locations. *Schistidium crenatum* is only known from two samples in Russia (Váňa *et al.*, 1988), *S. sordidum,* which is a synonym for *S. apocarpum subsp soridium* and *S. apocarpum subsp. Canadense* are found in sub-Antarctic and high latitude environments (Amann *et al.*, 1918). *Schistidium platyphyllum* and *S. platyphyllum subsp. Abrupticostatum* are semi aquatic species commonly found in high latitude Northern Hemisphere locations (Lockhart *et al.*, 2012). Finally, *S. grandirete* is an Arctic species

(Blom, 1996). In general, all *Schistidium* species in the sister clade to *S. antarctici* originate from cold permafrost climates, with the exception of *S. sinensiapocarpum*. Furthermore, few morphologically shared features are present between the moss species (Milyutina *et al*., 2010). The location of cold climate moss species as a sister group to *S. antarctici* may be a result of vicariance of a single common ancestor. However, due to the large degree of geographical separation between *S. antarctici* and its sister taxa, the grouping may be a result of convergent evolution in the ITS regions as a result of cold climates. Although the ITS1 and ITS2 regions do not code for protein, conserved secondary structures between plants suggest a functional role (Mai *et al.*, 1997), which is therefore subject to confounding environmental pressures. A detailed investigation of the effects of cold climates on ITS secondary structure would be useful in identifying confounding factors impacting on phylogenetic relationships.

4.4.2 Ribosomal protein subunit 4

In contrast to the support values from analysis using ITS, analysis using *rps*4 resulted in high support near the tree root with low support towards the tree tip. Furthermore, since studies of the *Schistidium* genus have primarily focused ITS, *rps*4 data are limited. Nevertheless, sequence data was available for many of the same species used in the analysis of ITS. Analysis using *rps*4 revealed *S. antarctici* as a paraphyletic group, with a single sample of *S. antarctici* grouped outside of the *S. antarctici* species clade. Support for the placement of taxa within each clade was low. However, support for the separation of all samples into two distinct clades was high, suggesting high confidence in the placement of the single *S. antarctici* sample outside of all others. Unfortunately, ITS data was not available for this sample. However, ITS and *rps*4 data from *S. antarctici* samples originating from the same location do not group outside of the main *S. antarctici* clade. As such, this suggests that the

single outlier *S. antarctici* sample may have been misidentified. *Schistidium antarctici* unlike *B. pseudotriquetrum* and *C. purpureus* is a quite distinct species, being originally named *Grimmia antarctici* (Cardot, 1906) before being reclassified as *S. antarctici* (Savicz-Lyubitskaya, 1965). However, it is occasionally confused with the species *Schistidium andinum*, which is known only from the Antarctic Peninsula (Ochyra *et al.*, 2008) and *C. purpureus*. If the outlier *S. antarctici* sample is a misidentified sample of *C. purpureus* it would be grouped within the out-group. As this was not the case, the sample may be a misidentified sample of *S. andinum*. If this is the case, it represents a new record for *S. andinum* in the Prince Charles Mountains. However, comparison to *S. andinum* samples from the Antarctic Peninsula would be required for confirmation.

4.4.3 Relationship of Schistidium antarctici within the Schistidium genus

The genus *Schistidium* is a poorly understood genus and primarily consists of cold and temperate climate species, which were once part of the overarching genus *Grimmia.* Attempts have been made to classify *Schistidium* into distinct groups (Ochyra *et al.*, 2008). A number of groupings have been suggested including: the separation of the genus *Schistidium* into the subdivision *Platyphylloideae* and *Apocarpiforms* (Kindberg, 1898), separation of *Schistidium* into the subdivision *Apocarpa* and *Conferta* (Vilhelm, 1922) and separation into the five subdivisions: *Robust*, *Conferta*, *Tenera*, *Atrofusca* and *Apocarpa* (Blom, 1996). However, Bayesian MCMC analysis of the *Schistidium* genus, based upon all available ITS and *rps*4 data did not resolve clades in agreement with any of the suggested division and sub-divisions mentioned above. Analysis using ITS revealed fine detail towards node tips, while *rps*4 revealed higher level information about the grouping of clades. However, further analysis incorporating both loci in a coalescent-based approach is required for confidence at all taxonomic levels. The phylogenetic relationships resolved are in most agreement with the taxonomic divisions and sub-divisions suggested by Blom (1996) and support the findings of

Milyutina *et al*., (2010), who constructed a phylogeny of the *Schistidium* genus using ITS and a maximum Likelihood method of analysis.

