DISPERSAL AND GENETIC EXCHANGE OF LICHEN POPULATIONS BETWEEN THE MARITIME ANTARCTIC AND SOUTHERN SOUTH AMERICA (WITH A FOCUS ON HUMAN IMPACT)

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ABSTRACT

This manuscript-based thesis is divided into four chapters. Chapter one is an introduction to lichens and the Antarctic. It introduces the goal of the thesis and the problems related with lichen systematics and the lack of knowledge about Antarctic lichens. The Antarctic is one of the last wildernesses, isolated from the other continents by the Antarctic Circumpolar Current, the Subantarctic Front, the Antarctic Polar Front, and the Drake Passage. Terrestrial life in Antarctica is restricted to widely separated and small ice-free areas that cover only 0.3% of the continent. Colonization of the Antarctic is a challenge for many taxa and is related to their ability for long-range dispersal and their adaptation to the harsh climate. Antarctic terrestrial ecosystems are significantly threatened by climate change, invasive species, and their interactions. Glacial retreat caused by higher than average temperatures exposes new habitats that can be easily colonized from local biota, but non-native species can also be favored by the new climatic conditions. In addition, propagule movement mediated by humans can introduce new species or change the population structure of many taxa. The terrestrial biota is comprised almost exclusively by “lower organisms” (invertebrates, bryophytes, algae, lichenized fungi, and microorganisms). Lichens are the dominant component, and the most important primary producers. Lichens are symbiotic associations consisting of a fungus (mycobiont) and one or more photosynthetic (photobiont) partners. They can disperse sexually or vegetatively. There are several problems related to the symbiotic nature of lichens that do not facilitate easy identification; although molecular data offers additional evidence, species delimitation in lichens is still not straightforward. The true number of species is underestimated due to the presence of cryptic species and species pairs. Recommended universal fungal barcode sequences (e. g. ITS) sometimes fail to delimit species pairs. Thus, it is necessary to identify fast-evolving markers that allow for the delimitation of closely related species before proceeding with the analysis of lichen populations. The goal of this thesis is to elucidate the so far unknown genetic structure among Antarctic lichen populations because of the

immediate consequences for conservation strategies. The thesis focuses not only on patterns of differentiation and gene flow, but also investigates the question of human-mediated propagule transfer into Antarctica and among Antarctic sites. This project provides data on the genetic structure of Antarctic lichens that is urgently needed to develop conservation strategies in the face of global warming and increased human activities in the region. Due to the fact that it is not possible to apply all of the unspecific fingerprinting methods to lichens, microsatellites or simple sequence repeats (SSRs) are one of the best tools to investigate the genetic structure of lichen populations. SSRs offer the possibility to discriminate the lichen partners, but species-specific microsatellites have been developed for only a few species. Regarding the Antarctic, only one species has been studied with SSRs.

The second chapter describes new methods and tools to delimit closely related species of lichens and provides fast evolving markers to characterize their genetic structure. The chapter introduces the lichen species analysed in this thesis and the problems related to their correct identification by morphological methods and molecular data. Chapter two explains the sampling methods for lichen populations and the localities from small areas in which the species pairs occur together. Then the methods used to generate and validate fungal specific microsatellites that cross-amplify species pairs are described. This chapter focuses on the species pair *Usnea antarctica* and *U. aurantiacoatra* because they are the most common lichens in the Maritime Antarctic. An internal transcribed spacer (ITS) marker do not discriminate between these species, and some authors have suggested to synonymize them. Unpublished results from another Antarctic species pair, *Placopsis antarctica* and *P. contortuplicata*, are included to confirm the capability of SSRs to discriminate closely related lichen species. This thesis is the first study to generate SSRs that cross amplify species pairs, using BLAST to compare one genome against the other to obtain markers with the same length in flanking regions. The *de novo* developed SSRs are able to discriminate the two closely related species, and can detect variability at the population level. In the end of the chapter, ITS sequences, microsatellites, and SNPs are used to delimit the species of *Usnea antarctica* and *U. aurantiacoatra*. The chapter exposes the importance of a correct species delimitation and the ability of SSRs and SNPs to delimit the Antarctic *Usnea* species pair compared with the recommended universal fungal barcode sequence ITS.

The third chapter studies the genetic diversity and differentiation among lichen populations to find the effects of dispersal strategies and migration history on the population genetic structure of Antarctic lichens. Samples from South America and the Maritime Antarctic were analysed to identify possible human-mediated gene-flow among continents and Antarctic localities. Chapter three presents population genetic analyses of three lichen species with different dispersal strategies (sexual,vegetative) based on a high number of samples. *Usnea* aurantiacoatra reproduces sexually and occurs disjunctively in South America and Antarctica. It is used to study intercontinental gene flow from South America to the maritime Antarctic. Surprisingly, the sterile, sorediate *Usnea antarctica* could only be found in Antarctica and is probably endemic there. In order to study gene flow between the continents also for an asexual lichen species, *Cetraria aculeata* was used. The two vegetative species (C*. aculeata* and *U. antarctica*) displayed lower levels of genetic diversity than *U. aurantiacoatra*. Low levels of genetic differentiation within the Antarctic populations and higher levels of genetic differentiation within the Patagonia ones indicate a long-lasting presence of *U. aurantiacoatra* in Antarctica and dispersal from there to South America. Genetic differentiation between populations of *U. antarctica* are comparable to the ones found in *U. aurantiacoatra*. Low diversity and strong genetic differentiation of *C. aculeata* in the Antarctic populations confirmed that the species colonized the Antarctic from Patagonia. Glacial refugia have been identified on Navarino Island and in the South Shetland Islands. At the moment, there is no evidence of migration or ongoing gene flow to the Antarctic.

Chapter four reports the main conclusions. Microsatellites are suitable tools to discriminate species pairs and to study the genetic structure of lichen populations. Phylogeographic history better explains the population genetic structure of each species than mode of propagation. Contrasting patterns of genetic differentiation provide evidence for glacial *in situ* survival of *Usnea antarctica* and *U. aurantiacoatra* in the Antarctic. There is no clear evidence of human mediated propagule movement between South America and Antarctica. The strong genetic structure of *C. aculeata* calls for protective measures to avoid gene flow between isolated populations and to prevent local extinctions.

ABBREVIATIONS

AFLP = Amplified Fragment Length Polymorphism

CTAB = Cetyltrimethylammonium bromide

DA = Discriminant Analysis

DAPC = Discriminant Analysis of Principal Components

HiSeq = High-throughput Sequencing System Illumina, sequencing by synthesis, 75 - 150 bp read length

ITS = Internal Transcribed Spacer

LGM = Last Glacial Maximum

MiSeq = High-throughput Sequencing System Illumina, sequencing by synthesis, 75 - 300 bp read length

MYA = million years ago

NGS = Next Generation Sequencing

PCs = Principal Components

PCA = Principal Component Analysis

PCR = Polymerase Chain Reaction

RADseq or RAD sequence = Restriction Associated DNA Sequencing

RFLP = Restriction Fragment Length Polymorphism

SNP = Single Nucleotide Polymorphism

SSR = Simple Sequence Repeat, microsatellite

CHAPTER 1: INTRODUCTION

1.1. BACKGROUND

Lichens are perplexing organisms. Biologists have had a troublesome time in defining their nature. The first to correctly recognize lichens as a symbiotic organism was Simon Schwendener in 1869 (Honegger 2000). Today, we know that lichens are a symbiosis between a mycobiont and algae and/or cyanobacteria as photobionts (Ahmadjian 1993). Recently, highly coevolved basidiomycete yeasts were also discovered in the cortex of many lichens (Spribille et al. 2016). Lichens can also be parasitized by other fungi (e.g. Millanes et al. 2016) and can provide habitat and shelter for other organisms, such as mites and tardigrade species (Green et al. 1999; Øvstedal & Lewis-Smith 2001). In any case, we are far from understanding the mechanisms that regulate this symbiosis. First, mycobionts are difficult to grow in the laboratory. Even when axenic cultivations succeed, their growth is slow and the un-lichenized mycobionts remain relatively amorphous (Nash III 2008). Second, the co-cultures of lichen partners need to be carefully designed to keep the growth of both symbionts in balance. Then, the resynthesis of thallus structures from pure cultures of both symbionts requires special conditions and substrates (e.g. filters, artificial soil), which help to mimic the conditions found in nature (Muggia et al. 2018). In addition, no experiments have tested the survival success of lichens after removing some partners (Wilkinson 2018).

Lichens are successful organisms. As a result of the symbiosis of both a photobiont and a mycobiont, they now occur in many habitats where separately they would be rare or absent. Lichens are found widespread across terrestrial

ecosystems; they are the conspicuous components in alpine, subalpine, and high latitude habitats (Feuerer & Hawksworth 2007). Lichens are poikilohydric and poikilothermic organisms with an outstanding ability to tolerate extreme conditions, like low and rapidly fluctuating temperatures and intra-cellular water contents. Consequentially, they are the dominant terrestrial life-form in Antarctica (Øvstedal & Lewis Smith 2001)

The lichen symbiosis typically involves a close physiological integration of both partners (Nash III 2008). The photobiont provides carbohydrates (mostly sugar alcohols) to the mycobiont while the latter provides shelter in the lichen thallus, for example by producing secondary substances functioning as sunscreen and herbivore defense (Eisenreich et al. 2011; Solhaug & Gauslaa 2012). More diversification is encountered in the fungal component, which usually dominates the association and produces diverse growth forms, sexual structures, and secondary substances (Grube & Winka 2002). As occurs in most fungi, many lichenized ascomycetes have a sexual and an asexual life cycle. In lichens, usually only the mycobiont expresses the full sexual and, to a certain degree, also asexual reproduction. Sexual reproduction of the photobiont is suppressed in the lichenized state. Small-sized meiotic and mitotic fungal spores are considered ideal vehicles for long-distance dispersal by wind (Tibell 1994); however, the principal problem with lichenization is the necessity of fungal spores to meet their proper photosynthetic partner for the re-establishment of the symbiosis. In addition to the typical sexual (teleomorph) and asexual (anamorph) fruiting structures of the individual symbiont, lichenized ascomycetes have evolved a number of vegetative propagules, by which both partners are distributed (Nash III 2008) to overcome this problem. In addition, vegetative strategies allow both partners to spread together and to start a new thallus when the growing conditions are suitable.

Most lichens were originally described as morphospecies (Hausdorf & Hennig 2010). The occurrence of specific metabolites has also been widely applied to circumscribe lichen species because morphological characters are often scarce or show considerable phenotypic plasticity. The use of molecular phylogenetic approaches based on the analysis of DNA at times suggest relationships that differ from those that arise from traditional systematics (Cornejo & Scheidegger 2015). Multilocus DNA sequence datasets are not often able to distinguish hypothesized morphospecies in several groups of lichens (Lohtander et al. 1998; Myllys et al.

2001; Articus et al. 2002; Altermann et al. 2014). On the other hand, traditional systematics sometimes fails to correctly delimit species when dealing with “cryptic species” or “species pairs”. Cryptic species are usually well defined by molecular data, but they are not supported by differences in morphological characters and do not possess any evident features to separate them. This situation not only occurs in microscopic fungi, but also is widespread among macromycetes (Crespo & Pérez-Ortega 2009). In some cases, the overestimation of species diversity based on morphology and chemistry has occurred, while at the same time new lineages, often represented by undescribed species, have also been reported (Mark et al. 2016).

Du Rietz (1924) was the first to develop the concept of species pairs. Later, Poelt (1970) went back to the idea of a sexual and a vegetative lineage of lichens that are indistinguishable except for their mode of reproduction. Vegetative lineages produce soredia or isidia (small propagules that contain both fungal and algal cells), but in rare circumstances can produce fruiting bodies under ecologically favorable conditions, although apothecia and their ascospores may be poorly developed. The driver forcing lichens to switch reproductive strategies has been identified as conflicting reproductive (favouring sexual reproduction) and nutritional (favouring vegetative reproduction that disperses both symbiotic partners) requirements imposed by the obligate symbiotic lifestyle of these fungi (Buschbom & Barker 2006; Buschbom & Mueller 2006). However, this phenomenon appears more in sorediate/fertile species pairs, while isidiate species usually form distinct lineages in phylogenetic studies (Lumbsch & Leavitt 2011). The idea of the species pair in Poelt’s sense has been intensively discussed (Tehler 1982; Mattsson & Lumbsch 1989; Crespo & Perez-Ortega 2009). The idea, which suggests that “sorediate species may harbor lower genetic diversity because they may indeed represent evolutionary dead ends or clones” (Del-Prado et al. 2016), is known as the dead-end theory. However, genetic variation has been documented in several asexual lineages, and sexual taxa have been shown to have arisen from asexual ancestors (Buschbom & Barker 2006; Cornejo et al. 2009; Lendemer & Harris 2014; Widhelm et al. 2016). Tripp (2016), using a model-based reconstruction of transitions between sexual and asexual reproduction of 23 phylogenetic studies, demonstrated that asexual lineages are able to undergo speciation, give rise to sexual lineages, and are likely to be evolutionarily old. Hence, regarding the species pair question, the idea of asexual lineages as an evolutionary dead-end must be rejected.

A considerable amount of molecular research has tested the reciprocal monophyly of species pairs, resulting in conflicting conclusions. In fact, the detection of slow genetic drift in a lichen (Printzen et al. 2003) has made it likely that ancestral polymorphisms might distort the outcome of phylogenetic analyses of closely related lichen species. Methods for delimiting closely related lichens have had to struggle with this potential problem of incomplete lineage sorting between recently diverged species due to a young age in geological terms (Wirtz et al. 2012). Cornejo and co-authors (2018) examined 25 studies about the *Xanthoparmelia* genus; 19 of those 25 studies rejected the species pair because specimens with different reproductive modes were intermingled within one single monophyletic clade. Several studies that rejected the occurrence of a species pair were based on the application of the nuclear ribosomal locus ITS, while others applied up to four loci. Altermann and co-authors (2014) attempted to recover six previously identified phylogenetic groups within *Letharia columbiana* and *L. vulpina* (Kroken & Taylor 2000) using 15 gene loci, and were unable to resolve all lineages. Together, these studies question the validity of the species pair concept in lichens and it has been claimed to be obsolete (Cornejo et al. 2018). Consequently, some negative findings led to taxonomical revisions synonymizing names for both species pair counterparts (e.g. Tehler et al. 2013; Messuti et al. 2016). On the other hand, species pairs have been supported in other studies because strong support was found for reciprocal monophyly between the sexual and asexual counterparts. Lendemer and Harris (2014) detected the species pair *Porpidia degelii* (H. Magn.) Lendemer vs. *P. albocaerulescens* (Wulfen) Hertel & Knoph in each single-locus tree. Saag and co-authors (2014) used five concatenated loci in *Vulpicida* J.-E. Mattsson & M.J. Lai to resolve reciprocal monophyly. In Cornejo and Scheidegger (2015), a three-loci phylogeny unravelled the species pairs *Lobaria kurokawae* Yoshim. (sexual) vs. *L. retigera* (Bory) Trevis. (asexual), and *L. sachalinensis* Asah. (sexual) vs. *L. kazawaensis* (Asah.) Yoshim. (asexual), although three other putative pairs within the *L. meridionalis*-group showed only a weak indication of reciprocal monophyly similarly to previous results (Wei et al. 2016). In Widhelm et al. (2016), the morphology and chemistry of two species pairs were concordant with monophyletic clades in a seven-loci phylogeny. Together, these examples indicate a simple point: that large, multi-locus datasets may need to be used to delimit recently diverged sister species. Only in one particular case (that of Lendemer & Harris 2014), the monophyly of species pairs was reflected in each

analysed locus in contrast to the concatenation of several loci; in contrast, several loci were needed to unravel species pairs in all other cases (Miadlikowska et al. 2011; Altermann et al. 2014; Saag et al. 2014; Cornejo & Scheidegger 2015; Wei et al. 2016). Leavitt et al. (2016) recently recovered consistent species phylogenies for the *Rhizoplaca melanophthalma* complex using datasets comprising between 100-1000 kb loci and a total of 16.8 Mb, whereas reconstructions using 25-100 kb loci differed among each other. This study supports results from simulation studies (Ogilvie et al. 2016) showing that genome-wide datasets might be necessary to delimit recently diverged taxa.

