Population Genetics and Photobiont Selectivity in Antarctic Lichens

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Abstract

This thesis examines population genetic structure and migration indices of an Antarctic endemic lichen *Buellia frigida* as well as algal selectivity of the lichens *Buellia frigida*, *Umbilicaria aprina* and *Umbilicaria decussata* in the Ross Sea Region of Antarctica. My aim was to determine where current populations may have originated (i.e. ancient or recent introductions to Antarctica) and if the level of algal selectivity potentially affects colonisation.

Chapter 2 reviews historical climate change in Antarctica, particularly in the Ross Sea Region, and introduces the basic characteristics of lichens. It also outlines how molecular analyses of lichen can aid in determining the genetic structure of populations and add to the growing data set which highlights sites in the Ross Sea Region (e.g. Dry Valleys) as glacial refugia.

To examine population differentiation between populations in this region it was necessary to develop polymorphic markers capable of delineating individuals. Chapter 3 focuses on the development of five microsatellite markers for this purpose. The initial data for these microsatellites suggested they were suitable for individual genotyping and characterization of *B. frigida* population structure being mycobiont specific with a high degree of polymorphism. A method of decreasing inhibiting factors present in lichen cells is also provided.

In Chapter 4 regional genetic differentiation was revealed by an analysis using microsatellite markers developed in Chapter 3 over 11 populations

in five regions of the Ross Sea Region. The identification of three populations with high allelic richness and sites of high dispersal indicated three regions as putative refugia (Terra Nova Bay, Dry Valleys and Beardmore Glacier). Terra Nova Bay has not previously been highlighted putatively as refugia. Limited mixing between adjacent geographical areas (such as the Dry Valleys and Ross Island) was identified, with migration likely to be influenced by wind currents.

The photobionts of the lichens *Buellia fr*igida, *Umbilicaria aprin*a and *Umbilicaria decussata* were examined using ITS rDNA sequence analysis in chapter 5. This identified that over a latitudinal gradient of roughly 10˚, there was a single haplotype present in the majority (>95%) of samples. This haplotype was nearly identical to haplotypes from as far afield as Svalbard showing a consistency in photobiont selection over a very wide geographical range and may be micro-climate specific. Other haplotypes present were specific to single geographical areas, and mutation may play the major role in this.

Collectively, these findings suggest that despite potentially high dispersal of propagules, populations of lichen species in the Ross Dependency show differentiation among locations and are potentially limited in their dispersal to different habitats. This may be the result of high selectivity for the photobiont. I conclude that lichen populations in the Ross Sea Region have originated from ancient, refugial populations rather than being populated via recent dispersal from northern continents. Dispersal within this region is likely to be restricted by ice-covered areas and relichenisation is potentially limited to particular photobiont strains which

are suited to the micro-climatic conditions found in this region. In order to minimise the consequences of anthropological disturbance, we recommend the continued protection of areas (e.g. Dry Valleys) housing high lichen and photobiont diversity.

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Chapter 1

Thesis Introduction

1.1 Introduction

Interest in Antarctic science has grown increasingly as perceptions have changed regarding its connection to the rest of the planet. It is now understood that along with unique environmental conditions, Antarctica has an important effect on worldwide climate conditions and vice versa. It helps to drive ocean circulation via cold dense bottom water, affects ocean isotopic composition, controls high latitude albedo (reflection), and sea level change (Barker et al. 1999). The unusually extreme climate and conditions in Antarctica means that there has also been increasing research efforts concentrated on terrestrial organisms in Antarctica, particularly on their adaptations, their evolution and biodiversity especially with respect to their origins. Such information is obviously also of great importance when looking at the response to climate change and the dynamics of present day ecosystems (SCAR Oct 4, 2004).

Terrestrial organisms are confined to the approximately 4% of Antarctica that is not covered with an ice-sheet. In the few ice free areas the terrestrial vegetation is composed mainly of lichens, bryophytes, algae and cyanobacteria. The extent of the ice free areas have changed throughout the millennia. It was originally suggested that the ice-sheet completely covered terrestrial areas in the Ross sea region (Ship et al. 1999).

However recent geographic data show that despite cyclical glacial events, in the Dry Valleys Region at least there has been long periods of time (10-12 Ma) where terrestrial ice free conditions could of existed (Sugden et al. 2006). Also glaciovulcanism infers that at lower altitudes ice free areas may have existed even in glacial maxima, at least in the maritime Antarctic, contrary to common conclusions (Smellie at al. 2008). All of this means that there are interesting questions as to the origins of the present vegetation. Careful study of the characteristics for the flora and fauna of these ice-free areas has the potential to provide information on the environmental conditions past and present. In particular, as lichens are the major macro-vegetation in Antarctica, they can provide insight into historical events as well as current levels of genetic diversity in lichen species. For example, the distribution of lichens in Antarctica has already provided information on the influence of temperature and micro-environmental conditions on lichen (Green et al. 2011a), as well as insights into possible glacial refugia in Continental Antarctica (Green et al. 2011b).

Lichens have been studied for over 200 years (Micheli et al. 1757). However, perhaps due to the difficulties in discovering what lichens actually “are,” as well as being a somewhat unfamiliar organism to the lay person, there are still many gaps in our knowledge of lichens. In the 1880’s the dual nature of lichens (photobiont and mycobiont) was discovered (Honegger 1996). Something of a hiatus occurred until revival of interest occurring in the 1970’s, and gradually the idea of lichens being a somewhat primitive plant has changed to viewing them as a complex

and sophisticated mini-ecosystem, with many diverse characteristics (Armstrong 1987; Buschbom 2007; Honegger and Zippler 2007; Cassie and Piercey-Normore 2008). Lichens, despite our historic paucity of knowledge, have been used as dyes, medicine, occasionally as a food source, and in recent years as an air pollution indicator (Hawkesworth and Rose 1970). Available research now provides insights into reproductive methods, photobiont and mycobiont relationships with each other and their environment, and species' distributions worldwide (Nash 1996).

Lichens, being a symbiotic association between two (or three) organisms, must necessarily have all symbionts together before the lichen can form a viable thallus. Various methods of symbiont dispersal occur, ranging from the mycobiont reproducing sexually via spores and the photobiont asexually via dispersal of single cloned cells (alga), to asexual dispersal of thallus fragments or specialised structures called isidia/soredia, which may or may not contain the photobiont (Ahmadjian 1993). Separate dispersal of the two symbionts requires the symbionts to be in physical proximity to each other, in a suitable habitat, for relichenisation to occur (Nash 1996).

These factors make population studies more complicated in lichens than most other organisms and this may explain the scarcity of research into dispersal methods and biogeography in lichen species worldwide. Nevertheless, the general synopsis is that colonisation of lichens is not as successful and as far reaching as the aerial spores seen in spore traps would infer, and species which do not disperse via ascospores (instead relying on asexual soredia and thallus fragments) are restricted geographically (Werth 2010).

While lichens are the major macro-vegetation of terrestrial Antarctica there is little information on how lichen populations came to be in their present locations in the Ross Sea Region. It is possible that populations are the result of long distance dispersal (Romeike et al. 2002; Munoz et al. 2004), or alternatively colonisation from isolated refugia as in the case of the Antarctic springtail *Gomphiocephalus hodgsoni* (McGaughran et al. 2011). If current populations are radiations from refugia, they must necessarily have survived in ice-free areas despite many glacial cycles.

Original work on ice sheet characteristics, predicted a complete cover of terrestrial areas in the Ross sea region (Ship et al. 1999). However, more recent geographic data show that despite cyclical glacial events, in the Dry Valleys, and perhaps elsewhere, there have been long periods (10-12 Ma) where terrestrial ice free conditions could have existed (Sugden et al. 2006). Also, glaciovulcanism infers that at lower altitudes, ice free areas may have existed even in glacial maxima, at least in the maritime Antarctic, contrary to common assumptions (Smellie at al. 2008).

It is also questionable whether lichens in continental Antarctica are obligate for one particular species of photobiont, and if so, rather than limiting speed and location of colonising populations, they are able to adapt under extreme conditions and utilise the available photobiont species. The photobiont in many lichens in Antarctica, comes from the genus *Trebouxia* which until recently was not thought to occur free living (Ahmadjian 1988). However, circumstantial evidence deems it likely they do indeed exist *ex situ* from lichens (Kroken and Taylor 2000; Beck et al. 2008). In species where reproduction occurs via spores, *in situ*

relichenization with a suitable photobiont must take place for thallus development. Populations of these lichens occur in many locations in the Ross Sea Region, and it is possible that in these challenging conditions any *Trebouxia* species, either free living or within other thalli, may be taken up by the mycobiont. However, if the photobiont species is obligate in these lichens, a specific *Trebouxia* species must be in contact before relichenisation can occur, and colonisation of new populations may therefore be limited. Unfortunately, there are only limited data on photobiont usage by Antarctic lichens. However, recent studies have shown a tendency towards lower selectivity which is in contrast to the selectivity in temperate and tropical regions (Romeike et al. 2002; Wirtz et al. 2003; Domaschke et al. 2012; Perez-Ortega et al. 2012). In more extreme Antarctic environments, it is possible that lichens will utilise local photobionts which are also likely to be better adapted to the local environment, rather than restricting themselves to a specific photobiont (Fernandez-Mendoza et al. 2011).

Prior to beginning my thesis research in 2006 knowledge of population genetics for Antarctic lichens was extremely limited. This is unfortunate because such information would inform our knowledge of the origins and potential dispersal pathways of lichens in Antarctica. Many of the species are thought to be of Gondwanan origin (Green et al. 2011b), but they may have been recent dispersals from northern areas. Furthermore knowledge of the interaction of the mycobiont with the photobiont in this extreme environment was unknown. In temperate environs this interaction can play an important role in the colonisation of new habitats. In light of global

climate changes new habitat may rapidly become available in Antarctica. In order to understand how these habitats may be colonised, and the impact of climate change on present populations, we need to understand the present day symbiotic relationships in lichens. In order to address this gap in knowledge I: 1) examined population genetic structure of *Buellia frigida* in the Ross Sea Region of Continental Antarctica and 2) extend the studies on photobiont selectivity in temperate environs by focusing on photobiont selectivity of three lichen taxa from the Ross Sea Region in Continental Antarctica: *Buellia frigida*, an Antarctic endemic, *Umbilicaria aprina*, with a bi-polar distribution and *Umbilicaria decussata*, with a cosmopolitan distribution.

1.2 Thesis Organisation

This thesis utilises two molecular methods to address questions of population genetic structure and photobiont selectivity in three lichen species (*Buellia frigida*, *Umbilicaria aprina* and *Umbilicaria decussata*) all commonly found in the Ross Sea Region. Chapter Two provides an introduction to the symbiosis of lichens, reviews the available literature, and outlines the connections between the previous research and my thesis research. Chapter Two also provides detailed descriptions of the three target species.

Chapter Three focuses on the development of microsatellite markers for the endemic lichen *Buellia frigida*, to enable investigation into the population structure and origin of Antarctic lichens. Microsatellites are co-dominant with high target specificity and, once developed, are able to be

efficiently amplified for large sample numbers, making them ideal to use in a symbiotic organism where one symbiont is targeted. Methods detailing extraction and purification of DNA and the development of microsatellite primers are also outlined. Five primer sets were characterised, with initial data for these microsatellites suggesting they are suitable for individual genotyping and characterisation of population structure. All loci were polymorphic with 8 to 16 alleles per locus in a sample set of 59 lichens.

The complexity of community interactions is less complicated in Continental Antarctica than in all other areas of the globe, therefore it is seen as an ideal model for investigation into resilience to global climate change. The Ross Dependency is an important part of the Antarctic Continent, largely due to its disproportionately high incidence of ice-free land in relation to the rest of Antarctica. This provides access to geological and biological data and in this area the most widespread and diverse macro-flora are lichens. Prior to beginning my thesis research, there were no molecular studies in the Ross Dependency which addressed population dynamics of lichens. Chapter Four uses the above microsatellites to assess population structure of the endemic Antarctic lichen *Buellia frigida* in five regions over roughly 10˚ of latitude along the Transantarctic Mountains and Ross Island, West coast of Ross Sea. Two geographic locations, Dry Valleys and Beardmore Glacier, stand out as possible sites of refugia based on previous literature (Stevens et al. 2003, Green et al. 2011b). The five microsatellite markers gave polymorphic information which was analysed with several mathematical models. These were useful

in highlighting population structure and information was gained concerning population differentiation and migration.

My final research chapter (Chapter Five) tests the hypothesis that lichens from extreme environments are less selective in their choice of photobionts. I examined photobiont sequences in lichens from the Ross Sea Region of Antarctica using the internally transcribed region of ribosomal DNA (ITS). The sequence data from the targeted lichen species (*Buellia frigida*, *Umbilicaria aprina*, *Umbilicaria decussata*), were analysed using Maximum likelihood, and a tree was constructed which gave information on relationships to algal samples within the collected samples. This tree was inclusive of photobiont sequences mined from Genbank which included sequences from within Antarctica and as far afield as Switzerland. The information gained was used to indicate relationships between photobionts and their fungal partners in the Ross Sea Region.

The final chapter (Chapter 6) summarises the thesis, and then outlines future research directions.

Chapter 2

General Introduction

2.1 Introduction

Interest in Antarctic Science has grown as perceptions have changed regarding its connection to the rest of the globe. It is now understood that along with unique environmental conditions, the Antarctic has an important effect on worldwide climate conditions and vice versa.

The Antarctic region is defined as comprising all land, sea and ice south of 60^{o} latitude. Antarctica itself, an almost circular continent with a mountain range (Transantarctic Mountains) dividing it into two parts, East and West Antarctica. This division also approximates the division into the Eastern (EAIS) and Western (WAIS) Antarctic ice sheets. South of 60^{o} latitude the sub-antarctic islands have a relatively mild climate and much richer floras and faunas than Antarctica. The main Antarctic continent is traditionally divided into two climatic and vegetation zones. The first is the **maritime Antarctic** which comprises the west side of the Antarctic Peninsula plus the closely adjacent islands (South Shetlands). Vegetation in this zone is dominated by lichens and mosses and the only two higher plants found in Antarctica also occur there especially in the north.

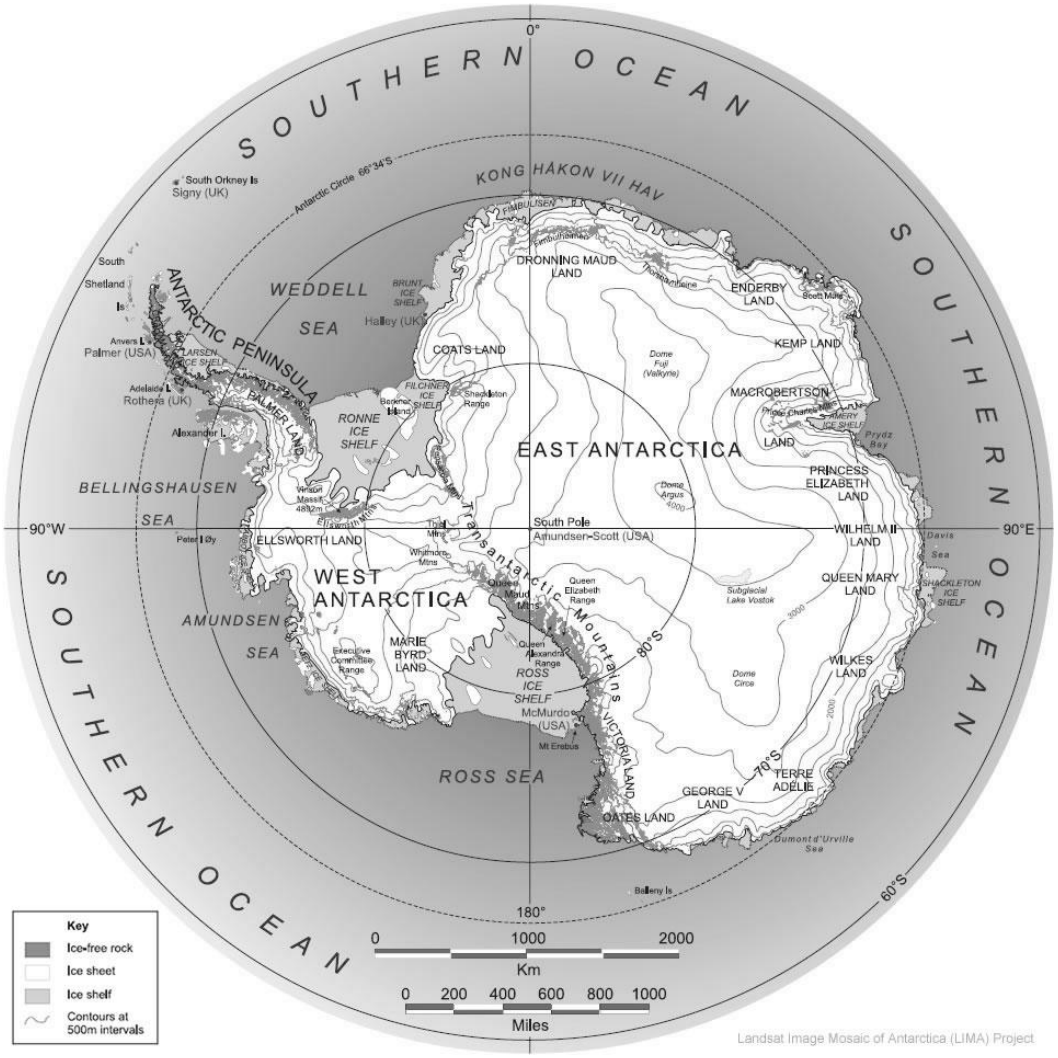

Figure 2.1: Antarctica: All land and Islands south of 60˚ latitude.

Source http://geology.com/world/antarctica-map.jpg

The second zone is **continental Antarctica** and this comprises the remainder and includes all the main continental regions. The terrestrial vegetation is entirely composed of lichens and bryophytes and these, themselves, are confined to the very small areas of ice free ground. The majority of Antarctica lies underneath a thick ice sheet, greater in depth than four kilometres in some places, making much of the continent terrain inaccessible for terrestrial organisms; however some coastal and mountainous areas are ice free for most of the year, particularly some

areas of the Transantarctic Mountains, which divide East and West Antarctica. Those areas which are ice and snow free for at least part of the year often have some vegetation, either bryophytes or more commonly lichens. In addition invertebrate communities exist in similar areas to flora, although restricted to non-flying insects and protozoans. How these lichen populations came to be where they are is still of some debate, with the two main theories being long distance dispersal (Romeike et al. 2002; Munoz et al. 2004) and radiation from historic ice free areas such as nunataks (ice free mountain tops) (Romeike et al. 2002)

2.2 Antarctic Glacial History

The Antarctic continent and Ice sheet have a major influence on earth's climatic conditions. The ice sheet contains the majority of the fresh water present on earth; it helps to drive ocean circulation via cold dense bottom water, affects ocean isotopic composition, controls high latitude albedo (reflection), and sea level change (Barker et al. 1999). The spread and depth of the ice sheet has changed throughout the millennia so it is not clear as to where and when terrestrial organisms were able to colonise ice free areas. Fossil remains show Antarctica was part of Gondwanaland and was at one stage populated by terrestrial organisms such as dinosaurs and forests. It is thought that in the Eocene and Oligocene epochs, around 45-30 million years ago (Ma), the Antarctic continent was in its current position, with the split between South America and the Antarctic peninsula occurring shortly after (41-24 Ma). This had a dramatic effect, resulting in the Antarctic circumpolar current being formed (~34 Ma), with Antarctic

Ocean currents no longer forced north to mix with warmer currents, thus creating a frozen continent. Although often given as the main reason for the development of the extensive glaciation on Antarctica there was also an almost coincident fall in atmospheric carbon dioxide levels and this is also postulated as being the main driver (Pearson and Palmer 2000). The dramatic fall in carbon dioxide appears to have been driven by the photosynthesis of the aquatic fern, *Azolla* in the Arctic; the so-called *Azolla* event (Speelman et al. 2009)

2.21 Ross Sea Glacial History

Estimates of glacial cycles have been reached primarily through ^{14}C dating of available terrestrial sites and oxygen isotope analysis of ice and marine environs. Recently materials which include flora and fauna have given more insight into events particularly on events since the last glacial maximum (LGM) ~ 15,000 yrs before present. It is agreed, that after the LGM, de-glaciation of some of the land areas which are currently ice-free occurred, but there are disparities between dates even within identical areas (Ingolfsson et al. 1998). It is not therefore surprising that with time spans greater than this, the thickness of the ice-sheet, extent of cover, and existence of any glacial refugia, are still in debate (site map fig 2.2). Publications giving information on ice sheet characteristics originally predicted a complete cover of terrestrial areas in the Ross sea region (Mercer 1973, Denton 1989, Ship et al. 1999). However recent geographic data show that there has been cyclical events so that at least in the Dry Valleys Region there were long periods of time (10-12 Ma) when terrestrial

ice free conditions could have existed (Sugden et al. 2006). Also, glaciovulcanism infers that lower altitude ice free areas may have existed even during glacial maxima in the maritime Antarctic, contrary to common conclusions (Smellie at al. 2008). There are varying ages given for the dry Valleys and nunataks in the Terra Nova area using cosmogenic dating (5.3-20MA and ~7.5 MA respectively) (Armienti and Baroni 1999; Oberholzer et al. 2003). Ricker hills which lie inland of Granite Harbour show via cosmogenic dating that the ice sheet was at least to 500m between 1.125 and 1.375 MA (Strasky et al. 2009). However it is much more likely to be considerably less as evidence indicates that as recently as 6-12 Ka there was ice sheet cover in this area (McKay et al. 2008, Hall and Denton 2000). There is no clear indication of rock exposure in the Beardmore region, however according to cosmogenic data it is likely to be >4MA (Ackert and Kurz 2004).

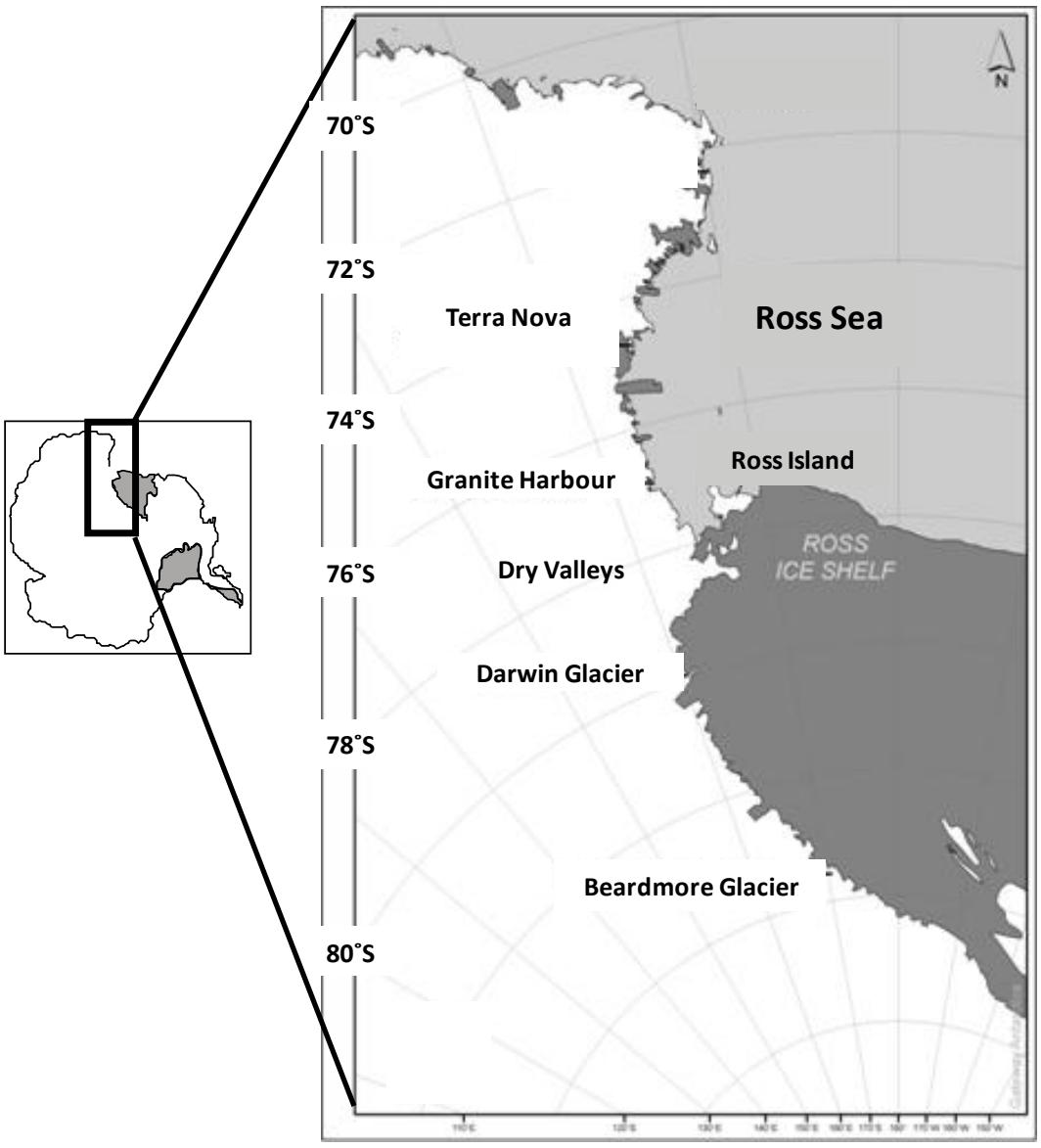

Figure. 2.2: Study sites along the Transantarctic Mountains in the Ross Sea region of Antarctica. Adapted from the Latitudinal Gradient Project (LGP)

Recent studies looking at genetic variation in some terrestrial fauna in the Transantarctic mountains have estimated, via divergence times, that some fauna may have actually been present in refugia throughout the cyclic increase and decrease of Antarctic ice sheets, indicating that at least some areas may have been ice free since the development of the EAIS

(Stevens et al. 2006; Convey et al. 2008). Some studies, for example those on moss species show no such signature (Skotnicki et al. 2000). However Continental Antarctic has only a small number of bryophytes, and lichens are able to exist in areas which are too taxing for moss species. Therefore lichens are more likely to provide information on the presence of long term refugia in the Ross Sea Region.

2.3 Lichens

Lichens may not be visually obvious components of bio-diversity; in fact they are easy to overlook in many ecosystems. However, they are key taxa, particularly in environments where vascular plants are limited or are unable to grow. Lichens have been calculated to dominate about 10% of terrestrial ecosystems, almost all where higher plants are excluded, because of low water storage or low temperatures (Honegger 2009).They are a large constituent of the total biomass in such conditions and therefore are key factors in nutrient recycling. They provide shelter and food for micro-organisms, and break down mineral constituents of rock surfaces, therefore being pre-cursors to the formation of soil in newly exposed rock habitats (Lindsay 1978). It is of interest to ascertain how lichen populations are structured genetically, how they came to be where they are and how they may respond to environmental changes. Undoubtedly, the potential for diversity and dispersal of lichen species in any environment is, in a large way, equivalent to understanding the dynamics of the relationships between lichen symbionts. These relationships, whilst well studied in a comparatively small number of

species, are still uncertain for many. With the widely varying dynamics that occur over different lichen species, it is clear that information from lichens in temperate environs does not necessarily extrapolate to more extreme environs.