5 CONCLUSION

Antarctic mosses are the main floral component of the Antarctic ecosystem and survive on the coldest, windiest and most isolated continent on the planet. In this study, the aim was to construct a phylogeny of the three common Antarctic mosses: *Bryum pseudotriquetrum*, *Ceratodon purpureus* and *Schistidium antarctici* and evaluate the ability of the molecular markers ITS and *rps*4 to delimit the three species. Coalescent-based species delimitation on samples of *B. pseudotriquetrum* allowed for the identification of four separate cryptic species, originating from the Bunger Hills, Windmill Islands, Prince Charles Mountains and Northern Hemisphere. In line with taxonomic protocol, we suggest that the first described specimens from the Northern Hemisphere remain as *B. pseudotriquetrum*, while populations from the Bunger Hills revert to *Bryum korotkevich*. Further taxonomic work is required prior to the reclassification of populations from the Windmill Islands. However, the place holder names of *B. pseudotriquetrum var. Windmill* and *B. pseudotriquetrum var. Prince Charles* prior to further investigation is recommended. Finally, work remains to identify populations of *B. pseudotriquetrum* not sampled in this study; in particular, greater sampling and replicates are required from the Northern Hemisphere and non-Antarctic Southern Hemisphere locations.

Bayesian MCMC analysis of *C. purpureus* samples revealed that populations from the Antarctic Peninsula are more closely related to populations from the Northern Hemisphere, whilst populations from East Antarctica are most closely related to populations from Australia and Heard Island. This evidence corroborates evidence of the introduction of moss

species by human and bird colonies, which will require specific investigation in future studies. Moreover, the use of multiple loci in future analysis will improve tree support.

Analysis of *S. antarctici* ITS and *rps*4 data separately revealed *S. antarctici* as a monophyletic group, indicative of a single species. Additionally, analysis revealed that East Antarctic *S. antarctici* populations are most closely related to Arctic and cold climate *Schistidium* species, which may be a result of convergent evolution. Future work will require the use of multilocus data for coalescent-based species delimitation and to improve tree support. Moreover, the examination of ITS sequence changes in response to cold climates, will allow for the identification of confounding factors in phylogenetic analyses.

Lastly, the issue of Antarctic moss identification has been examined both through the identification of contaminating biological material and the reliability of the GenBank database for the identification of *C. purpureus*, *S. antarctici*, *B. recurvirostrum*, and *B. pseudotriquetrum* samples, using ITS and *rps*4. However, care must be taken as *rps*4 may not correctly identify samples of *B. pseudotriquetrum*.

In conclusion, Antarctic mosses represent a unique group of plants which survive in some of the harshest conditions on earth. Due to these harsh conditions, their identification has proven difficult and although much work remains to be done, this study demonstrates that *rps*4 and ITS are effective for identifying phylogenetic relationships in Antarctic mosses, for the coalescent-based species delimitation of cryptic populations and for the identification of the mosses examined in this study. Furthermore, this investigation has elucidated phylogenetic relationships among the three most common East Antarctic mosses, improving our ability to

use these Windmill Islands mosses as proxies for the effects of climate change on Antarctic terrestrial ecosystems.

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APPENDIX

Table 8. *Schistidium* ITS sequence data incorporated into the Bayesian MCMC analysis of the *Schistidium* genus. Sequence Identities (ID), Accession numbers and source are listed.