Hence, although molecular data offers additional evidence, species delimitation in lichens is still not straightforward. The published studies have so far almost exclusively relied on DNA sequence data from relatively few loci. Incomplete lineage sorting (Printzen et al. 2003) may lead to incongruence between gene and species trees (Aguileta et al. 2008) and between morphological and molecular species concepts (e.g., Myllys et al. 2001; Lohtander et al. 2009). These problems could be overcome by increasing the number of studied gene loci or by using faster evolving markers. The delimitation of species is of basic importance because species are the fundamental units in biology. Depending on the organismal group and the specific focus of researchers, different species concepts have focused on phenotypic and ecological differences, reproductive features, limitations of gene flow, or phylogenetic and/or genealogical relatedness of individuals (morphological, ecological, biological, phylogenetic, etc., species concepts). De Queiroz (2007) recently highlighted that, despite their apparent differences, more or less all species concepts agree in defining species as separately evolving population-level lineages and summarized their commonalities in the general lineage concept of species. Uncertain species delimitations can undermine population-level studies of lichens. “If several unrecognized species are included in a study, the assumptions of null-models (e.g. panmixia or certain modes of range expansion) may be violated” (Printzen et al. 2013).

Antarctica is the most remote, cold, and isolated continent covering 14 million square kilometers. Antarctica broke its Gondwanan connection with South America over 40 million years ago (Mya; Scher & Martin 2006). By 42 Mya, the separation of

Antarctica from South America started forming the Drake Passage, establishing the Antarctic Circumpolar Current and the Subantarctic Front and Antarctic Polar Front (32 Mya). By 28 Mya, those currents and the Drake Passage were in their present positions (Lawver & Gahagan 2003). After, significant cooling events at roughly 37 Mya were followed by repeated cycles of glaciation throughout the Oligocene and into the Miocene (Liebrand et al. 2017). Additional rounds of warming and cooling followed (Crame 1999), with a major round of ice expansion 14–17 Mya, the formation of the Western Antarctic Ice Shelf 5–6 Mya, and glaciation 2.4 Mya (Halanych & Mahon 2018). The most common biota reported today were not present at the Gondwana breakup, and the distribution of the present-day species are the result of recent processes (dispersal, local extinction, local adaptation) rather than continental drift (Halanych & Mahon 2018). Although vicariance certainly was associated with the Drake Passage formation, the presence of the same or sister taxa on either side of the Drake Passage is likely the result of more recent dispersal rather than an old vicariance event caused by continental drift (Halanych & Mahon 2018). In fact, the Drake passage together with the currents prevent the colonization of most vascular plants and keeps a floristic similarity of mosses and lichens in both regions, which has led to levels of endemism between 35 and 100% in different organismal groups (Rogers 2007). Glacial activity on the continent drove phylogeographic patterns for species by causing isolation (Stevens & Hogg 2003; Thatje et al. 2005). Allopatric fragmentation due to the continued glaciation cycles explains the overall structure of biodiversity patterns in several animals (Thatje et al. 2005; Stevens et al. 2007; Wilson et al. 2007; Baird et al. 2011; Havermans et al. 2011; Harder et al. 2016; Soler-Membrives et al. 2017). Briefly, several factors have historically interacted to shape today's mosaic of genetic diversity in Antarctica: isolation and recolonization, allopatric divergence among populations, low genetic diversity due to genetic drift, rare dispersal events, and the occasional occurrence of secondary contact zones (Domaschke et al. 2012; Nolan et al. 2006; Rogers 2007).

Terrestrial life in Antarctica is restricted to widely separated and small ice-free areas that cover only 0.3% of the continent (Convey & Stevens 2007). Terrestrial Antarctica has been divided into several zones based on climatic and biotic features, and generally three zones have been described: the sub-Antarctic, the Maritime Antarctic, and the continental Antarctic (Rogers 2007); however Terauds and co-authors (2012) distinguished 15 Antarctic biogeographic regions, combining the

published biogeographic studies with three spatial frameworks. Chown and Convey (2007) suggested the “Gressitt Line” between the Antarctic Peninsula and the rest of the continent as a biogeographical boundary comparable to the Southeast Asian “Wallace Line”. Indeed, numerous studies indicate that the Maritime Antarctic and the Continental Antarctic host distinct flora (Peat et al. 2007).

The colonization of the Antarctic is a challenge for many taxa and related to their ability for long-range dispersal and their adaptation to the harsh climate. Cold temperatures and little availability of ice-free terrain limit “temperate” taxa in establishing populations on Antarctica. Migration and gene flow between populations is limited by the small extension of habitable areas and often reduced dispersal abilities of organisms. Strong local and regional genetic differentiation has been observed in most Antarctic terrestrial organisms, for example mosses, flowering plants, nematodes, springtails, mites, midges, and even prokaryotes (Allegrucci et al. 2012; Chong et al. 2015; Courtright et al. 2000; McGaughran et al. 2010; Mortimer et al. 2011; Skotnicki et al. 2004; van de Wouw et al. 2008). Molecular studies highlight the complexity of Antarctic populations, with substantial landscape genetic diversity even over relativity limited spatial extents (Terauds et al. 2012). However, the local genetic diversity registered in Antarctica is lower compared to other latitudes (Halanych & Mahon 2018), due to climatic pressure and the selection for well-adapted genotypes to the Antarctic habitat. From a biological perspective, the Antarctic thus presents an assemblage of widely spaced “habitat islands” (Bergstrom & Selkirk 1997) with sufficiently long continuity to support considerable genetic diversity (Convey et al. 2014). In other continents, habitat fragmentation is a threat to biodiversity (Liu et al. 2018). Habitat fragmentation is defined as the breaking-up of habitats into smaller and isolated patches that hamper ecological flows across a landscape (Wu 2009). Positive species interactions suffer from habitat fragmentation (Hagen et al. 2012; Peh et al. 2014), especially in case of mutualistic interactions (Magrach et al. 2014), leading to decreased complementarity (Smith & Knapp 2003). “The effects of habitat fragmentation on populations, communities, and ecosystems can take years to decades before becoming apparent, suggesting that patches will continue to lose species and see declines in ecosystem functions for considerable time periods” (Wilson et al. 2016). In addition, the rarity of natural dispersal events has, until recently, allowed the Antarctica’s biota to evolve and diversify in relative isolation (Barnes et al. 2006). Patterns shaped by natural dispersal, colonization, and

diversification in Antarctica are now at risk of being overridden by impacts associated with changing climates and rapidly increasing human movement both into the region and between its distinct eco-regions (Chown et al. 2015).

Antarctic terrestrial ecosystems are significantly threatened by climate change, invasive species, and their interaction (Walther et al. 2002; Frenot et al. 2005). Climate change is affecting all regions in several ways. The Western Antarctic regions (south of the Pacific Ocean) are warming rapidly (Shepherd et al. 2018), while the Eastern Antarctic regions (south of the Indian Ocean and Australia) are experiencing a cooling trend in terrestrial environments and notable increases in snowfall (Winkelmann et al. 2012; Thomas et al. 2015). The Antarctic Peninsula and Bellingshausen Sea have experienced the most rapid regional warming during the last decades of the past century (Hansen et al. 1999; Vaughan et al. 2001). However, a recent analysis has shown a more complex situation. Mean annual temperatures in the Antarctic Peninsula rose at a rate of 0.32 ± 0.20 °C per decade from 1979 to 1997 and have fallen since then −0.47 ± 0.25 °C per decade from 1998 to 2014 (Turner et al. 2016). The climatic modifications that are taking place are affecting the water and soil availability and could radically alter biodiversity in the continent's ice-free areas on a near-immediate time scale, such as by increasing growth rates and activity periods, therefore increasing abundance and richness (Chown et al. 2015). A connection or genetic exchange between isolated and locally adapted lineages could lead to a homogenization of some Antarctic species. Changing of species abundance and range in both the Antarctic Peninsula and the sub-Antarctic region have been reported as marked ecological changes (Rogers 2007).

Glacial retreat caused by the higher average temperature exposes new habitats that can be easily colonized from local biota, but non-native species can also be favored by the new climatic conditions. A recent study by Duffy and co-authors (2017) predicted the possibility for the most common invasive species to invade the Antarctic. While the harsh climate will continue to be a limiting factor for invasive species to establish in Antarctica, their model predicts new colonization by cold-tolerant species. In any case, alien species have been introduced both purposefully and accidentally and growing numbers of alien species from virtually all organismal groups have been recorded in terrestrial Antarctic ecosystems (Frenot et al. 2005), among them bacteria (Hughes 2003), Collembola (Greenslade & Convey 2012), Diptera (Volonterio et al. 2013), Oligochaetes (Hughes & Worland 2010), flowering

plants (Olech 1996), fungi (Farrell et al. 2011), and lichens (Olech 1996; Osyczka 2010). There is a pressing need to reassess the extent of Antarctica's biological isolation (Fraser et al. 2018).

Antarctica is one of the last wildernesses. At the end of the 19th century with the Heroic Age of Antarctic Exploration, humans started to explore the continent, and at the same time whaling factories were set up in Sub Antarctic Islands like South Georgia and in the South Shetlands. Later, after the Antarctic Treaty System of 1960, many Nations started scientific activities on the continent. Today, we count at least 100 active research facilities (all-year and summer stations, field camps and refuges) in the Antarctic Treaty area. In addition, in the last two decades, tourism has become more popular and cheaper. Increasing numbers of scientists and tourists with multiple landings in different Antarctic regions have facilitated propagule movement into Antarctica and among different bioregions (Lee & Chown 2011). Several studies have examined which categories of visitors: tourists, scientists, and science support personnel carry the highest propagule loads, and indicated that personal clothing items produce a relatively high risk (Cowan et al. 2011). Although the relationship between propagule pressure and invasion success remains largely elusive (Wittmann et al. 2014), successful propagule translocation into the Antarctic and gene transfer among Antarctic bioregions must be accepted as a fact. Intra-regional propagule movement can lead to biological homogenization.

The Antarctic is unique because the terrestrial biota is comprised almost exclusively by "lower organisms" (invertebrates, bryophytes, algae, lichenized fungi, and microorganisms; Øvstedal & Lewis Smith 2001). Only two phanerogams, *Deschampsia antarctica* and *Colobanthus quitensis*, have colonized the Maritime Antarctic (Vera 2011), while bryophytes and lichens are highly diverse. Thanks to their dispersal ability and the unique symbiosis, lichens are the dominant life-form throughout much of the Maritime Antarctic and virtually all of continental Antarctica with more than 400 species reported from south of 86° in latitude. About 34% of Antarctic lichens are endemics, showing isolation of lichen biota over geological timescales. In fact, during glaciations, some species of lichens and mosses have survived in local refugia in Victoria Land (Green et al. 2011) and other "cryptic" oases in the coastal continental Antarctic (Convey et al. 2008). High levels of endemism amongst the Antarctic lichens may depend on one hand by their physical isolation from other terrestrial habitats in the region. On the other hand, Antarctic extreme

conditions have selected for a specialized endemic community over time (Singh et al. 2015).

The wide distributional ranges of many Antarctic lichens and the resulting logistical challenges of sampling along intercontinental transects have so far discouraged systematically designed studies on topics such as spatial genetic variation, genetic isolation, and genetic dispersal among the Antarctic and South American lichen populations. In the past, biogeographical patterns of microorganisms were not often investigated because researchers accepted the wide distribution ranges of species occurring on different continents (Lumbsch & Leavitt 2011). This was due to a common belief among mycologists and lichenologists that the "everything is everywhere: but the environment selects" (Baas Becking 1934) hypothesis. Finlay (2002) suggested that small eukaryotes lack endemism and have a global distribution. He based his hypothesis on studies of morphological species of ciliates (Fenchel & Finlay 2004). Recently, several studies in different groups of microorganisms, including fungi, demonstrated that some species that were previously assumed to be widely distributed are actually comprised of several taxa with more restricted distributions (e.g., Geiser et al. 1998; Kasuga et al. 1999; Koufopanou et al. 2001; Papke et al. 2003; Whitaker et al. 2003). These results indicate that a part of the undiscovered fungal diversity may be hidden in widely distributed groups of species that are currently classified as single species based on morphological similarities (cryptic species, Lumbsch & Leavitt 2011). As mentioned before, studies based only on morphological characters have been shown to vastly underestimate the diversity of lichenized fungi; studies need to include molecular data information, even if species delimitation in lichenized fungi remains a challenge in the molecular era. The Antarctic has always attracted the attention of lichenologists due to the fact that lichens are the most important primary producers of Antarctic terrestrial ecosystems. In general, what is mostly known of Antarctic lichens are species checklists collected in a few localities and based on morphological methods. There are very few studies that provide data on genetic diversity over larger spatial scales, but these studies use a small number of samples and a few nuclear DNA barcoding markers. Romeike and co-authors (2002) reported polymorphism in 22 thalli of *Umbilicaria* along a 5000 km transect. Ruprecht and co-authors (2012) investigated the photobiont diversity of 119 lecideoid lichen samples

around the Antarctic. Both studies found low genetic differentiation among different regions. Mutation rates and genetic drift are slow in lichens (Printzen et al 2003), which causes the problem of incomplete lineage sorting between recently diverged lichen species; DNA sequences will therefore detect demographic events on broad evolutionary time scales rather than human historic time scales. Thus, it is necessary to involve faster evolving markers in order to highlight mutations to study on-going evolutionary trends and to characterize different population structuring. Microsatellites (Simple Sequence Repeat, or SSRs) evolve much faster than genes and have successfully been used in the landscape genetics of lichens to detect genetic structuring at a local scale (Walser et al. 2004; Werth et al. 2007). In addition, microsatellites can be designed to be species-specific for mycobionts. Due to the symbiotic lifestyle of lichens, it is necessary to develop fast-evolving markers able to target the DNA of only one lichen partner.