2.4 What are Lichens?

Lichens are a symbiosis between a mycobiont (fungus) and a photobiont (algal and/or cyanobacteria), the photosynthetic partner. Symbiosis was defined initially in biological terms as the living together of unlike organisms (De Bary 1879). A.W Bennet, a physician and naturalist, actually first used the word in 1877 to describe the relationship in lichens (Bennet 1887). Whilst lichens are regarded as one of the best examples of a symbiotic association, there has been a tendency to constrict the definition to mean a mutualistic association ie: of mutual benefit to both partners. Perhaps somewhat surprisingly, there is still debate as to whether this symbiosis of lichens is mutually beneficial. It certainly appears that the mycobiont is getting the better deal as they are provided with photosynthetically produced carbohydrates they, alone, cannot synthesise. This allows the mycobionts to grow where sources for heterotrophic growth are scarce or absent, such as volcanic rock. The advantages for the photobiont is not so clear, although it is possible that the mycobiont provides protection from high UV and desiccation to the photobiont. As the fungi make many secondary products, as well as breaking down substrate via acidification etc (Nash 1996), the symbiosis provides minerals and

metabolites allowing the photobiont to be proliferate even in extreme conditions.

Although it is argued that lichens are not actually a symbiotic organism but an obligate heterotroph (Honegger 2009) and a parasitized photobiont, this adds little to the debate as parasitism was described as one of the better examples of symbiosis when it was first defined.

2.5 The Name of a Lichen

Lichens are referred to nowadays as lichenised fungi. By definition, they are classified as fungi and the name given to a lichen is the name for the fungus. This eliminates potential confusion as many lichenised fungi can associate with several different photobionts. Lichenisation is a very successful life strategy for fungi with almost 20% of all fungal species being lichenised with the vast majority in the Ascmycetes.

2.6 Structure

In almost all cases the fungus (mycobiont) makes up the majority of the lichen and determines the form of the lichen (the complete lichen is called a thallus). The photosynthetic partner (photobiont) is then arranged in a variety of ways within the thallus in a wide variety of arrangements. The vast majority of lichens (around 95%) are bipartite, and the mycobiont has a single photosyntheitc partner which can be a green alga (85% of lichens), a cyanobacterium (around 10% of lichens) and a small number of lichens have photobionts from other algal groups. Another 4-5% of lichens are tripartite, the so-called cephalodiate species and have a green algal

photobiont arranged as in bipartite species and a cyanobacterium that is confined to a special structure, the cephalodium, that can occur within the tissue of the lichen thallus or on its upper or lower surface (Nash 2007). Within the cephalodia the cyanobionts are apparently become predominantly heterotrophic and are specialised for nitrogen fixation.

All lichen species in this investigation are bipartite with green algal photobionts.

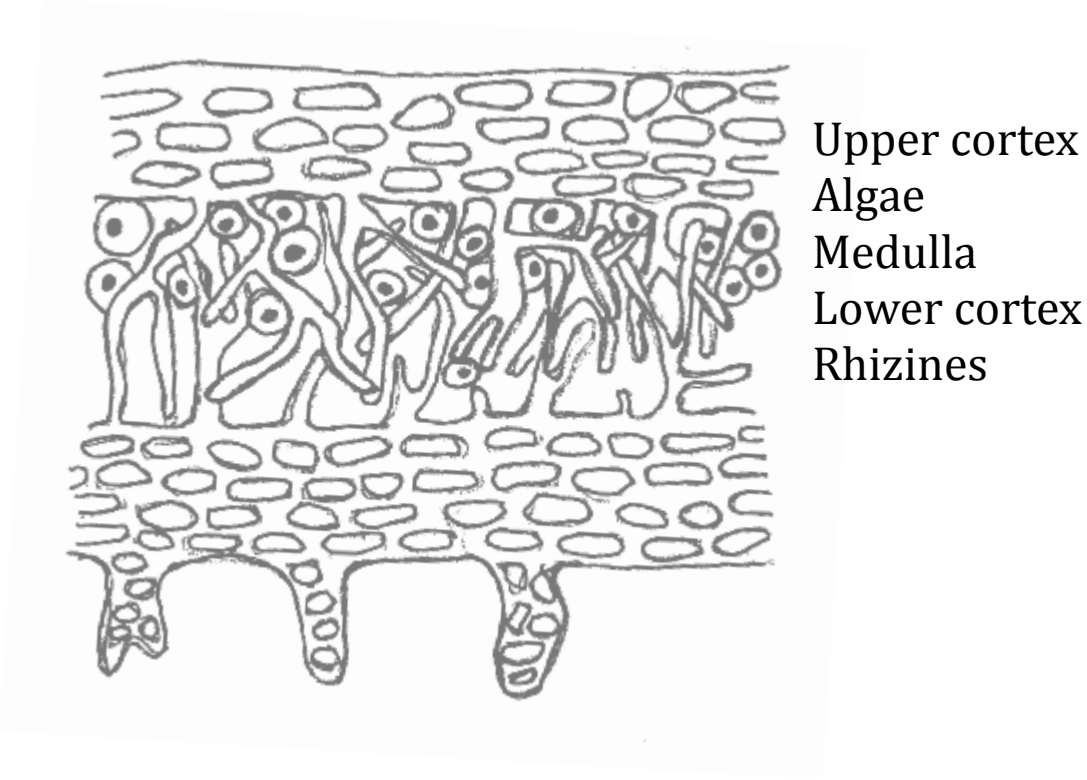

Figure 2.3: The basic structure of lichens, with the mycobiont constituting the upper cortex, medulla, lower cortex and rhizines if present.

Lichens show a wide range of morphological forms (Nash 2007) but all the species in this study are classified as being **foliose** or **crustose**. Foliose lichens are constructed with a thallus that can be normally easily divided into separate layers and which has a clear upper and lower surface (Fig.

2.3). Within the thallus there are several layers with the upper and lower layers being the cortices (singular, cortex). The upper/outer surface layer (cortex) is formed from tightly packed filaments, and helps to reduce light penetration to the photobiont and also protects the lichen from other organisms (Budel and Scheidegger 1996). The lower cortex is often covered in rhizinae that have an attachment function. The main part of the lichen between the cortices is the medulla and this is typically composed of loosely packed fungal filaments. The photobiont cells are arranged in a layer just beneath the upper cortex. The more open nature of the medulla is to allow easy diffusion of carbon dioxide to the photobiont cells thus allowing photosynthesis to proceed. The structure of the lichen displays the photobiont in a manner that allows light for photosynthesis whilst apparently providing shelter from the external elements (e.g. ultra-violet rays) whilst maintaining the algal cells in a moist (water vapour saturated) environment within the thallus. In crustose lichens there is no lower cortex, but other structures are the same.

Both symbionts adopt very different forms in the lichen thallus than if they were to grow separately. Grown outside the lichen the mycobiont forms a mass of undifferentiated mycelium and the photobiont a featureless group of unicellular organisms.

2.7 Relichenization and Dispersal

Relichenisation refers to the process of forming a lichen thallus from the contact of a mycobiont and a suitable photobiont (Büdel and Scheidegger 1996). Lichens which disperse via ascospores must necessarily reform a

lichen thallus via relichenization. The uptake of a suitable photobiont appears to dictate the formation of the thallus (Honegger 1998) with the mycobiont able to ‘wait’ as a mass of hyphae until the photobiont is found (Ott 1987).

It is possible that there are other ways a mycobiont can procure carbohydrates when a suitable photobiont is not immediately available. ‘Piggy-backing’ describes a process where the fungi ‘borrows’ an alga from an existing thallus, until able to capture its own viable photobiont (Piercey-Normore and DePriest 2001). Ott (1987), observed hyphae from a germinated spore of *Xanthoria parietina*, once in contact with its symbiont, excreting a jellylike substance, thus holding the alga in a suitable habitat until a thallus was formed. As this symbiont was so rarely found free living, Ott (1987) postulates that the *X. parietina’*s normal stage of development includes the step where a tangled mass of hyphae developed from the germinating spore, intersperse with commonly available coccal alga, forming an areolated crust which colonises the substrate hindering other species, and allowing the fungi time till a suitable symbiont is available. Another strategy was the ability of *X. parietina* to take algae from soredia that have the suitable symbiont, independant of the fungal type (Ott 1987).

Lichens have varying methods of dispersal, with lichens ranging from anamorphic (fully vegetative reproduction including both bionts e.g soredia), through to teleomorphic (fully sexually, reproducing via fully fungal ascospores), and many and any combinations in between.

Sexually produced structures (ascospores) are a product of the fungus alone and are very small and light and have been found in air columns many kilometres from source populations (Pearce et al. 2009). Asexual dispersal methods include ***soredia*** which are small ball-like structures that contain both mycobiont and photobiont. While larger than ascospores the soredia can also travel fairly large distances, although this appears to be mostly on a local and regional scale (Marshall 1996). A second asexual dispersal method is thallus fragments which seem to be restricted to local dispersal (Heinken 1999; Cassie and Piercey-Normore 2008).

The sexually produced and high flying ascospores face the important challenge of finding a compatible photobiont (Romeike et al. 2002) and going through the process of re-lichenisation in order to establish a new thallus. Although this would appear to pose substantial difficulties, Sanders and Lucking (2002) found this process very successful in tropical species. This is highlighted by population genetic studies on two species *Cliostomum corrugatum* and *Lobaria pulmonaria* which show differing gene flow, the former with high gene flow between regions and the latter with population differentiation over even regional distances. *Cliostomum corrugatum,* is dispersed via mycobiont spores, and *Lobaria pulmonaria* via soredia and possibly ascospores. Accordingly, lichen dispersal and relichenisation selectivity is very closely linked with genetic potential.

2.8 Selectivity and Specificity in Lichens

The lichen symbiosis can demonstrate both specificity and selectivity towards each symbiont, with specificity referring to the association of all

symbionts towards each other, and selectivity referring to the taxonomic range of potential partners of any of the two or three symbionts (Beck et al. 2002). In this thesis, I use selectivity to refer to the association of the mycobiont for the photobiont as suggested by Beck et al. (2002), particularly as the photobiont species is unknown in many lichens and many *Trebouxia* species associate with multiple mycobionts.

Because of the difficulty in identifying photobiont species within the lichen or in culture, the majority of modern publications use molecular techniques to identify the inhabitant (photobiont) of the lichen symbiosis. Sequencing the internally transcribed spacer (ITS) region is a common method of gathering enough genetic information to delineate selectivity. Due to the popularity of this method there is a significant database of ITS sequences available for comparison between sequences.

It has been found that temperate lichen species tend to have a high selectivity for the photobiont at least to species level (Kroken and Taylor 2000; Dahlkild et al. 2001; Helms et al. 2001; Tibell 2001; Beck et al. 2002; Helms et al. 2003; Yahr et al. 2004; Piercey-Normore 2005; Hauck et al. 2007). This is thought to reflect the moderate environmental conditions which allow many free living photobionts to be available for relichenisation. However, this level of selectivity even in these environments is variable, with differences in selectivity found within mycobiont genera (Kroken and Taylor 2000; Guzow-Krzeminska 2006). It has been suggested that microclimatic conditions may add an additional layer of complication to which photobiont is present in the lichen symbiosis (Yahr et al. 2006; Domashke et al. 2012). In both polar regions,

differences were found in the level of selectivity in similar environments (cold, arid) with higher selectivity seen in the Antarctic than northern polar regions, postulated to be due to founder effects of long distance colonisation (Domaschke et al. 2012). However, this theory is dependent on supposition of long distance dispersal having taken place exclusive of existing refugial populations, and with the samples in these studies taken from islands at lower Antarctic latitudes they are likely to have differing colonisation events to higher continental latitudes. In the wider Antarctic, it is still unclear whether long distance dispersal is solely, if at all, responsible for colonisation, particularly as in recent years doubt has been raised as to whether the entire Antarctic land mass was under ice during cyclical glacial maxima (Sugden et al. 2006; Stevens et al. 2007; McGaughran et al. 2008; Smellie at al. 2008; De Wever et al. 2009; Convey et al. 2008; Green et al. 2011b).

There is little other information regarding selectivity of lichens in Antarctic systems as a whole, particularly those areas of higher latitudes in Antarctica. Available data suggests that in colder environments selectivity is lower. This is thought to be related to the need for higher flexibility in such environments as available photobionts are not likely to be present in high numbers, unlike in temperate conditions. A study looking at diversity in four *Umbilicaria* species in the Antarctic Peninsula shows a low selectivity towards the photobiont (Romeike et al. 2002), with unusually low selectivity also found in the lichen *Lepraria borealis* collected from Alexander Island with an inland nunatak at 72˚ latitude (Engelen et al. 2010). Additionally, a study investigating *Nostoc* (cyanobacteria)

containing lichen in the maritime Antarctic found a low incidence of specificity with the conclusion that the ecological conditions favoured versatile mycobionts (Wirtz et al. 2003). In the greater continental Antarctic region there are even fewer studies. Two studies of lichen in the Ross Sea region exist, with these studies finding lower selectivity or specificity to pre-dominate over a selection of lichen species (Perez-Ortega et al. 2012, Ruprecht et al. 2012). However, of these studies, one investigated only one area of the Dry Valleys while the other focused on habitat correlation between lichens and their environment. As the Ross Sea region contains the majority of ice free areas along a latitudinal gradient in the East Antarctic, there is need for a comprehensive study investigating photobiont selectivity across a latitudinal gradient.

2.9 Population Genetics of Lichens

Population genetic studies seek to understand genetic structure within and among populations and are based on Mendelian principals, as well as processes of mutation, drift, selection and gene flow. In this way information gathered about the relationships of individuals within and among populations can be used to ascertain evolutionary history and biogeography as well as give insight into dispersal methods.

The mycobiont was selected as the target organism for studying the population genetic structure in this study. The reasons for this are: 1) the mycobiont constitutes by far the major structural part of the selected lichen species; and 2) the mycobiont dictates the dispersal of the lichen for relichenisation either as spores or soredia.

Studies of population genetics in lichens are relatively few in comparison with plants and animals. This paucity of data is possibly attributable to complications with the symbiotic nature of lichens, as well as the greatly varying reproductive and dispersal methods (Dyer and Murtagh 2001; Buschbom 2007; Werth 2010). Initially, studies were based on chemotype variation in which gene flow was observed between neighbouring thalli. However, this method was seen to be unreliable due to non-neutral selectivity as well as too low in resolution for population analyses (Culberson et al. 1988; Werth 2010). Fingerprinting methods such as AFLP, RAPD, RFLP, have been used with success in the past. However, the bi-partite or tri-partite nature of lichens mean the mycobiont must be isolated and sterile cultures grown (Dyer and Murtagh 2001; Honegger et al. 2004 Seymour et al. 2005). This is very time consuming and in many cases the mycobiont is very difficult to grow and prone to contamination, as random fungal spores are prevalent in many situations (Printzen and Ekman 2003). Consequently species specific markers are preferable, and specific ribosomal markers have been used with success. Unfortunately, many problems also arise with this technique; insufficient variability for population genetic studies, difficulties in interpreting multi-copy DNA, and linked loci (Zoller at al. 1999; Alvarez and Wendel 2003; Opanowicz and Grube 2004). The development of microsatellite markers in the late 1980's has in many ways provided an answer for such difficulties in the symbiotic lichen, as they are species specific, polymorphic and co-dominant (Jarne and Lagoda 1996). Due to potential analysis pitfalls such as null alleles, and assumption of incorrect mutation models, careful analysis must

ensue, nevertheless they are potentially good markers for population genetic studies (Jarne and Lagoda 1996; Queloz et al. 2009). Comparative focus on fungal genomes has shown they have a high incidence of microsatellites, with di-nucleotides next to mononucleotides in abundance (Lim et al. 2004). Despite these positive qualities there are still relatively few microsatellites developed even for general fungi and this may be in part be due to the initial cost in time and effort of microsatellite marker development (Jarne and Lagoda 1996; Dutech et al. 2007). However due to the even greater lack of microsatellite development for lichen mycobionts, those studies of microsatellites in fungal species are useful for comparative purposes (Barnes et al. 2002; Lim et al. 2004; Rubini et al. 2005; Breuillin et al. 2006; Dutech et al. 2007; Gauthier et al. 2007; Nkuekam et al. 2009; Queloz et al. 2009).

Thus far, one set of microsatellite markers have been developed for the northern hemisphere lichen species *Lobaria pulmonara* which in consequence has begun to be widely studied (Walser et al. 2003; Walser 2004; Walser et al. 2005; Widmer et al. 2010; Dal Grande et al. 2012). This lichen's distribution is characterised in large part by its reproductive and dispersal method, and shows that using microsatellite markers is a viable technique for studying the symbionts of lichen.

There are few genetic studies of population structure in the mycobiont of Antarctic lichens, with any method of genetic analysis, with those available being focused on the Peninsula (Dyer and Murtagh 2001; Murtagh et al. 2002), and none for continental Antarctica. As data on mosses and particularly invertebrates in Continental Antarctica have become available,

there is interesting potential for comparison (Skotnicki et al. 2000; Courtright et al. 2000; Fanciulli et al. 2001; Stevens et al. 2003; Stevens et al. 2006; Stevens et al. 2007; McGaughran et al. 2009). In addition to genetic studies, biodiversity studies highlight ecological habitat and colonisation in lichen (Green et al. 2011a).

2.10 Taxonomy of the Targeted Lichens

2.10.1 Mycobiont

Lichen forming fungi are widespread nutritional specialists that acquire carbon from living algal/cyanobacterial cells (Honegger 2009). There are several groups of fungi that have lichenized fungi, the *Basidiomycetes* (0.4% of lichenized fungi), and the *Deuteromycetes* (1.6%). However, the largest phylum is the *Ascomycetes* at an estimated 98% of all lichenized fungi. Ascomycetes are named after the reproduction strategy, producing spores within sac like structures called ascus. Out of the 46 orders in the Ascomycotina 16 include lichenised taxa. However, one of the orders with the highest number in the Ascomycetes is the Lecanorales (Nash 1996). This order is divided into at least 25 families, about 160 genera, and 8000–10,000 species. The species in this study belong to two of those families, the Physciaceae Zahlbr. (1898), and Umbilicariaceae Chevall. (1826). Physciaceae are characterised by ascus and ascospore types, such as ascospore wall thickness, excipulum type, and hypothecium pigmentation. Umbilicariaceae are characterised by a foliose thallus characteristically attached at one point (umbilicate) (Øvstedal and Smith 2001).

2.10.1.1 Endemic species: *Buellia frigida* Darb. (1910)

This is an endemic species to Antarctica found on rock in exposed conditions from sea level to high inland altitudes, and as such is one of the most common and widespread lichens in Continental Antarctica (Øvstedal and Smith 2001). It is a crustose lichen, from grey to blackish in colour with usually convex sessile apothecia, to 1mm in diameter, and has 8-15 ascospores. Thalli have been seen to occur as large as 20cm diameter, but more commonly are smaller (Øvstedal and Smith 2001).

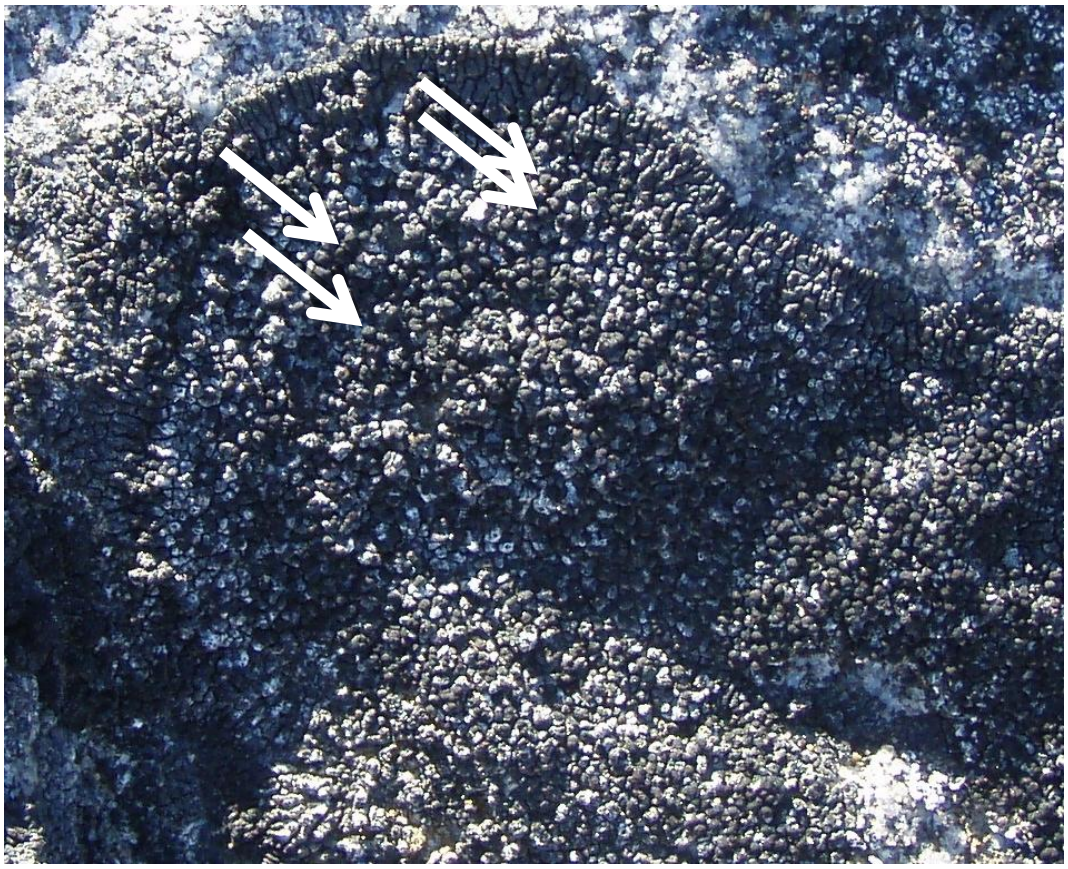

Figure 2.4: *Buellia frigida*, arrows denote apothecia which are present in high numbers. Sample collected: Gondwana, Terra Nova Bay.

2.10.1.2 Bi-polar species: *Umbilicaria aprina* Nyl. (1869)

The thallus is monophyllous (consisting of a single lobe, often undulate or folded) 5-10 cm across. The upper surface is pale to dark grey-brown grey, and is smooth and sometimes weakly ridged. The lower surface is dark grey to black with simple rhizines and thallospores (propagule), with apothecia not been seen. Typically found in habitats flushed by melt water and margins of melt streams, from low to high altitudes, coastal to inland. Distribution is widespread in Antarctica and is found in North Europe/America and East African mountains (Øvstedal and Smith 2001).

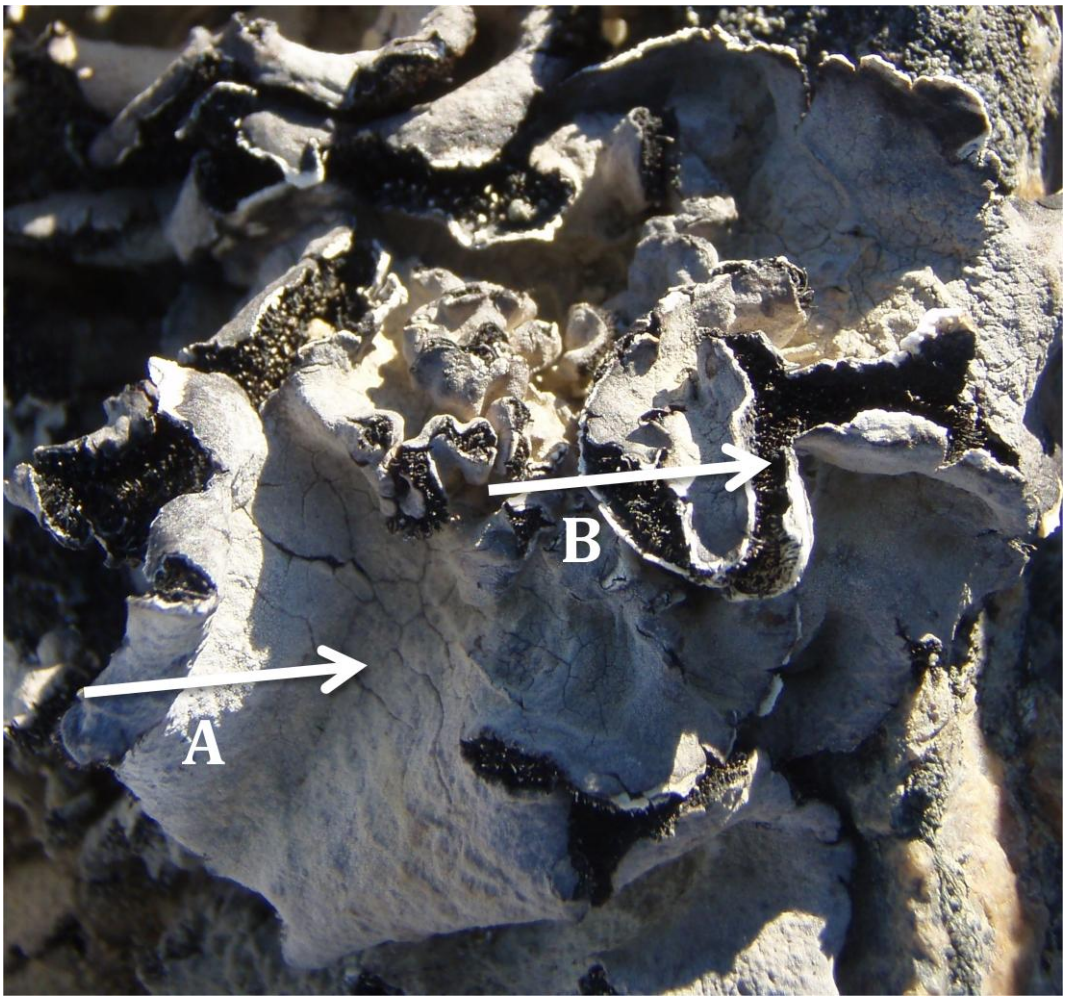

Figure 2.5: *Umbilicaria aprina*, arrows show: A) surface of lichen; and B) lower surface showing presence of rhizines. Sample collected: Gondwana, Terra Nova Bay.

2.10.1.3 Cosmopolitan species: *Umbilicaria decussata* (Vill.) Zahlbr. (1942)

The thallus is usually monophyllous ~3cm diameter but sometimes up to 10cm. Upper surface is grey to black and is reticulately ridged to the margin. The lower surface is black with thallospores but without rhizines. The apothecia are up to 3mm but rare, and asci are 8-spored. Widespread on dry rock particularly in exposed conditions locally abundant at sites in

Continental Antarctica and often associated with *Buellia frigida*. It is cosmopolitan in colder regions from coastal to high altitudes inland (2000m) (Øvstedal and Smith 2001).