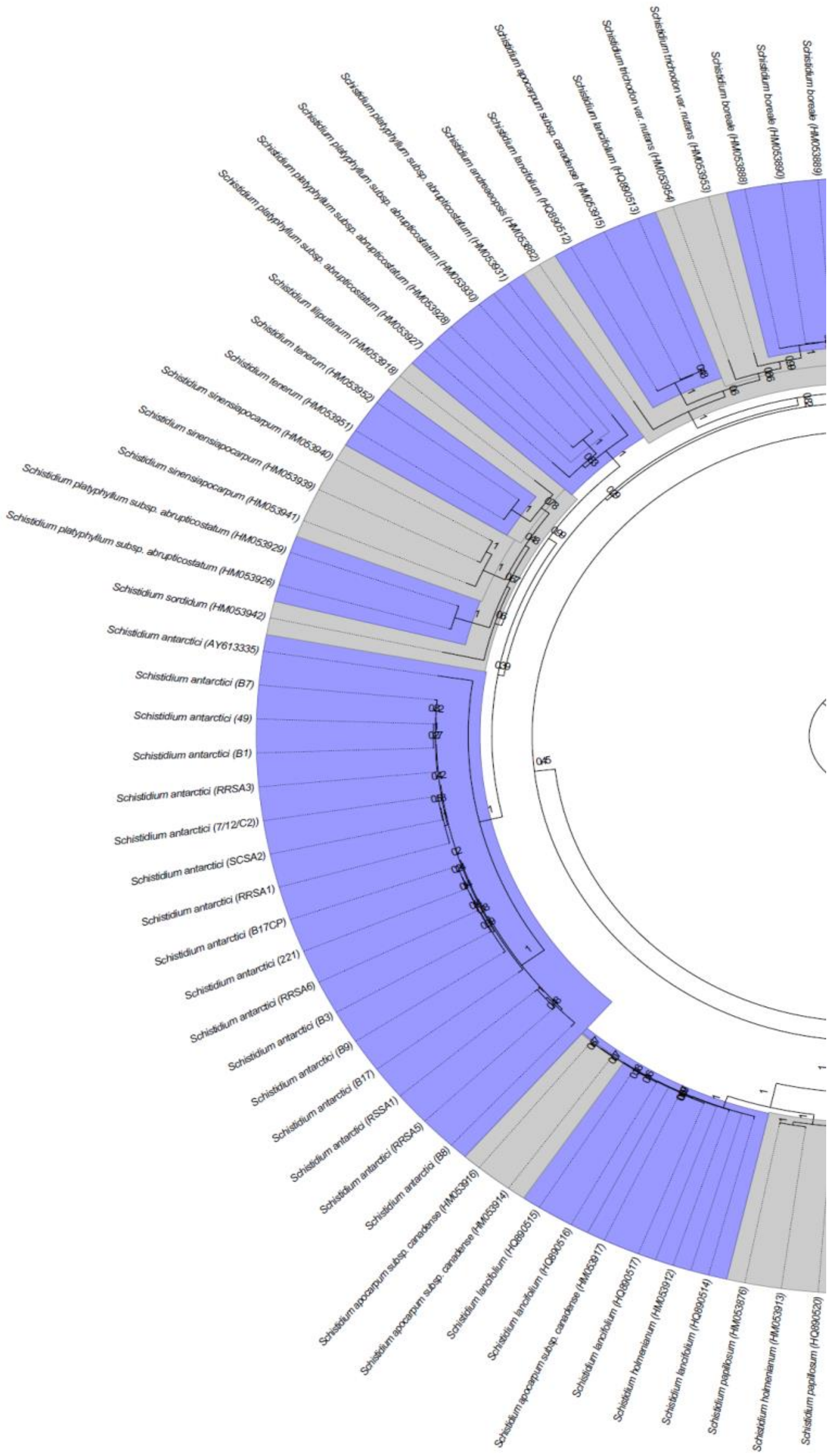
Sequence ID	Accession Number	Source
S. agassizii	HM053879	(Milyutina *et al*., 2010)
S. agassizii	HM053878	(Milyutina *et al*., 2010)
S. amblyophyllum	HM053880	(Milyutina *et al*., 2010)
S. andreaeopsis	HM053881	(Milyutina *et al*., 2010)
S. andreaeopsis	HM053882	(Milyutina *et al*., 2010)
S. antarctici	AY613335	(Skotnicki *et al*., 2012)
S. apocarpum	JQ040700	(Skotnicki *et al*., 2012)
S. apocarpum subsp. Canadense	HM053915	(Milyutina *et al*., 2010)
S. apocarpum subsp. Canadense	HM053917	(Milyutina *et al*., 2010)
S. apocarpum subsp. Canadense	HM053916	(Milyutina *et al*., 2010)
S. apocarpum subsp. Canadense	HM053914	(Milyutina *et al*., 2010)
S. apocarpum subsp. Canadense	HM053883	(Milyutina *et al*., 2010)
S. atrofuscum	HM053887	(Milyutina *et al*., 2010)
S. atrofuscum	HM053886	(Milyutina *et al*., 2010)
S. boreale	HM053890	(Milyutina *et al*., 2010)
S. boreale	HM053889	(Milyutina *et al*., 2010)
S. boreale	HM053888	(Milyutina *et al*., 2010)
S. confertum	HM053879	(Milyutina *et al*., 2010)
S. confertum	HM053891	(Milyutina *et al*., 2010)
S. confertum	HM053892	(Milyutina *et al*., 2010)
S. crassipilum	EU343802	(Hernández-Maqueda *et al.*, 2008)
S. crenatum	HQ890505	(Ignatova *et al*., 2009)
S. cryptocarpum	HM053893	(Milyutina *et al*., 2010)
S. dupretii	HM053895	(Milyutina *et al*., 2010)
S. dupretii	HM053894	(Milyutina *et al*., 2010)
S. flaccidum	HM053899	(Milyutina *et al*., 2010)
S. flaccidum	HM053896	(Milyutina *et al*., 2010)
S. flaccidum	HQ890511	(Ignatova *et al*., 2009)
S. flaccidum	HQ890510	(Ignatova *et al*., 2009)
S. frigidum	HM053907	(Milyutina *et al*., 2010)
S. frigidum	HM053906	(Milyutina *et al*., 2010)
S. frigidum	HM053905	(Milyutina *et al*., 2010)
S. frigidum	HM053904	(Milyutina *et al*., 2010)
S. frisvollianum	HM053908	(Milyutina *et al*., 2010)
S. frisvollianum	HM053909	(Milyutina *et al*., 2010)
S. grandirete	HM053910	(Milyutina *et al*., 2010)
S. grandirete	HM053911	(Milyutina *et al*., 2010)
S. holmenianum	HM053912	(Milyutina *et al*., 2010)
S. holmenianum	HM053913	(Milyutina *et al*., 2010)
S. lancifolium	HQ890512	(Ignatova *et al*., 2009)