Microsatellites are powerful and widely used genetic markers. Their co-dominant nature and high level of polymorphism make them invaluable for genotyping purposes as well as for standard population genetic analyses (Gonthier et al. 2015). The first lichen SSRs were developed for the mycobionts of *Lobaria pulmonaria* (Walser et al. 2003), and then the photobiont *Dictyochloropsis reticulata* (Dal Grande et al. 2009; Widmer et al. 2012). Recently, more SSR markers have been developed for lichen fungi: (i.e., *Bryoria* Section *Implexae* - Nadyeina et al. 2014; *Cetraria aculeata* - Lutsak et al. 2016; *Lobaria pindarensis* Devkota et al. 2014; *Nephroma laevigatum* and *N. parile* - Belinchón et al. 2014; *Peltigera dolichorhiza* - Magain et al. 2010; *Protoparmeliopsis muralis* - Guzow-Kreminska & Stocker-Wörgötter 2013; *Rhizoplaca melanophthalma* - Lindgren et al. 2016*; Usnea subfloridana* - Tõrra et al. 2014). Thanks to these markers, the genetic diversity of lichens at the landscape scale can be investigated (Werth et al. 2015). Most of the studies based on SSRs are related to one species, evaluating the difference in populations at a local scale covering small areas. These studies investigated the distribution and the dispersal ecology of lichens and the number of SSRs. In general, the studies do not exceed the use of 10 markers. Out of all the Antarctic lichens, only *Buellia frigida* has been studied with SSRs; this study found evidence for genetic differentiation among locations in the Ross Dependency, suggesting that there is the existence of a potential ‘‘source’’ for the refugial populations within this region (Jones et al. 2012, 2013, 2015).

In the last decade, the advent of next-generation sequencing (NGS) drastically changed the scale of molecular datasets for systematic analyses and revolutionized our ability to assess evolutionary histories of organisms (Kraus & Wink 2015; Wachi et al. 2018; Zimmer & Wen 2015). Direct sequencing NGS methods, such as de-novo genome sequencing (Ellegren 2014), re-sequencing (Stratton 2008), or RNAseq of expressed genes (Ozsolak & Milos 2011; Wickett et al. 2014) can provide whole genome-scale data but may still be limited by the quality and the amount of DNA extracted from lichen thalli. Therefore, these methods are rarely applied to population studies, which require the sequencing of many individuals. However, a subset of the genome often contains sufficient polymorphisms to answer questions of phylogenetic or population genomic studies. Hence, many NGS methods for systematic analyses are designed to be economical and generate reduced genome representation datasets (Allendorf 2017; Davey et al. 2011). One of these methods is genotype-by-sequencing and its altered approach, which is known as restriction associated DNA sequencing (RADseq; Baird et al. 2008).

1.2. OBJECTIVES

The goal of this thesis is to elucidate the so far unknown genetic structure among Antarctic lichen populations because of the immediate consequences for conservation strategies. The project focuses not only on patterns of differentiation and gene flow but also on the question of human-mediated propagule transfer into Antarctica and among Antarctic sites. Although there is clear evidence that humans are actively moving non-native biota into Antarctica and among Antarctic regions, human transfer of lichen propagules is at present only a hypothesis. This thesis provides indispensable data on the genetic structure of Antarctic lichens that is urgently needed to develop conservation strategies in the face of global warming and increased human activities in the region.

To generate the data, lichen populations were systematically sampled in South America, the Falkland Islands, in the South Shetland Islands, and the Antarctic Peninsula. Also, several lichen species with different propagation strategies (sexually – vegetatively) needed to be included as target species. The dispersal strategy affects the chances of passive dispersal, establishment at a new locality, and hence the genetic differentiation among populations. Lichens with large thalli were preferred because they are easy to detect and collect in the field and considering that it is difficult to obtain DNA from a lichen thallus, their large size allowed multiple DNA extractions. More importantly, the target species were reported in both continents: South America and Antarctica in order to detect gene flow.

To understand gene flow and variability among different populations, classical methods based on Sanger Sequencing of mitochondrial or nuclear barcoding markers are not suitable because they do not highlight mutations affecting individuals in rapid time periods and at the local level in lichens. For this reason, to study the genetic structure of several lichen populations it was necessary to develop more quickly evolving markers to capture local genomic variation. Microsatellites are the best tool: they do not require high-quality DNA to be tested, and SSRs are cheap to analyse given they do not involve Sanger sequencing. Besides, SSRs can be multiplexed to obtain a lot of information from a single sample in one PCR reaction. Those markers must be fungal specific in order to avoid the amplification of the

wrong partner and the markers must cross-amplify among a species pair to delimit two species.

After characterizing the Antarctic lichen populations, it is considerably more challenging to identify human-mediated gene-flow among populations, because this process involves not only observable transfer of propagules (Huiskes et al. 2014), but also unobservable local establishment. Indirect evidence must therefore come from population genetic data. As a first step this would involve finding genetic similarities among populations and identifying individuals with identical genotypes as potential migrants. But even with fast-evolving markers, the occurrence of genetically identical genotypes in different populations is in itself not sufficient evidence for human impact. The fundamental difficulty is to distinguish human transfer of propagules from transport by natural vectors such as wind or birds. The only way to overcome these problems is a quasi-experimental sampling design in which sites with demonstrably high human impact are compared with “low impact” sites. Ideally, these sites should be equidistant from possible source populations, but isolated enough from each other to prevent unlimited gene flow among them. They should also show similar ecological conditions (bedrock, macroclimate) to avoid the confounding effects of selection. Finally, access to these sites must be logistically feasible. The South Shetland Islands offer the best area to test and detect human impact. The biggest Islands are King George and Livingston. Both islands are ca. 900 km away from the southernmost tip of South America and human impact is very different on both islands. King George Island currently supports ten stations for scientific research, a permanent civilian settlement of ca. 100 people (Villa Las Estrellas), and an airfield (“Teniente Marsh”) with frequent support flights from Punta Arenas (Chile) and Río Gallegos (Argentina) in Patagonia. Touristic activities have been growing and include events such as an annual “Antarctic marathon” on Fildes Peninsula. The number of annual visitors has been going up to around six times higher than the average on Livingston Island. Livingston Island has only two permanent stations and one seasonal field station, no other settlements, and no airfield. Tourism was almost negligible before 1993 and has since stagnated to around 5000 visitors. Noticeably, tourism has not much affected the ice-free areas on Byers and Hurd Peninsula, while on King George Island it is concentrated in the largest ice-free areas around Admiralty and Maxwell Bay. Both islands have similar macroclimate and bedrock (mostly andesitic lavas). Movement of scientists between both islands is considerably

rarer than between Patagonia and other islands. Considering the distance of ca. 100 km between both islands, natural as well as human-mediated rates of gene flow among lichen populations on both islands are assumed to be low. In order to detect human mediate gene flow it is important to compare populations of the same species collected in both islands against populations collected in other localities where humans are almost absent or produce lower impacts. On the other hand, the comparison of Antarctic populations against populations from South America is necessary to detect intercontinental gene flow and identify possible migrants. A high number of sampling localities and species is a necessary condition to detect gene flow; in fact, not all the species could occur in all the sampling localities.

The thesis is based on the null hypothesis of no genetic differentiation among Antarctic lichen populations and unlimited gene flow between South America and Maritime Antarctic. Consequently, the two fundamental hypotheses to be tested are:

1. Antarctic lichen populations are genetically differentiated.
2. Genetic connectivity among Antarctic and South American lichen populations is higher in places with higher human impact.

Specific questions to be answered include:

- Are levels of genetic differentiation among populations high enough to suggest the presence of ecotypes or cryptic species in different parts of the sampling region?
- Does the level of genetic isolation differ among sexually and asexually dispersing species?
- Are lichen populations genetically isolated or is there evidence for gene flow, particularly from South America into the Maritime Antarctic? Is it possible to identify migrants?
- Does the level of genetic isolation differ between sites with high and low human impact?
- What are the conservational implications for Antarctic lichens?

1.3. STRUCTURE

This manuscript-based thesis comprises data and results from three articles published in peer-reviewed journals and one manuscript currently in review. Unpublished results of the *de novo* developed microsatellites for the Antarctic *Placopsis* species pair have been included in this thesis as a confirmation of the reliability of microsatellites to delimit species pairs and detect local genetic variability.

This study is crucial in understanding the origin and structure of the Antarctic lichen communities. It will use methods from population genetics to elucidate the genetic structure among Antarctic lichen populations. Different species will be analysed. As reported before, there are several problems related with the symbiotic nature of lichens that do not facilitate their identification; although molecular data offers additional evidence, species delimitation in lichens is still not straightforward. The true number of species is underestimated due to the presence of cryptic species and species pairs. Besides, it is not possible to apply all of the genetic population techniques to lichens. Microsatellites offer the possibility to discriminate the lichen partners, but species-specific microsatellites have been developed for only a few species and regarding the Antarctic, only one species has been investigated with SSRs. Before proceeding with the analysis of lichen populations, it is mandatory to develop a method able to successfully assign samples to the right species and to delimit closely related species. For this reason, the results of this thesis are presented in two chapters. The first concentrates on the development of SSR markers for species delimitation, the characterization of population diversity, and the clear delimitation of the species studied by me. The second chapter relies on these results to analyze the genetic diversity among Antarctic and South American lichen populations. A conclusion follows.

Chapter two deals with the development of tools to delimit closely related lichen species by generating fast evolving markers that are also able to characterize the genetic structure of lichens. At the beginning, it introduces the lichen species and the problems related to their correct identification by morphological methods and molecular data. The second chapter reports the methods of sampling lichen

populations and the localities from small areas in which the species pairs occupy the same spots. Then, it describes the method used to generate and validate fungal-specific microsatellites that cross-amplify species pairs. SSRs are fast evolving markers that highlight mutations suitable to study on-going evolution and to characterize different population structures. Finally, molecular data, microsatellites, and single nucleotide polymorphisms (SNPs) obtained with restriction associated DNA sequencing (RADseq) were used to delimit the species pair. A discussion exposes the importance of a correct species delimitation and the efficacy of SSRs and SNPs to solve the problem of species pairs compared with the recommended universal fungal barcode sequence ITS. This chapter is based on publications 1, 2, and 3 and unpublished material is added to confirm the reliability of SSRs to delimit species. Out of all the studies on species delimitation of lichens, the methods developed in this chapter are very innovative because it is the first study that uses SSRs and SNPs for species delimitation, giving suitable tools to discriminate species pairs.

Chapter three studies the genetic diversity among lichen populations to find out the effects of dispersal strategies and the migration history on the population genetic structure of Antarctic lichens. Then, populations from South America and the Maritime Antarctic were analysed to identify human-mediated gene-flow among continents and Antarctic localities. The chapter presents the analyses of three lichen species with a high number of samples. Two species help to understand continental gene flow from South America to the Maritime Antarctic, while one species suspected to be endemic in the Antarctic contributes to the characterization of the Antarctic lichen community. Then a discussion explains the effects of dispersal strategy and migration history on the population genetic structure of Antarctic lichens. The discussion examines the gene flow between South America and the Maritime Antarctic with a particular interest on human activities as a vehicle for propagule movement between South America to Antarctica. It is the first study to characterize stands from several Antarctic localities with microsatellites and to track gene flow from South America. Chapter three is based on publications 1, 2, and 4.

Chapter four summarizes the important findings and briefly discusses the conservation implications for Antarctic lichens.

1.4. LIST OF INCLUDED PUBLICATIONS

This thesis is composed of the following manuscripts published in peer-reviewed journals:

PUBLICATION 1: Elisa Lagostina, Francesco Dal Grande, Sieglinde Ott, and Christian Printzen. 2017. Fungus-Specific SSR Markers in the Antarctic Lichens *Usnea antarctica* and *U. aurantiacoatra* (*Parmeliaceae*, Ascomycota). Applications in Plant Sciences 5 (9).

PUBLICATION 2: Elisa Lagostina, Francesco Dal Grande, Mikhail Andreev, and Christian Printzen. 2018. The use of microsatellite markers for species delimitation in Antarctic *Usnea* subgenus *Neuropogon*. Mycologia, 110(6), 1047-1057.

PUBLICATION 3: Felix Grewe, Elisa Lagostina, Huini Wu, Christian Printzen, and H. Thorsten Lumbsch. 2018. Population genomic analyses of RAD sequences resolves the phylogenetic relationship of the lichen-forming fungal species *Usnea antarctica* and *Usnea aurantiacoatra*. MycoKeys, (43):91-113.

PUBLICATION 4: Elisa Lagostina, Mikhail Andreev, Francesco Dal Grande, Felix Grewe, Aline Lorenz, H. Thorsten Lumbsch, Riccardo Rozzi, Ulrike Ruprecht, Leopoldo García Sancho, Ulrik Søchting, Mayara Scur, Nora Wirtz, and Christian Printzen. (under review) Effects of dispersal strategy and migration history on genetic diversity and population structure of different Antarctic lichens. Journal of Biogeography

1.5. AUTHOR'S CONTRIBUTION

This doctoral thesis was supported by Deutsche Forschungsgemeinschaft (DFG) in the framework of the priority program "Antarctic Research with comparative investigations in Arctic ice areas" by grant PR 567/18-1.

During December 2015 and January 2016, I took part in the Antarctic expedition to the Argentinian Carlini Station on King George Island (South Shetland).

During the Summer of 2017, I was a visiting researcher for six weeks at the Field Museum in Chicago supported by a Polar Research Grant of the Field Museum supervised by Dr. H. Thorsten Lumbsch.

The original idea of the project was proposed by Dr. Christian Printzen; I did not take part in the project proposal. Due to the difficult logistics in reaching the sampling areas, many authors contributed to the field collections: Dr. Mikhail Andreev, Dr. Felix Grewe, Dr. Aline Lorenz, Dr. Christian Printzen, Dr. Mayara Scur, Dr. Nora Wirz, and I sampled in the Maritime Antarctic. Dr. Christian Printzen, Dr. Ulrike Ruprecht, Prof. Ulrik Søchting, and Dr. Indra Starke-Ottich sampled in South America and the Falklands. Dr. Francesco Dal Grande and Prof. Imke Schmitt provided bioinformatics support and server access.

The author's contribution to each of the published manuscripts is presented in the following table. The contribution to each part of the scientific process is scored between 1-3 for each category. The categories include: 1 primary activity, 2 equal contributions with other authors, and 3 secondary contribution within a team.

	Field collection	Laboratory work and data preparation	Methodological design	Statistical analysis	Writing
Publication 1	1	1	2	1	1
Publication 2	2	1	1	1	2
Publication 3	2	2	3	3	3
Publication 4	2	1	1	1	1

CHAPTER 2:

DEVELOPING METHODS AND TOOLS TO CHARACTERIZE THE GENETIC STRUCTURE OF LICHENS AND TO DELIMIT SPECIES OF CLOSELY RELATED LICHENS

2.1. CHAPTER STRUCTURE

The symbiotic nature of lichens does not facilitate their identification. Although molecular data offers additional evidence, species delimitation in lichens is still not straightforward. The true number of species is underestimated due to the presence of cryptic species and species pairs. The delimitation of species is of basic importance because species are the fundamental units in many fields of biology. Unfortunately, there is not a unique definition of species, even if all definitions agree on the common view that species are (a segment of) separately evolving metapopulation lineages. However, the way that those metapopulations diverge changes according to the organismal group and the specific focus of the research. All definitions agree that evolutionary processes are the driving forces of speciation. Since the evolutionary

rate is not constant or continuous, there are different conclusions about when two metapopulations start to be considered as two distinct species according to the field of interest (de Queiroz 2007). Besides, it is not possible to apply all of the genetic population techniques to lichens because most methods cannot discriminate the DNA from the different symbionts. So, before proceeding with the analysis of lichen populations, it is mandatory to develop a method able to successfully assign samples to the right species and to delimit closely related species.