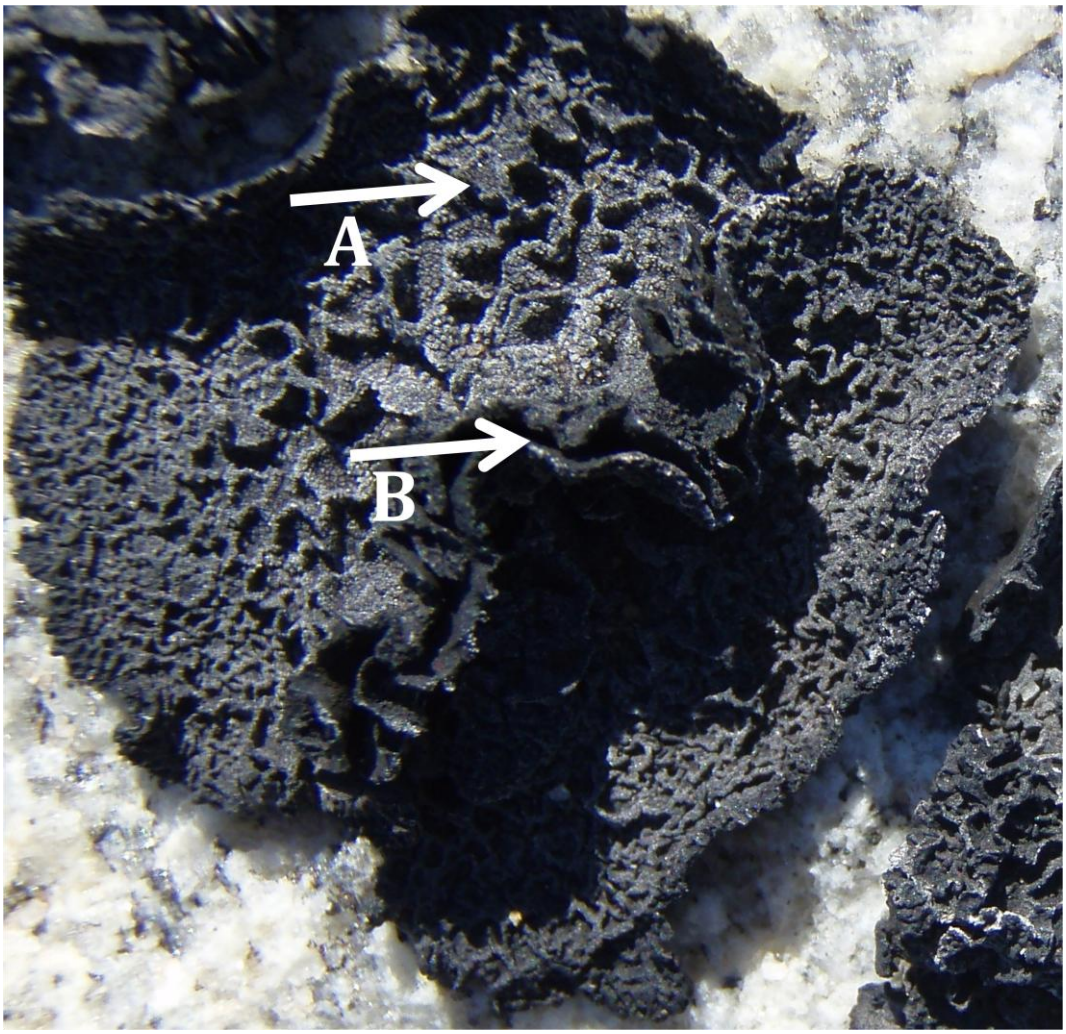

Figure 2.6: *Umbilicaria decussata* showing: A) surface showing reticulate ridges; and B) lower surface smooth with no rhizines. Sample collected: Gondwana, Terra Nova Bay.

2.10.2 Photobiont

Lichens have either an alga or cyanobacteria (or both) which are encased in the fungal filaments, as an inhabitant. Cyanobacteria contain photosynthetic pigments such as chlorophyll a and phycocyanin in the internal membrane which captures light, giving the energy required for carbon synthesis. Algae also capture light through photosynthesis, converting inorganic substances H_2O and CO_2 to organic matter using photosynthetic pigments such as chlorophyll a and chlorophyll b. Algae and cyanobacteria occur in many habitats but both require fairly moist conditions. Algae can look like structured plants in some forms e.g seaweeds, but both cyanobacteria and algae lack the structures that higher plants are characterised by, such as roots, leaves, flowers and seeds.

2.10.2.1 Algae

Green algae are photosynthetic eukaryotes; they have double membrane plastids which contain chlorophyll a and b, as well as accessory pigments such as beta carotene and xanthophylls. The plastids which store starch have a stellate structure that link nine pairs of microtubules in the flagella base. When cell walls are present they are normally made up of cellulose (Lewis and McCourt 2004).

The majority of lichens in Antarctica use either *Trentepohlia* or *Trebouxia* as the algal symbionts (*Trebouxia* is the most widespread photobiont worldwide), with these being in the phylum Chlorophyta (Øvstedal and

Smith 2001; Ahmadjian 2004). The three lichens in this study use *Trebouxia* Puymaly (1924) species as the algal symbiont.

Trebouxia are unicellular, usually spherical, containing a single large star like chloroplast which contains a central single pyrenoid (Fig 2.7). It reproduces by autospores (non-motile spores produced inside its parent cell and develops into the same shape as the parental cell before it is released) by biflagellate zoospores (Ahmadjian 2004).

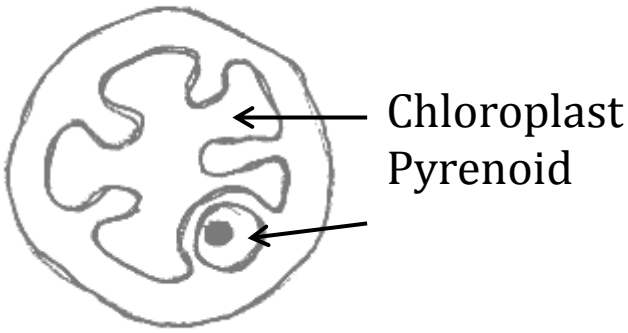

Figure 2.7: Basic structure of a *Trebouxia* cell, with chloroplast and single pyrenoid.

2.10.2.2 *Trebouxia* Characteristics

Very few species of lichenised fungi disperse algal cells with the ascospores (Marshall 1996, Nash 1996), making it necessary to find the photobiont *in situ*. Although the *Trebouxia* genus is extremely common in lichenised fungi, there has been little understanding of its characteristics, particularly in its ability to exist in a free–living state (Bubrick 1984, Ahmadjian 1988, 1993). Ahmadjian (2004) concluded that *Trebouxia* live as a heterotroph (photosynthesising and being provided with nutrients via

haustoria) and are unable to live independently. The inability to occur in a free living state can be seen to have a major effect on lichens to relichenize following sexual reproduction and dispersal. However, physical evidence and conclusions drawn by inference have thrown doubt on this finding and it is likely that *Trebouxia* species are indeed able to exist in a free living state for some time period (Bubrick 1984, Sanders 2005).

Photobionts were not thought to sexually reproduce, at least when they are in a symbiosis with the mycobiont. However, there is now some schools of thought which suggest that when in a free living state sexual recombination could indeed occur (Kroken and Taylor 2000). The major benefit of sexual reproduction is the capacity for genetic recombination, giving organisms a greater chance of survival with changing environments, such as enhancing fitness for a particular eco type (Hurst and Peck 1996).

Chapter 3

Isolation & Characterisation of Microsatellites in the Lichen Buellia frigida Darb. (Physciaceae), an Antarctic Endemic

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3.1 Introduction

Antarctica has a predominantly cryptogamic flora dominated by lichens (roughly 400 species of lichens, 50 liverworts, 100 mosses); only two species of higher plants occur and only on the warmer Antarctic Peninsula (Romeike et al. 2002). There is rapid decline in species numbers with increasing latitude, and on the main Antarctic continent there are approximately 100 species of lichen, 20 species of moss, and no vascular plants recorded (Green et al. 2007). At the Last Glacial Maximum, Antarctic ice sheets were substantially larger than at present, and there is growing interest in whether the present-day flora survived glaciation in refugia or represent the result of recent long-distance dispersal (Convey et al. 2008). Previous molecular studies of lichens in Antarctica have used markers such as ITS (Dyer and Murtagh 2001; Romeike et al. 2002), which provide little information on population structure. As lichen symbiont genomes are difficult and time consuming to separate (Hill 1994), a specific fungal marker has many advantages, particularly if large sample sets are required. Microsatellites are rapidly evolving, are characterized by high levels of polymorphism, and are highly specific, making them ideal to use with symbiotic organisms, particularly as mitochondrial DNA evolves too slowly in many lichenized fungi to measure population structure (Walser et al. 2003). Here, we describe microsatellite markers for a common Antarctic endemic, *Buellia frigida* Darb. (Physciaceae), found in coastal and mountainous locations. Its widespread distribution within Antarctica makes it ideal for studies of population structure, which can

provide insights into population connectivity and the evolutionary history of Antarctica.

3.2 Methods

Fungal genomic DNA was extracted from 35 apothecia, cut from a single dried thallus of *B. frigida* collected from Cape Hallett, Ross Dependency, Antarctica (72°19′23″S, 170°13′36″E) (Fig. 3.1).

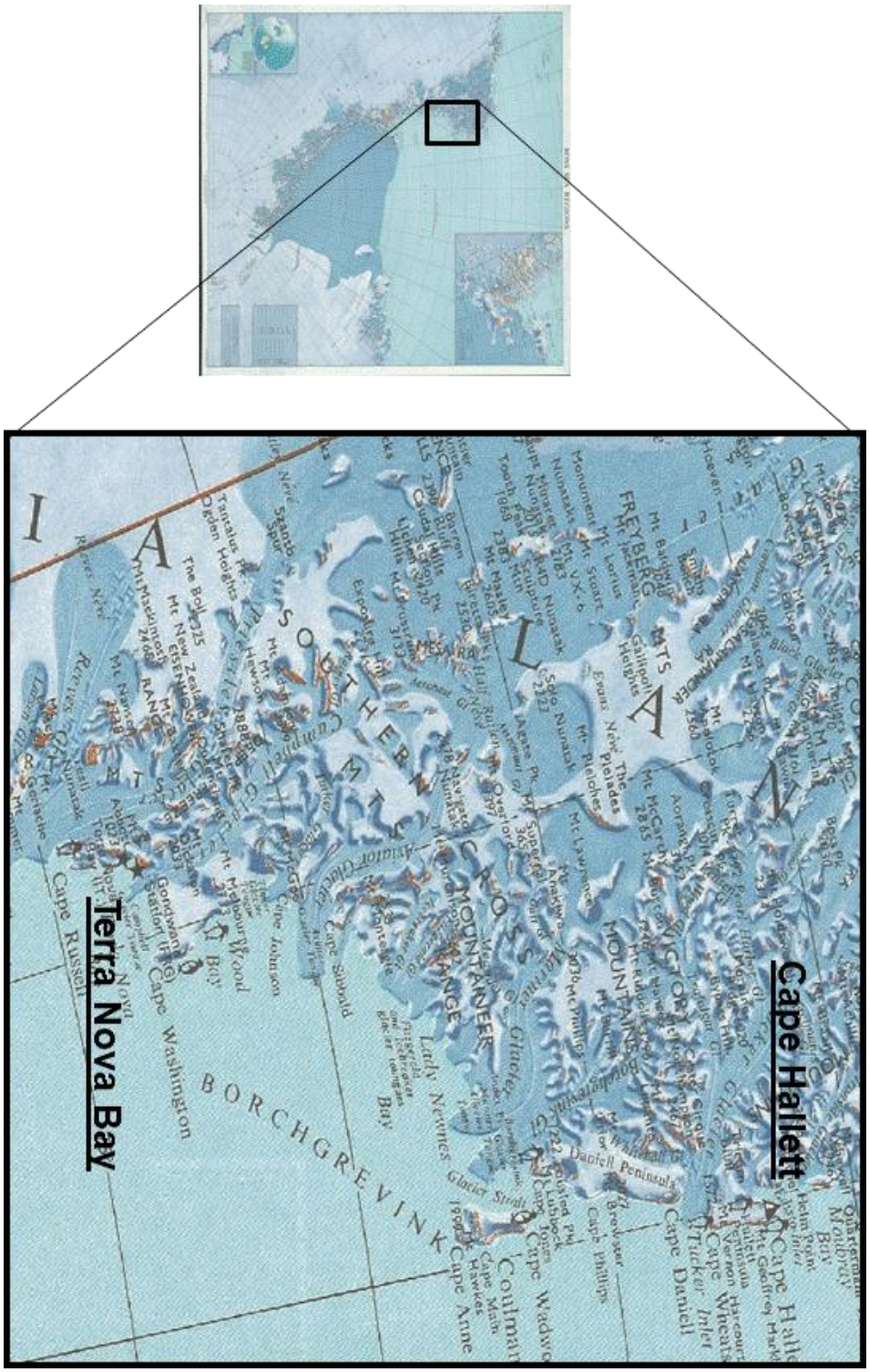


Figure 3.1: Ross Sea Region, Antarctica showing collection site for *Buellia frigida* sample used for microsatellite development. Adapted from Infomap 135, Department of Survey and Land Information.

Apothecia were chosen because this area is relatively free of algal material. DNA was isolated using a DNeasy mini plant extraction kit (QIAGEN, Valencia, California, USA) following the manufacturer's protocol with the addition of being shaken overnight at 55°C in lysis buffer. DNA concentration was estimated by comparison with a 100 bp ladder (Invitrogen Life Science, Auckland, New Zealand) on a 1% agarose gel. DNA purity was assessed by PCR amplification using universal ITS primers (ITS1/ITS4; White et al. 1990) to check for contaminants. A microsatellite library was constructed according to Edwards et al. (1996) and Karagyozov et al. (1993), with DNA digested by *Rsa*I and *Hae*III (separately) for 1 h at 37°C (consisting of 1 µg template DNA, 20 U of enzyme, and 1× buffer), followed by ligation to the *Eco*R1 adapter used by Karagyozov et al. (1993) (a 21-mer 5′-CTCTTGCTTGAATTCGGACTA-3′, and a phosphorylated 25-mer 5′-pTAGTCCGAATTCAAGCAAGAGCACA-3′). The ligated fragments were amplified using PCR in 50 µL reactions using the following reagents: the 21-mer 5′-CTCTTGCTTGAATTCGGACTA-3′ at 2 µM, 50 ng fragments, 200 µM dNTPs, 10 µl of 1× J buffer (67mM Tris-HCl (pH 8.8), 16mM $(NH_4)_2SO_4$, 6.7mM $MgCl_2$, 6.7 µM EDTA and 10mM 2-mercaptoethanol) (Jeffreys et al. 1988), and 1 U *Taq* polymerase (Invitrogen). A PTC-100 thermocycler (MJ Research, Ramsey, Minnesota, USA) was used with the following cycling conditions: 30 cycles of 30 s each at 94°C, 60°C, and 72°C. The product was denatured, then incubated in 1 M sodium phosphate with the microsatellite oligonucleotides ($(GT)_{15}$ and $(GA)_{15}$) previously bound to nylon membranes as in the enrichment method described by Edwards et

al. (1996). Hybridized DNA fragments were eluted from the membranes by boiling at 100°C for 5 min, precipitated by addition of 2.5 volumes of ethanol and 5 µL of 5% linear acrylamide, then centrifuged at 16000 × g for 20 min. The supernatant was discarded, the pellet washed with 70% ethanol, and the fragments resuspended in 25 µL TE buffer. This was followed by a second PCR (as above) using 1 µL of the ligated fragments and a second round of enrichment. The DNA was then digested with *Eco*RI, ligated into pGEM-T vector, and transformed in *Escherichia coli*. Positive colonies were plated and 26 of 110 clones were chosen for sequencing using primer M13F (Invitrogen) on an ABI 3130XL automated DNA sequencer (Applied Biosystems, Carlsbad, California, USA). Of these, only five had perfect repeats and contained suitable sequences for primer development. Primers for microsatellite analysis were chosen using the online Primer3 (Rozen and Skaletsky 2000; http://www.ncbi.nlm.nih.gov/tools/primer-blast) with the following conditions: optimal melting temperature (T_m) 60°C, optimal fragment size 200 bp, GC content between 20–80%.

To avoid bias toward only haploid tissue (e.g., thalli), DNA was extracted from whole samples (N = 59), including apothecia from Gondwana (Fig 3.1), Terra Nova region, Antarctica (74°35′23″S–74°46′30″S, 164°00′53″E–164°13′12″E), using a high alkaline PEG reagent DNA extraction protocol (Chomczynski and Rymaszewski 2006), with the following modifications: the sample was ground with liquid nitrogen before addition of 20–40 µL of PEG reagent, shaken at 55°C for 1 h, the lysate was spun at 4000 rpm for 20 s, and an aliquot of 2 µL supernatant added to 58 µL of H_2O. A

modified dialysis technique based on Freifelder and Better (1982) was used to reduce inhibitors by preferentially soaking them into agarose. The elute from PEG extraction was soaked into the top of 1.2% agarose (in 1:10 TE) in 0.2 mL thin-walled tubes with a pinhole-pierced bottom in a 1.5 mL Eppendorf tube overnight or spun for 10 min at 4000 rpm; 20–40 µL of 1:10 TE was added and nonabsorbed DNA removed from the surface after vortexing. This wash was spun at 4000 rpm for 15 s and the supernatant recovered. To assess the purity of the whole lichen DNA extract, universal ITS primers (ITS1/ITS4; White et al. 1990) were used in PCR. The resultant products were of similar size to published ITS sequences of the mycobiont (≈580 bp) and photobiont (≈650 bp), and were sequenced to confirm they corresponded with recognized species in GenBank.

Radioactive microsatellite products were analyzed on sequencing gels. A ^{33}P-γATP–labeled forward primer (0.1 µM) was used in 10 µL PCR reactions containing 1 µL of DNA (≈20 ng), 1× Platinum *Taq* buffer, 1.5 mM $MgCl_2$, 1 µM reverse primer, 200 µM dNTPs, and 0.5 U Platinum *Taq* DNA polymerase (Invitrogen) in a PTC-100 thermocycler (MJ Research) with initial denaturing at 94°C for 2 min, then 29 cycles of 94°C for 15 s, 64°C for 15 s, and 72°C for 1 min. The products were denatured at 90°C in a formamide-based loading buffer before being run on 6% denaturing polyacrylamide gels at 55 W for ≈3 h. The gel was then transferred to 3 mm filter paper, vacuum dried for 2 h, and exposed to Kodak XAR film for 1–3 d. All samples were run with a 10 bp ^{33}P-labeled ladder (Invitrogen) for reference.

3.3 Results

Primers (Table 3.1) were tested on three lichen species: *B. frigida* (N = 59), *Umbilicaria aprina* Nyl. (N = 79), *U. decussata* (Vill.) Zahlbr. (N = 40), and a photobiont species present in lichens, *Trebouxia jamesii* (Hildreth & Ahmadjian) Gärtner. Primers produced a product in *B. frigida* and *U. aprina* (with ≈30% success; locus range in Table 3.2), but not in *U. decussata* or the photobiont *T. jamesii*. Of the original 59 *B. frigida* samples, 39 provided a product of four or more of the five alleles and were used in further analyses. Expected heterozygosity was calculated in GenAlEx 6.4 (Peakall and Smouse 2006), and all loci were polymorphic, with a high mean expected heterozygosity (H_e = 0.833). Tests were performed for linkage disequilibrium (P > 0.05 in all cases, corrected for multiple comparisons), and observed heterozygosity departed significantly from expected heterozygosity under Hardy–Weinberg equilibrium (P < 0.0001 in all cases) using GENEPOP version 4.0 (Raymond and Rousset 1995). It is possible that this excess of homozygotes indicates *B. frigida* are selfing as in other Antarctic species (Dyer and Murtagh 2001) either with heterogeneous origins (>1 propagation event) or mutation events leading to heterozygotes with null alleles. Alternatively, the excess of homozygotes could have resulted from PCR bias toward haploid thallus tissue.

Table 3.1: Characterisation of five microsatellite loci in the mycobiont of *Buellia frigida*. Shown for each primer pair are the repeat motif, forward and reverse sequence, annealing temperature (T_a), size of original cloned sequence, number of alleles (*A*) and Genbank accession numbers.

Locus	Primer Pair	Repeat Motif	T_a (C^o)	Size (bp)	*A*	GenBank Ac. No.
Buef1	AGCAGGTATCCTCGTGCTGT ACCTCAATTCAGGCATGCAG	$(GT)_{21}$	60.5	189	16	JN205305
Buef2	ACTGCAGAGCATGTGGTGAC CCCATCTCAGCCAGAAGGTA	$(CA)_{15}$	60.0	186	10	JN205306
Buef3	GCATGGTGTACAACTCCCTGT CTTTGCCTCTGCAAACCAGT	$(GT)_{14}$	60.2	168	11	JN205307
Buef4	AAGCTGCCTGTCTCCAAGAA ACGTGAGTCCTCACCCTTGT	$(CA)_{15}$	59.9	151	8	JN205308
Buef5	GGGGACAAACCCGTAGAGA AGCAGGTATCCTCGTGCTGT	$(CA)_{21}$	59.9	164	13	JN205309

Table 3.2: Results of initial primer screening in one population (N=59) of *Buellia frigida*. Shown are the ranges of repeat size, observed and expected heterozygosities and *P*- values for Hardy-Weinberg equilibrium (exact probability method) tests.

Population (N=39)	Size (bp)	H_o	H_e	HWE	Size (bp)+
Buef1	172-220	0.121	0.899	<0.0001*	178-204
Buef2	174-200	0.091	0.719	<0.0001*	174-208
Buef3	164-194	0.257	0.867	<0.0001*	158-186
Buef4	140-222	0.091	0.799	<0.0001*	140-172
Buef5	146-188	0.071	0.879	<0.0001*	148-176

* Indicates observed heterozygosity departed significantly from expected heterozygosity under HWE (*P* <0.01)

+ Indicates *Umbilicaria aprina* allele range

3.4 Conclusions

The initial data for these microsatellites suggest they are suitable for individual genotyping and characterization of *B. frigida* population structure being mycobiont specific with a high degree of polymorphism enabling detection of targeted individuals. We conclude that the markers can potentially provide insight into population structure and gene flow, and they are currently being used to analyze population genetic structure in *B. frigida* across a latitudinal gradient in the Ross Sea sector of Antarctica (See Chapter Four).

Chapter 4

Microsatellite Analyses of the Antarctic Endemic Lichen *Buellia frigida* Darb. (Physciaceae) Suggests Dispersal Limitation and the Presence of Glacial Refugia in the Ross Dependency, Antarctica

To be submitted under the same title as: T.C. Jones[1], I.D. Hogg[1], R.J. Wilkins[1], T.G.A Green[1,2]

4.1 Introduction

In Continental Antarctica, terrestrial vegetation is restricted to organisms such as liverworts, bryophytes and lichens (Green et al. 2007). Lichens are the major macro vegetation (~90 taxa), with populations present even on inland nunataks that are devoid of any other vegetation (Øvstedal & Lewis Smith 2001). How these populations as well as the more diverse coastal and slope populations originated is currently under debate (Romeike et al. 2002). Specifically, are they recent dispersals from relict nunatak populations remaining from previous, warmer millennia, as per other organisms such as Collembola (McGaughran et al. 2011), or have they arrived via long distance dispersal from populations elsewhere following glacial retreat? Lichen species from the Antarctic Peninsula have shown some evidence of both long distance dispersal from temperate zones as well as recent dispersal of existing residents (Romeike et al. 2002; Munoz et al. 2004).

In the Ross Dependency of western Antarctica, it was once thought probable that the extensive glaciations of the Last Glacial Maximum (LGM) roughly 15,000 years ago had completely eliminated all terrestrial life (Ship et al. 1999). However, it is now known, based on cosmogenic dating, that some areas have remained ice-free for much longer periods, perhaps millions of years (Storey et al. 2010). There is also growing genetic evidence for ancient origins and survival in glacial refugia for Antarctic flora and fauna (Janetschek 1967; Stevens & Hogg 2003; De Wever et al. 2009; Jones et al. 2013). Furthermore, recent investigations have suggested that species assemblages of lichens in the Queen Maud Mountains (83-84°S) have more

in common with those of the Antarctic Peninsula than with continental species (Green et al. 2011a). This evidence, taken together with the new geological evidence (Storey et al. 2010), strongly suggests that some areas of the Ross Dependency have, despite previously extensive glaciations, allowed the long-term survival of Gondwanan flora and fauna (e.g. Stevens et al. 2007).

Lichens are a symbiosis between fungi (mycobiont) and a photosynthetic organism (photobiont). Photobionts include green algae or cyanobacteria (bipartite) or both (tripartite). In crustose lichens like *Buellia frigida* studied here, the thallus is adhered to the substrate by the mycobiont, with the photobiont generally in a layer in the upper surface of mycobiont filaments enabling photosynthesis to occur. Reproductive methods include dispersal of mycobiont ascospores from reproductive structures (apothecia) and subsequent re-establishment with a suitable photobiont (relichenization), or dispersal of both mycobiont and photobiont together via vegetatively produced soredia or dispersal of whole fragments. However, the latter method is rare in continental Antarctic lichens. The capacity for long-distance dispersal is reflected in the high incidence of ascospores found in aerial traps (Marshall 1996). This suggests that wind can carry viable spores over large distances (Buschbom 2007, Lattman et al. 2009). However, it is unknown if this translates to successful relichenization and development of viable thalli in Antarctica.

Population genetic theory predicts higher levels of genetic diversity for refugial populations compared to founder populations or those that have gone through a bottleneck event (Comps et al. 2001). Accordingly, the

application of molecular techniques to assess population genetic structure may provide an effective means of assessing lichen dispersal pathways in Antarctica and to resolve their association with the landscape (i.e. ancient or recent). Microsatellites have previously been used to assess populations with known refugial origins (Comps et al. 2001, Guo 2012). Although it is challenging to assign definitive causes for observed population genetic structures, it is possible to interpret results in light of prior knowledge of an organism's life history as well as other evidence of putative refugia (e.g. physical records).

While there are limited studies of effective lichen dispersal, previous studies have found that the reproductive mode has a large influence on the colonisation distance of lichens. Sexually-produced ascospores are very small and light and have been found in air columns many kilometres from source populations (Werth et al. 2006). Soredia, which contain both the algal and fungal components, can also travel large distances (Armstrong 1990), although they are larger and heavier than the individual components alone. The soredia appear to be mostly successful on a local and regional scale (Walser 2004), while fragments of intact lichens (which are also reproductively viable) seem to be restricted to local dispersal (Heinken 1999; Cassie 2008). Successful colonisation is further compromised by thallus fragments having a higher degree of propagule attachment, and soredia having a lower incidence of attachment. The widely-dispersing ascospores potentially face the largest challenge of having to find a compatible photobiont to begin the process of relichenization (i.e. re-establishing the symbiotic relationship). However, studies of species which differ in dispersal

mode, such as *Cliostomum corrugatum* (via ascospores) and *Lobaria pulmonaria* (via vegetative propagules), show differing levels of gene flow. The former has high gene flow among locations and the latter shows population differentiation, indicating dispersal by ascospore can equate to efficient long distance migration (Lattman et al. 2009; Walser 2004, respectively). A further constraint in continental Antarctica is that habitats must be available for colonisation and these are likely to be restricted both spatially and temporally (Green et al. 2011b).