Table 8. (Continued)

S. lancifolium	HQ890513	(Ignatova *et al*., 2009)
S. lancifolium	HQ890516	(Ignatova *et al*., 2009)
S. lancifolium	HQ890517	(Ignatova *et al*., 2009)
S. lancifolium	HQ890514	(Ignatova *et al*., 2009)
S. lancifolium	HQ890515	(Ignatova *et al*., 2009)
S. liliputanum	HM053918	(Milyutina *et al*., 2010)
S. marginale	HM053919	(Milyutina *et al*., 2010)
S. marginale	HM053920	(Milyutina *et al*., 2010)
S. maritimum	HM053924	(Milyutina *et al*., 2010)
S. maritimum	HM053922	(Milyutina *et al*., 2010)
S. maritimum subsp. Piliferum	HM053923	(Milyutina *et al*., 2010)
S. papillosum	HM053925	(Milyutina *et al*., 2010)
S. papillosum	HM053875	(Milyutina *et al*., 2010)
S. papillosum	HQ890520	(Ignatova *et al*., 2009)
S. platyphyllum subsp. Abrupticostatum	HM053931	(Milyutina *et al*., 2010)
S. platyphyllum subsp. Abrupticostatum	HM053929	(Milyutina *et al*., 2010)
S. platyphyllum subsp. Abrupticostatum	HM053926	(Milyutina *et al*., 2010)
S. platyphyllum subsp. Abrupticostatum	HM053930	(Milyutina *et al*., 2010)
S. platyphyllum subsp. Abrupticostatum	HM053928	(Milyutina *et al*., 2010)
S. platyphyllum subsp. Abrupticostatum	HM053927	(Milyutina *et al*., 2010)
S. platyphyllum	HM053877	(Milyutina *et al*., 2010)
S. pruinosum	HM053932	(Milyutina *et al*., 2010)
S. pruinosum	HM053933	(Milyutina *et al*., 2010)
S. pulchrum	HQ890521	(Ignatova *et al*., 2009)
S. rivulare	HM053937	(Milyutina *et al*., 2010)
S. rivulare	HM053936	(Milyutina *et al*., 2010)
S. rivulare	HM053935	(Milyutina *et al*., 2010)
S. rivulare	HM053934	(Milyutina *et al*., 2010)
S. robustum	HM053938	(Milyutina *et al*., 2010)
S. sinensiapocarpum	HM053939	(Milyutina *et al*., 2010)
S. sinensiapocarpum	HM053940	(Milyutina *et al*., 2010)
S. sinensiapocarpum	HM053941	(Milyutina *et al*., 2010)
S. sordidum	HM053942	(Milyutina *et al*., 2010)
S. strictum	HM053944	(Milyutina *et al*., 2010)
S. subjulaceum	HQ890522	(Ignatova *et al*., 2009)
S. tenerum	HM053951	(Milyutina *et al*., 2010)
S. tenerum	HM053952	(Milyutina *et al*., 2010)
S. trichodon var. nutans	HM053954	(Milyutina *et al*., 2010)
S. trichodon var. nutans	HM053953	(Milyutina *et al*., 2010)
S. umbrosum	HM053956	(Milyutina *et al*., 2010)
S. umbrosum	HM053955	(Milyutina *et al*., 2010)
S. viride	HM053957	(Milyutina *et al*., 2010)
S. viride	HM053958	(Milyutina *et al*., 2010)

Table 9. *Schistidium rps*4 sequence data incorporated into the Bayesian MCMC analysis of the *Schistidium* genus. Sequence Identities (ID), Accession numbers and source are listed.