It appears clear that to solve the phylogenetic relationships of lichen species it is necessary to use a different approach with fast evolving markers that can discriminate between lichen symbionts. Microsatellites cope with this problem because they are designed to be species-specific for mycobionts. In addition, microsatellites can evaluate the genetic composition of populations better than nuclear or ribosomal DNA sequences and can be a proper tool for the purpose of the thesis.

Chapter two is divided into five sections:

- Target species;
- Sampling localities and methods;
- Development and validation of fungal-specific microsatellites;
- How to delimit species pairs;
- Discussion.

First, I will introduce the species of interest. Then, I will explain the sampling methods used to collect samples of species pairs in the same locality. To exclude the confounding effects of geographical population structure, the samples used in this chapter were restricted to maritime Antarctic populations where the two morphotypes grow together. Later methods to develop and validate fungus-specific microsatellites from the raw genome will be explained. In the end, the problematic species pair *Usnea* was analysed with molecular data, microsatellites (SSRs), and single nucleotide polymorphisms (SNPs) obtained with restriction associated DNA sequencing (RADseq). All the main highlights of the chapter will be discussed in the last section.

Chapter one is based on publications 1, 2, and 3 and unpublished material is included to confirm the reliability of SSRs to delimit species. The methods used to study species delimitation of lichens in this chapter are very innovative because it is the first time using SSRs and SNPs for species delimitation to give a clear delimitation.

2.2. TARGET SPECIES

The aim of the thesis is to evaluate genetic structure and gene flow between the Maritime Antarctic and South America in varied species of lichens with different dispersal strategies. For this reason, three target species known to be present in both continents have been selected: *Cetraria aculeata* (asexual via fragmentation), *Usnea antarctica* (asexual via soredia), and *U. aurantiacoatra* (sexual). Microsatellites were also developed for another Antarctic species pair, *Placopsis antarctica* (asexual via soredia) and *P. contortuplicata* (sexual). The preliminary unpublished results from the two *Placopsis* species have been added to this chapter to prove that SSRs can clearly delimit species pairs.

Cetraria aculeata (Schreb.) Fr. (fig. 1-E) is a terricolous lichen species, forming shrubby tufts in a wide variety of biomes and microhabitats worldwide (Kärnefelt 1979; Fernández-Mendoza et al. 2011). It is a morphologically variable species, with high variation in size, the coloration of its terete to the slightly flattened branches, the structure of the pseudocyphellae of the cortex that facilitate gas exchange, and the frequency of thallus projections. This variability has led to the description of numerous species and infraspecific taxa, which display, however, continuous variation (Kärnefelt 1979; Lutsak et al. in press 2020). *Cetraria aculeata* mostly reproduces asexually via thallus fragmentation. *C. aculeata* is a bipolar lichen species that colonized Antarctica from Patagonia (Fernández-Mendoza & Printzen

2013). A previous study based on nuclear DNA pointed out that populations of *C. aculeata* on King George Island displayed lower genetic diversity than Patagonian populations, indicating limited gene flow among Antarctica and South America (Domaschke et al. 2012); although, the authors indicated a possible artefact in their results due to the unequal geographic sampling.

Usnea antarctica Du Rietz and ***U. aurantiacoatra*** (Jacq.) Bory (fig. 1-A & B) are the most common lichens in the Maritime Antarctic. The two species occupy similar habitats but show different dispersal strategies, morphology, and distribution. They constitute a species pair in which *Usnea antarctica* usually propagates vegetatively by soredia, while *U. aurantiacoatra* has apothecia and thalli that are usually larger. *Usnea antarctica* and *U. aurantiacoatra* belong to the *Neuropogon* group within *Usnea*. Except for two species with a bipolar range into the Northern hemisphere (*U. sphacelata* and *U. lambii*), the distributions of most species in this group are restricted to the high Andes and southernmost South America, the Falkland Islands, Australasia, and Antarctica. The greatest abundance and species diversity occurs in ice-free areas of the Antarctic Peninsula, where *Usnea* species may develop stands covering a few to several hundred hectares (Walker 1985; Øvstedal & Smith 2001; Seymour et al. 2007). The success of *U. antarctica* and *U. aurantiacoatra* in the Antarctic environment is shown by the fact that both are common species and easy to collect. *Usnea antarctica* is reported to have the highest relative coverage within a deglaciation gradient in a study area on King George Island followed by *U. aurantiacoatra* (Rodriguez et al. 2018). *Usnea aurantiacoatra* is absent from the continental Antarctic, whereas *U. antarctica* has been recorded. Phylogenetic analyses performed on several Southern Hemisphere *Usnea* species reported a close relationship within the two species, and consider them a single group. Some authors have suggested that the two species might constitute a species pair, in which *U. aurantiacoatra* represents the fertile and *U. antarctica* the sterile counterpart, but phylogenetic studies indicate that they could be conspecific (Wirtz et al. 2012). For this reason, some authors have suggested synonymizing the two species (Lumbsch & Wirtz 2011; Wirtz et al. 2012). In addition, chemical analysis of *Usnea* thalli cannot discriminate between the two morphotypes.

Placopsis antarctica D.J. Galloway, R.I.L. Sm. & Quilhot and ***P. contortuplicata*** I.M. Lamb (fig. 1-C & D) constitute a species pair where *P. contortuplicata* propagates sexually with apothecia and *P. antarctica* propagates vegetatively with soredia. Phylogenetically, the two species are clearly separated into two different clades. The *Placopsis* genus has the highest species diversity in the Southern Hemisphere. *Placopsis* has two photobionts: algae of the genus *Stichococcus* and cyanobacteria of the genus *Nostoc*. *Placopsis* is a crustose genus, spreading into a rosette form whitish to greenish in color; cyanobacteria are separated in cephalodia, an orange structure. Three species of *Placopsis* have been reported in the Antarctic region: *P. contortuplicata*, *P. parellina* (Ny.) I. M. Lamb, and *P. pycnotheca* I. M. Lamb (Øvstedal & Lewis Smith, 2001). However, a study of Galloway and co-authors (2005) found that that Antarctic collections named *P. parellina* do not actually belong to this species. For this reason, they suggested that samples collected as *P. parellina* in the Antarctic should be renamed as *P. antarctica*.

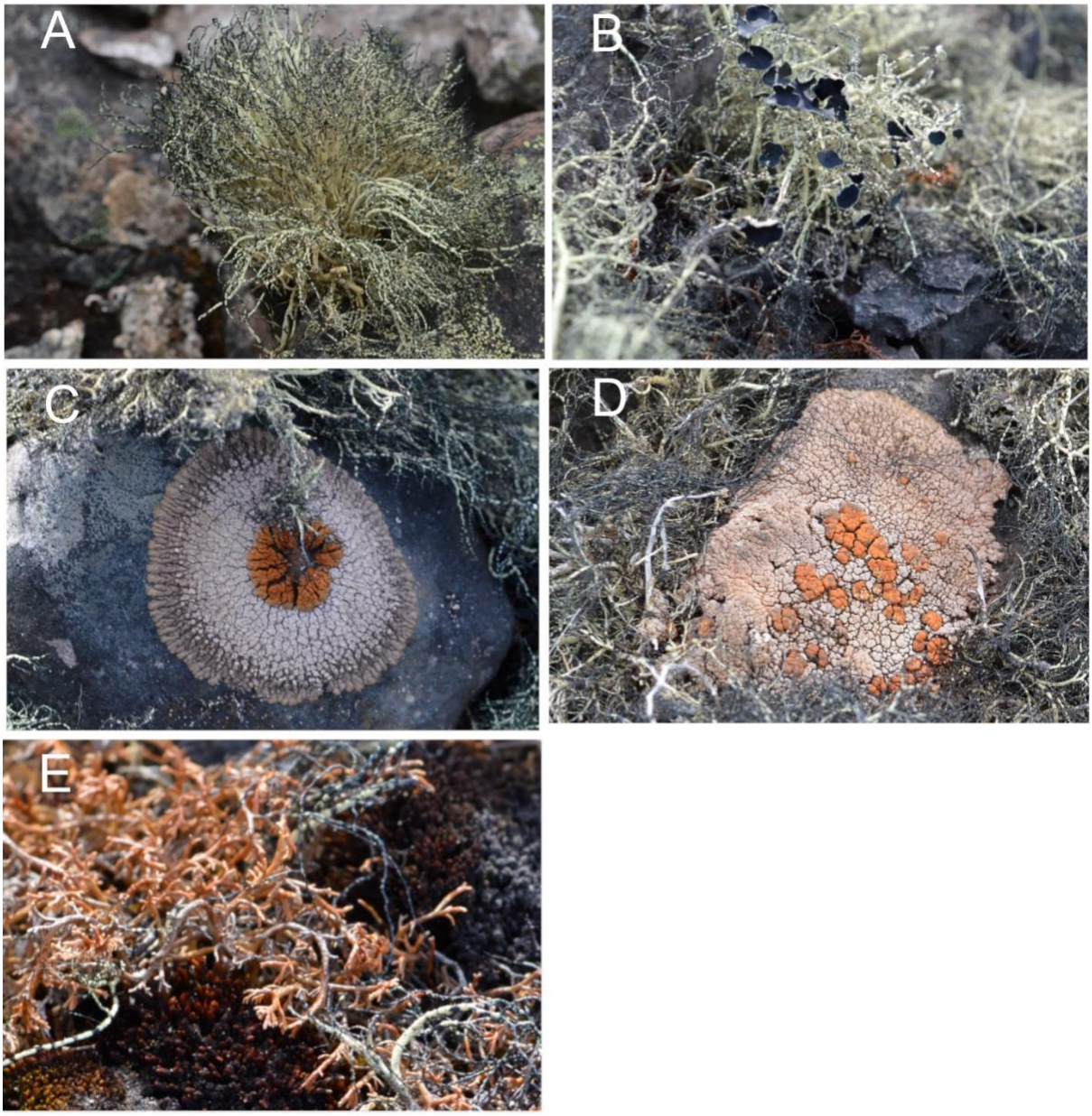

FIGURE 1: Pictures of studied species in King George Island. Photos by Elisa Lagostina.

A. *Usnea antarctica*. B. *Usnea aurantiacoatra.* C. *Placopsis antarctica*. D. *Placopsis contortuplicata.* E. *Cetraria aculeata*.

2.3. SAMPLING: LOCALITIES AND METHODS

The innovation from previous studies adopted in publications 1, 2, and 3 was to evaluate the species pair relationship from stands of *Usnea antarctica* and *U. aurantiacoatra* collected from the same localities. On King George Island, the two species share the same localities and sometimes the same growing spots. Elephant Island stands were chosen to increase the variability of the dataset by including a distant locality (fig. 2 & table 1). An average of 20 samples were taken from each locality. Thalli were collected at a distance of at least 50 cm from each other to avoid sampling clones. Each thallus was isolated in a sampling bag and properly dried to avoid mold. Samples were shipped from the Maritime Antarctic by cargo-ship. The samples were stored at -20°C and defrosted for a short time for analyses. Samples used in the publications were deposited at the Herbarium Senckenbergianum of Frankfurt am Main.

TABLE 1: List of sampling localities and coordinates, day of sampling, and collectors of samples used to develop microsatellites.

Stand	Species	Stand ID	Stand Size	Continent	Country/Region	Locality	Latitude (°)	Longitude (°)	Sampling Date	COLLECTOR(S)
1	*Usnea antarctica*	Elephant Island	19	Antarctica	Elephant Island	Stinker Point	-61.222283	-55.359683	February 8, 2016	M. Andreev
2	*Usnea antarctica*	King George Island 1	19	Antarctica	King George Island	Carlini Station	-62.237000	-58.658200	December 18, 2015	E. Lagostina & B. Kanz
3	*Usnea antarctica*	King George Island 2	17	Antarctica	King George Island	Glacial Point	-62.239383	-58.653360	December 27, 2015	E. Lagostina & B. Kanz
4	*Usnea antarctica*	King George Island 3	19	Antarctica	King George Island	Penguinera	-62.252900	-58.649516	December 22, 2015	E. Lagostina & B. Kanz
5	*Usnea antarctica*	King George Island 4	19	Antarctica	King George Island	Bellingshausen	-62.190260	-58.926733	April 8, 2016	M. Andreev
1	*Usnea aurantiacoatra*	Elephant Island	18	Antarctica	Elephant Island	Stinker Point	-61.222283	-55.359683	February 4, 2016	M. Andreev
2	*Usnea aurantiacoatra*	King George Island 1	19	Antarctica	King George Island	Carlini Station	-62.237000	-58.658200	December 18, 2015	E. Lagostina & B. Kanz
3	*Usnea aurantiacoatra*	King George Island 2	21	Antarctica	King George Island	Glacial Point	-62.239383	-58.653360	December 27, 2015	E. Lagostina & B. Kanz
4	*Usnea aurantiacoatra*	King George Island 3	18	Antarctica	King George Island	Penguinera	-62.252900	-58.649516	December 22, 2015	E. Lagostina & B. Kanz
5	*Usnea aurantiacoatra*	King George Island 4	15	Antarctica	King George Island	Bellingshausen	-62.203616	-58.992750	April 8, 2016	M. Andreev
1	*Placopsis antarctica*	Elephant Island	19	Antarctica	Elephant Island	Stinker Point	-61.222283	-55.359683	February 1, 2016	M. Andreev
2	*Placopsis antarctica*	King George Island 1	8	Antarctica	King George Island	Carlini Station	-62.237000	-58.658200	December 18, 2015	E. Lagostina & B. Kanz
3	*Placopsis antarctica*	King George Island 2	17	Antarctica	King George Island	Fildes Peninsula	-62.190283	-58.926733	February 15, 2018	M. Andreev
4	*Placopsis antarctica*	King George Island 3	12	Antarctica	King George Island	Meseta de la Cruz	-62.206000	-58.953983	January, 2015	A. Beck
5	*Placopsis antarctica*	Robert Island	18	Antarctica	Robert Island	Coppermine Peninsula	-62.371850	-59.717067	January, 2015	A. Beck
6	*Placopsis antarctica*	Livingston Island 1	22	Antarctica	Livingston Island	Mt Reyna Sofia	-62.669100	-60.381030	February 24, 2018	C. Printzen
7	*Placopsis antarctica*	Livingston Island 2	20	Antarctica	Livingston Island	Sally Rocks	-62.701390	-60.416390	February 27, 2018	C. Printzen
8	*Placopsis antarctica*	Livingston Island 3	7	Antarctica	Livingston Island	Nunatak	-62.681020	-60.344190	March 3, 2018	C. Printzen
9	*Placopsis antarctica*	Livingston Island 4	9	Antarctica	Livingston Island	Punta Hesperides	-62.643260	-60.372500	March 6, 2018	C. Printzen
10	*Placopsis antarctica*	Livingston Island 5	23	Antarctica	Livingston Island	Cerro Munigaza Byer	-62.653500	-61.007400	July, 2015	A. Beck
11	*Placopsis antarctica*	Livingston Island 6	19	Antarctica	Livingston Island	Nunatak Clark Byer	-62.667750	-60.912317	July 7, 2015	A. Beck
12	*Placopsis antarctica*	DI	20	Antarctica	Deception Island	Ventana del Chileno	-62.965750	-60.715200	December 4, 2015	A. Beck
13	*Placopsis antarctica*	DI2	19	Antarctica	Deception Island	Crater Lake	-62.986850	-60.675567	December 5, 2015	A. Beck
1	*Placopsis contortuplicata*	King George Island 1	17	Antarctica	King George Island	Meseta la Cruz	-62.206000	-58.953983	January, 2015	A. Beck
2	*Placopsis contortuplicata*	King George Island 2	18	Antarctica	King George Island	Valle Klotz	-62.196333	-58.993367	January, 2015	A. Beck
3	*Placopsis contortuplicata*	King George Island 3	16	Antarctica	King George Island	Fildes Peninsula	-62.232000	-59.010200	February 15, 2018	M. Andreev
4	*Placopsis contortuplicata*	King George Island 5	11	Antarctica	King George Island	Glacial Point	-62.239383	-58.653360	December 27, 2015	E. Lagostina & B. Kanz
5	*Placopsis contortuplicata*	King George Island 6	18	Antarctica	King George Island	Penguinera	-62.252900	-58.649516	December 22, 2015	E. Lagostina & B. Kanz
6	*Placopsis contortuplicata*	King George Island 7	14	Antarctica	King George Island	Tres Hermanos	-62.460833	-58.714444	December 30, 2015	E. Lagostina & B. Kanz
7	*Placopsis contortuplicata*	Livingston Island 1	25	Antarctica	Livingston Island	Pico Radio	-62.665110	-60.394360	February 25, 2018	C. Printzen
8	*Placopsis contortuplicata*	Livingston Island 2	20	Antarctica	Livingston Island	Nunatak	-62.681020	-60.344190	March 3, 2018	C. Printzen
9	*Placopsis contortuplicata*	Livingston Island 3	20	Antarctica	Livingston Island	Mt Reyna Sofia	-62.669100	-60.381030	March 7, 2018	C. Printzen