Buellia frigida Darb. is a crustose lichen which grows on rock surfaces in ice-free areas of Antarctica. It is bipartite with its photobiont being species of the green alga *Trebouxia* (Seppelt 1978). It is distributed widely across Antarctica, from the Antarctic Peninsula to exposed rocky coasts and inland rock exposures of continental Antarctica. It reproduces sexually via ascospores with many apothecia present on the thallus surface. Thalli can range from a few millimetres to > 50cm wide (Øvstedal & Smith 2001). The larger thalli may be well over 1000 years old with the growth rate of *B. frigida* in the Dry Valleys (78°S) having been estimated to be less than 1mm per century (Sancho et al. 2007).

In-depth studies focusing on genetic variation in lichens have largely utilised nuclear ribosomal DNA fungal-specific markers and these generally show little variation at the population level. Polymorphic RAPDs have also been used on pure fungal cultures or photobiont free thalli (Murtagh et al. 2000). However, these markers are problematic due to difficulties in growing pure cultures when dealing with large sample sizes, and are prone to contamination (Printzen & Eckman 2003). Microsatellites which are highly

specific, selectively neutral, co-dominant and polymorphic can make ideal markers (Lim et al. 2004). Accordingly we used previously developed microsatellites (Jones et al. 2012) to assess the population structure of the common endemic lichen *Buellia frigida*, along a 10^{o} latitudinal gradient in the Ross Dependency of Antarctica. As the growth rate of *B. frigida* in Continental Antarctica is very slow in comparison with temperate species (e.g. *Lobaria pulmonara* has a generation time of 35 years and growth rate > $4.5mm^{-1}$ (Scheidegger & Goward 2002) we expect generation time in *B. frigida* to be relatively longer (e.g >50 years. Consequently, slow mutating ribosomal ITS markers are not likely to be informative for delineating differences among individuals. In these instances, faster-mutating markers such as microsatellites are preferred in order to detect signals of historic population structure in present day populations. Resulting microsatellite data then can be analysed using Nei's (1978) fixation index and allelic richness which have been traditional measures of assessing population differentiation using allelic frequencies. These measures are still valid, although there has been further development of equations (e.g. G'ST, DEST) for the assessment of microsatellite data (e.g. Jost 2008; Meirmans & Hedricks 2011).

In order to test the hypothesis that genetic differentiation would be limited among populations (indicating a recent association with the landscape), we analysed microsatellite data for *B. frigida* sampled over a latitudinal gradient in the Ross Sea region of Antarctica. If we fail to reject this hypothesis we would conclude that the populations do not have a long-term association with the Antarctic landscape and are, thus of relatively recent origin in Antarctica.

4.2 Methods

Specimens of *Buellia frigida* Darb. (Physciaceae, Ascomycota) were collected during the 2006-2011 Antarctic summer field seasons (October-February) from sites covering a latitudinal gradient of roughly 10º (74°36´S-84°00´S) in the Ross Dependency, Antarctica (Fig. 1). Sites were chosen based on logistics for the Latitudinal Gradient Project (LPG) – a framework developed under the Scientific Committee for Antarctic Research (SCAR) for understanding ecosystems and environmental impacts of climate change along the Victoria Land Coast. Samples at each site were collected randomly (based on a grid system), within an area accessible by day walks from either field camps or helicopter landing areas. Portions of thalli were scraped from rocks and boulders using a scalpel, into sterile cryogenic tubes via acid free paper. DNA was extracted following the protocol described in Jones *et al.* (2012). Radioactive microsatellite products were size separated using polyacrylamide gel electrophoresis. For each of the five primer sets (described in Jones et al. 2012), 0.1 µM of 33P-γATP labelled forward primer was used in 10 µl PCR volumes containing 1 µl of DNA (≈20 ng), 1x Platinum taq buffer, 1.5 mM $MgCl_2$, 1µM reverse primer, 200 µM dNTPs and 0.5 U Platinum Taq DNA polymerase (Invitrogen, Life Science, Auckland, NZ), in a PTC-100 thermocycler (MJ Research, Ramsey, Minnesota, USA) with initial denaturing at 94˚C for 2 min then 29 cycles of 94˚C 15 s, 64˚C 15 s and 72˚C for 1 min. The products were denatured at 90˚C in a formamide-based loading buffer before being loaded onto 6% (14.25g acrylamide, 0.75g BIS, 125 g Urea, 175ml distilled H2O) denaturing polyacrylamide gels (40 x

20cm, 0.4mm thick, formed by pouring in a mix of 5 ml TBE, 45ml 6% acrylamide:bisacrylamide (19:1), 50% urea with 500ul 10% Ammonium PerSO4, with 50ul TEMED added to initiate polymerisation). Electrophoresis was at a constant wattage of 55W (which resulted in the gels reaching a temperature of ~50^{o}C) for ~ 3hrs using a 1x TBE buffer solution. The gel was then transferred to 3mm filter paper and vacuum dried for 2 hours. The gel was exposed to Kodak XAR film for 3-5 days before development. All samples were run with a 10 bp ^{33}P labelled ladder (Invitrogen, Life Science, Auckland, NZ) at three positions for reference. Bands were assigned sizes according to the ladder and entered into Microsoft Excel 2007. Data were visually inspected in Excel, and GenAlex 6.4 (Peakall & Smouse, 2006) was then used to convert data from allele size to the required formats for statistical analyses. Measures such as linkage disequilibrium, fixation statistics (F), as well as observed and expected heterozygosity, were calculated initially in Arlequin v. 3.11. Microchecker (Van Oosterhout et al. 2004) was used to test all loci, and all populations for deviations from Hardy Weinberg Equilibrium (HWE), as well as the probable cause of any deviations. FreeNA (Chapius & Estoup 2007) was used to estimate allele frequencies corrected for the presence of null alleles. These allele frequencies were then analysed in FreeNA to estimate observed and expected heterozygosity across each marker and population along with mean number of alleles and mean effective number of alleles (Table 1). Different sample sizes can lead to under/overestimated allelic differences, as there is a greater chance of picking non representative examples with higher or lower sample numbers, and rarefaction minimises this difference. Basic

allelic richness (*A*) was estimated in HP-RARE 1.0 (Kalinowski 2005) which uses rarefaction to estimate allelic richness with sample numbers of incongruent sizes. Higher *A* can be seen as an indication of refugia due to contraction and subsequent expansion giving low frequency variants which have not been lost due to population genetic processes (Marchelli & Gallo 2004). Previous work has shown that differences in *A* among populations as low as 0.5 can be significant, reflecting the sensitivity of this measure in indicating past events (Comps et al. 2001, Takahashi et al. 2005, Nkuekam et al. 2009). In addition to allelic richness for all loci, private alleles were analysed separately in HP-RARE 1.0 (Kalinowski 2005) to highlight any unique populations (Kalinowski 2004).

Wright's relative measure of genetic diversity, the fixation index F_{ST} and extrapolations of this such as G_{ST} have been the standard measure of genetic diversity for allozymes and sequence data. However, assessing the differentiation among populations is highly affected by the amount of variation within individuals and populations as this can 'hide' variation (Leng *et al.* 2011). Given that some methods are preferable over others in particular situations, several studies have recommended a combined approach to measuring genetic differentiation (Bird et al. 2011; Leng et al. 2011; Meirmans & Hedricks 2011). Accordingly, we used F_{ST}, G_{ST} and estimated D (D_{EST}) to facilitate comparisons with other similar studies. We used single stepwise mutation (SSM) to calculate diversity indices of both D and G_{ST} statistics based on Leng et al. (2011). F_{ST} values were calculated in FreeNA using the EM algorithim (Chapius & Estoup 2007). Standardised values of G_{ST} (G'_{ST}) values were estimated according to Meirmans and

Hedrick (2011) equation 1: $G'_{ST} = G_{ST}\ (k\text{-}1+H_S)/(k\text{-}1)(1\text{-}H_S)$, in statistical freeware R (http://www.r-project.org).

Migration (M) was estimated in MIGRATE-N v3.3.1 (Beerli 2009) using the analysis strategy Maximum Likelihood (ML), single step evolution model, constant mutation rate for all loci, a burn in per chain of 1000, with M values generated from F_{ST} calculations. D_{EST} values were estimated in DEMEtics v.0.8-5 (Gerlach et al. 2010). Significance for both F_{ST} and D_{EST} was calculated using 1000 bootstrap replicates. Pairwise comparisons between populations were made using the following indices: a) F_{ST}; b) D_{EST}; c) D_{EST} (from uncorrected frequencies); and d) G'_{ST} (Table 2). Significance of D_{EST} was calculated using 1000 bootstrap replicates in DEMEtics, uncorrected D_{EST} was calculated in SMOGD v.1.2.5 (Crawford 2010). F_{ST} confidence intervals were calculated using 1000 bootstrap replicates in FreeNA. Relationships between geographic distance (shortest distance over land) and estimates of genetic dissimilarity ($G'_{ST,}$ D_{EST}) were determined using Mantel tests in Arlequin (V3.1.1).

4.3 Results

A total of 164 individual *Buellia frigida* samples were obtained from populations sampled in the Ross Dependency from Terra Nova Bay (74˚ S) to the Queen Maud Mountains (Southern Transantarctic Mountains, 84˚ S) (Fig. 4.1). We excluded samples that showed no amplification or where only one or two loci amplified, leaving 111 individuals that showed a visible microsatellite at 3 or more of the 5 loci. As a precaution, a phylogenetic analysis was performed including the individuals with one or two loci. However, these individuals provided no additional insights and were excluded from further analyses. The number of microsatellite alleles per population per locus varied from four to a maximum of 16, with all populations polymorphic.

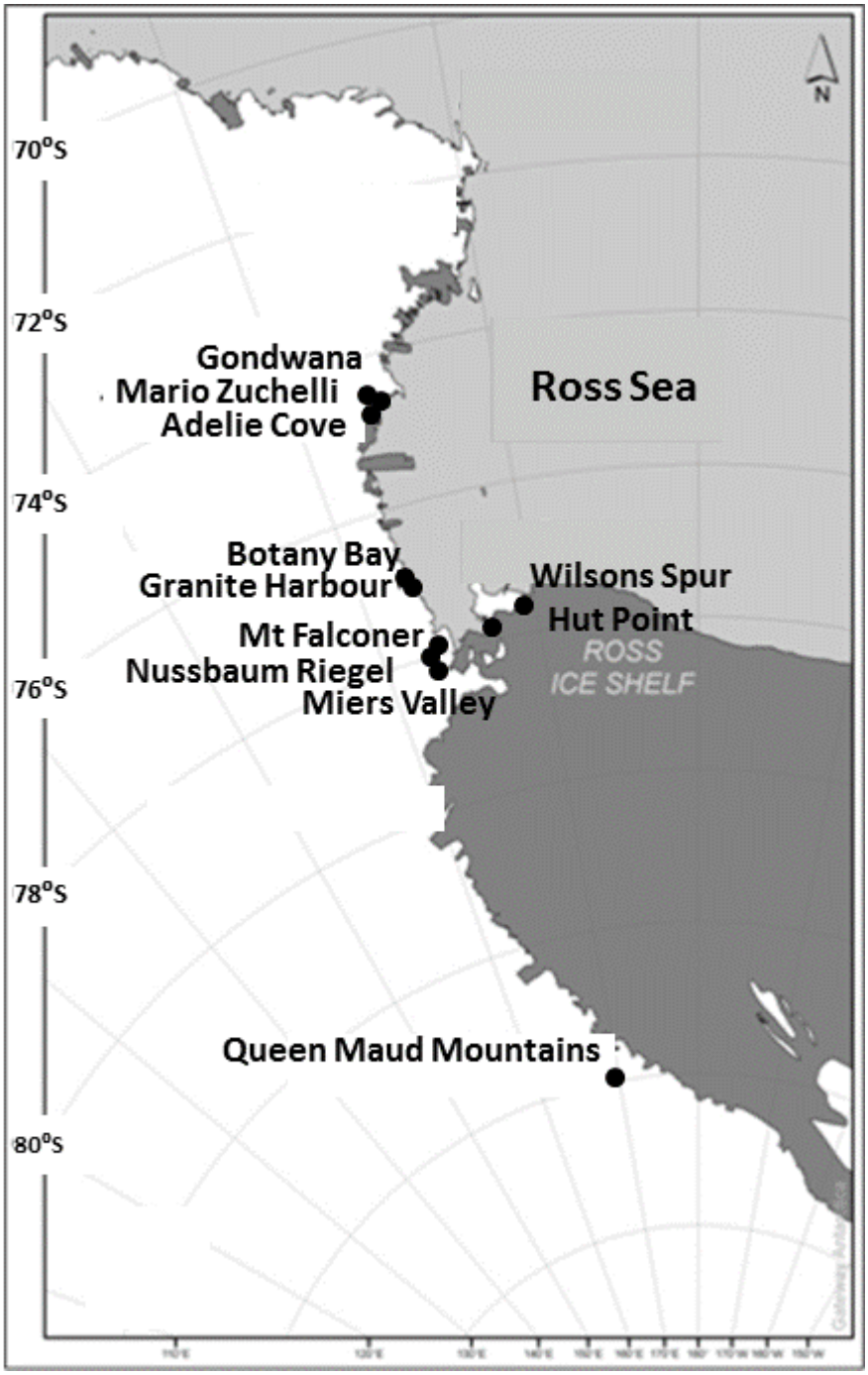


Fig 4.1: Locations of sample sites in the Ross Sea Region. Map adapted from LGP website.

[+]TN = **Terra Nova**; (Gondwana (74º 36', 164º 12'), Mario Zuchelli (74º 43', 164º 04') and Adelie Cove (74º 45', 164º 02)), GH = **Granite Harbour**; Granite Harbour (77º 00', 162º 31') and Botany Bay (77º 00',162º 32'), RI =

Ross Island; (Hut Point (77º 49', 166º 38') and Wilsons Spur (77º 31', 169º 17')), DV = **Dry Valleys**; (Mt Falconer (77º 34', 163º 09'), Nussbaum Riegel (77º 41', 162º 51') and Miers Valley (78º 02', 163º 89'), and QMM = **Queen Maud Mountains** (84º 00', 171º 00').

Linkage disequilibrium was present in most populations and most loci (data not shown). However, loci from the Queen Maud Mountains were the exception, particularly Buef1 relative to the other loci. Standard of allelic fixation and inbreeding were conducted in Arlequin v3.1 which showed a high degree of variation within individuals (F_{IT}=0.779, F_{IS}=0.771) and low differentiation among populations (F_{ST}=0.038). Measures of heterozygosity, both observed (H_o) and expected (H_e), were very low, indicating an excess of homozygotes in all populations. The high incidence of homozygotes could be due to several factors including haploid thalli tissue, self-fertlisation within populations, or the presence of null alleles. Microchecker results indicated the presence of null alleles in all populations and at most loci, (with the exception of 2 loci, Buef1, Buef5 at Queen Maud Mountains). Consequently, basic allele data were transformed and the estimated frequencies of null alleles are shown in Table 4.1.

From these data, the harmonic mean of alleles (A) showed that 6-14 alleles were present across populations with mean effective alleles (N_e) ranging from 2.7 to 6.7. Allelic richness as estimated in HP-RARE 1.0 was highest in the Dry Valleys and lowest at Ross Island. However, the number of private alleles (corrected for sample size), was highest for the Dry Valleys followed by the Queen Maud Mountains (Table 4.1).

Table 4.1: Summary of all populations sampled of *Buellia frigida*. Number of individuals genotyped is provided for microsatellite loci (M_{SAT}) harmonic mean number of alleles (A), harmonic mean number of effective alleles (N_e), allelic richness (*A*), observed heterozygosity (Ho) and expected heterozygosity (H_e) under Hardy-Weinburg at each location. Allelic richness is calculated in HP-RARE. Effective alleles and Ho were calculated in Microsoft Excel using equations $h=1-\Sigma p_i^2$ and $A_e=1/(1-h)$. Expected heterozygosity was calculated in Arlequin.

Region	N_{MSAT}	NULL	A	N_e	*A*	A_r	Ho	He
TN	40	0.50	11	3.67	3.84	1.10	0.728	0.856
GH	27	0.31	12	5.68	3.84	1.05	0.824	0.854
RI	12	0.54	6	3.46	3.59	0.95	0.692	0.827
DV	27	0.41	14	6.69	4.07	1.71	0.850	0.892
QMM	6	0.41	6	2.70	3.38	1.48	0.630	0.769

Differentiation indices based on the transformed allele frequencies showed the least differentiation between Terra Nova and Granite Harbour, and the greatest between the Dry Valleys and Queen Maud Mountains. D_{EST} was highest between the Dry Valleys and the Queen Maud Mountains (0.739) and lowest between Terra Nova and Granite Harbour (0.105). G_{ST} was highest between Ross Island and Queen Maud Mountains (0.344) and lowest between Granite Harbour and Ross Island (0.082) (Table 4.2). D_{EST} was calculated both with and without transformed allele frequencies to

assess differences in variability between the two. Both calculations showed similar patterns but D_{EST} (untransformed) for the Dry Valleys showed substantially lower differentiation, perhaps highlighting the amount of null alleles present at this site.

Table 4.2 Population pairwise comparsons of: a) F_{ST}; b) D_{EST}; c) D_{EST} (uncorrected); and d) G'_{ST}. Significance of D_{EST} was calculated using 1000 bootstrap replicates in DEMEtics, uncorrected D_{EST} was calculated in SMOGD. Fst confidence intervals were calculated using 1000 bootstraps in FreeNA. G'_{ST} values were calculated according to Meirmans and Hedrick (2011). Confidence intervals and significance values are shown in the upper diagonal with relevant index values in the lower diagonal.

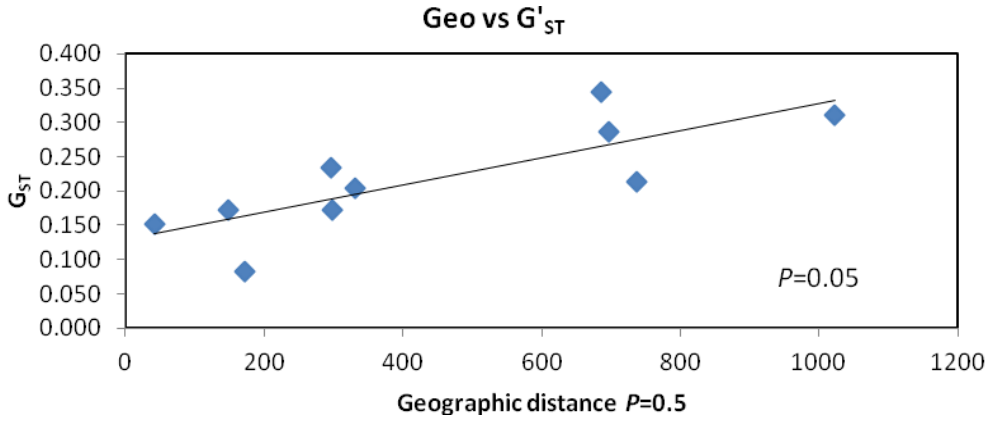
F_{ST}	TN	GH	RI	DV	QMM
TN	-	0-0.017	0-0.216	0.01-0.062	0.014-0.112
GH	0.004	-	0-0.021	0.013-0.071	0.018-0.14
RI	0.008	0.009	-	0.002-0.057	0.022-0.116
DV	0.031	0.038	0.029	-	0.27-0.15
QMM	0.060	0.076	0.071	0.085	-
D_{EST}					
TN	-			*	*
GH	0.105	-		*	
RI	0.279	0.226	-	*	*
DV	**0.480**	**0.383**	**0.443**	-	
QMM	**0.477**	0.487	**0.582**	0.739	-
D_{EST} (uncorrected)					
TN	-				
GH	0.082	-			
RI	0.112	0.231	-		
DV	0.315	0.282	0.299	-	
QMM	0.438	0.399	0.538	0.713	-
G'_{ST}					
TN	-				
GH	0.173	-			
RI	0.234	0.084	-		
DV	0.203	0.152	0.172	-	
QMM	0.312	0.213	0.344	0.286	-

Migration values (*M*) suggest migration occurs between all regions, with highest migration rates from Terra Nova to the Dry Valleys (1.764) and lowest from Queen Maud Mountains to Ross Island (0.463) (Table 4.3).

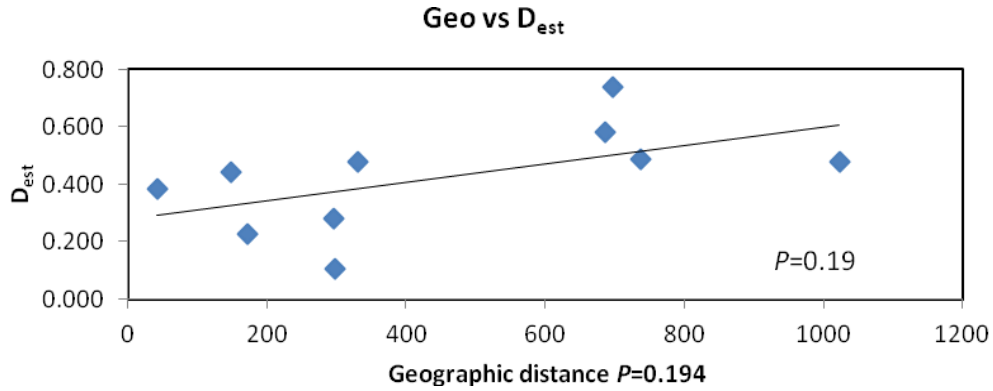
Table 4.3: Maximum likelihood estimates of migration between populations using MIGRATE-N. likelihood percentiles as confidence intervals.

Direction of migration	*M*	90% Confidence intervals
TN - GH	1.543	1.348-1.753
TN - RI	1.329	1.151-1.524
TN - DV	1.764	1.557-1.987
TN - BG	1.440	1.255-1.644
GH - TN	1.219	1.092-1.356
GH - RI	0.649	0.557-0.750
GH - DV	0.737	0.640-0.845
GH - BG	0.863	0.756-0.979
RI - TN	0.796	0.673-0.933
RI - GH	1.300	1.143-1.472
RI - DV	0.590	0.486-0.709
RI - BG	0.635	0.527-0.757
DV - TN	0.818	0.708-0.939
DV - GH	0.805	0.696-0.925
DV - RI	0.597	0.504-0.701
DV - BG	1.157	1.025-1.299
BG - TN	0.579	0.499-0.668
BG - GH	0.861	0.762-0.968
BG - RI	0.463	0.391-0.542
BG - DV	0.695	0.607-0.792

Mantel tests using G'_{ST} and D_{EST} measures showed a positive linear relationship between geographic distance and genetic differentiation (Fig. 4.2). This relationship was statistically significant for G'_{ST} (P = 0.05) but not for D_{EST} (P = 0.19)).



a)



b)

Figure 4.2: Mantel relationships for geographic distance between regions (shortest distance over land in kilometres) relative to estimates of genetic dissimilarity for *Buellia frigida* calculated using (a) G'_{ST} and (b) D_{EST}. *P* values are shown at bottom right of each figure.

Based on Migration Statistics (*M*) potential dispersal routes and centres of refugia are shown in Figure 4.3. These data suggest that while migration is

occurring among all sites, the main dispersal routes are from Terra Nova Bay southwards, and northwards from Granite Harbour, Ross Island and the Queen Maud Mountains.

4.4 Discussion

We found evidence for genetic differentiation among locations for *Buellia frigida* in the Ross Dependency of Antarctica. The patterns of differentiation and the presence of higher allelic diversity at some locations suggest the existence of potential source or refugial populations within this region. Initial analysis of microsatellite data showed an excess of homozygotes, no differentiation among locations and high estimates of linkage disequilibrium. This would generally indicate a self-fertilising or clonal species with high levels of gene flow among locations. However, following transformation of our dataset for null alleles, we found evidence for strong differentiation among populations of *B. frigida*. While Mantel tests only showed significant geographical co-relation using G_{ST}, differentiation indices calculated using transformed data detected differences among populations, generally in accordance with increasing geographic distance. F_{ST} and D_{EST} (both corrected and un-corrected) supported an isolation-by-distance, with the exception of the Dry Valleys, which were genetically distinct from neighbouring locations (e.g.??). The Dry Valley region showed both the highest allelic richness and the highest number of private alleles, suggesting this area may be associated with refugial populations (*sensu* Hewitt 2006). Direct comparison of allelic richness inferred in this study with those found in other lichen species is difficult due to a paucity of microsatellite-based migration statistics in the literature. However, as the allelic richness found here is similar to that found in other fungi such as the symbiotic fungus *Tuber magnatum* and the fungus *Ceratocystis pirilliformis,* (Rubini et al. 2005; Nkuekam et al.

2009), we are confident that the values obtained can be usefully compared across the populations in our study.

The Queen Maud Mountains showed a low *A* in comparison with the Dry Valleys (3.38 vs. 4.07, respectively). Thus, it is likely that populations in this vicinity may have experienced a bottleneck event. This area at the southern extent of the Transantarctic Mountains is geographically isolated due to vast expanses of snow and ice fields and most exposed ground in this area is likely to have been covered during glaciation events. However, the Queen Maud Mountains also had the second highest private *A* statistic. Private alleles are used as an indicator of population demography as population growth/expansion is often accompanied by a rise in new rare mutations (Marchelli & Gallo 2004). Thus, although a bottleneck event may have occurred in the Queen Maud Mountains, there is strong evidence that extensive population growth has ensued. Other work highlighting Queen Maud Mountains as an area of high species diversity may further support recent population expansion for taxa in this area (Green et al. 2011a). Three of the loci at this site showed unique allele sizes suggesting that recent colonisation from more northerly areas of the Ross Dependency is unlikely. Instead, the Queen Maud Mountains may have supported refugial populations in historical times, as has been suggested by previous work on lichen species diversity (Green et al. 2011a). The migration statistics suggest some migration from the Dry Valleys to the Queen Maud Mountains. Hence, while it appears lichens from the Dry Valleys are contributing to the genetic diversity at Queen

Maud Mountains, these populations are not panmictic as substantial differentiation still exists.

Interestingly, there is evidence for migration from the Queen Maud Mountains to Granite Harbour. However, migration is lower from Queen Maud Mountains to Ross Island. Indeed, migration statistics showed lowest migration to Ross Island, in comparison to all the other studied areas. This may be an artefact of low sample numbers at Ross Island, although sample sizes are similar to those from Queen Maud Mountains. Ross Island also had the second lowest allelic richness, the lowest private allele richness and the largest difference between observed and expected heterozygosity. Overall, it appears that there is less variation on Ross Island despite its proximity to the diverse Dry Valleys, which may reflect its relatively recent origin (500K-4.5MA) (Armstrong 1978). However, samples were collected from low coastal sites on the island, and because the Ross Ice Sheet is postulated to have extended to 300m above present sea level until the end of the last glacial maxima (Conway *et al.* 1999), it is likely that these populations are recent colonists.

Our migration statistics show that *B. frigida* from Terra Nova have the highest rates of migration to all other areas of this study. However despite this there is also high migration from some southern populations northward.