Sequence ID	**Accession Number**	**Source**
S. antarctici	JQ040706	(Skotnicki *et al*., 2012)
S. antarctici	JQ040705	(Skotnicki *et al*., 2012)
S. antarctici	JQ040704	(Skotnicki *et al*., 2012)
S. apocarpum	JQ040707	(Skotnicki *et al*., 2012)
S. apocarpum	GU809069	(Liu *et al*., 2011)
S. apocarpum	GU809063	(Liu *et al*., 2011)
S. apocarpum	GU809062	(Liu *et al*., 2011)
S. apocarpum	AJ845208	(Streiff, 2004)
S. apocarpum	JQ040708	(Skotnicki *et al*., 2012)
S. crassipilum	AJ553984	(Hedderson *et al.*, 2004)
S. liliputanum	HM989818	(Liu *et al*., 2011)
S. papillosum	AJ553985	(Hedderson *et al*., 2004)
S. strictum	GU809064	(Liu *et al*., 2011)
S. strictum	GU809065	(Liu *et al*., 2011)
S. strictum	GU809066	(Liu *et al*., 2011)
S. strictum	GU809067	(Liu *et al*., 2011)
S. trichodon	HM989819	(Liu *et al*., 2011)
S. trichodon	HM989817	(Liu *et al*., 2011)
S. trichodon	HM989816	(Liu *et al*., 2011)
S. rivulare	GU809068	(Liu *et al*., 2011)


Schistidium boreale (HM053889)
Schistidium boreale (HM053890)
Schistidium boreale (HM053888)
Schistidium trichodon var. nutans (HM053953)
Schistidium trichodon var. nutans (HM053954)
Schistidium lancifolium (HQ890513)
Schistidium apocarpum subsp. canadense (HM053915)
Schistidium lancifolium (HQ890512)
Schistidium andreaeopsis (HM053882)
Schistidium platyphyllum subsp. abrupticostatum (HM053931)
Schistidium platyphyllum subsp. abrupticostatum (HM053930)
Schistidium platyphyllum subsp. abrupticostatum (HM053928)
Schistidium platyphyllum subsp. abrupticostatum (HM053927)
Schistidium lilliputanum (HM053918)
Schistidium tenerum (HM053952)
Schistidium tenerum (HM053951)
Schistidium sinensiapocarpum (HM053940)
Schistidium sinensiapocarpum (HM053939)
Schistidium sinensiapocarpum (HM053941)
Schistidium platyphyllum subsp. abrupticostatum (HM053929)
Schistidium platyphyllum subsp. abrupticostatum (HM053926)
Schistidium sordidum (HM053942)
Schistidium antarctici (AY613335)
Schistidium antarctici (B7)
Schistidium antarctici (49)
Schistidium antarctici (B1)
Schistidium antarctici (RRSA3)
Schistidium antarctici (7/12/C2)
Schistidium antarctici (SCSA2)
Schistidium antarctici (RRSA1)
Schistidium antarctici (B17CP)
Schistidium antarctici (221)
Schistidium antarctici (RRSA6)
Schistidium antarctici (B3)
Schistidium antarctici (B9)
Schistidium antarctici (B17)
Schistidium antarctici (RSSA1)
Schistidium antarctici (RRSA5)
Schistidium antarctici (B8)
Schistidium apocarpum subsp. canadense (HM053916)
Schistidium apocarpum subsp. canadense (HM053914)
Schistidium lancifolium (HQ890515)
Schistidium lancifolium (HQ890516)
Schistidium apocarpum subsp. canadense (HM053917)
Schistidium lancifolium (HQ890517)
Schistidium holmenianum (HM053912)
Schistidium lancifolium (HQ890514)
Schistidium papillosum (HM053876)
Schistidium holmenianum (HM053913)
Schistidium papillosum (HQ890520)