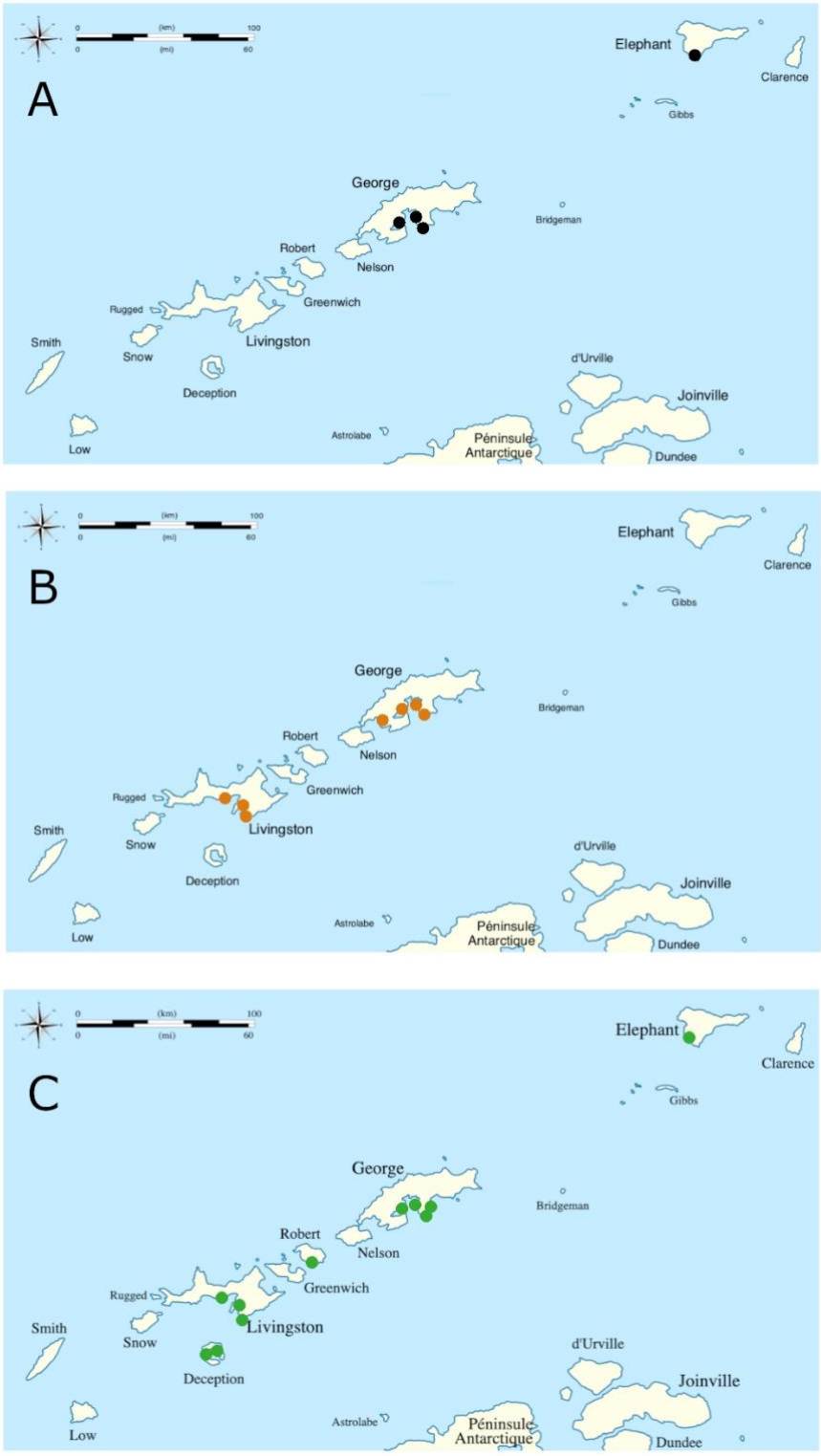

FIGURE 2: Maps of the sampling localities in the South Shetland Islands. A: black dots represent stands of *Usnea antarctica* and *U. aurantiacoatra*. B: orange dots represent stands of *Placopsis contortuplicata.* C: green dots represent stands of *Placopsis antarctica*.

2.4. DEVELOPMENT AND VALIDATION OF FUNGAL-SPECIFIC MICROSATELLITES

2.4.1. SPECIES IDENTIFICATION AND RELATED PROBLEMS

First the target species were morphologically identified. Then, the identification of some samples was confirmed by sequencing the Internal transcribed spacer (ITS) barcoding gene region of both symbiosis partners. BLAST was then used to compare genes in GenBank. For the mycobionts, the primers ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990) were used. For the photobionts *Trebouxia* sp. ITS1T (Kroken & Taylor 2000) and ITS4 (White et al. 1990) were used, and for the photobionts *Stichococcus* sp. Al1500af (Helms et al. 2001) and ITS4 (White et al. 1990) were used. Polymerase chain reactions (PCRs) were performed in 25 µL volume using Illustra PureTaq ready-to-Go PCR beads (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) containing 5 µL of DNA extract and 0.4 nM of each primer. Cycling conditions included initial denaturation at 94 C for 5 min; five cycles of 94 C for 30 s, 54 C for 45 s, and 72 C for 60 s; 33 cycles of 94 C for 30 s, 48 C for 30 s, and 72 C for 60 s; and a final elongation step at 72 C for 10 min. The PCR products were sequenced by Macrogen Europe (Amsterdam, The Netherlands), and the sequences were assembled, edited, and manually aligned with Geneious 10 (Kearse et al. 2012).

Photobionts were identified at the genus level by using BLAST on the sequences in GenBank. The photobionts were checked in order to confirm the identification of the lichens and to know which species the microsatellites must be tested against to confirm the fungal specificity (see next section).

With morphology, it was possible to distinguish *Usnea antarctica* from *U. aurantiacoatra* based on the presence of soredia (preferred over the presence of apothecia because asexual species can also produce apothecia in some conditions). However, molecular identification based on ITS alone has not been able to separate the two species, and some authors have suggested synonymizing them (Lumbsch &

Wirtz 2011; Wirtz et al. 2012). For that reason, the species sequenced in GenBank are not reliable because it is not possible to know exactly to which species the name refers. In addition, chemical analysis of *Usnea* thalli did not discriminate between *U. antarctica* and *U. aurantiacoatra* (see publication 2 for more details). For those reasons, all of the samples were first identified morphologically based on the presence/absence of soredia. Later, their identification was confirmed with a molecular approach based on sequencing the ITS region.

Considering Antarctic *Placopsis* sp., it was possible to discriminate the species pair with morphology due to the presence/absence of soredia. In addition, ITS is a good marker and able to discriminate the two species thanks to the presence of several mutations. However, in GenBank there are only three ITS sequences named *Placopsis contortuplicata* and two sequences called *P. parellina*. Of those two: one *P. parellina* has the same sequence of *P. contortuplicata*, while the other is the real *P. antarctica* with the previous name *P. parellina*. Indeed, in this case using BLAST on samples in GenBank did not confirm the identification, particularly in the case of the P*. antarctica* samples.

2.4.2. DNA EXTRACTION, LIBRARY PREPARATION, AND GENOME ASSEMBLY

To sequence the genomes, it was necessary to extract 1–5 µg of DNA, thus a three day extraction protocol was performed to extract high quality DNA from the target species. Total genomic DNA was extracted from the thalli of *Usnea antarctica, U. aurantiacoatra,* and *Placopsis contortuplicata* from different samples collected in King George Island in the austral summer of 2015/2016. Around twenty milligrams of thallus were pre-treated with acetone to remove secondary metabolites and ground with liquid nitrogen using a sterilized mortar and pestle. DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Cubero & Crespo 2002) as reported in publication 1. DNA samples from all three species were shipped to LGC genomics for library preparation and were sequenced with a V3 MiSeq Illumina (2 x 300 bp). In a second run, *Placopsis antarctica* was sequenced with a HiSeq 2500 Illumina (2x100 bp). *P. antarctica* DNA was extracted with the same CTAB

protocol and sent to Macrogen Korea. A TruSeq Nano DNA Kit was used to make the library. The reads obtained for each genome are summarized in table 2.

TABLE 2: List of total raw reads for the four sequenced lichens metagenome.

Species	Total raw reads
Usnea antarctica	3,098,758
Usnea aurantiacoatra	1,755,882
Placopsis antarctica	53,165,666*
Placopsis contortuplicata	12,917,692

* Sequenced with Illumina HiSeq.

Raw reads were first checked to remove the Illumina adapter, and then were quality (PHREAD = 26) and length- (>150 bp) filtered using Trimmomatic version 0.35 (Bolger et al. 2014). Forward and reverse reads were assembled with Paired-End reAd mergeR (PEAR) software (Zhang et al. 2014). The resulting overlapping, paired, and singleton reads were then assembled with SPAdes version 3.9 (Nurk et al. 2013). Assemblathon2 (Bradnam et al. 2013) was used to check the N50 and genome size of each lichen. Scaffolds were taxonomically binned using Metawatt (Strous et al. 2012). Scaffolds assigned to the phylum Ascomycota (table 3) were used to search for mycobiont-specific microsatellite motifs.

TABLE 3: Reports of the Ascomycota genomes: number of scaffolds, scaffold size, N50, and obtained genome size for each species from Assemblathon2.

Species	n° of scaffolds	Scaffolds size	N50	Genome size
Usnea antarctica	5,854	50,854 - 718 bp	5,596	32,130,291
Usnea aurantiacoatra	6,402	31,278 - 718 bp	5,706	27,329,760
Placopsis antarctica	4,636	230,836 - 544 bp	17,790	32,040,444
Placopsis contortuplicata	2,216	224,439 - 699 bp	26,604	30,870,760

To extract DNA for microsatellites, analyses were performed using a faster protocol based on a kit. In the case of *Usnea* species, the total DNA was extracted from young terminal branches without signs of infection by parasitic fungi. For *Placopsis* species, DNA was extracted from one areole without any visible contamination. Branches or areoles were collected in 2-mL reinforced tubes with metal beads inside and ground with the Bead Ruptor 24 (Omni International, Kennesaw, Georgia) in three cycles of 25 s at a speed of 4.20. Before each cycle, tubes were dipped in liquid nitrogen for 15 s. DNA was extracted with the Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Microsatellite primers were tested on eight samples of *Usnea* sp. or *Placopsis* sp.; in total, 30 SSRs were designed for both *Usnea* and 25 SSRs for both *Placopsis*. A touch down PCR protocol was performed to increase the PCR yield (reported in publication 1) and PCR products were sequenced to confirm that all samples had the same size flanking regions and that there was variability in the microsatellite regions. Finally, 23 SSR markers (table 4) were selected for the *Usnea* species pair and were multiplexed in 3 PCR reactions; 15 SSR markers (table 5) were selected for the *Placopsis* species pair and were multiplexed in 2 PCR reactions.

2.4.3. DEVELOPMENT AND VALIDATION OF FUNGUS-SPECIFIC MICROSATELLITE PRIMERS

The obtained genomes of both *Usnea* were the lowest quality ones. However, the possibility to compare the two genomes was an advantage. Later, thanks to the successful amplification rate reported in the species pair *Usnea antarctica-U. aurantiacoatra*, another species pair *Placopsis contortuplicata-P. antarctica* was chosen for designing SSRs, which also had the samples with the best-obtained genome.

The protocol used to develop the microsatellites was reported in publication 1 and was unmodified for the *Placopsis* species. Briefly, the software MISA (MIcroSAtellite identification tool; Beier et al. 2017) was used to identify SSRs, after testing the flanking region *in silico*: each SSR repeat from one genome was compared in BLAST against the other genome. In addition, as reported in publication

2, long sequences containing SSRs were tested to confirm that primers were specific to Ascomycetes. Sequences at least 1000 bp long were searched in GenBank using a BLASTn approach and 21 of 23 contigs held genes with sequences similar to Ascomycota.

To confirm fungal specificity, SSRs were tested on pure cultures of the photobionts. DNA extraction followed the same protocol used for lichens and PCR conditions are reported in publication 1. For *Usnea,* four strains of *Trebouxia jamesii* (Hildreth and Ahmadjian) Gärtner isolated in pure culture were selected. Each strain was taken from a different species of *Usnea*: *U. antarctica*, *U. aurantiacoatra*, *U. trachycarpa* (Stirt.) Müll. Arg and *U. lambii* (Imshaug) Wirtz & Lumbsch. In the case of *Placopsis*, the SSR primers were tested on two algal strains of Antarctic *Stichococcus* isolated from *P. contortuplicata* and three bacteria of the genus *Nostoc* isolated from *Placopsis* sp. of Antarctic and South American samples. PCRs were unsuccessful for all the photobionts and fungal specificity of the SSRs was confirmed.

Because it has recently been shown that Basidiomycetes may be obligate partners of ascomycetous lichen symbioses (Spribille et al. 2016), it was checked whether SSR markers were specific to Ascomycetes by comparing the contigs from which they were developed against the National Center for Biotechnology Information (NCBI) database using the BLASTn approach. Twenty-one out of the 23 contigs (ca. 3000–35000 bp in length) contained genes with sequences most similar to Ascomycota. Two of the five shortest contigs did not show close similarity to any sequences deposited in GenBank.