This argues against long distance dispersal occurring solely from continents further north. Migration routes may in part relate to wind movements in this area of the Ross Dependency. While there is mixing of

air flow from various influences (Katabatic, cyclones, barrier winds), there does seem to be evidence of strong wind flow from katabatic drainage (flow of cold dense air) from the Terra Nova Bay region, reaching >200km beyond the Bay (Seefeldt & Cassano 2008). With cyclones known to move from north to south, dispersal of lichen propagules from Terra Nova to more southern areas seems likely. There is also evidence for seasonal wind flow from the Beardmore Glacier (Queen Maud Mountains) up the Ross Ice Shelf and around Ross Island towards Granite Harbour (Seefeldt et al. 2003; Seefeldt & Cassano 2008), which would help explain the higher migration to this area rather than directly to Ross Island. However, dispersal from Queen Maud Mountains to more northerly sites is likely to be limited due to the distance between this location and other suitable habitat. While there are ice-free areas in the Brown Hills area which may provide stepping stones for dispersal further north, in reality the Brown Hills have been found to be very dry - a limiting factor for lichen survival that highlights the region as a dispersal barrier (C. Colesie et al. unpubl. data). Surprisingly, there is high migration from the Dry Valleys to the Queen Maud Mountains. We postulate that the strength and timing of wind flow in a southerly direction may explain the higher level of migration from the Dry Valleys to the Queen Maud Mountains if ascospores are indeed carried further and are not dependant on the Brown Hills area providing a stepping stone.

Despite high potential of air-dispersed ascospores for *B. frigida*, colonisation of different areas is likely to be restricted if appropriate wind direction and velocity are not occurring when ascospore settlement is

likely. A high proportion of mycobiont input is necessary for establishment of lichen populations (Beck et al. 2002). In cases where there are only infrequent winds connecting areas (e.g. Dry Valleys and Ross Island), lower levels of dispersal between those areas would be expected. This may also be exacerbated due to a lack of suitable habitats or dispersal corridors, or by the need for lichens to relichenize and find a suitable partner (e.g. Chapter 5). The much higher incidence and settlement of local spores (which may be more suited to local conditions; *sensu* Fisher *et al.* 2005), is also likely to overwhelm any spores from more distant sources.

Definitive identification of refugial locations is challenging because the precise ages of surface exposure in the Ross Dependency are uncertain. However, there are estimates which show reasonable correlation between proposed refugia and population genetic data. Dry Valleys and Terra Nova nunataks have been dated at 5.3-20 Ma and ~7.5 Ma, respectively, using cosmogenic dating (Armienti & Baroni 1999; Oberholzer et al. 2003). In contrast, the Ricker Hills ice sheet, which lies inland of Granite Harbour, extended to at least 500 m above sea level between 1.1 and 1.4 Ma, making it likely that Granite Harbour was covered in ice to at least that time (Strasky et al. 2009) and possibly as recently as 6-12 Ka (McKay et al. 2008, Hall & Denton 2000). Ross Island is between 500 Ka to 4.5 Ma (Armstrong 1978). Hence, is likely to have a longer exposure time than Granite Harbour, with more time for colonisation from diverse sources. However, as samples from this location were taken very near the coast, it is possible that they too were under ice during the most recent glacial

maxima, and have since been populated from either higher-altitude Ross Island sites or from further afield. Long distance colonisation of Ross Island from the Dry Valleys has been inferred for Collembola in the Ross Dependency (Stevens & Hogg 2003; McGaughran et al. 2011). While there is no clear indication for the duration of exposed rock in the Queen Maud Mountains, cosmogenic data suggest that the area has had ice-free areas for at least 4 Ma (Ackert & Kurz 2004); making it possible this area has also served as a refugium.

Collectively, our microsatellite data suggest moderate levels of genetic differentiation among populations of *Buellia frigida*, despite high levels of migration occurring among geographic areas. We provide further support for the notion that the Queen Maud Mountains and Dry Valley areas have served as ancient refugia. We also suggest that the Terra Nova Bay vicinity may be an additional refugial area and subsequent source of dispersal within the Ross Dependency.

Although temperate lichen species with similar dispersal mechanisms show panmixis between regions of the same continent (Buschbum 2007, Lattman et al. 2009), populations in the Ross Dependency are not panmictic, perhaps due to the relatively recent and limited nature of the current ice-free areas. It is also likely that past glaciation events have isolated, and maintained differentiation among, populations. Refugial populations will continue to be an important source of colonists to ice free areas as dispersal appears to occur on regional scales within the Ross Dependency.

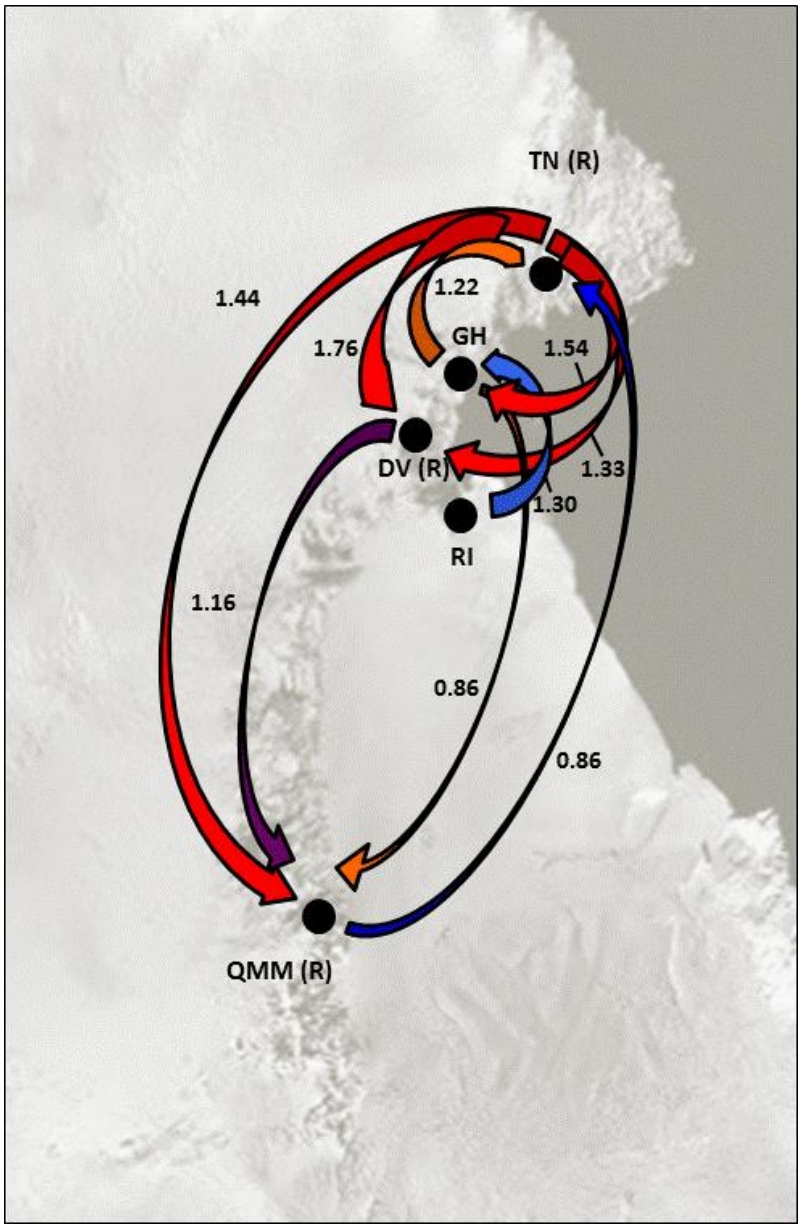


Figure 4.3 Hypothesised dispersal routes from each population according to migration statistics, with only the largest migration routes shown from each region (for ease of visual interpretation). Arrows indicate direction and *M* values above 0.85 are included by each arrow. Potential refugial sites are indicated, based on allelic richness.

Chapter 5

Photobiont Selectivity for Lichens and Evidence of a Glacial Refugium in the Ross Sea Region, Antarctica

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5.1 Introduction

Lichens are an important component of terrestrial ecosystems and dominate where vascular plants reach their physiological limits such as in desert, high alpine and polar regions (Lindsay 1978). Structurally, lichens represent a mutualistic symbiosis between lichenised fungi (mycobiont) and a photobiont (green algae and/or cyanobacteria) (Honegger 2009). Taxonomy is based on the mycobiont although the photobiont retains its own species designation and corresponding phylogeny (Greuter et al. 2000). As a polyphyletic group, lichens show a wide range of physical structures, modes of dispersal, and relationships with photobionts (Sanders 2005; Nash 2008; Honegger 2009). The lichen mycobiont can demonstrate both specificity and selectivity towards its photobiont- with specificity referring to the association of both partners towards each other, and selectivity to the taxonomic range of potential partners from the perspective of one symbiont only (Beck et al. 2002).

Lichen reproduction and dispersal is varied, with some species dispersing mainly via asexual structures (soredia and isidia) which are composed of both symbionts, and others dispersing via sexually produced fungal spores (Ahmadjian 1993). Lichens dispersing using only fungal spores, must relichenise *in situ* (germinate and select suitable photobiont associations) necessitating a suitable photobiont being available in the habitat to be colonised. In temperate and tropical regions, lichens tend to be highly selective towards the photobiont (Helms et al. 2001; Beck et al. 2002; Yahr et al. 2004; Hauck et al. 2007). In contrast, studies from both Polar

Regions have shown a tendency towards lower selectivity (Romeike et al. 2002; Wirtz et al. 2003; Domaschke et al. 2012; Pérez-Ortega et al. 2012). In these more extreme environments, it is possible that lichens will utilise local photobionts, which may also be better adapted to the local environment, rather than restricting themselves to a specific photobiont (Fernãndez-Mendoza et al. 2011). Lower selectivity may also simply be a direct consequence of survival under extreme conditions. For example, with a potentially lower rate of photobiont cell encounter, the colonising fungi may use whatever photobionts are available.

In Antarctica, knowledge of photobiont usage by lichens is limited, although previous studies have suggested low selectivity by mycobionts. For example, in the maritime Antarctic, Wirtz et al. (2003) found that lichens with cyanobacterial photobionts effectively used species that were locally available. Similarly, in the small, southern Dry Valleys of the Ross Sea Region, a low level of selectivity was found for *Buellia frigida* although *Umbilicaria aprina* showed some evidence of selectivity (Pérez-Ortega et al. 2012). A further study, focusing on the lecideoid species across continental Antarctica (Ruprecht et al. 2012), also found differences in selectivity among species with most showing low selectivity towards the photobiont. Here, we extended these studies by focussing on three lichen taxa along a latitudinal transect in the Ross Sea Region, Western Antarctica: *Buellia frigida*, an Antarctic endemic, *Umbilicaria aprina*, a bi-polar species, and *Umbilicaria decussata*, a cosmopolitan species.

The most common photobionts of Antarctic lichens are from the genus *Trebouxia* (Aoki et al. 1998; Romeike et al. 2002). *Trebouxia* is a

monophyletic taxon with more than thirty currently identified species, all of which were once thought to exclusively exist as photobionts within lichens (Ahmadjian 2004). However, there is now evidence that they can also occur independently and the fact that most Antarctic lichen species disperse via spores (Seymour et al. 2005), and therefore relichenise *in situ*, would seem to support this. Photobionts are difficult to identify to species level as many structures are suppressed in the lichenised state, and culturing is an expensive and time consuming process, with low success rates (Helms 2001).The use of molecular techniques to determine photobiont identity is a practical alternative and has become increasingly popular, particularly where specific primers allow complete lichens (inclusive of both bionts) to be analysed and can minimise the risk of amplifying non-target organisms. The internal transcribed spacers (ITS) in the ribosomal coding region have been successfully used in previous studies of the lichen photobionts (e.g. Romeike et al. 2002; Nelsen and Gargas 2009; Fernandez-Mendoza et al. 2011). High resolution in this region of rDNA also allows for comparison with available sequences in databases such as Genbank (Helms et al. 2001). Furthermore, there are algal-specific ITS markers, which amplify *Trebouxia* species, as well as other algal taxa ensuring that potential photobionts are not overlooked (Dahlkild et al. 2001).

Our aim was to determine if lower photobiont selectivity is a common feature for lichens in Antarctica. Specifically, using DNA sequences from the ITS region, we examined whether: 1) lichens would show a low level of

selectivity along a latitudinal gradient within the Ross Sea region; and 2) photobionts would be locally derived.

5.2 Materials and Methods

5.2.1 Field Sampling

Lichens were collected in 2006-2011 in the Antarctic summer period from sites over a latitudinal gradient of roughly 10º (74°36´S-84°00´S) in the Ross Sea Region of the Ross Dependency, Antarctica (Fig. 1). Sites were chosen in alignment with the Latitudinal Gradient Project (LPG) a framework developed under the Scientific Committee for Antarctic Research (SCAR) for understanding ecosystems and environmental impacts of climate change along the Victoria Land Coast. Lichens were collected using self-weighted random sampling, within an area accessible by day walks from either field sites or helicopter access points. Portions of thalli were removed from specimens of *Buellia frigida* Darb. (n = 157), *Umbilicaria aprina* Nyl. (n = 78), and *U. decussata* (Vill.) Zahlbr. (n = 40) as follows: *Buellia frigida,* a crustose lichen, was removed by scraping visibly distinct thalli from rocks with a clean scalpel blade onto acid free paper and into sterile cryogenic microcentrifuge tubes; *U. aprina* and *U. decussata,* both foliose lichens, were collected by removing portions from the outer edge of thalli with clean tweezers into sterile cryogenic microcentrifuge tubes. Thalli within and among regions varied in size, with *B. frigida* ranging from 0.5 cm to > 25 cm and for *U. aprina* and *U. decussata* ranging from 0.5 cm to > 10 cm. However, most samples of *B. frigida* were taken from thalli < 5 cm in diameter, and for *U. aprina* and *U.*

decussata most samples were < 4 cm in diameter. Samples were air dried on site and transported back to New Zealand for DNA extraction.

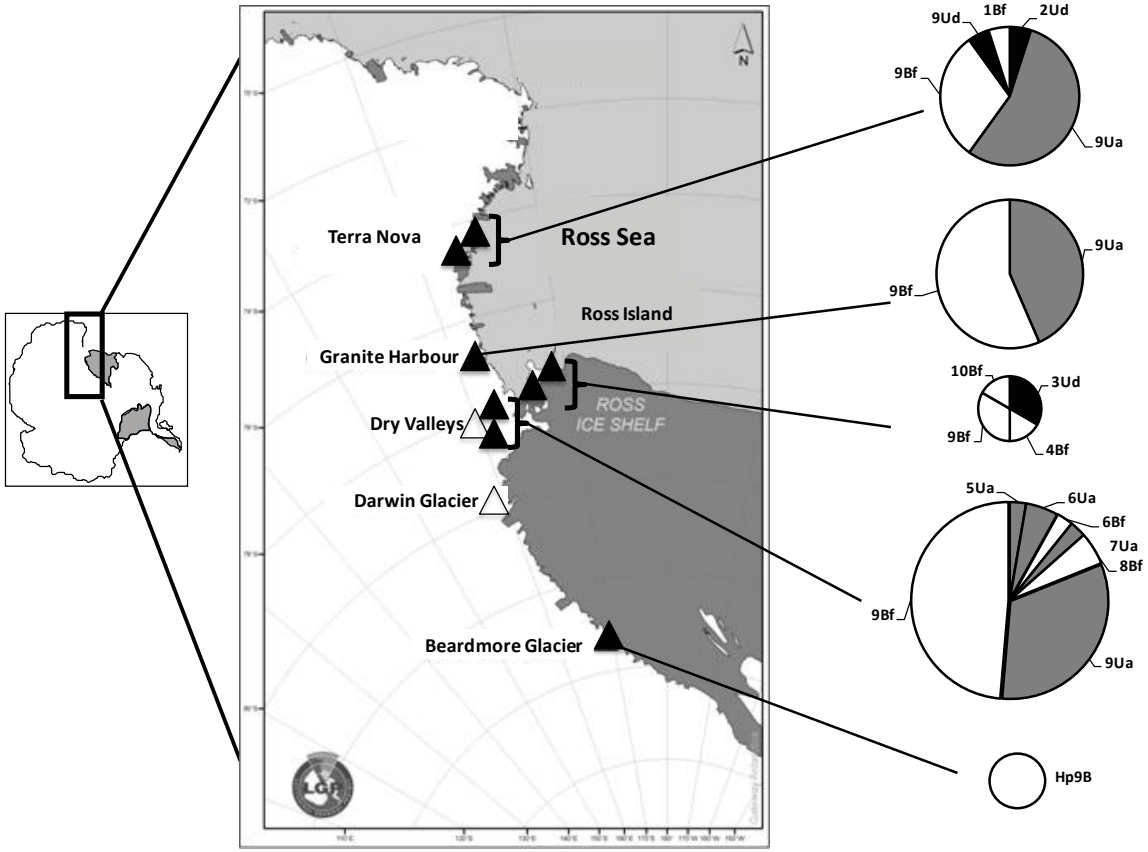


Fig. 5.1: The Ross Sea region with sites of lichen sample collection indicated. Solid triangles represent sites where at least one of the targeted lichen species was found and collected. Empty triangles represent sites where no targeted lichen species were found (Linnaeus Terrace, Dry Valleys and Foggy Dog, Darwin Glacier). Circles represent percentage composition of haplotypes Hp1-10 derived relative to geographic locations in the Ross Sea region. Numbers indicate haplotype and different shades correspond to lichen species, *Umbilicaria aprina* (Ua) = grey, *Buellia frigida* (Bf) = white and *Umbilicaria decussata* (Ud) = black. Circle diameter indicates the relative number of photobionts sequenced in each region.

5.2.2 DNA extraction and sequencing

DNA was extracted from whole lichens (mycobiont and photobiont) using a high alkaline PEG reagent protocol (Chomczynski and Rymaszewski 2006) with the following modifications: the sample was ground with liquid nitrogen before addition of 20-40 µl of PEG reagent, then shaken at 55^{o}C for one hour, the lysate was then spun at 4,000 rpm for 20 s and an aliquot of 2 µl supernatant added to 58 µl of ultra-pure H_2O. As the PEG extraction did not remove all inhibitors a modified dialysis technique based on Freifelder (1982) was used by preferentially soaking the extractions into agarose. The aliquot and MqH_2O from the PEG extraction was soaked into the top of 1.2% agarose (in 1/10 TE) in 0.2 ml thin walled tubes with a pin hole pierced bottom in a 1.5 ml eppendorf tube overnight or spun for ten minutes at 4,000 rpm (revolutions per minute); 20-40 µl of 1:10 TE was then added and the non-absorbed DNA was removed from the surface by vortexing. The removed wash was spun at 4,000 rpm for 15 s and the supernatant recovered.

To assess the composition of the DNA extract (that two products were present), universal Internal Transcribed Spacer (ITS) primers (ITS5/ITS4; White et al. 1990) were used in the PCR. One µl of the above extract was used in a 25 µl reaction volume with a final concentration of 0.2 µM of each primer, 0.2 mM of the four dNTP's, 2 mM $MgCl_2$, 1% BSA and 1 U Platinum Taq Polymerase. PCR amplification was carried out in a PTC-100 thermocycler (MJ Research, Ramsey, Minnesota, USA) with initial denaturing at 94°C for 2 min then 29 cycles of 94°C 40 s, 50°C 40 s and

72°C for 2 min followed by a final elongation step of 10 min at 72°C. The resultant products were run on a 2% TAE agarose gel mixed with ethidium bromide alongside a 100bp ladder (Invitrogen Life Science, Auckland, New Zealand) and were of similar size to GenBank ITS sequences of the mycobiont (≈580 bp) and photobiont (≈650 bp). The bands were then excised from the gel and purified using a Zymoclean GEL DNA recovery kit according to the manufacturer's instructions, and sequenced on an AB 3130XL automated DNA Sequencer at the University of Waikato DNA sequencing facility. A BLAST search in GenBank was used to confirm that sequences corresponded with recognised species. Sequencing reactions were performed using the primer ITS4 (White et al. 1990). Once the presence of both symbionts was established, a 5' end algal specific primer was used 1AKLf (Dahlkild et al. 2001), along with the 3' end unspecific primer ITS4 (White et al. 1990), to amplify the photobiont only and the above process of gel extraction repeated. Initially sequencing reactions were performed using ITS1AKL and ITS4. These corresponded well with single reads using ITS4 so the majority of sequences are single read only. All sequences have been deposited in the project "Antarctic lichen photobionts" (ANTLA) in the Barcode of Life Datasystem (BOLD) (Ratnasingham and Hebert 2007) database (http://www.barcodinglife.org) and cross referenced to GenBank.

5.2.3 Phylogenetic Analyses

ITS sequences (n=91) were initially aligned using Geneious Pro 5.3.4 (Drummond et al. 2011) using both Geneious and Muscle algorithms, as well as manually checking base alignments. As the 5.8S rDNA region both anchored the alignment and contained some variation it was also retained. All ambiguous sections were removed. Sequences were compared to those in GenBank using nucleotide BLAST searches, both to ascertain they were algal sequences and to find similar sequences. Eighteen comparative *Trebouxia sp.* sequences were downloaded from GenBank (*T. jamesii* (x5), *T. aboricola* (x3), *T. asymmetrica*, *T. decolorans* (x2), *T. impressa* (x2), *T.* sp (x5), (Table 5.1). These were chosen based on geographic source, alignment with BLAST searches (Table 5.1) and from previous studies of particular interest (e.g Romeike et al. 2002; Nyati et al. 2004; Des los Rios et al. 2005; Ruprecht et al. 2012).

Table 5.1: *Trebouxia* species taken from GenBank with associated accession numbers, Authors and location of source is also provided.

Trebouxia species	Accession number	Authors	Source	Location
	AJ431584	Romeike *et al.* 2002	*U. decussata*	Ant. Peninsula
T. Jamesii	AJ431589	Romeike *et al.* 2002	*U. decussata*	Ant. Peninsula
	AJ431588	Romeike *et al.* 2002	*U. decussata*	Ant. Peninsula
	GQ375320	Domaschke *&* Printzen 2012	*Cetraria aculeata*	Antarctica
	GQ375319	Domaschke *&* Printzen 2012	*Cetraria aculeata*	Norway
T. aboricola	AJ969529.1	Nyati *et al.* 2004	*Xanthoria ligulata*	New Zealand
	AJ249481	Friedl *et al.* 2000	*Pleurosticta acetabulum*	Taxon: 53268
	Z68703	Friedl *et al.* 2000	Clone “arbace_c3”	Taxon 53268
T. asymmetrica	AF345889.1	Piercey-Normore *&* DePriest 2001	*Diploschistes albescens*	UTEX 2507
T. decolorans	AJ969545.1	Nyati *et al.* 2004	*Xanthoria parietina*	France
	AJ969601	Nyati *et al.* 2004	*Xanthoria parietina*	Switzerland
T. impressa	AJ249576	Friedl *et al.* 2000	*Melanelia glabra*	Taxon 13788
	AJ318780	Romeike *et al.* 2002	*U. kappenii*	Ant. Peninsula
Trebouxia sp.	AJ431583	Romeike *et al.* 2002	*U. decussata*	Lagoon Island Ant.
	AJ431580	Romeike *et al.* 2002	*U. antarctica*	Harrow Peak, Ant.
	AJ431579.1	Romeike *et al.* 2002	*U. Antarctica*	Ant. Peninsula
	AM159204	Nyati *et al.* 2004	Environmental sample	Switzerland
	AY667580.1	De los Rios *et al.* 2005	Environmental sample	Granite Harbour, Ant.

Arlequin v 3.11 was used to test genetic distances among populations using pairwise comparisons of F_{ST} with the significance p-values assessed with 10098 permutations. Data sets of original sequences and then original sequences with downloaded sequences, were entered into DnaSP v5 (Librado and Rozas 2009) considering gaps as fifth state, invariable sites removed, with a 9999 permutation test giving both haplotype diversity and nucleotide diversity for population data (split into five regions) and species data (split by mycobiont host) (Table 5. 2).

Table 5.2: Nucleotide diversity (Jukes and Cantor 1969) and Haplotype diversity (Hd) within populations and species

	Number of Haplotypes	Haplotype diversity (Hd)	Nucleotide Diversity (π)	Number of samples
Comparison of Species				
Buellia frigida	6	0.223	0.00449	51
Umbilicaria aprina	4	0.211	0.00076	36
Umbilicaria decussata	3	0.833	0.0581	4
Total				91
Comparison of Populations				
Terra Nova	5	0.195	0.01155	20
Granite Harbour	1	0	0	23
Dry Valleys	5	0.341	0.00114	37
Ross Island	4	0.867	0.08274	6
Beardmore Glacier	1	0	0	5
Total				91

Data collapsed into haplotypes via DnaSP were entered into jModelTest v0.1.1 (Posada 2008) using likelihood scores and AIC selection to ascertain model of evolution. Trees were built using the photobiont sequence for each lichen species in Geneious, using neighbour joining methods and the mutation model Tamura & Nei (1993) with 1024 bootstraps. Maximum likelihood algorithms were conducted in GARli v2.0 (Zwickl 2006) using parameters of the evolution model, with 1024 bootstrap replicates. A majority rule consensus tree (Fig. 5.2) was constructed in PAUP* (Swofford 2002) using 1024 bootstraps, with branches having greater than 50% bootstrap support labelled. All phylogenetic trees were viewed and edited in FigTree V1.3.1. (Rambaut 2008).

5.3 Results

Successful amplification and consequent sequencing was challenging and despite repeated attempts only 91 useable sequences were obtained from the 275 samples initially amplified. Those samples from which a clear sequence was obtained, include: 51 *Buellia frigida*; 36 *Umbilicaria aprina*; and four *Umbilicaria decussata*. Although these three species are commonly found in the Ross Sea Region, distribution can be patchy. For example, none of the species was found at Linnaeus Terrace site within the Dry Valleys or from the Foggy Dog site near Darwin Glacier, despite previous reports of *B. frigida* in the Darwin Glacier area (R. Türk pers. comm.). *Umbilicaria aprina* and *U. decussata* were not found at the same location except in the vicinity of Gondwana Station and Terra Nova Bay

(Fig. 5.1), where all three species were common. We found a total of 10 haplotypes for *Trebouxia*, with one major haplotype (Hp9) present in 78 of the 91 samples and included photobiont sequences from all three lichen species. The other haplotypes had 1-79 base pair changes from this major haplotype. *B. frigida* had the highest number of haplotypes with six from 51 sequences (Hd=0.223), *U. aprina* had four from 36 sequences (Hd=0.211), and *U. decussata* three from four sequences (Hd=0.833).

jModelTest results indicate Tamura Nei plus Gamma (TrN+G) as the model of evolution with a –lnL of 2479. 94 with variable base frequencies, equal transversion rates, and variable transition rates. Substitution rates are rAC=0.5241, rAG=3.0502, rAT=1.4302, rCG=1.3519, rCT=4.1154, rGT=1.0000 and the shape parameter alpha of the gamma-distribution was 0.3640. Neighbour joining (data not shown) and maximum likelihood (ML) trees showed very similar topology despite different evolution models. The majority rule tree from the ML analysis with all haplotypes (inclusive of outgroups from GenBank), showed that photobionts from *U. aprina* and all but two from *B. frigida* were very similar and occurred at one major node that included three *Trebouxia* sequences from GenBank (Fig. 5.2). Two sequences corresponded to photobiont sequences from the Antarctic region (Mt Melbourne AJ31580, and an environmental photobiont sequence from Granite Harbour AY667580.1), and the third to an environmental photobiont sequence from Switzerland (AM159204). One outlier, the *B. frigida* photobiont sequence Hp10 aligned with a photobiont from *Xanthoria ligulata* (sourced from New Zealand) and the position of the other (Hp4) was partially unresolved and was basal to other photobiont

sequences in the major clade (node 3, Fig. 5.2). The photobionts from *U. decussata* were included with *T. jamesii* (node 1). Node 1 included photobionts from other *U. decussata* samples from the Antarctic Peninsula (AJ431584, AJ431589, AJ431588) and *T. jamesii* from *Cetraria aculeata* from Antarctica (GQ375320) and Svalbard (GQ375319). One photobiont sequence from *U. decussata* (Hp9) occurred in the main group of sequences (node 2, Fig.5. 2).