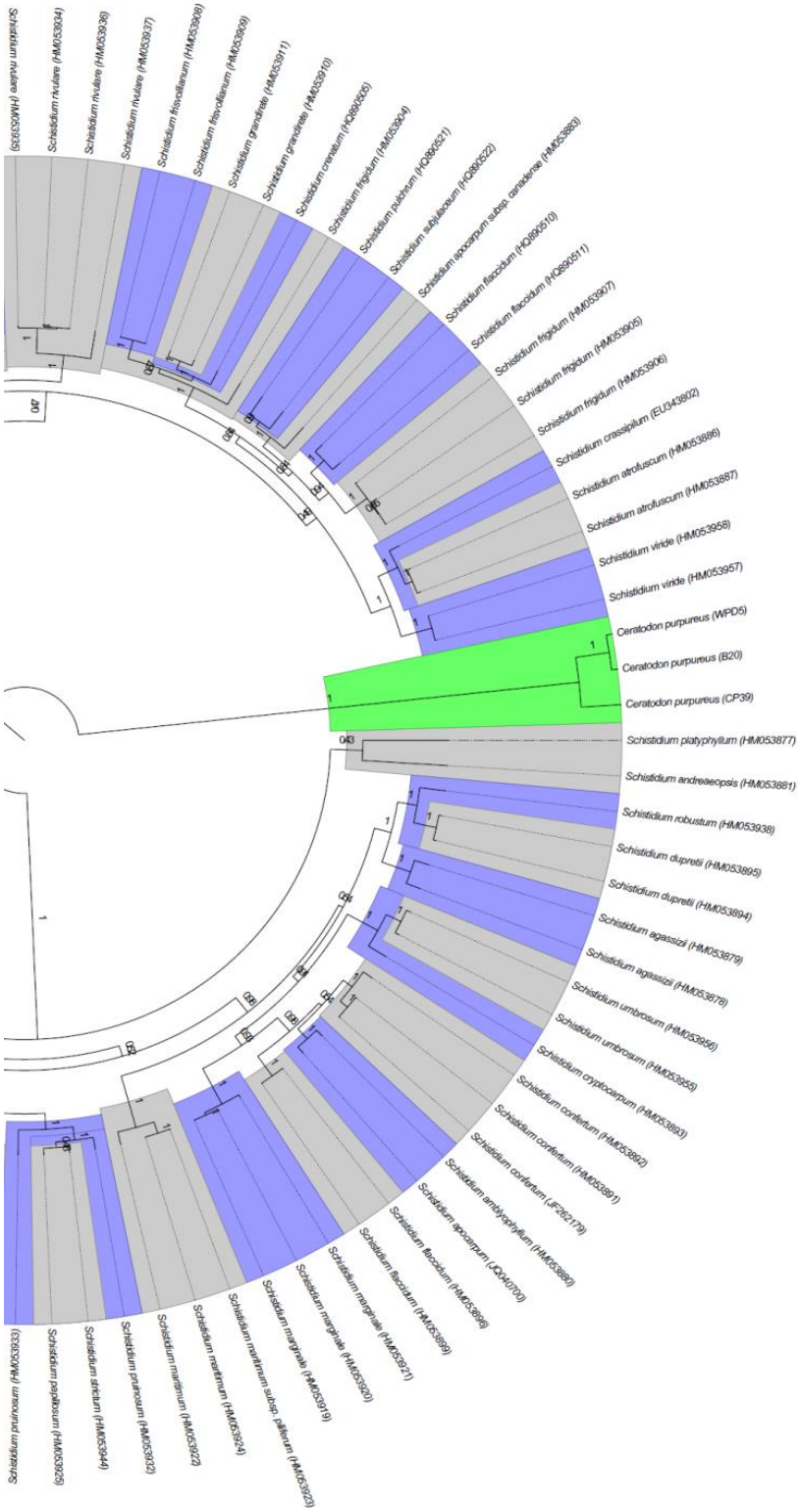


Figure 23. Tree generated via Bayesian MCMC analysis of ITS sequence data originating from samples of *S. antarctici*, and *Schistidium* sequences available on GenBank and rooted using ITS sequence data from *C. purpureus.* The species name of each sample is listed followed by the corresponding GenBank accession number or sample identification code. Distinct clades are highlighted in alternating blue and grey, with the out-group highlighted in green. Species groups are listed (far right) and correspond to the highlighted clade left of the species name. Values present on branches represent posterior probabilities of support for each node and scale bar representing evolutionary changes in branches.