To reduce the cost of fluorescent primers and to easily multiplex the reactions, a fluorescent dye–associated tag was attached to the forward primers. Four different tails were selected to multiplex the reactions (FAM: GCCTCCCTCGCGCCA, VIC: GCCTTGCCAGCCCGC, NED: CAGGACCAGGCTACCGTG, PET: CGGAGAGCCGAGAGGTG). In this way labelled universal primers begin to be incorporated into PCR fragments in early PCR cycles, tailed forward primers are exhausted in early cycles, and subsequent PCR cycles incorporate fluorophores into PCR fragments (fig. 3). The advantage is to simultaneously co-amplify and analyse multiple loci with similar-sized alleles in a single PCR reaction and to reduce the cost of fluorescent primers (Blacket et al. 2012). To multiplex eight SSR markers it was

necessary to design PCR products that did not overlap; 100 bp of difference was the minimum requirement.

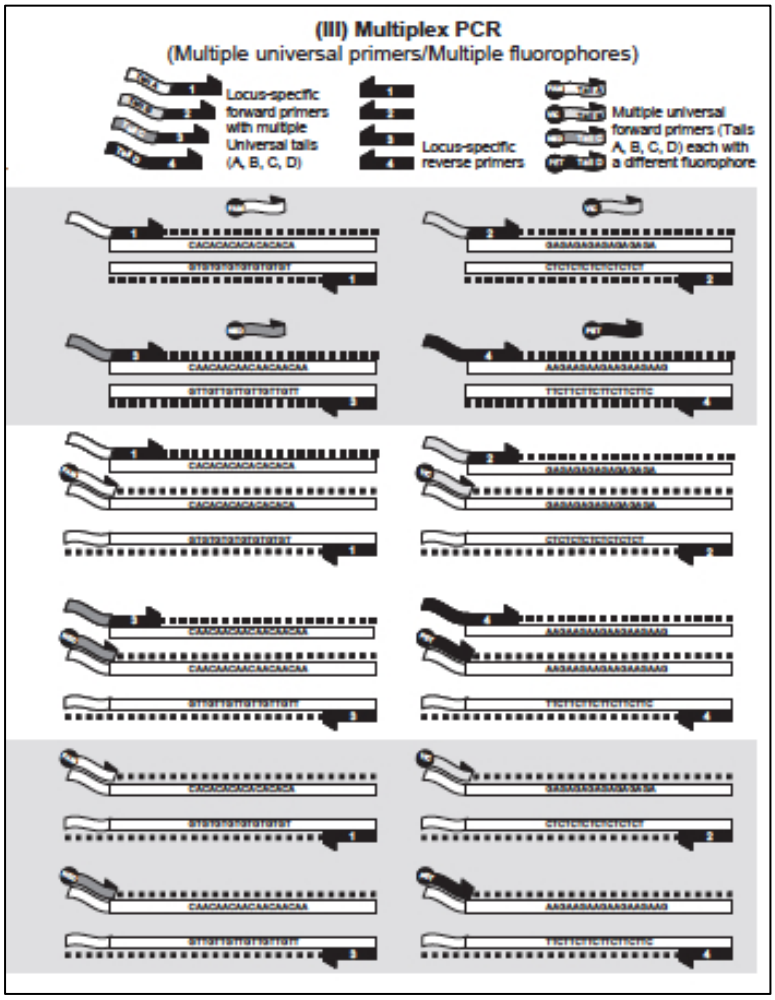

FIGURE 3: Amplification of PCR fragments with fluorescently-labelled high annealing temperature universal primers and locus-specific tailed forward primers (partial figure 1 from Blacket et al. 2012).

TABLE 4: Characteristics of 23 fungus-specific microsatellite primers cross amplified for *Usnea antarctica* and *U. aurantiacoatra*. In the table are reported the locus name, the primer sequences, the SSR repeat motif, the tail attached at the forward primers (A:GCCTCCCTCGCGCCA; B: GCCTTGCCAGCCCGC; C: CAGGACCAGGCTACCGTG; D: CGGAGAGCCGAGAGGTG), the fluorescent dye, the SSR size range (including the size of the tail), and the numbers of repeats.

	Locus	Primer Sequence (5'-3')	Repeat motif	Tail	Fluorescent dye	Size range	No. of SSR repeats
MM1	Ua1	F: GCATCTGGGCTCTTGGACTT R: CATTTGCAGGCAGTCCATCG	$(CTT)_n$	A	FAM	235-271	3-15
	Ua2	F: GGGATAACTCGCTATGGCCC R: ACACCCTGATCGATCAAACCA	$(CTT)_n$	B	VIC	256-283	6-15
	Ua3	F: AAGCACACGCAAAGCTTCAG R: CGGAGGTCTGAATGTCGGAG	$(CTG)_n$	C	NED	248-275	4-13
	Ua4	F: CTTTCACTGTCCTGCCCTGT R: GAGACCCCGTGTCCAATCAG	$(CCT)_n$	D	PET	277-304	2-13
	Ua5	F: GGAAGGGGAAGGGGAGAGAT R: GGTGGGCAACTGGAATGGTA	$(CTT)_n$	A	FAM	555-573	8-14
	Ua6	F: TTGAGCCGCCACAAGAGATT R: ATCGGCCAATTGATACCCCG	$(AAG)_n$	B	VIC	337-349	4-8
	Ua7	F: AAGACGGACATTCCACCACC R: ACCGCTCTGGCTACCTCTTA	$(GGA)_n$	D	PET	552-573	2-9
	Ua8	F: AAGAAGCCAGCTTTGACGGA R: GCTTGTCTCAGGCAGGATGA	$(TGT)_n$	A	FAM	393-402	6-9
MM2	Ua9	F: AACGGAGCTTCCTTCCATTGA R: ACAACACAGACAACCCCGAA	$(CATC)_n$	A	FAM	285-297	8-11
	Ua10	F: GACTTTACGGCCCACATCCA R: TTTCCATGTGGCTTGGAGGG	$(AGA)_n$	B	VIC	284-314	4-14
	Ua11	F: TCGCATTATTCGTGCAAGCG R: GTAATATCCGCTCGCCCACA	$(TATG)_n$	C	NED	254-274	5-10
	Ua12	F: AGGCGCTGTGTGAGAACC R: AGCAAGACCAAGAAGGCGAG	$(CGAA)_n$	D	PET	232-256	3-9
	Ua13	F: CCAAGCCAACCTCAGACCAT R: CGACGTCTCCTTCCATAGCC	$(CAG)_n$	A	FAM	393-408	4-9
	Ua14	F: GTCAGCCCATCTACCGTACG R: TGGGTTGGGAAAGGAAGTGT	$(GAA)_n$	B	VIC	386-419	5-16
	Ua15	F: CGCAAACAGTACAACCGGAA R: GCCACAACAAAGGTGACGAC	$(GCT)_n$	C	NED	341-347	5-7
	Ua16	F: GTTTGGAAGACCACCGGCTA R: CCAAGCACACCCTGACATCT	$(AGT)_n$	D	PET	334-355	10-17
MM3	Ua17	F: ATGACGTGCTGTAGGTGTGG R: GTGTCAAGTGTCGAGCAGGA	$(CTGGTA)_n$	A	FAM	403-415	4-6
	Ua18	F: AGGGAGTTCTGCAGGGGATA R: AGTGATTGATGCTCCGGTGG	$(GAA)_n$	B	VIC	355-388	6-17
	Ua19	F: AGCCATTTTTCCGAGGTCGT R: GCTTTGTTGCGCTTCACTGA	$(GAC)_n$	C	NED	349-367	6-12
	Ua20	F: GATCACTCTTCGAGCTCCCG R: CCAGAGTACCTTCCGTTGCA	$(AAGC)_n$	D	PET	405-421	6-10
	Ua21	F: TTCCCGAGCTCCAATCACAC R: CCATATCCCGTCCTCGCAAA	$(TCC)_n$	A	FAM	260-272	7-11
	Ua22	F: TGGTCCACTTTAGCCAGTCAT R: TCTGCCCTTGACATCTTTGACA	$(ATG)_n$	C	NED	272-290	8-14
	Ua23	F: TAGTGCGAGGCCTGATGTTC R: ACCGAAAAGGCTTGGACGAT	$(CTT)_n$	D	PET	244-253	6-9

TABLE 5: Characteristics of 15 fungus-specific microsatellite primers cross amplified for *Placopsis antarctica* and *P. contortuplicata.* In the table are reported the loci names, the primer sequences, the SSR repeat motif, the tail attached at the forward primers A:GCCTCCCTCGCGCCA; B: GCCTTGCCAGCCCGC; C: CAGGACCAGGCTACCGTG; D: CGGAGAGCCGAGAGGTG), the fluorescent dye, and the SSR size in the genome of *Placopsis antarctica*.

	Locus	Primer Sequence (5'-3')	Core Motif	Tail	Fluorescent dye	Size
MM1	MSP24	F: ATAGCACCAAGACCAACGCA R: CGCGCCCCCATAAAAATCTG	(CCCT)n	A	FAM	308
	MSP9	F: GATGGGTGCAGTAAGGCCTT R: GCTGCCCGATCATCCATACA	(TCT)n	B	VIC	276
	MSP2	F: CGGCGTGGTGATGGAAGATA R: CCGTCTACTGTGCCCAAGTT	(GCT)n	C	NED	251
	MSP16	F: AGTAATGGCGGTGTGACAGG R: CATCATGGGTCGTGCAACAC	(GAG)n	A	FAM	421
	MSP15	F: ACGAAGATCCGCCTATCGAA R: ACTGGTCTAAAGGGCTGCTT	(ATA)n	B	VIC	381
	MSP6	F: TCCAGCGAAAATCCAGCAGT R: GGGCCCAAATGCAATGTCAT	(AAG)n	C	NED	396
	MSP18	F: AGGTAAATTGGCGCAGACCA R: GGTGGGAGCGATGACTTCAA	(GAT)n	D	PET	358
MM2	MSP19	F: ATTAACAGCCACCCCGTCTC R: AGACCTGACTTTCCAAGCGG	(CTT)n	A	FAM	206
	MSP20	F: CGCAGTACCCGCATCTTTTG R: CAGGCCCTGGAAGGATTTGT	(CAG)n	B	VIC	216
	MSP11	F: CACGGGGCTTTCGATGAGAA R: TAAGACCCATCCGACACCCT	(GCG)n	C	NED	282
	MSP22	F: CCTGCTCCCCTTTACTTCCC R: GGAGGTCATCAAGTCGCGAT	(CAC)n	D	PET	253
	MSP13	F: CCAGCCTCTAATTGACCCCG R: AAGACTCGGCCGAAACAGAG	(CCA)n	A	FAM	348
	MSP14	F: CTAGGGATTTCTAGGGCGCG R: AATGTCAATCTCACCGCCGT	(ATC)n	B	VIC	373
	MSP23	F: CTTCCCTCGGCTCAAGGTTT R: TGAAAGGGCTTGTGGAGGTG	(TGGA)n	C	NED	400
	MSP26	F: TCTGGGGTGCTATGAGTGGA R: AAATCTCCGCCCGTGTTCAT	(GGAT)n	D	PET	378

The variability of each microsatellite locus was measured by counting the number of alleles for each SSR marker. Publication 2 reports that the rate of success of amplifications across *Usnea* species was confirmed by the low numbers 0.5% of null alleles for all the King George and Elephant Island stands. Analyses of alleles

from publication 1 is reported below, merged with data from *Placopsis* population analyses (table 6). A total of five populations of *P. antarctica* collected in the South Shetland Islands (Elephant, King George, Robert, Livingston, and Deception) and two populations of *P. contortuplicata* (sampled only in King George and Livingston Islands) were used to check the variability of the *Placopsis* SSR markers. The number of alleles for each stand were measured using GenAlEx (Peakall & Smouse 2006, 2012).

TABLE 6: Number of samples, numbers of alleles, and number of effective alleles for each stand of *Usnea antarctica* and *U. aurantiacoatra* and populations of *Placopsis antarctica* and *P. contortuplicata*.

Population	n° of Samples	n° of Alleles	n° of Effective Alleles
U. antarctica King George C1	20	2.609	1.704
U. antarctica King George P3	19	2.609	1.849
U. aurantiacoatra King George C1	20	3.217	2.035
U. aurantiacoatra King George P3	18	3.652	2.344
P. antarctica Elephant	19	2.000	1.423
P. antarctica King George	42	3.267	1.784
P. antarctica Robert	18	1.933	1.470
P. antarctica Livingston	99	3.133	1.702
P. antarctica Deception	39	2.067	1.522
P. contortuplicata King George	97	3.600	1.605
P. contortuplicata Livingston	65	2.400	1.446

Microsatellites markers were variable within all species tested and the variability in terms of the numbers of alleles was a bit higher for the sexual species in *Usnea*. This result was not surprising since one advantage of sexual reproduction is the capability to increase variability inside populations.

The size of the flanking region was about the same number of bases in both closely related species. The markers showed high genetic variability even within a small geographic area.

2.5. HOW TO DELIMIT SPECIES PAIRS

In this section are described three different molecular methods used to delimit the species pair *Usnea antarctica* and *U. aurantiacoatra*. It is the first work that applies microsatellites and SNPs to lichens to delimit closely related species.

2.5.1. SPECIES DELIMITATION USING A FUNGAL UNIVERSAL BARCODING GENE

The ITS markers of 179 Antarctic *Usnea* samples were sequenced with primers ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990). The ITS of 86 samples of *Usnea antarctica* and 93 samples of *U. aurantiacoatra* were analysed with a haplotype network (fig. 4) with the TCS algorithm implemented in popART (Clement et al. 2002), as reported in publication 2. Due to the misidentification of sample EL36 (described as *U. antarctica* and later identified as *U. aurantiacoatra* with a parasitic fungus), which was explained in the discussion of publication 2, the haplotype network shown in this chapter has been recalculated. The total ITS alignment was 666 bp long and included an intron of 216 bp in length that was 226 bp from the 3′ end of the small subunit of the ribosomal RNA. The alignment included 33 polymorphic sites, 15 of them in the intron. Figure 4 shows the newly run haplotype network. It shows 33 haplotypes, and *U. aurantiacoatra* was genetically more variable (20 haplotypes) than *U. antarctica* (14 haplotypes). Sorediate and esorediate morphs do not form reciprocally monophyletic groups on the haplotype network, and even share one haplotype. Nevertheless, they appear relatively well clustered.

The debate around the species status of *U. antarctica* and *U. aurantiacoatra* arose because phylogenetic trees and haplotype networks did not resolve the two as mutually exclusive monophyletic lineages (Lumbsch and Wirtz 2011; Wirtz et al. 2012). The haplotype network (fig. 4), based on a dataset that includes a polymorphic type I intron near the end of the ribosomal small subunit (18S) that has

not previously been studied in this group, confirms this finding. Similar to previous studies, it shows the two morphotypes as nonmonophyletic lineages sharing one haplotype with each other.