Umbilicaria decussata photobionts were at two nodes (nodes 1 and 2; Fig. 5.2), while *U. aprina* photobionts were found only within one node (node 2). *Buellia frigida* had 49 out of 51 photobionts within one node (node 2). However, the remaining two photobionts, (Hp4 and Hp10) were basal to node 2 (Hp4) and contributed to the formation of node 4 (Hp10). A BLAST search of these outlying *B. frigida* sequences showed that Hp4 showed closest sequence similarity to a *Trebouxia sp.* sequence from a North American environmental sample (AM159208.1; 94% identity (687/733) and 10/733 gaps (1%)), while Hp10 aligns with an uncultured *Trebouxia* photobiont AJ969582 from an Australian sample of *Xanthoria parietina* (98% identity (776/790) and 4/790 gaps (1%)). Only one haplotype (Hp9) was present in all locations —all other haplotypes were unique to individual sampling locations (Fig. 5.1).

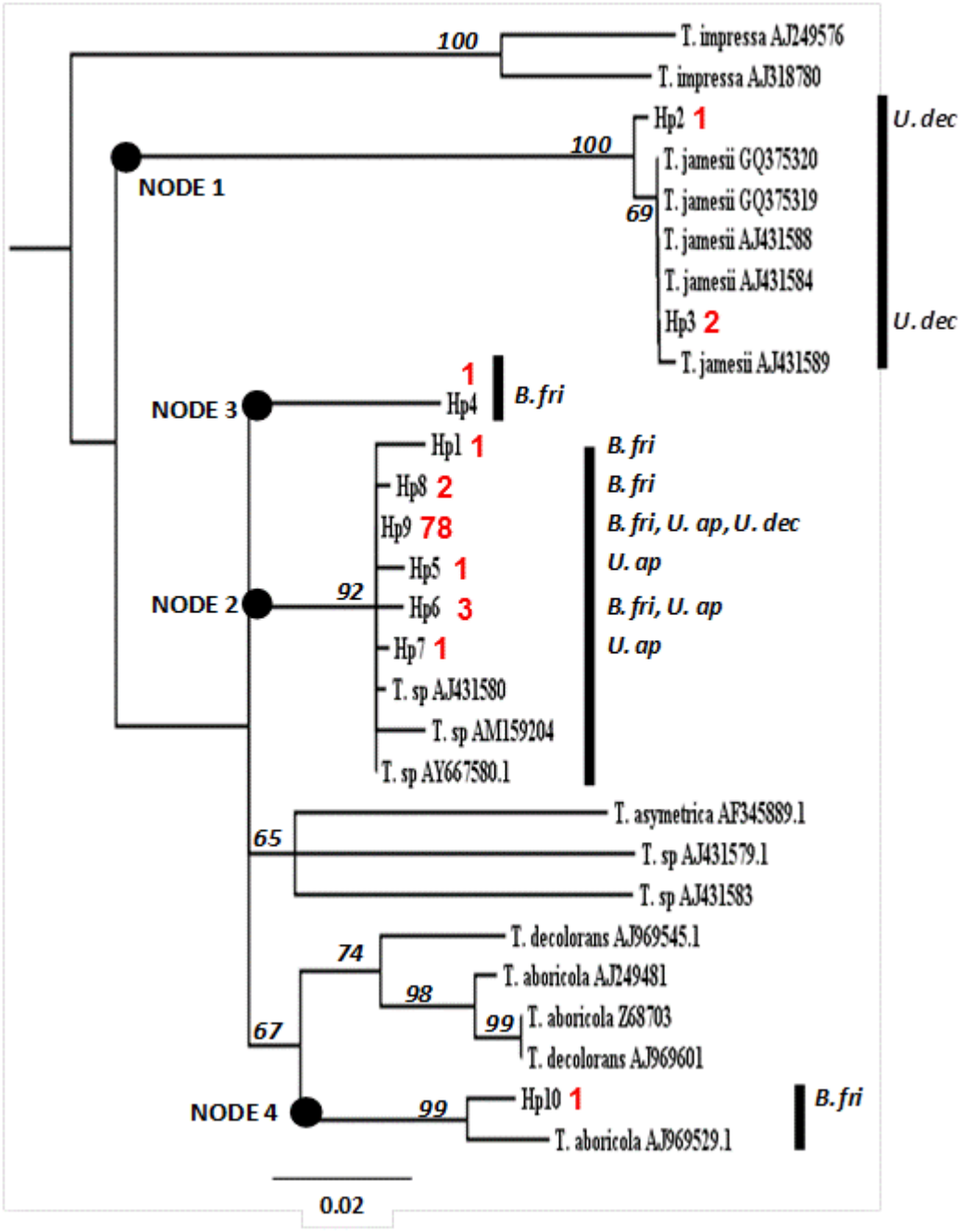


Fig. 5.2: PAUP* Majority Rule consensus tree from Maximum Likelihood analysis, constructed for all used GenBank sequences and photobiont sequences from this study and includes all Antarctic samples and outgroups. Nodes are numbered (1 to 4) and contain sequences from both origins. Haplotypes 1, 4, 8 and 10 were found only in *Buellia frigida* (Nodes 2, 3, and 4); Haplotypes 5 and 7 only in *Umbilicaria aprina* (Node 2) and Haplotypes 2 and 3 only in *Umbilicaria decussata* (Node 1). *B. Fri* = *Buellia frigida*, *U. ap* = *Umbilicaria aprina* and *U. dec* = *Umbilicaria decussata.*Numbers beside haplotypes indicate number of samples of that haplotype.

5.4 Discussion

Using ITS sequences, we found evidence for species-specific photobiont selectivity in lichens from the Ross Sea Region, as well as local distribution of photobionts within a geographic area. There was also evidence that the level of selectivity found within these lichen species may influence their distribution within the Ross Sea Region. For example, photobionts for both *Umbilicaria aprina* and *Buellia frigida* were primarily associated with a single and widespread haplotype (Hp9). Furthermore, *Umbilicaria aprina* showed no variation in the species diversity of its photobionts, and all of the observed haplotypes were specific to one node (node 2). Previous studies have also found *U. aprina* to be highly selective, with Pérez-Ortega et al. (2012) finding only two very similar haplotypes in their sample of 19 specimens. In comparison, the *U. decussata* photobionts showed very high haplotype diversity, despite the lower sample size for this species. They belonged to two very different nodes which likely represent different photobiont species. *Umbilicaria decussata*, although associated primarily with *T. jamesii* (node 1) also had, Hp9, the most common photobiont haplotype found in the Ross Sea region. Even within node 1 representing *T. jamesii* there is a high degree of haplotype variability in photobionts from *U. decussata*. High photobiont species diversity has been previously found for *U. decussata* from the Antarctic Peninsula as well as other *Umbilicaria* species (Romeike et al. 2002).

Photobiont ITS sequences from *B. frigida* were included in a clade with previously sequenced *Trebouxia* species, with the majority (n=43)

belonging to the most common photobiont haplotype (Hp9). However, two other divergent haplotypes were also found, Hp4 basal to node 2 (albeit with low bootstrap support) and Hp10 forming a well-supported cluster with *T. aboricola* (node 4) from a New Zealand sample of the lichen *Xanthoria ligulata.* We initially thought that another lichen species may have been accidentally collected. However, analysis of the mycobiont confirmed the lichen host was indeed *B. frigida*. One possible explanation for the association with such divergent photobionts is through "algal switching" whereby the mycobiont can utilise an alternative algal species under some environmental conditions (Piercey-Normore and DePriest 2001; Peksa and Skaloud 2011).This phenotypic plasticity can even occur following the establishment of soredia (i.e. when both mycobiont and photobiont have dispersed together), and has been previously highlighted as a means of adapting to local conditions (Peksa and Skaloud 2011).

Higher photobiont diversity within a single lichen species has been suggested as being indicative of lower selectivity by the mycobiont which could give enhanced colonisation ability as found in cosmopolitan species such as *Protoparmeliopsis muralis* (Guzow-Krzeminska 2006), and *Lecanora rupicola* (Blaha et al. 2006). However, we were unable to find evidence of this enhanced colonisation ability in the Ross Sea region. Here, the cosmopolitan *U. decussata* despite low selectivity and high photobiont diversity was considerably less widespread than the endemic *B. frigida*. In contrast, *B. frigida* showed high selectivity and associated almost exclusively with Hp9, the most common photobiont in the Ross Sea region. We suggest that this higher selectivity may actually be

advantageous in the extreme Antarctic environment where the lichens occur only in favourable microhabitats (Green et al. 2011a). However, we also note that despite photobiont selection being essential for establishment, the actual ability of a lichen to compete and survive will have a strong mycological aspect including genotypic and phenotypic components (Honegger 2009).

The majority of photobiont sequences (95%) were all part of a single large clade and aligned with existing GenBank algal sequences collected from lichens as well as from environmental samples. In one case, they aligned with a Genbank sequence taken from an environmental sample in Switzerland, showing a consistency in photobiont selection over a very wide geographical range suggesting linkage to similarities in micro-environmental conditions rather than to latitude (Fernandez Mendoza 2011; Green et al. 2011a). From our study, this haplotype was the most common strain of this *Trebouxia* species in the Ross Sea Region and may be selected to the current or geologically-recent climatic conditions. The presence of haplotypes from Switzerland as well as Antarctica at one node (node 2) indicates that this is not a unique Antarctic species and is perhaps an undescribed, cold-adapted species (Pérez-Ortega et al. 2012).

The endemic lichen *Buellia frigida* is widespread throughout the Ross Sea Region and the vast majority of samples (45 out of 51) shared the same haplotype. However, it does associate with other haplotypes and particularly so in the Dry Valleys region where it associated with 5 of the 10 haplotypes. Pérez-Ortega et al. (2012) also found high haplotype diversity (n=6) in *B. frigida* from the smaller, southern Dry Valleys area.

This pattern also existed in *U. aprina* which despite low selectivity in general showed the highest haplotype diversity in this area. The reason for higher haplotype diversity for photobionts in this area is unclear. However, one possibility is that the higher haplotype diversity may the effect of local photobionts accumulating mutations that are selected for during repeated glaciation events, resulting in an increased genetic diversity within the Dry Valleys. Alternatively, mutations could also be accumulating due to the longevity of the lichens in this arid microclimate. Previous genetic studies on springtails (Collembola) and mites (Acari) (Stevens et al. 2007; McGaughran et al. 2008) have highlighted the Dry Valleys as a possible glacial refugium. Accordingly, this area would seem to house high genetic diversity for both flora and fauna and its continued preservation should be a conservation priority. In particular, the maintenance of such genetic variability may facilitate the longer-term evolutionary responses of these populations to environmental changes (*sensu* Jump et al. 2009).

On-going monitoring of lichens and their photobionts as part of developing terrestrial observation networks in Antarctica (e.g. Nielson et al. 2011; Chown et al. 2012), may provide a sensitive biological indicator of environmental changes (Green et al. 2011a). It is clear that Antarctic terrestrial biota are likely to be permanently disrupted by anthropological activities such as transfer of soil material or the physical disturbance of populations (Stevens and Hogg 2003, Cowan and Ah Tow 2004, Cowan et al. 2011) and in order to minimise these consequences, we recommend the continued protection of areas (e.g. Dry Valleys) housing high lichen and photobiont diversity.

Chapter 6
General Conclusions

6.1 Summary

The genetic structure of lichen populations is to a large extent dictated by dispersal characteristics and opportunities (DePriest 2004; Walser et al 2005). However, this is complicated in lichens by their symbiotic nature (see Chapter 2). Indeed it appears each lichen species could have different dispersal capabilities when the combination of photobiont selectivity and dispersal method is taken into consideration. Studying this structure in Antarctica is further complicated by the logistical challenges of sampling in such an extreme and remote environment. Accordingly, there have been few genetic studies undertaken for lichens in the Ross Sea Region of Antarctica (Romeike et al 2002; Wirtz et al 2003). Lichens are an important component of nutrient cycling, particularly in areas with few inputs from vascular plants such as high altitude (e.g. Alpine) and latitude habitats such as the Antarctic. Understanding the structure of lichen populations in such areas adds to a growing area of polar research on ice sheet dynamics from a biological perspective.

In this thesis I address this gap in our knowledge by focusing on the distribution and dynamics of present day Antarctic lichen populations, from both overall population structure and individual component (i.e. symbiont relationship) perspectives. Specifically, my research provides data regarding likely dispersal routes of current lichen populations (Chapter 4),

along with data on the level of selectivity by the mycobiont for the photobiont in these lichen species (Chap 5). Specifically, I was interested in addressing whether selectivity for the algal photobiont was a potentially limiting factor for relichenisation. Furthermore, I have developed and provided microsatellite primers for a widespread lichen species (*Buellia frigida*) (Chapter 3).

Prior research on the population genetics of lichens has concentrated on methods (e.g. rDNA analysis) which provide limited results in identifying wide-scale patterns even in temperate environs, and despite recent development of microsatellites for one lichen species, these were unable to amplify target DNA for any of the lichens in this study. Accordingly, in Chapter 3, I developed important polymorphic markers which enable characterisation of a common and widespread Antarctic endemic. These were tested with the endemic *Buellia frigida* (the target species) as well as two additional lichen species *Umbilicaria aprina* and *Umbilicaria decussata,* and a common lichen photobiont *Trebouxia jamesii.* These markers proved to be fungal specific with a moderate amplification success rate and will be useful for future studies of *B. frigida*. Furthermore, I was able to develop DNA extraction methods which minimised the presence of DNA inhibitors that are prevalent in lichen taxa.

Chapter Four used these newly developed markers, and provided evidence for genetic structure among populations of the Antarctic endemic *Buellia frigida*. Putative refugial locations were identified, some of which had been suggested in prior research on invertebrates (McGaughran et al. 2011), and terrestrial vegetation (Green et al 2011b). One additional site

(Terra Nova Bay) was also highlighted as newly identified potential refugium.

Methods which identify the selectivity of lichens for their photobionts are well established in temperate environs. However, prior to my thesis research there was a paucity of information on these relationships in Antarctica. Thus, Chapter Five examined selectivity among the three target lichen species using ITS sequence analysis and a range of phylogenetic methods. This tested the hypothesis that the high latitudes of Antarctica would demonstrate low levels of selectivity owing to limited choice of potential symbionts. Originally, selectivity was thought to decrease with concomitant latitude increase. However, Chapter Five showed instead that the three lichen species associated primarily with one photobiont strain, despite evidence of other photobiont species being available in some of these localities (Perez-Ortega et al. 2012; Ruprecht et al. 2012). There was also evidence to suggest that levels of selectivity may be species-specific and some haplotypes were specific to individual regions. Furthermore, the association of the mycobionts with local photobionts may be important, as one common haplotype was found at all locations and in all three species.

Conclusions and Future Research

The application of molecular techniques such as the analyses of microsatellite DNA loci used in this thesis to assess the population genetic structure of lichens, has provided an effective means of assessing inter-habitat dispersal. This data have provided insight into the evolutionary

history of lichens in Antarctica, and indicated that, while dispersal is currently occurring, there are areas where refugial signatures (such as higher allele variability and variation in allele sizes from different locations) are clear. Application of a variety of analyses on microsatellite data provided valuable information that can remain hidden when data is highly polymorphic. Thus, Chapter Four showed that differentiation between populations is highly affected by the amount of variation within individuals and populations (Leng et al. 2011; Meirmans and Hedrick 2011).

Signatures of refugia identified here add to growing biological data which indicate ice-sheet cover did not eliminate the resident biota as Antarctica moved to its current position (Stevens et al. 2007; McGaughran et al. 2008). In particular, the Dry Valley region showed both the highest allelic richness and the highest private alleles, putting strong emphasis on this region as a refugial area (*sensu* Hewitt 1996) and further highlighting it as a target for further research. The Beardmore Glacier area also stands out as worthy of additional study. For example, microsatellite loci for *Buellia frigida* in the Queen Maud Mountains were markedly different in allele size compared to other locations and linkage disequilibrium was not present in these loci, unlike all other populations of this study. Furthermore, these same loci showed no evidence of null alleles at the Queen Maud Mountain site despite null alleles being prevalent at all other sites. Possible explanations include lower wind velocities which would clear snow, unlike other intermediate sites. These explanations have been invoked in previous biodiversity studies to explain why the Queen Maud Mountains

are so different from other regions in the Ross Dependency (Green et al. 2011b). However, further investigation is warranted.

Previous studies on photobionts of selected lichen species in the Antarctic have suggested low selectivity by mycobionts (Wirtz et al. 2003; Perez-Ortega et al. 2012; Ruprecht et al. 2012). I suggest that this is not necessarily so, with one species (*Umbilicaria aprina*) showing associated photobionts at only one node of the phylogenetic tree, constructed from Maximum Likelihood analysis, and indeed the majority of photobiont sequences (95%) from all three lichens form a single node. This node included existing GenBank algal sequences collected from lichens as well as environmental samples, from both Antarctica and similar environments globally. *Umbilicaria decussata* photobionts were included at two nodes unlike the other two species, which may be connected to this lichens' global distribution, a characteristic associated with low selectivity, rather than its location in the Antarctic (Guzow-Krzeminska 2006).

The hypothesis that selectivity is wholly related to latitude and its accompanying extreme conditions, with lichens at higher latitudes of the Antarctic being less selective than temperate counterparts, was not supported. In fact, there continued to be a species-specific signature, with some species showing a higher level of selectivity than others. However, there appeared to be an influence of local conditions, with a departure from high selectivity at just one species rich locality for the endemic lichen *Buellia frigida*. The majority of photobiont sequences (95%) were all part of a single large clade and aligned with existing GenBank algal sequences collected from lichens as well as from environmental samples from other

continents suggests further examination of the connection between microclimate and photobiont haplotype would be fruitful. The correlation between photobionts from Antarctica and other cold environments is interesting as it poses the possibility that micro-climate is potentially dictating which species of photobiont is available to the mycobiont beyond latitudinal considerations. Rather, it is likely that similarities between microclimates are the major driving force in determining which photobiont is fit and available in local conditions.

With the beginnings of a global perspective on relationships amongst symbionts (e.g. selectivity level for photobiont) being established (Helms et al. 2001; Yahr et al. 2004; Blaha et al. 2006; Doering and Piercey-Normore 2009), further exploration of this topic would be informative. Furthermore, as lichens are an essential component of Antarctic terrestrial ecosystems, on-going monitoring of lichen photobionts as part of developing terrestrial observation networks in Antarctica (e.g. Wall et al. 2011; Chown et al. 2012), are likely to provide valuable indicators of environmental changes (Green et al. 2011a).

In this thesis, only one lichen species (the endemic *Buellia frigida*) was examined for both the mycobiont and photobiont. Even so it is apparent that both symbionts of this lichen species show a signature of refugia in the Ross Sea Region, particularly in the Dry Valleys region where the majority of diversity exists. It is unknown if these data can be extrapolated to other lichen species in the Antarctic. Information on both symbionts from a range of bi-polar or cosmopolitan lichens would help to enhance our understanding on whether the finding from my study are more

generally applicable, or if the dispersal techniques and proposed colonisation characteristics of *B. frigida* are endemic-specific.

With the advent in recent years of rapid elucidation of genomes (i.e. next generation sequencing), it would be beneficial to identify SNP markers for the lichen species analysed herein. Initially, this would require optimisation to identify symbiont specific markers. However, once identified rapid subsequent analysis could ensue. *U. decussata* would be an ideal candidate for further study. In Antarctica, this genus is likely to be different from other genera in similar habitats (Sancho et al. 1992). Furthermore, as *U. decussata* is cosmopolitan, relationships between this species and its photobiont could give additional information on specificity in different environments, from temperate to polar. As demonstrated by my thesis research there is a strong possibility that photobionts are linked with microenvirons which could potentially be explored further. One of the greatest challenges may be the difficulties in amplification of this species. I was unable to extract DNA from algal-free material of *U. decussata* for microsatellite marker development. However, this may be possible. Concern that insufficient mycobiont DNA of this species was extracted or that it was sheared so only multi-copy sequences such as the ITS region would amplify, induced trialling the amplification of another available marker polyketide synthase (PKS). This was successfully amplified (appendix 5). Hence, poor DNA quality was not sufficient to explain the lack of microsatellite amplification in the mycobiont.

Based on my thesis research, I suggest that the following three lines of research would be profitable in the future: 1) investigate the relationship

between photobiont species and microclimate both in Antarctica and further afield; 2) evaluate population genetic structure and level of selectivity for a range of lichen photobionts across Antarctica and using additional lichen species; and 3) determine the global population structure and dispersal patterns using the cosmopolitan lichen *Umbilicaria aprina.*

Collections of three lichen species sampled as part of my thesis research cover a latitudinal gradient of 10˚ S. These will remain an important resource for future efforts, and have been deposited in the Waikato Herbarium facilities at the University of Waikato.

References

Ackert Jr RP, Kurz MD (2004) Age and uplift rates of Sirius Group sediments in the Dominion Range, Antarctica, from surface exposure dating and geomorphology. Global and Planetary Change 42:207-225

Ahmadjian V (1988) The lichen algaTrebouxia: does it occur free-living? Plant Systematics and Evolution 158:243-247

Ahmadjian V (1993) The Lichen Symbiosis. John Wiley, Chichester 15: 250

Ahmadjian V (2004) *Trebouxia*: Reflections on a Perplexing and Controversial Lichen Photobiont, in: J Seckbach (ed), Symbiosis, Springer Netherlands 373-383

Alvarez I, Wendel JF (2003) Ribosomal ITS sequences and plant phylogenetic inference. Molecular Phylogenetics and Evolution 29:417-434

Aoki M, Nakano T, Kanda H, Deguchi H (1998) Photobionts isolated from Antarctic lichens. Journal of Marine Biotechnology 6: 39-43

Armienti P, Baroni C (1999) Cenozoic climatic change in Antarctica recorded by volcanic activity and landscape evolution. Geology 27:617-620

Armstrong RA (1987) Dispersal in a population of the lichen *Hypogymnia physodes*. Environmental and Experimental Botany 27: 357-363

Armstrong RA (1990) Dispersal, Establishment and Survival of Soredia and Fragments of the Lichen, *Hypogymnia physodes* (L.) Nyl. New Phytologist 114:239-245

Armstrong RL (1978) K-Ar dating: Late Cenozoic McMurdo Volcanic Group and dry valley glacial history, Victoria Land, Antarctica. New Zealand Journal of Geology and Geophysics 21:685-698

Barker PF, Barrett PJ, Cooper AK, Huybrechts P (1999) Antarctic glacial history from numerical models and continental margin sediments. Palaeogeography, Palaeoclimatology, Palaeoecology 150:247-267

Barnes I, Gaur A, Burgess T, Roux J, Wingfield BD, Wingfield MJ (2002) Microsatellite markers reflect intra-specific relationships between isolates of the vascular wilt pathogen Ceratocystis fimbriata. Molecular Plant Pathology 2:319-325

Beerli P (2009) How to use MIGRATE or why are Markov chain Monte Carlo programs difficult to use. Population genetics for animal conservation 17:42-79

Beck A, Friedl T, Rambold G (1998) Selectivity of photobiont choice in a defined lichen community: inferences from cultural and molecular studies. New Phytologist 139:709-720

Beck A, Kasalicky T, Rambold G (2002) Myco-photobiontal selection in a Mediterranean Cryptogam community with *Fulgensia fulgida*. New Phytologist 153: 317-326

Bennett AW (1887) On the Affinities and Classification of Alga. Journal of the Linnean Society of London, Botany 24:49-61

Bird CE, Karl SA, Smouse PE, Toonen RJ (2011) Detecting and measuring genetic differentiation. In: Koenemann S, Held C, Schubart C, (eds) Phylogeography and population genetics in Crustacea. CRC Press Crustacean Issues Series p. 31-55

Blaha J, Baloch E, Grube M (2006) High photobiont diversity associated with the euryoecious lichen-forming ascomycete *Lecanora rupicola* (Lecanoraceae, Ascomycota). Biological Journal of the Linnean Society 88: 283-293

Breuillin F, Dutech C, Robin C (2006) Genetic diversity of the Chestnut blight fungus *Cryphonectria parasitica* in four French populations assessed by microsatellite markers. Mycological Research 110:288-296

Bubrick P, Galun M, Frensdorff A (1984) Observations on free-living *Trebouxia* De Puymaly and *Pseudotrebouxia* Archibald, and evidence that both symbionts from *Xanthoria parientina* (L.) can be found free-living in nature. New Phytologist 97:455-462Budel B, Scheidegger C (1996) Thallus morphology and anatomy. Lichen biology:37-64

Budel B, Scheidegger C (1996) Thallus morphology and anatomy. Nash T (ed) In Lichen biology:37-64

Buschbom J (2007) Migration between continents: geographical structure and long-distance gene flow in *Porpidia flavicunda* (lichen-forming Ascomycota). Molecular Ecology 16: 1835-1846

Cassie DM, Piercey-Normore MD (2008) Dispersal in a sterile lichen-forming fungus, *Thamnolia subuliformis* (Ascomycotina: Icmadophilaceae). Botany 86: 751-762

Chapuis M-P, Estoup A (2007) Microsatellite Null Alleles and Estimation of Population Differentiation. Molecular Biology and Evolution 24:621-631

Chomczynski P, Rymaszewski M (2006) Alkaline polyethylene glycol-based method for direct PCR from bacteria, eukaryotic tissue samples, and whole blood. BioTechniques 40: 454-458

Chown SL, Lee JE, Hughes KA et al. (2012) Challenges to the future conservation of the Antarctic. Science 337: 158-159

Colesie C, Scheu S, Green TGA, Weber B, Wirth R, Büdel B (2012) The advantage of growing on moss: facilitative effects on photosynthetic performance and growth in the Cyanobacterial lichen *Peltigera rufescens*. Oecologia 3:599

Comps B, Gömöry D, Letouzey J, Thiébaut B, Petit, RJ (2001). Diverging trends between heterozygosity and allelic richness during postglacial colonization in the European beech. Genetics 157:1: 389-397

Convey P, Gibson JAE, Hillenbrand C-D, Hodgson DA, Pugh PJA, Smellie JL, Stevens MI (2008) Antarctic terrestrial life – challenging the history of the frozen continent? Biological Reviews 83: 103-117