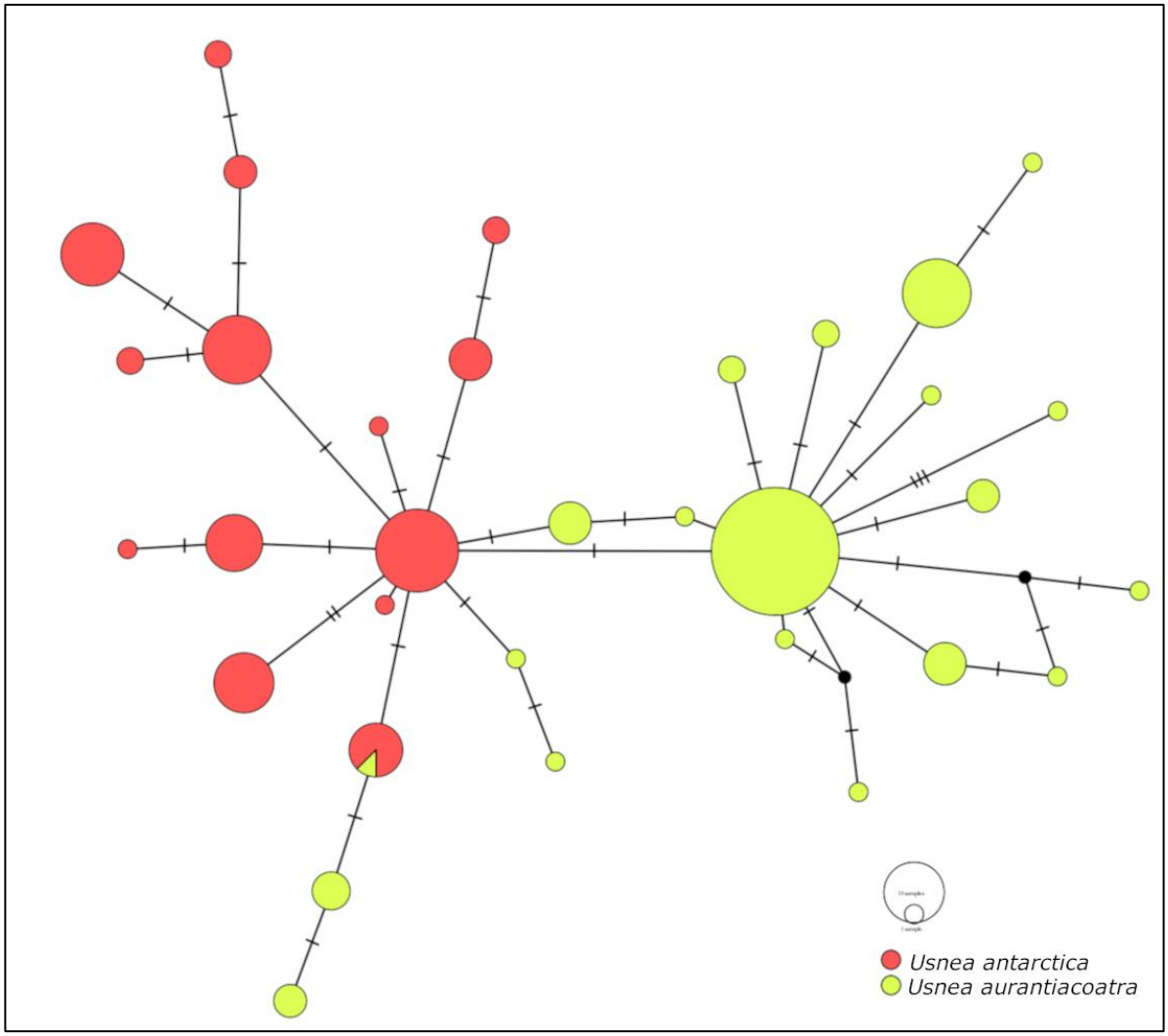

FIGURE 4: Haplotype network of 86 *Usnea antarctica* and 93 *U. aurantiacoatra* based on an alignment of 666 bp from partial 18S-ITS1-5.8S-partial ITS2 sequences. Red circles represent *U. antarctica* and green circles represent *U. aurantiacoatra*. Long lines connect linked haplotypes and small horizontal lines represent the number of modifications between haplotypes. The size of the dots is correlated with the number of samples belonging to each haplotype.

2.5.2. SPECIES DELIMITATION USING MICROSATELLITES

A total of 95 samples from each Antarctic *Usnea* morphotype was tested to finally resolve the phylogenetic relationship of the species pair. The unpublished analysis of the species pair *Placopsis antarctica - P. contortuplicata* was added to this chapter to confirm the ability of microsatellites to separate morphotypes into two discriminate species. The dataset was made by using 15 SSRs from stands of *P. contortuplicata* collected in King George and Livingston Islands and 13 stands of *P. antarctica* collected in King George, Robert, Livingston, and Deception Islands (fig. 2 & table 1).

A Discriminant Analysis of Principal Components (DAPC) was made with the R package adegenet 2.1.0 (Jombart 2008; Jombart & Ahmed 2011). DAPC was chosen as the best method to confirm the ability of the SSR markers to discriminate the two species. It was preferred over classical multivariate analyses (PCA, DA) because the purpose was to identify groups and, in contrast to other multivariate methods, DAPC attempts to maximize among-group variation (Jombart et al. 2010). A DAPC for the *Usnea* species pair was run with 40 PCs and 1 DA based on the dataset of publication 2, with sample EL36 reassigned to *U. aurantiacoatra*. The DAPC for the two *Placopsis* species was run with 20 PCs and 1 DA.

Publication 2 reports the success of the microsatellite amplification, and a few null alleles were reported (0.5%) from a total of 4370 alleles. In the discussion of publication 2, sample EL36 was reassigned because it was a misidentified *Usnea antarctica*. A DAPC was tested with 1 DA and 40 PCs based on the corrected dataset from publication 2 (fig. 5).

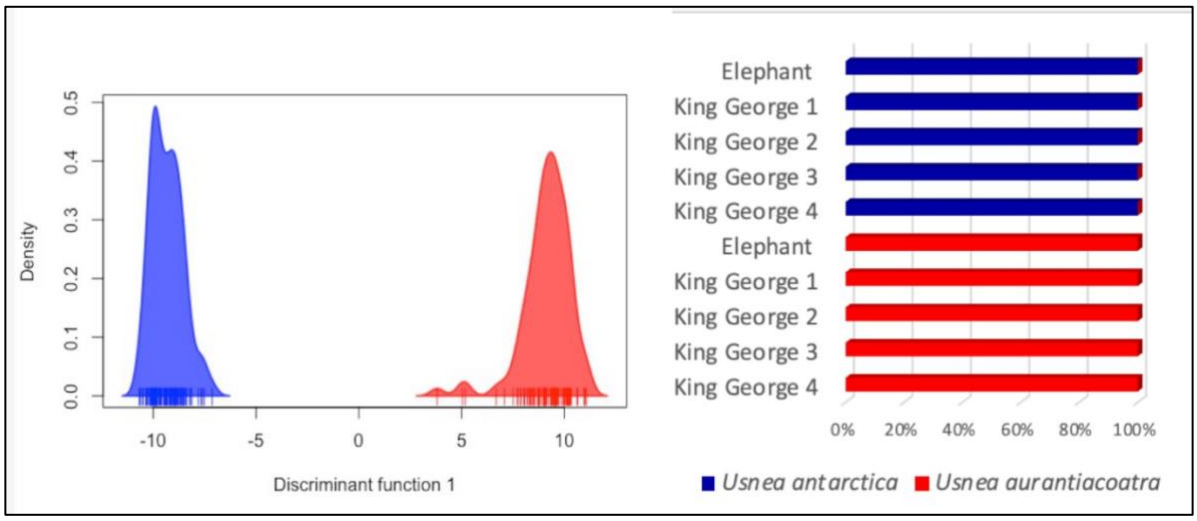

FIGURE 5: DAPC with 40 PCs and 1 DA, based on the samples of publication 2. *Usnea antarctica* is in blue and *U. aurantiacoatra* is in red. On the left is the graph density over Discriminant Function 1. On the right is the sample distribution into clusters.

The *Placopsis* species pair was also successfully amplified; 2.7% null alleles were reported from a total of 5580 alleles. A DAPC was run with 1 DA, and 20 PCs (fig. 6).

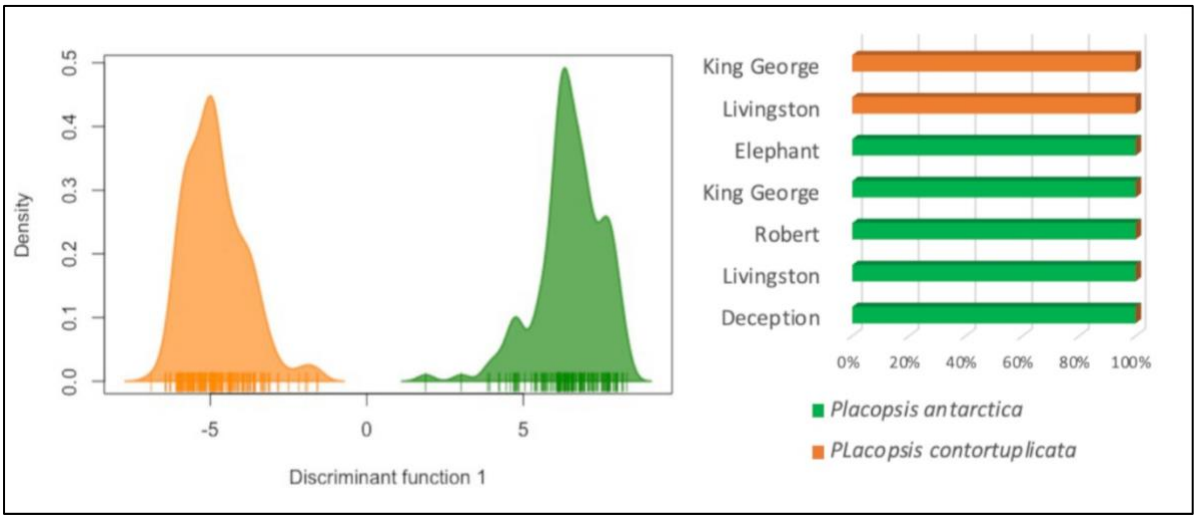

FIGURE 6: DAPC with 20 PCs and 1 DA, from 13 stands of *Placopsis antarctica* (in green) and 9 stands of *P. contortuplicata* (in orange) from South Shetland Island. On the left is the a graph density over Discriminant Function 1. On the right is the sample distribution into clusters.

2.5.3. ANOTHER APPROACH FOR SPECIES DELIMITATION: RAD SEQUENCES

Publication 3 used a different method to investigate the *Usnea* species pair based on SNPs obtained with NGS technologies. A small number of samples used in publication 2 were selected to undergo DNA extraction again and were sequenced with Illumina MiSeq. The final dataset included 105 samples: 58 identified as *U. antarctica* and 47 samples of *U. aurantiacoatra*. Out of all *U. antarctica* samples, 9 and 12 samples came from Primavera and Esperanza bases, respectively (see chapter 2 for sample information). On the other hand, *U. aurantiacoatra* was collected only in King George and Elephant Islands.

DNA was extracted again because RADseq needs high-quality DNA and a concentration of 200 pg. In order to reach the minimum amount of DNA required, an entire branch of *Usnea* sp. was manually grinded with a mortar and pestle and liquid nitrogen, and then the DNA was extracted with a ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) without modifications.

Library preparation is reported in publication 3. In short, for the RADseq library production, DNA isolations were pooled with sequence adapters (Rubin & Moreau 2016), subsequently digested with the restriction enzyme ApeKI (New England Biolabs, Ipswich, MA, USA) and ligated using T4 ligase (New England Biolabs). Up to 48 samples with compatible barcodes were pooled and selected for fragments of sizes between 300 and 500 bp using the BluePippin DNA size selection system (Sage Science, Beverly, MA, USA). The pooled libraries were amplified using the REDTaq ReadyMix (Sigma-Aldrich, St. Louis, MO, USA) prior to sequencing on an Illumina MiSeq using the MiSeq Reagent Kit v3 for 150 cycles (Illumina, San Diego, CA, USA) to produce single-end sequences with a length of 150 bp. The pyRAD assembler was used to generate loci. This process used a combination of the ipyRAD (https://github.com/dereneaton/ipyrad/blob/master/docs/index.rst) and pyRAD (Eaton & Ree 2013) pipelines with an additional mapping step that filtered for lichen-fungal loci with a reference sequence. An average of 21.8% (sd = 2.9%) of all loci were mapped to the *de novo* assembled *Usnea strigosa* lichen fungus reference genome (because the genome completeness of both *U. antarctica* and *U. aurantiacoatra* was not sufficient for this analysis – see previous section for genome details) and, of these loci, an average of 85.4% (sd = 5.5%) were included into the

final pyRAD dataset. Two samples of *U. antarctica* (EL59, EL281) and two samples of *U. aurantiacoatra* (EL415, EL437) were removed from the analysis due to lower numbers of loci. All remaining 101 samples in the final dataset had on average of 4,143 (sd = 1,316) loci (Supplemental material 2 in publication 3).

A DAPC was newly run to evaluate the capability of RADseq to discriminate species pairs and to compare the ability of SSRs and RADseq to discriminate species pairs. The DAPC was conducted by using the first 50 principal components and all DA-eigenvalues (fig. 7).

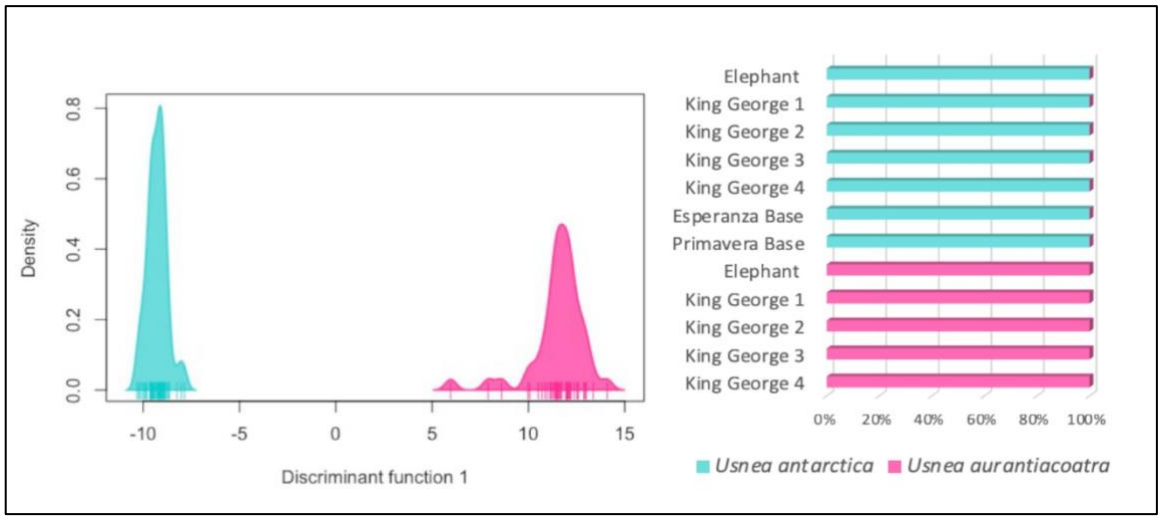

FIGURE 7: DAPC with 50 PCs and 1 DA, based on samples from publication 3. *Usnea antarctica* is in light blue and *U. aurantiacoatra* is in pink. On the left is the a graph density over Discriminant Function 1. On the right is the sample distribution into clusters.