Conway H, Hall BL, Denton GH, Gades AM, Waddington ED (1999) Past and Future Grounding-Line Retreat of the West Antarctic Ice Sheet. Science 286:280-283

Courtright E, Wall D, Virginia R, Virginia RA, Frisse LM, Vida JT Thomas WK (2000) Nuclear and mitochondrial DNA sequence diversity in the Antarctic nematode *Scottnema lindsayae*. Journal of Nematology 32:143

Cowan DA, Ah Tow L (2004) Endangered Antarctic environments. Annual Review of Microbiology 58: 649-690

Cowan DA, Chown SL, Convey P, Tuffin M, Hughes K, Pointing S, Vincent WF (2011) Non-indigenous microorganisms in the Antarctic: assessing the risks. Trends in Microbiology 19:540-548

Crawford NG (2009) SMOGD: software for the measurement of genetic diversity. Molecular Ecology Resources 10:556-557

Crittenden PD. (2002) Molecular and physiological diversity in the bipolar lichen-forming fungus *Xanthoria elegans*. Mycological Research 106:1277-1286

Culberson CF, Culberson WL, Johnson A (1988) Gene Flow in Lichens. American Journal of Botany 75:1135-1139

Dahlkild A, Kallersjo M, Lohtander K, Tehler A (2001) Photobiont Diversity in the *Physciaceae* (Lecanorales). The Bryologist 104: 527-536

Dal Grande F, Widmer I, Wagner H, Scheidegger C (2012) Vertical and horizontal photobiont transmission within populations of a lichen symbiosis. Molecular Ecology

De Bary A (1879) The phenomenon of symbiosis. Karl J. Trubner, Strasbourg, Germany

Denton GH, Bockheim JG, Wilson SC, Wilson SC, Stuiver M. (1989) Late Wisconsin and early Holocene glacial history, inner Ross Embayment, Antarctica. Quaternary Research 31:151-182

Des Los Rios A, Sancho LG, Grube M, Wierzchos J, Ascaso C (2005) Endolithic growth of two *Lecidea* lichens in granite from continental Antarctica detected by molecular and microscopy techniques. New Phytologist 165: 181-190

De Priest PT (2004) Early Molecular Investigations of Lichen-Forming Symbionts: 1986-2001. Annual Review Microbiology 58: 273-301

De Wever A, Leliaert F, Verleyen E, Vanormelingen P, Van der Gucht K, Hodgson DA, Sabbe K, Vyverman W (2009) Hidden levels of phylodiversity in Antarctic green algae: further evidence for the existence of glacial refugia. Proceedings of the Royal Society B: Biological Sciences

Doering M, Piercey-Normore MD (2009) Genetically divergent algae shape an epiphytic lichen community on Jack Pine in Manitoba. The Lichenologist 41:69-80

Domaschke S, Fernãndez-Mendoza F, Garcia MA, Martãn MP, Printzen C (2012) Low genetic diversity in Antarctic populations of the lichen forming Ascomycete *Cetraria aculeata* and its photobiont. Polar Research 31

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A (2011) Geneious v5.4. Available from http://www.geneious.com/ Accessed 3 June 2011

Dutech C, Enjalbert J, Fournier E, Delmotte F, Barres B, Carlier J, Tharreau D, Giraud T (2007) Challenges of microsatellite isolation in fungi. Fungal Genetics and Biology 44: 933-949

Dyer P, Murtagh G (2001) Variation in the ribosomal ITS-sequence of the lichens *Buellia frigida* and *Xanthoria elegans* from the Vestfold Hills, eastern Antarctica. Lichenologist 33: 151-159

Edwards K, Barker J, Daly A, Jones C, Karp A (1996) Microsatellite libraries enriched for several microsatellite sequences in plants. Biotechniques 20: 758-760

Engelen A, Convey P, Ott S (2010) Life history strategy of Lepraria borealis at an Antarctic inland site, Coal Nunatak. The Lichenologist 42:339-346

Fanciulli PP, Summa D, Dallai R, Frati F (2001) High levels of genetic variability and population differentiation in *Gressittacantha terranova* (Collembola, Hexapoda) from Victoria Land, Antarctica. Antarctic Science 13:246-254

Fernãndez-Mendoza F, Domaschke S, Garcãa M A, Jordan P, Martãn MP, Printzen C (2011) Population structure of mycobionts and photobionts of the widespread lichen *Cetraria aculeata*. Molecular Ecology 20: 1208-1232

Freifelder D, Better M (1982) Dialysis of small samples in agarose gels. Analytical Biochemistry 123: 83-85

Friedl T, Besendahl A, Pfeiffer P, Bhattacharya D (2000) The distribution of group I introns in lichen algae suggests that lichenization facilitates intron lateral transfer. Molecular Phylogenetics and Evolution 14: 342-352

Gauthier N, Dalleau-Clouet Cc, Fargues J, Bon MC (2007) Microsatellite Variability in the Entomopathogenic Fungus *Paecilomyces fumosoroseus*: Genetic Diversity and Population Structure. Mycologia 99:693-704

Gerlach G, Jueterbock A, Kraemer P, Deppermann J, Harmand P (2010) Calculations of population differentiation based on GST and D: forget GST but not all of statistics! Molecular Ecology 19:3845-3852

Green TGA, Schroeter B, Sancho LG (2007) Plant life in Antarctica. In: Pugnaire FI, and Valladares F [eds.], handbook of functional plant

ecology, second edition 389-433. Marcel dekker inc. New york, New York, USA

Green TGA, Sancho LG, Pintado A, Schroeter, B (2011a) Functional and spatial pressures on terrestrial vegetation in Antarctica forced by global warming. Polar Biology 34: 1643-1656

Green TGA, Sancho LG, Türk R, Seppelt R, Hogg I (2011b) High diversity of lichens at 84˚S, Queen Maud Mountains, suggests preglacial survival of species in the Ross Sea region, Antarctica. Polar Biology 34:1211-1220

Greuter W, Mcneill J, Hawksworth DL, Barrie FR (2000) Report on Botanical Nomenclature: Saint Louis 1999 XVI International Botanical Congress, Saint Louis: Nomenclature Section, 26 to 30 July 1999, Englera 3-253

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52: 696-704

Guo, Q. (2012). Incorporating latitudinal and central–marginal trends in assessing genetic variation across species ranges. Molecular Ecology. 21:22 5396-5403

Guzow-Krzeminska, B (2006) Photobiont flexibility in the lichen *Protoparmeliopsis muralis* as revealed by ITS rDNA analyses. Lichenologist 38: 469-476

Hall BL, Denton GH (2000) Extent and Chronology of the Ross Sea Ice Sheet and the Wilson Piedmont Glacier along the Scott Coast at and Since the Last Glacial Maximum. Geografiska Annaler Series A: Physical Geography 82

Hauck M, Helms G, Friedl T (2007) Photobiont selectivity in the epiphytic lichens *Hypogymnia physodes* and *Lecanora conizaeoide,* Lichenologist 39: 195-204

Hawksworth F, Rose D (1970) Qualitative scale for estimating sulphur dioxide air pollution in England and Wales using epiphytic lichens. Nature 227:145-148

Heinken T (1999) Dispersal Patterns of Terricolous Lichens by Thallus Fragments. The Lichenologist 31:603-612Helms G, Friedl T, Rambold G, Mayrhofer H (2001) Identification of photobionts from the lichen family *Physciaceae* using algal-specific ITS rDNA sequencing. Lichenologist 33: 73-86

Helms G, Friedl T, Rambold G, Mayrhofer H (2001) Identification of Photobionts from the lichen family Physciaceae using algal-specific ITS rDNA sequencing. The Lichenologist 33:73-86

Helms G, Friedl T, Rambold G (2003) Phylogenetic relationships of the Physciaceae inferred from rDNA sequence data and selected phenotypic characters. Mycologia 95:1078-1099

Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. Biological Journal of the Linnean Society 58:247-276

Hill DJ (1994) The nature of the symbiotic relationship in lichens. Endeavour 18:96-103

Honegger R (1991) Functional Aspects of the Lichen Symbiosis. Annual Review of Plant Physiology and Plant Molecular Biology 42: 553-578

Honegger R (1996) Mycobionts. Nash T (ed) In: Lichen biology. Cambridge University Press

Honegger R (1998) The Lichen Symbiosis: What is so Spectacular about it? The Lichenologist 30:193-212

Honegger R, Zippler U, Gansner H, Scherrer S (2004) Mating systems in the genus *Xanthoria* (lichen-forming ascomycetes). Mycological Research 108:480-488

Honegger R, Zippler U (2007) Mating systems in representatives of *Parmeliaceae, Ramalinaceae* and *Physciaceae* (Lecanoromycetes, lichen-forming ascomycetes). Mycological Research 111: 424-432

Honegger R (2009) Lichen-forming fungi and their photobionts, in: Plant Relationships, 2nd ed. H B Deising (ed), Springer Berlin Heidelberg 305-333

Hurst LD, Peck JR (1996) Recent advances in understanding of the evolution and maintenance of sex. Trends in Ecology & Evolution 11:46-52

Ingolfsson,O, Hjort C,,Berkman PA, Bjorck S, Colhoun E, Goodwin ID, Hall B, Prentice ML (1998) Antarctic Glacial History Since the Last Glacial Maximum: An Overview of the Record on Land. Antarctic Science 10: 326-344

Jarne P, Lagoda PJL (1996) Microsatellites, from molecules to populations and back. Trends in Ecology & Evolution 11:424-429

Jeffreys AJ, Wilson V, Neumann R, Keyte J (1988) Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. Nucleic Acids research, 16, 10953-10971

Jones TC, Green T, Hogg ID, Wilkins RJ. (2012) Isolation and characterization of microsatellites in the lichen *Buellia frigida* (Physciaceae), an Antarctic endemic. American Journal of Botany 99:e131

Jost L (2008) GST and its relatives do not measure differentiation. Molecular Ecology 17:4015-4026

Jukes TH, Cantor CR (1969) Evolution of protein molecules, in: Mammalian Protein Metabolism, Munro, HN (ed) Academic, New York Vol 3: 21-132

Jump AS, Marchant R, Penuelas J (2009) Environmental change and the option value of genetic diversity. Trends in Plant Science 14: 51-58

Kalinowski ST (2004) Counting alleles with rarefaction: private alleles and hierarchical sampling designs. Conservation Genetics 5:539-543

Kalinowski ST (2005) hp:rare 1.0: a computer program for performing rarefaction on measures of allelic richness. Molecular Ecology Notes 5:187-189

Karagyozov L, Kalcheva ID, Chapman VM (1993) Construction of random small-insert genomic libraries highly enriched for simple sequence repeats. Nucleic Acids Research, 21, 3911-3912

Kroken S, Taylor JW (2000) Phylogenetic Species, Reproductive Mode, and Specificity of the Green Alga Trebouxia Forming Lichens with the Fungal Genus *Letharia*. The Bryologist 103:645-660

Lättman H, Lindblom L, Mattsson J-E, Milberg P, Skage M, Ekman S (2009) Estimating the dispersal capacity of the rare lichen *Cliostomum corrugatum.* Biological Conservation 142:1870-1878

Leng L, Zhang DEX (2011) Measuring population differentiation using GST or D? A simulation study with microsatellite DNA markers under a finite island model and non-equilibrium conditions. Molecular Ecology 20:2494-2509

Lewis LA, McCourt RM (2004) Green algae and the origin of land plants. Am. J. Bot. 91:1535-1556

Librado P, Rozas J (2009) DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451-1452

Lim S, Notley-McRobb L, Lim M, Carter DA (2004) A comparison of the nature and abundance of microsatellites in 14 fungal genomes. Fungal Genetics and Biology 41:1025-1036

Lindsay DC (1978) The role of lichens in Antarctic ecosystems. Bryologist 81: 268-276

Marchelli P, Gallo LA (2004) E The combined role of glaciation and hybridization in shaping the distribution of genetic variation in a Patagonian southern beech. Journal of Biogeography 31:451-460

Marshall WA (1996) Aerial Dispersal of Lichen Soredia in the Maritime Antarctic. New Phytologist 134:523-530

McGaughran A, Hogg ID, Stevens MI (2008) Patterns of population genetic structure for springtails and mites in southern Victoria Land, Antarctica. Molecular Phylogenetics and Evolution 46: 606-618

McGaughran, A., Torricelli, G., Carapelli, A., Frati, F., Stevens, M. I., Convey, P., & Hogg, I. D. (2010). Contrasting phylogeographical patterns for springtails reflect different evolutionary histories between the Antarctic Peninsula and continental Antarctica. Journal of Biogeography 37:1 103-119

McGaughran A, Stevens MI, Hogg ID, Carapelli A (2011) Extreme Glacial Legacies: A synthesis of the Antarctic Springtail phylogeographic Record. Insects 2:62-82

McKay R, Dunbar G, Naish T, Barrett PJ, Carter L, Harper M (2008) Retreat history of the Ross Ice Sheet (Shelf) since the Last Glacial Maximum from deep-basin sediment cores around Ross Island. Palaeogeography, Palaeoclimatology, Palaeoecology 260:245-261

Meirmans PG, Hedrick PW (2011) Assessing population structure: F. Molecular Ecology Resources 11:5-18

Mercer J (1973) Cainozoic temperature trends in the Southern Hemisphere: Antarctic and Andean glacial evidence. Palaeoecology of Africa 8:85-114

Micheli, Haller, Linnaeus, Watson, Dillenius (1757) An Historical Memoir concerning a Genus of Plants Called Lichen, by Micheli, Haller, and Linnaeus; And Comprehended by Dillenius under the Terms *Usnea, Coralloides*, and *Lichenoides*: Tending Principally to Illustrate Their Several Uses. Communicated by Wm. Watson, M. D. F. R. S. Philosophical Transactions (1683-1775) 50:652-688

Munoz J, Felicisimo AM, Cabezas F, Burgaz AR, Martínez I (2004) Wind as a Long-Distance Dispersal Vehicle in the Southern Hemisphere. Science 304:1144-1147

Nash TH (1996) Lichen Biology. Cambridge University Press, Cambridge, GBR

Nash TH (2007) Lichen Biology. Second Edition. Cambridge University Press, Cambridge, GBR

Nelsen MP, Gargas A (2009) Symbiont flexibility in *Thamnolia vermicularis* (Pertusariales: Icmadophilaceae). Bryologist 112: 404-417

Nielsen UN, Wall DH, Adams BJ, Virginia RA (2011) Antarctic nematode communities: observed and predicted responses to climate change. Polar Biology 34:1701-1711

Nkuekam GK, Barnes I, Wingfield MJ, Roux J (2009) Distribution and population diversity of *Ceratocystis pirilliformis* in South Africa. Mycologia 101:17-25

Nyati S, Scherrer S, Honegger R (2004) Photobiont diversity in the genus *Xanthoria*, in: T Randlane & A Saag (eds), Book of Abstracts of the 5th IAL Symposium Lichens in Focus Tartu University Press 65

Oberholzer P, Baroni C, Schaefer JM, Orombelli G, Ochs SI, Kubik PW, Baur H, Wieler R (2003) Limited Pliocene/Pleistocene glaciation in Deep Freeze Range, northern Victoria Land, Antarctica, derived from in situ cosmogenic nuclides. Antarctic Science 15:493-502

Opanowicz M, Grube M (2004) Photobiont genetic variation in *Flavocetraria nivalis* from Poland (Parmeliaceae, lichenized Ascomycota). Lichenologist 36:125-131

Ott S (1987) Sexual reproduction and developmental adaptations in Xanthoria parietina. Nordic Journal of Botany 7:219-228

Øvstedal DO, Smith RI (2001) Lichens of Antarctica and South Georgia. A guide to their Identification and Ecology. Cambridge University Press, Cambridge

Peakall ROD, Smouse PE (2006) Genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes. 6: 288-295

Pearce DA, Bridge PD, Hughes KA, Sattler B, Psenner R, Russell NJ (2009) Microorganisms in the atmosphere over Antarctica. Fems Microbiology Ecology 69:143-157

Pearson PN, Palmer MR (2000) Atmospheric carbon dioxide concentrations over the past 60 million years. Nature 406:695-699

Peksa O, Škaloud P (2011) Do photobionts influence the ecology of lichens? A case study of environmental preferences in symbiotic green alga *Asterochloris* (Trebouxiophyceae). Molecular Ecology 20: 3936-3948

Perez-Ortega S, Ortiz R, Green TGA, de los Rios A (2012) Lichen myco- and photbiont diversity and their relationships at the edge of life (McMurdo Dry Valleys, Antarctica). FEMS Microbiology Ecology (in press) DOI: 10.1111/j.1574-6941.2012.01422.x

Piercy-Normore MD, DePriest PT (2001) Algal switching among lichen symbioses. American Journal of Botany 88: 8 1490-1498

Piercey-Normore MD (2005) The lichen forming ascomycete *Evernia mesomorpha* associates with multiple genotypes of *Trebouxia jamesii*. New Phytologist 169:331-344

Posada D (2008) jModelTest: Phylogenetic Model Averaging. Molecular Biology and Evolution 25: 1253-1256

Printzen C, Ekman S (2003) Local population subdivision in the lichen *Cladonia subcervicornis* as revealed by mitochondrail cytochrme oxidase subunit 1 intro sequences. Mycolgia 95:399-406

Queloz V, Duo A, Sieber TN, Grunig CR (2009) Microsatellite size homoplasies and null alleles do not affect species diagnosis and population genetic analysis in a fungal species complex. Molecular Ecology Resources 10:348-367

Rambaut A (2008) FigTree v111: Tree figure drawing tool Available: http:// treebioedacuk/software/figtree/ Accessed feb-April 2012

Ratnasingham S, Hebert PDN (2007) BOLD: The Barcode of Life Data System (http://www.barcodinglife. org). Molecular Ecology Notes 7:355-364

Raymond M, Rousset F (1995) GENEPOP (Version 1.2): Population Genetics Software for Exact Tests and Ecumenicism. Journal of Heredity 86: 248-249.

Romeike J, Friedl T, Helms G, Ott S (2002) Genetic diversity of algal and fungal partners in four species of *Umbilicaria* (Lichenised Ascomycetes) along a transect of the Antarctic peninsula. Molecular Biology and Evolution 19: 1209-1217

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132:365-386

Rubini A, Paolocci F, Riccioni C, Vendramin GG, Arcioni Sl (2005) Genetic and Phylogeographic Structures of the Symbiotic Fungus *Tuber magnatum*. Applied and Environmental Microbiology 71:6584-6589

Ruprecht U, Lumbsch HT, Brunauer G, Green TGA, Turk, R (2012) Insights into the diversity of *Lecanoraceae* (Lecanorales, Ascomycota) in Continental Antarctica (Ross Sea region). Nova Hedwigia 94: 287-306

Sancho L, Kappen L, Schroeter B (1992) The lichen genus Umbilicaria on Livingston Island, South Shetland Islands, Antarctica. Antarctic Science 4:189-196

Sancho LG, Allan Green TG, Pintado A (2007) Slowest to fastest: Extreme range in lichen growth rates supports their use as an indicator of climate change in Antarctica. Flora - Morphology, Distribution, Functional Ecology of Plants 202:667-673

Sanders WB, Lucking R (2002) Reproductive Strategies, Relichenization and Thallus Development Observed in situ in Leaf-Dwelling Lichen Communities. New Phytologist 155:425-435

Sanders WB (2005) Observing microscopic phases of lichen life cycles on transparent substrata placed *in situ*. Lichenologist 37: 373-382

Scheidegger C, Goward T (2002) Monitoring lichens for conservation: red lists and conservation action plans. In: Monitoring with Lichens—Monitoring Lichens. NATO Science Series 7: 163-181

.

Seefeldt MW, Tripoli GJ, Stearns CR (2003) A high-resolution numerical simulation of the wind flow in the Ross Island region, Antarctica. Monthly Weather Review 131:435-458

Seefeldt MW, Cassano JJ (2008) An analysis of low-level jets in the greater Ross Ice Shelf region based on numerical simulations. Monthly Weather Review 136:4188-4205

Seymour FA, Crittenden PD, Dyer P (2005) Sex in the extremes:lichen forming fungi. Mycologist 19: 51-58

Ship S, Anderson J, Domack E (1999) Late Pleistocene Holocene retreat of the West Antarctic Ice-Sheet system in the Ross Sea: Part 1 Geophysical results. Geological Society of America Bulletin 111:1486-1516

Skotnicki M, Ninham J, Selkirk P (2000) Genetic diversity, mutagenesis and dispersal of Antarctic mosses a review of progress with molecular studies. Antarctic Science 12:363-373

Smellie JL, Johnson JS, McIntosh WC, Esser R, Gudmundsson MT, Hambrey MJ, van Wyk De Vries B (2008) Six million years of glacial history recorded in volcanic lithofacies of the James Ross Island Volcanic Group, Antarctic Peninsula. Palaeogeography, Palaeoclimatology, Palaeoecology 260:122-148

Smith JJ, Ah Tow L, Stafford W, Cary C, Cowan DA (2006) Bacterial Diversity in Three Different Antarctic Cold Desert Mineral Soils. Microbial Ecology 51: 413-421

Speelman EN, Van Kempen MML, Barke J, Brinkhuis H, Reichart GJ, Smolders AJ, Roelofs J, Sinninghe Damsté JS (2009) The Eocene Arctic *Azolla* bloom: environmental conditions, productivity and carbon drawdown. Geobiology 7:155-170

Stevens MI, Hogg ID (2003) Long-term isolation and recent range expansion from glacial refugia revealed for the endemic springtail *Gomphiocephalus hodgsoni* from Victoria Land, Antarctica. Molecular Ecology 12: 2357-2369

Stevens MI, Greenslade P, Hogg ID, Sunnucks P (2006) Southern Hemisphere Springtails: Could Any Have Survived Glaciation of Antarctica? Mol Biol Evol 23:874-882

Stevens MI, Frati F, McGaughran A, Spinsanti G, Hogg ID (2007) Phylogeographic structure suggests multiple glacial refugia in northern Victoria Land for the endemic Antarctic springtail *Desoria klovstadi* (Collembola, Isotomidae). Zoologica Scripta 36: 201-212

Storey B, Fink D, Hood D, Joy K, Shulmeister J, Riger-Kusk M, Stevens MI (2010) Cosmogenic nuclide exposure age constraints on the glacial history of the Lake Wellman area, Darwin Mountains, Antarctica. Antarctic Science 22:603-618

Strasky S, Di Nicola L, Baroni C, Salvatore MC, Baur H, Kubik PW, Schluchter C, Wieler R (2009) Surface exposure ages imply multiple low-amplitude Pleistocene variations in East Antarctic Ice Sheet, Ricker Hills, Victoria Land. Antarctic Science 21:59

Sugden DE, Bentley MJ, Cofaigh CO (2006) Geological and geomorphological insights into Antarctic ice sheet evolution. Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences 364:1607-1625

Swofford DL (2002) PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods) Version 4. Sinauer Associates, Sunderland, Massachusetts, USA

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10: 512-526

Tibell L (2001) Photobiont Association and Molecular Phylogeny of the Lichen Genus Chaenotheca. The Bryologist 104:191-198

Walser J, Sperisen C, Soliva M, Scheidegger C (2003) Fungus-specific microsatellite primers of lichens:application for the assessment of genetic variation on different spatial scales in *Lobaria pulmonaria*. Fungal Genetics and Biology 40:72-82

Walser J (2004) Molecular evidence for limited dispersal of vegetative propagules in the epiphytic lichen *Lobaria pulmonaria*. Am. J. Bot. 91:1273-1276

Walser J, Holderegger R, Gugerli F, Hoebee SE, Scheidegger C (2005) Microsatellites reveal regional population differentiation and isolation in *Lobaria pulmonaria*, an epiphytic lichen. Molecular Ecology 14:457-467

Werth S (2010) Population Genetics of Lichen-forming fungi. The Lichenologist 499-519

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR-protocols a guide to methods and applications 315 – 322

Widmer I, Grande FD, Cornejo C, Scheidegger C (2010) Highly variable microsatellite markers for the fungal and algal symbionts of the lichen *Lobaria pulmonaria* and challenges in developing biont-specific molecular markers for fungal associations. Fungal Biology 114:538-544

Wirtz N, Lumbsch HT, Green TGA, Turk R, Pintado A, Sancho L, Schroeter B (2003) Lichen fungi have low cyanobiont selectivity in maritime Antarctica. New Phytologist 160: 177-183

Yahr R, Vilgalys R, Depriest PT (2004) Strong fungal specificity and selectivity for algal symbionts in Florida scrub *Cladonia* lichens. Molecular Ecology 13: 3367-3378

Yahr R, Vilgalys R, DePriest PT (2006) Geographic variation in algal partners of *Cladonia subtenuis* (Cladoniaceae) highlights the dynamic nature of a lichen symbiosis. New Phytologist 171:847-860

Zoller S, Lutzoni F, Scheidegger C (1999) Genetic variation within and among populations of the threatened lichen *Lobaria pulmonaria* in Switzerland and implications for its conservation. Molecular Ecology 8:2049-2059

Zwickl DJ (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. PhD dissertation, The University of Texas at Austin

Appendix 1
Vouchers

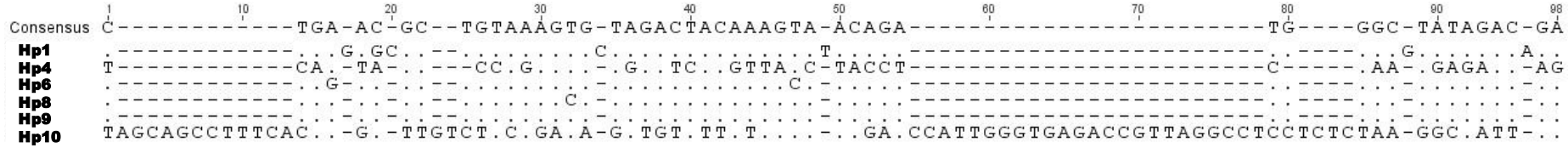
Vouchers of representative samples collected by Tracey Jones are deposited within the University of Waikato Herbarium and include latitude, longitude, altitude and substrate type (accession numbers WAIK22460, WAIK22459, WAIK22458, WAIK22457, WAIK22456, WAIK22455). A voucher of the sample the microsatellites were developed from was collected by Catherine Beard, and is held in the collection of Prof. Rod Seppelt, Australian Antarctic Division, and includes latitude, longitude, altitude and substrate type (accession number ADT No.25347).

Appendix 2

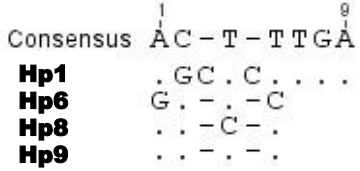
Photobiont Haplotype Sequence Differences

Appendix 2: Difference in sequences between algal haplotypes. Differences shown only, sequence is not contiguous.

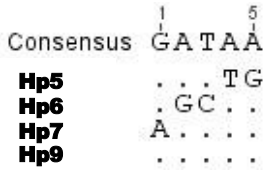
Buellia frigida



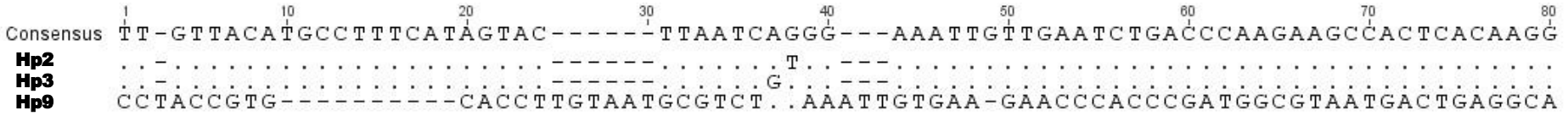
Buellia frigida without outlier sequences



Umbilicaria aprina



Umbilicaria decussata



Appendix 3

Buellia frigida

Microsatellite sequences from which primers were designed

Microsatelite marker Buef1

```
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TCTCTTGCTTGAATTCGGACTACCTGAGGAGCGCGCAGACATGCAGACACAGCAGAATGTCAGCAC
CAGCACTGTAAAGAACACCGCTATTTCTGTCAAGACTACAGATGATTTAGTGCAACCTGAGGACAC
CTGAACAGCAGGTATCCTCGTGCTGTTCAGTTGTCTCAAATTGCAGCCGGTCTTCTACAATTTTGA
CGGAGCAACGTCCTTTGCAGCGGTGTTGTCTACAGCATTCAGCTGTGTGTGTGTGTGTGTGTGTGT
GTGTGTGTGTGTGTGTGTGTCTCTACGGGTTTGTCCCCAGGTGCTGCATGCCTGAATTGAGGTGGT
GTAGACTTGCCTAGTCCGAATTCAAGCAAGAGAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCG
ACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATA
GCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACA
ACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAA
TTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCG
GCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGC
TGCGCTCGGTCGTTCGGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTATACGGTTATCCA
CAGAATCAGGGGATAACGCAGAAAGACCATGTGAGCAAAAAGGCCAGCAAAGGCCAGATCCGTAAA
AGGCCGTGGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCAAAAAATCGACGCT
CAG


No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len       tm     gc%   any    3' seq
LEFT PRIMER         22   20    59.90   55.00  4.00  0.00
AGCAGGTATCCTCGTGCTGT
RIGHT PRIMER       224   21    60.16   52.38  4.00  0.00
GGCAAGTCTACACCACCTCAA
SEQUENCE SIZE: 224
INCLUDED REGION SIZE: 224

PRODUCT SIZE: 203, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 2.00
EXCLUDED REGIONS (start, len)*: 126,42

    1 GCAACCTGAGGACACCTGAACAGCAGGTATCCTCGTGCTGTTCAGTTGTCTCAAATTGCA
                          >>>>>>>>>>>>>>>>>>>

   61 GCCGGTCTTCTACAATTTTGACGGAGCAACGTCCTTTGCAGCGGTGTTGTCTACAGCATT


  121 CAGCTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTCTCTACGGGTTTG
          XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

  181 TCCCCAGGTGCTGCATGCCTGAATTGAGGTGGTGTAGACTTGCC
                            <<<<<<<<<<<<<<<<<<<<<

ADDITIONAL OLIGOS
                    start  len       tm     gc%   any    3' seq

 1 LEFT PRIMER         22   20    59.90   55.00  4.00  0.00
AGCAGGTATCCTCGTGCTGT
   RIGHT PRIMER       210   20    61.21   50.00  8.00  2.00
ACCTCAATTCAGGCATGCAG
   PRODUCT SIZE: 189, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 2.00
```

Microsatelite marker Buef2

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GACAGGATGGCTGTGTGAGAGCTGCTGACTTGCAGCGGGCAGGCAAAGTGCAGAGTAAGACAATTG
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ACAGGCACGCACAACTGTACCTTCTGGCTGAGATGGGGCAGCAACAGAAGCTTCAGCAGGCTGATC
AGCAGCTTCTGTAGTAGGTGATGCTGCAGGTGCCGCAGCTGGAGGTGGTGGAGGAGGCAATGTGAC
CCCGTGTGCAGCCAAGACCTGCAGTCCCAATGAAATGGCTTAGTCCGAATTCAAGCAAGAGAATCA
CTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAG
CTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTG
AAATTGTTATCCGCTCACAAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGG
GTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAA
ACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGGAGAGGCGGTTTGCGTATTGGG
CGCTCTTTCCGCTTCCTCGCTCACTGAACTCGCTGCGCTCGGGTCGTTCGGCTGCGGCGAGCGGTA
ATCAGCTTCACTCAAAGGGCGGTAATACGGGTTATCCACCAGAAATCAGGGGGATAACGCAGGAAA
GAACATGGTGGAGCAAAAGGCCAGCCAAAAGGCCCAGGAACCCGGTAAAAGGCCCGCGTTGCTTGG
GCGTTTTTCCCATTAGGCCTCCGGCCTCCTCTGGGACCGAGCATTCCACAAATTCGACGCCTCTCA
GCCGAAGTGTGCCAAACCTCGCG

No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any    3' seq
LEFT PRIMER        180   20   59.90   55.00  6.00  3.00
ACTGCAGAGCATGTGGTGAC
RIGHT PRIMER       365   20   60.21   55.00  4.00  2.00
CCCATCTCAGCCAGAAGGTA
SEQUENCE SIZE: 499 INCLUDED REGION SIZE: 499

PRODUCT SIZE: 186, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 2.00
EXCLUDED REGIONS (start, len)*: 302,30

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   61 GTTAGTCTAGATGAAAAAGAAGACAACTCAGAACCTGCATGGAGCCCTGGGCTGTAGGGG


  121 AGGAGCAATGATGAGTCGAACCAGAGGACACATACCACTCAAAATATGCTGCAGTAACGA
                                                                  >

  181 CTGCAGAGCATGTGGTGACAGGATGGCTGTGTGAGAGCTGCTGACTTGCAGCGGGCAGGC
      >>>>>>>>>>>>>>>>>>

  241 AAAGTGCAGAGTAAGACAATTGAGATACAGGTATGTTTATGATTTGAGGATGCGAGTCAA


  301 ACACACACACACACACACACACACACACACACAGGCACGCACAACTGTACCTTCTGGCTGAG
       XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX              <<<<<<<<<<<<<<<

  361 ATGGGGCAGCAACAGAAGCTTCAGCAGGCTGATCAGCAGCTTCTGTAGTAGGTGATGCTG
      <<<<<

  421 CAGGTGCCGCAGCTGGAGGTGGTGGAGGAGGCAATGTGACCCCGTGTGCAGCCAAGACCT


  481 GCAGTCCCAATGAAATGGC
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Microsatelite marker Buef3

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CGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGC
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CAGAGTGCGAAACCCGACAGACTATAAAAGATACAGGCGTTCCCCCTGAGCTCCCCTCGTGCCGCT
CCTCCATGTCGACCTGCCGCTTACCGGAATCTGTTCGCTTTTCCTCCCTTCGGAAGCCG
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any    3' seq
LEFT PRIMER         13   21   59.91   52.38  6.00  3.00
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RIGHT PRIMER       180   20   60.43   50.00  6.00  2.00
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SEQUENCE SIZE: 228
INCLUDED REGION SIZE: 228

PRODUCT SIZE: 168, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 1.00
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                >>>>>>>>>>>>>>>>>>>>

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                                                          XXXXXXXXX

  121 TGTGTGTGTGTGTGTGTCTGCTCTTGCAAGAAAGTATGACTGGTTTGCAGAGGCAAAG
      XXXXXXXXXXXXXXXXXXX                    <<<<<<<<<<<<<<<<<<<

  181 CATGGTCATGACCAAGAGGGTGATGAAAACATTGATATAGGAAACGGG
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Microsatelite marker Buef4

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C

No mispriming library specified
Using 1-based sequence positions

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LEFT PRIMER	99	20	60.13	50.00	4.00	1.00	
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RIGHT PRIMER	249	20	59.60	55.00	8.00	0.00	
ACGTGAGTCCTCACCCTTGT							

SEQUENCE SIZE: 308
INCLUDED REGION SIZE: 308

PRODUCT SIZE: 151, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 2.00

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   61 CTGATGCTGAGCCGGATCCTGCAAAGAAAGCAACTCACAAGCTGCCTGTCTCCAAGAAGC
                                              >>>>>>>>>>>>>>>>>>>>

  121 AGCAGGTGCATGCACAAACTGATGCATGGTTGCGCGAGCGCACACACACACACACACACA

  181 CACACACACACCACACAACAGACTGACAAGACGAAACAGAGTTTCAGTCACAAGGGTGAG
                                                      <<<<<<<<<<<

  241 GACTCACGTGGGATGCAGCTTTGCAACAGCGTCAGGGGGGTAGCTGCAGTTGGCAGCCGC
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  301 AGCTGCGG
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Microsatelite marker Buef5

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GCGCTCCTCAGGTAGTCCGAATTCAAGCAAGAGAATCACTAGTGAATTCGCGGCCGCCTGCAGGTC
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GGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCG
CTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACCGGTTATC
CACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAGGGCCAGCAAAAGGCCAGGAACCG
```

TAAAAAGGCCGCGTTGCTGGCGTTTTCAATAGGCTCCGCCCCCTGACGAGCATCACAAAAATTCGA
CGCTCATGTCAGAGGTGGCGAAACCGGACAGGACTATAAAGATACAGGCGATTCCCCCTGGAAAGC
TCCCTCGTGGTGCTCTTCCTG

No mispriming library specified
Using 1-based sequence positions

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	40	19	59.91	57.89	3.00	0.00	GGGGACAAACCCGTAGAGA
RIGHT PRIMER	203	20	59.90	55.00	4.00	0.00	AGCAGGTATCCTCGTGCTGT

SEQUENCE SIZE: 261
INCLUDED REGION SIZE: 261

PRODUCT SIZE: 164, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 2.00
EXCLUDED REGIONS (start, len)*: 59,42

```
    1 GGCAAGTCTACACCACCTCAATTCAGGCATGCAGCACCTGGGGACAAACCCGTAGAGACA
                                            >>>>>>>>>>>>>>>>>>>XX

   61 CACACACACACACACACACACACACACACACACACACACACAGCTGAATGCTGTAGACAACA
      XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

  121 CCGCTGCAAAGGACGTTGCTCCGTCAAAATTGTAGAAGACCGGCTGCAATTTGAGACAAC

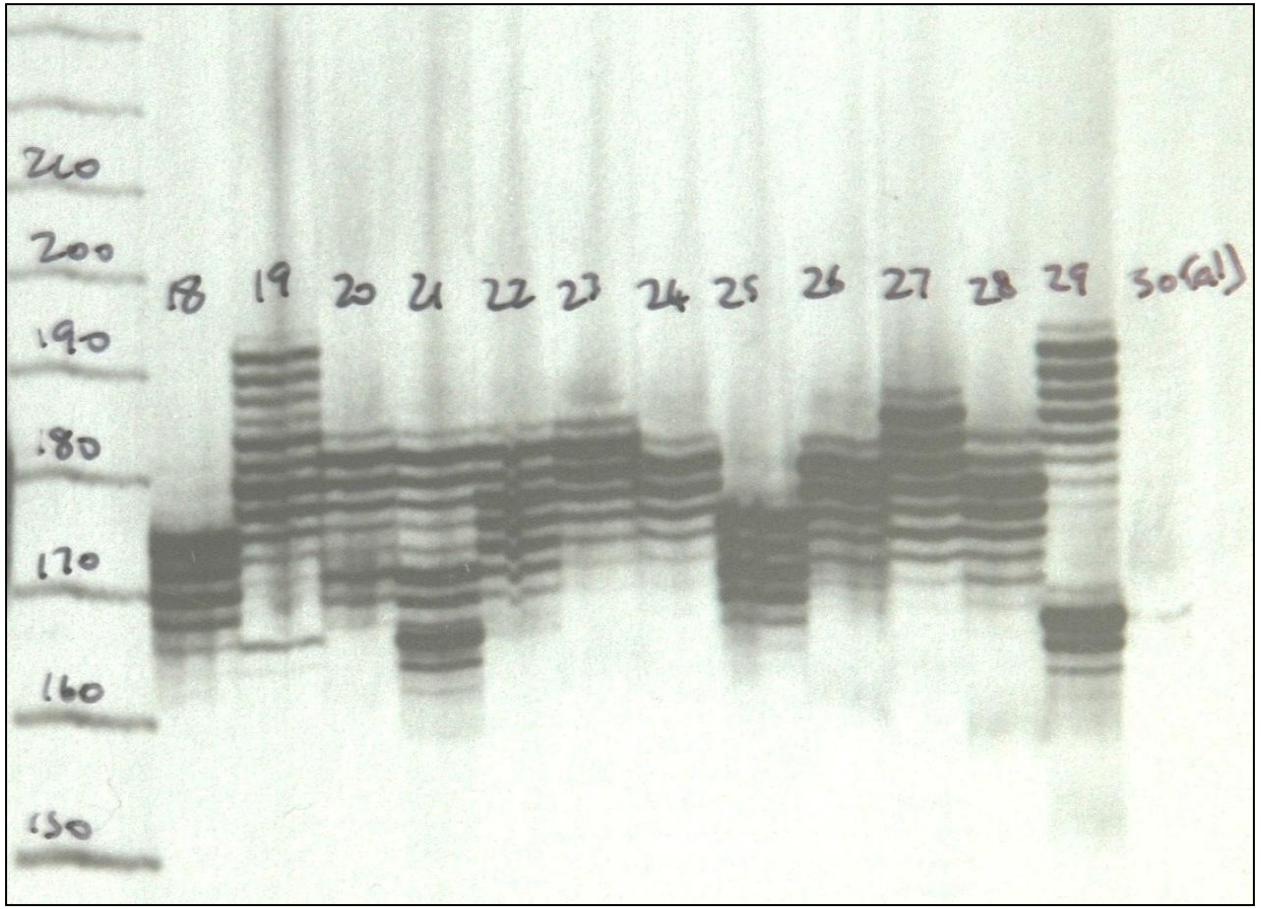

  181 TGAACAGCACGAGGATACCTGCTGTTCAGGTGTCCTCAGGTTGCACTAAATCATCTGTAG
         <<<<<<<<<<<<<<<<<<<

  241 TCTTGACAGAAATAGCGGTGT
```

Appendix 4

Autoradiograph of ^{33}P amplification products (*Buellia frigida*)

Appendix 4: L=Ladder, 1-12 *Buellia frigida,* 13-*Trebouxia jamesii* (algae) Autoradiograph of Microsatellite primer Buef3 (*Buellia frigida*, Granite Harbour)

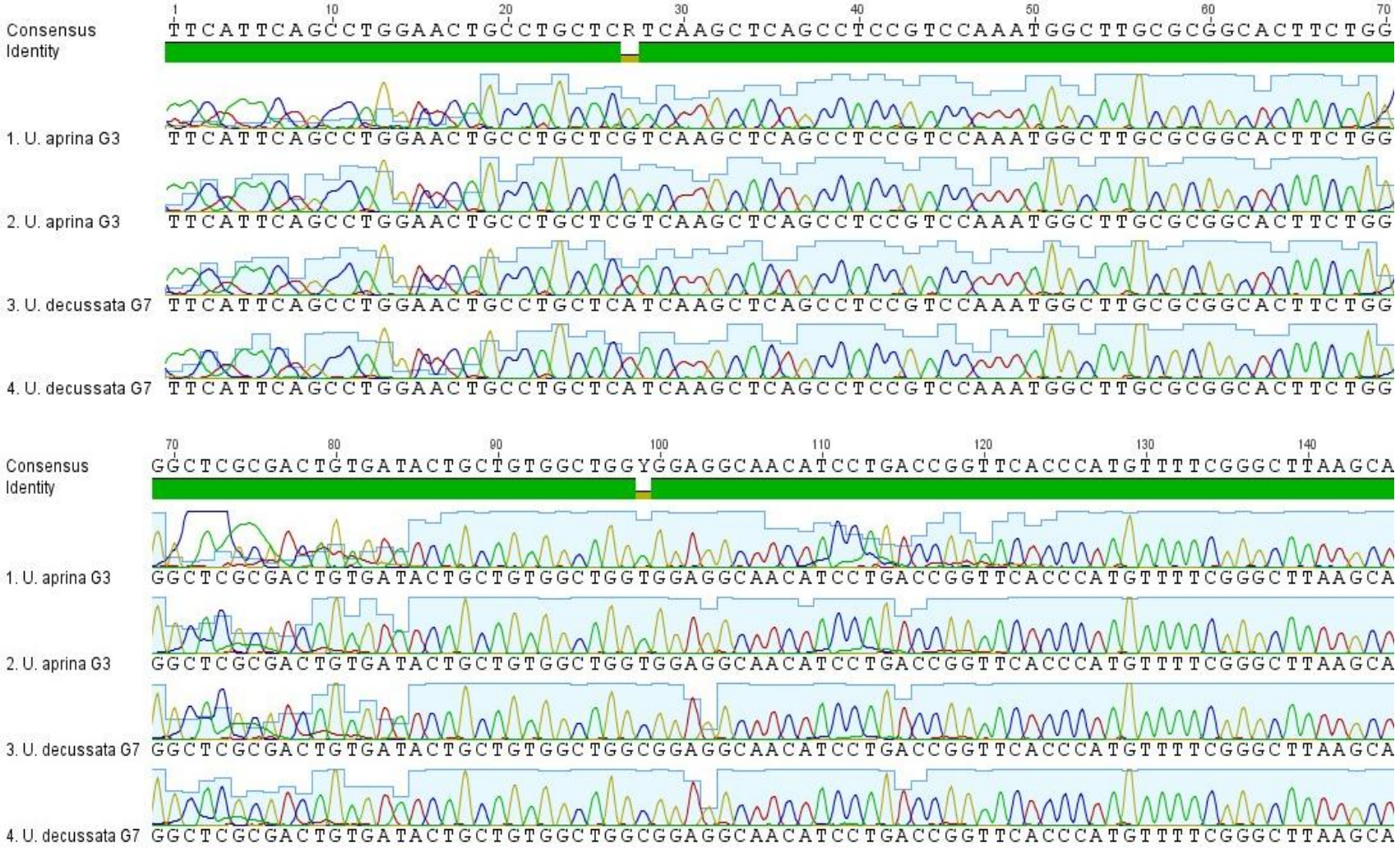


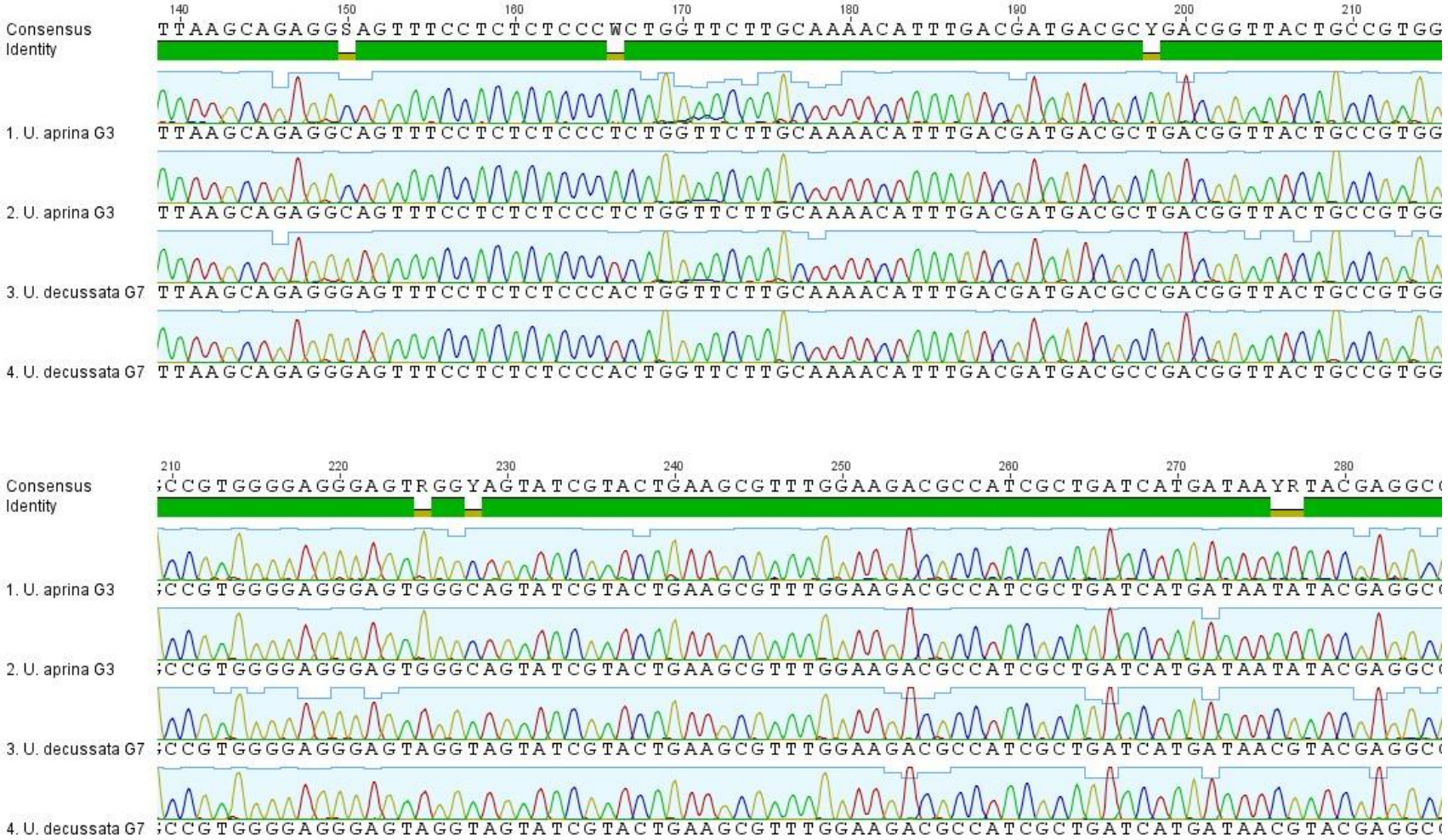
Appendix 5

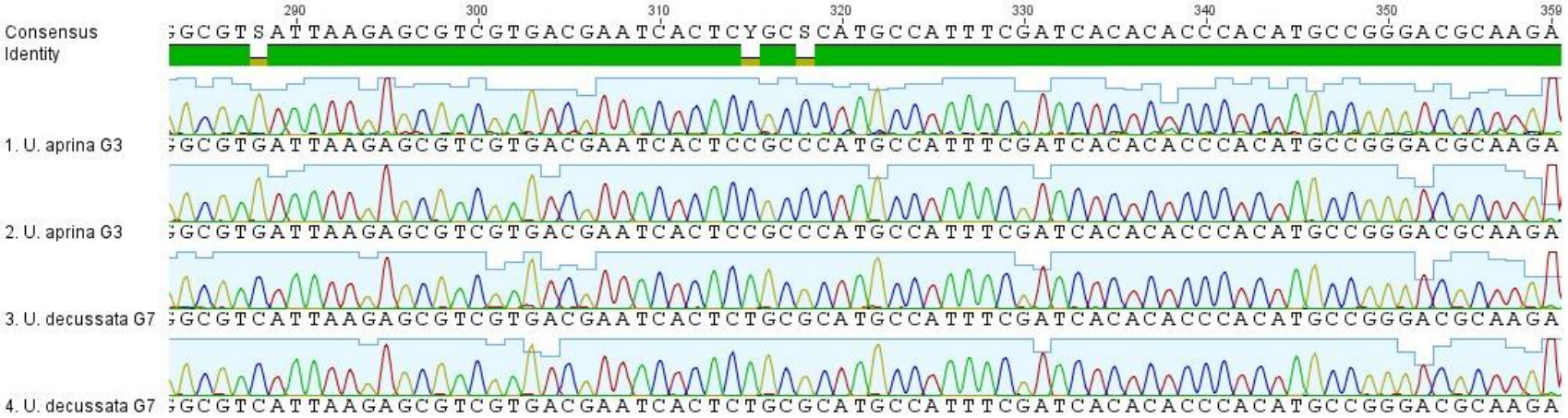
Umbillicaria aprina and Umbilicaria decusssata

Polyketide Synthase sequences

Appendix 5: PKS sequence of two Gondwana samples (*U. aprina* =G3 and *U. decussata*=G7)




140
150
160
170
180
190
200
210
Consensus
Identity
TTAAGCAGAGGSAGTTTCCTCTCTCCCWCTGGTTCTTGCAAAACATTTGACGATGACGCYGACGGTTACTGCCGTGG
1. U. aprina G3
TTAAGCAGAGGCAGTTTCCTCTCTCCCTCTGGTTCTTGCAAAACATTTGACGATGACGCTGACGGTTACTGCCGTGG
2. U. aprina G3
TTAAGCAGAGGCAGTTTCCTCTCTCCCTCTGGTTCTTGCAAAACATTTGACGATGACGCTGACGGTTACTGCCGTGG
3. U. decussata G7
TTAAGCAGAGGGAGTTTCCTCTCTCCCACTGGTTCTTGCAAAACATTTGACGATGACGCCGACGGTTACTGCCGTGG
4. U. decussata G7
TTAAGCAGAGGGAGTTTCCTCTCTCCCACTGGTTCTTGCAAAACATTTGACGATGACGCCGACGGTTACTGCCGTGG
210
220
230
240
250
260
270
280
Consensus
Identity
GCCGTGGGGAGGGAGTRGGYAGTATCGTACTGAAGCGTTTGGAAGACGCCATCGCTGATCATGATAAYRTACGAGGC
1. U. aprina G3
GCCGTGGGGAGGGAGTGGGCAGTATCGTACTGAAGCGTTTGGAAGACGCCATCGCTGATCATGATAATATACGAGGC
2. U. aprina G3
GCCGTGGGGAGGGAGTGGGCAGTATCGTACTGAAGCGTTTGGAAGACGCCATCGCTGATCATGATAATATACGAGGC
3. U. decussata G7
GCCGTGGGGAGGGAGTAGGTAGTATCGTACTGAAGCGTTTGGAAGACGCCATCGCTGATCATGATAACGTACGAGGC
4. U. decussata G7
GCCGTGGGGAGGGAGTAGGTAGTATCGTACTGAAGCGTTTGGAAGACGCCATCGCTGATCATGATAACGTACGAGGC


290
300
310
320
330
340
350
359
Consensus
Identity
1. U. aprina G3
2. U. aprina G3
3. U. decussata G7
4. U. decussata G7
GCGTSATTAAGAGCGTCGTGACGAATCACTCYGCSCATGCCATTTCGATCACACACCCACATGCCGGGACGCAAGA
GCGTGATTAAGAGCGTCGTGACGAATCACTCCGCCCATGCCATTTCGATCACACACCCACATGCCGGGACGCAAGA
GCGTGATTAAGAGCGTCGTGACGAATCACTCCGCCCATGCCATTTCGATCACACACCCACATGCCGGGACGCAAGA
GCGTCATTAAGAGCGTCGTGACGAATCACTCTGCGCATGCCATTTCGATCACACACCCACATGCCGGGACGCAAGA
GCGTCATTAAGAGCGTCGTGACGAATCACTCTGCGCATGCCATTTCGATCACACACCCACATGCCGGGACGCAAGA