2.6. DISCUSSION

The aim of the chapter is to provide tools able to delimit species pairs and characterize the genetic structure of Antarctic lichens. Uncertain species delimitations can undermine population-level studies of lichens. Recent studies based on molecular analyses have found increasingly more evidence that species pairs do exist (Cornejo & Scheidegger 2015), and this chapter confirms it. These are the two Antarctic species pairs that have been analysed: *Usnea antarctica* - *U. aurantiacoatra* and *Placopsis antarctica* - *P. contortuplicata*. The analyses are based on samples collected in the same localities in order to exclude the confounding effects of geographical population structure; the samples were restricted to the maritime Antarctic populations where the two morphotypes grow together (fig. 2 & table 1). Five populations of *Usnea* sp. and 22 populations of *Placopsis* sp. were tested to develop and validate fungus-specific microsatellites. Both species pairs have molecular identification problems due to a lack of information in GenBank. The fungal universal DNA barcode ITS can discriminate the *Placopsis* species pair but using BLAST on the ITS sequence in GenBank cannot confirm the identification. In contrast, for Antarctic *Usnea* the concept of species is still debatable because ITS cannot separate the species pair. This thesis is the first study to utilize the ability of microsatellites to solve the uncertain species delimitation of species pairs. In both species pairs, microsatellites easily separated them (fig. 5 & 6) and offered a fast tool to identify even small or young samples in which morphological structures are difficult to analyze.

Generally, few lichens have been investigated with microsatellites and the number of markers rarely exceeds ten (Werth et al. 2015). This chapter is the first to develop a high number of SSRs able to amplify closely related species: 23 fungal specific SSR markers have been developed for the species pair *U. antarctica* and *U. aurantiacoatra*, and 15 SSRs for *P. antarctica* and *P. contortuplicata*. Due to the symbiotic nature of lichens, microsatellites have been chosen because they can discriminate the mycobiont from photobionts, they do not require high-quality DNA to be tested, SSRs are cheap to analyze without involving Sanger sequencing, and they can be multiplexed to obtain a lot of information from a single sample in one PCR

reaction. Two innovations were introduced in this work. First, markers were developed in species pairs by using BLAST *in silico* to compare each SSR repeat from one species against its species pair genome. Those comparisons allowed the selection of only markers with the same length of flanking regions, and it gave an advantage of reducing sequencing time. It was possible to create cross-amplifying markers that can be used in other species as well because flanking regions are more conservative (see publication 1). Also, this method reduces the possibility of generating non fungal SSR markers, because an error during the fungal genome assembly must happen twice and those miss-assigned sequences must carry a microsatellite in order to be selected. Besides, the fungal specificity has been confirmed by amplifying the SSR primers in PCR with DNA from pure algal cultures isolated from different lichens. The PCR reactions did not amplify any SSR markers. Second, this work is the first report in lichenology to use SSRs to delimit species pairs.

The recent debate around the species status of *U. antarctica* and *U. aurantiacoatra* arose because phylogenetic trees and haplotype networks did not resolve the two as mutually exclusive monophyletic lineages (Lumbsch & Wirtz 2011; Wirtz et al. 2012). The haplotype network (fig. 4), based on a dataset including a polymorphic type I intron near the end of the ribosomal small subunit (SSR) that has not previously been studied in this group, confirms this finding. Similar to previous studies, it shows that the two morphotypes are non-monophyletic lineages sharing one haplotype with each other. The two morphotypes, however, are clustered in the network, a pattern confirmed by the DAPC result. Even if it is speculative, previous studies may also have suffered from the inclusion of misidentified samples. Two different methods based on SNPs and SSRs can discriminate the Antarctic *Usnea* species pair.

Phylogenetic and population genomic results from the SNP dataset clearly delimited *U. antarctica* and *U. aurantiacoatra* into two lineages, supporting the acceptance of two species (fig. 7). It confirms that closely related species are difficult to separate using sequence-based multi-locus approaches and that great care should be taken when interpreting results from molecular studies when it comes to testing for conspecificity. On the other hand, the microsatellite-based multi-locus rendered almost identical results (fig. 5), including 100% correct assignment of samples to their species.

In publication 3, three pairs of samples of *U. antarctica* have been reported to be very close relatives with high co-ancestry. Those three pairs were collected on Elephant Island (samples EL382 and EL409), King George Island (samples EL001 and EL409), and on the Antarctic Peninsula (samples EL713 and EL743), respectively, and may indicate almost immediately related clones. On Elephant Island and the Antarctic Peninsula, the pairs were collected in the same locations with a greater chance to pick up clones. However, the clonal pair from King George Island must have dispersed between Fildes and Potter Peninsula over ice or water boundaries prior to the collection. Contrarily, none of the individuals of *U. aurantiacoatra* expressed similarly close relationships. However, the same three pairs of samples tested with microsatellites did not show any evidence of clonality because in a total of 23 markers, three to six SSRs were different in each pair. This comparison suggests that both methods have a different resolution and SSRs are still a good method to check variability inside populations.

Microsatellite markers were variable within all *Usnea* and *Placopsis* species tested. The variability in terms of the number of observed alleles and effective alleles is a bit higher for the sexual species in *Usnea* species. This result is not surprising since one advantage of sexual reproduction is the capability to increase variability inside populations. Although lower genetic variability was detected in *U. antarctica* than in *U. aurantiacoatra*, this result probably reflects the higher effective population size of the sexually reproducing species. *U. antarctica* was surprisingly variable, contradicting the old idea that asexually reproducing, sorediate lichens constitute “clones or groups of clones” (Tehler 1982).

At the morphological level, this study confirms that the presence and absence of soredia can be used to safely discriminate between *U. antarctica* (with soredia) and *U. aurantiacoatra* (without soredia). The presence of apothecia, on the other hand, is a more unreliable character, because young thalli of *U. aurantiacoatra* often lack apothecia. Care must also be taken to not mistake galls on parasitized thalli of *U. aurantiacoatra* for soralia, as the single apparently wrongly assigned sample shows.

This last section is dedicated to a comparison of the two newly developed methods to characterize stands of Antarctic *Usnea*. Both SSRs and SNPs require a sequenced genome to perform the analysis. In the case of SSRs, the genome can

have lower coverage. In order to obtain SNPs from RADseq reads, a congeneric species (*U. strigosa*) was used without lowering the yield of the SNPs dataset, since the already generated genomes of both Antarctic *Usnea* did not have enough coverage (table 3). The reference genome comes from a congeneric species, allowing the application of RADseq to organisms without an already sequenced genome that come from a family where a species has been sequenced. For the first time, a genome generated from DNA extracted from the thallus instead of an axenic fungal culture was used to map a large number of fungal loci sufficient for population genomic methods. This widens the potential application of RADseq for intimate symbiotic organisms and includes studies where cultures of one symbiotic partner are not readily available. On the other hand, the samples involved in the SNP study were just a small part of the dataset analysed with microsatellites because the preparation of the library for RADseq required a high quantity of DNA. So, at the moment only lichens with big thalli can be analysed with RADseq without involving PCR to increase the DNA concentration.

Regarding the number of samples tested at the same time, in the case of SSRs each sample can be tested at the same time with 7-8 multiplexed markers, and 96 samples can be analysed for each plate. In contrast, the library preparation for RADseq has been developed for only 46 labels for 46 samples. In any case, both methods can be optimized by choosing different sizes and colors for multiplexed microsatellites or by changing the Illumina sequencer that can pool more samples together. Analysing a RADseq dataset requires a strong background in computing skills and bioinformatics (Hodel et al. 2016) and faster computers or servers to speed up the analysis of the large quantity of data. In contrast, SSRs are manually scored with user-friendly programs on a regular laptop. The obtained datasets of SSRs and SNPs can be analysed with the same software. This chapter has proved that both methods are valid to solve the phylogenetic relationship of species pairs.

CHAPTER 3:

GENETIC STRUCTURE AND GENE FLOW OF LICHEN-FORMING FUNGI IN THE MARITIME ANTARCTIC AND SOUTHERN SOUTH AMERICA

3.1. CHAPTER INTRODUCTION

This chapter studies the genetic diversity among lichen populations to find out the effects of dispersal strategies and phylogeographic history on the population genetic structure and diversity of Antarctic lichens. Then, stands from South America and the Maritime Antarctic were analysed to identify human-mediated gene-flow among continents and Antarctic localities. The chapter presents the analyses of three lichen species with a high number of samples to address very important phylogeographic questions about Antarctic lichens. Two species help to understand continental gene flow from South America to the Maritime Antarctic; while the other species, suspected to be endemic in the Antarctic, contributes to the characterization of the Antarctic lichen community.

Previous phylogenetic studies indicate that *Usnea antarctica* and *U. aurantiacoatra* belong to the *Neuropogon* group of *Usnea* and that the species of this group are mostly restricted to the southernmost region of South America, Australasia, and Antarctica, and have likely evolved there (Walker 1985; Wirtz et al. 2008, 2012). In contrast, *Cetraria aculeata* is a bipolar lichen species that colonized Antarctica from Patagonia during the Pleistocene (Fernández-Mendoza & Printzen 2013). Therefore, these three species are representative taxa to study the effects of dispersal strategies and phylogeographic history on the population genetic structure of Antarctic lichens, and to assess the likely effects of climate change and the impact of humans on lichens.

Information about the spatial genetic structure of lichens is therefore urgently needed to understand the joint effects of local human activities and global temperature increases on Antarctic terrestrial vegetation. As reported in publication 4, the main research questions can be summarized as follows:

- Are lichen populations genetically isolated, or does gene flow exist, particularly between southern South America and the Antarctic?
- How do the dispersal strategies influence the genetic structure of the mycobionts?
- What impact does the phylogeographic history have on the population genetic structure of Antarctic lichens?

This chapter uses methods from publication 1 and 2, but the results are mainly based on publication 4.

3.2. MATERIALS & METHODS

To understand gene flow and variability among different populations, several localities in South America and in the Antarctic were sampled. Many cooperation partners, already working with lichens, were involved to reduce the travel costs and to extend the area of the study. In order to ensure a standard in all the sampled populations, all partners were required to systematically sample different species of lichens with populations of at least 20 samples at least 50 cm apart. As reported in publication 4, sampling covered a wide range of localities in the Maritime Antarctic (61-64º S) and southern South America (50-55º S), including the Falkland Islands. Most samples were collected between 2015 and 2018. A few populations sampled between 2007 and 2014 and cryo-conserved at the Herbarium Senckenbergianum (FR) were added to the dataset. This chapter analyses 22 stands of *Usnea aurantiacoatra* and 16 stands of *Cetraria aculeata* in southern South America, Falkland, and the Maritime Antarctic, as well as 20 stands of *U. antarctica* in the South Shetland Islands and the Antarctic Peninsula (table 1 & fig. 2- A, B, C). Only *C. aculeata* and *U. aurantiacoatra* were collected in South America. Samples collected in South America for *Usnea antarctica* were identified morphologically and molecularly to belong to *U. aurantiacoatra*. Probably, *U. antarctica* is endemic in the Antarctic.

The final datasets are comprised of: 10 localities/22 stands/441 individuals for *U. aurantiacoatra*, 6 localities/20 stands/370 individuals for *U. Antarctica,* 10 localities/16 stands/266 individuals for *C. aculeate*, 5 localities/14 stands/254 samples for *Placopsis antarctica*, and 2 localities/10 stands/194 samples for *P. contortuplicata* (all listed in fig. 8 & table 7).

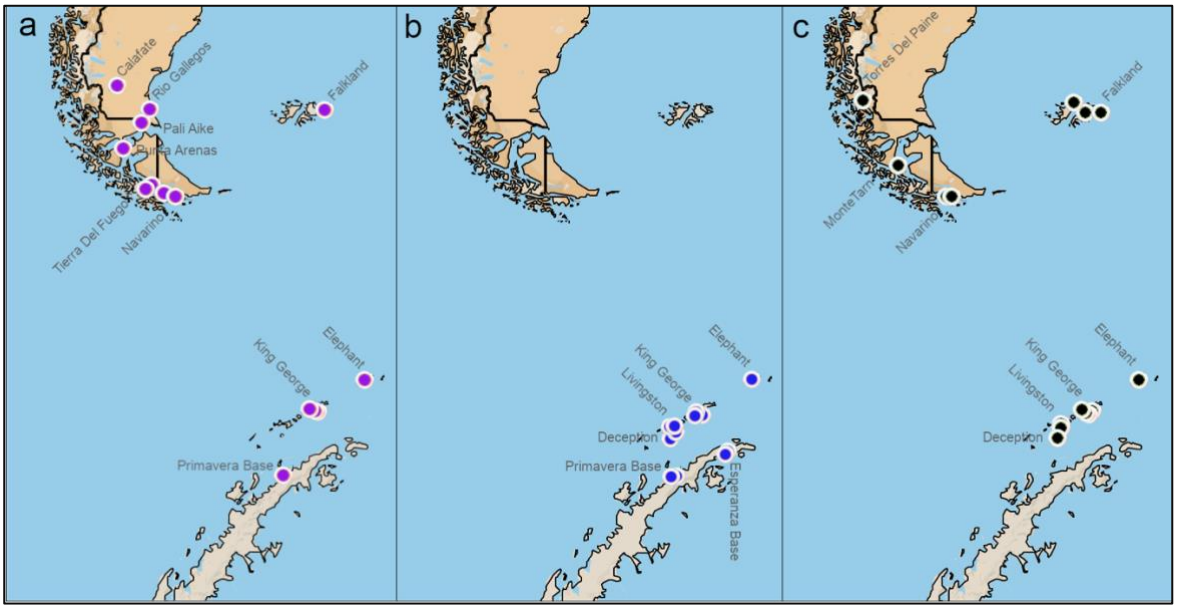

FIGURE 8: Sampling localities of each species. Dots represent stands: A. *Cetraria aculeata* (purple); B. *Usnea antarctica* (blue); C. *Usnea aurantiacoatra* (black).

To evaluate the effects of human impact on gene flow between South America and the Antarctic localities, the Antarctic localities were divided in two groups: higher human impact (King George Island) and lower human impact (Livingston Island, Elephant Island, and the Antarctic Peninsula), considering the human activity at each locality. King George Island and Livingston Island belong to the South Shetland Islands and are both ca. 900 km away from the southernmost tip of South America. As mentioned in the introduction, the human impact is very different on both islands. King George Island currently supports ten scientific stations, a permanent civilian settlement, and an airfield ("Teniente Marsh") with frequent support flights from Punta Arenas (Chile) and Río Gallegos (Argentina) in Patagonia. Touristic activities have consistently increased, and after 2002 the annual visitor numbers and five-year means have been up to six times higher than those for Livingston Island. Livingston Island has only two permanent and one seasonal field station; tourism was almost negligible before 1993 and has since stagnated around 5000 visitors. Movements of scientists between both islands are considerably rarer than between Patagonia and either island. Considering the distance of ca. 100 km between both islands, natural as well as human-mediated rates of gene flow among lichen populations on both islands are assumed to be low. On the other hand, Elephant Island and the Antarctic

Peninsula are subjected to low tourism, at least in the sampling area, and the number of permanent research stations is very low. All localities have similar macroclimate and bedrock.

TABLE 7: List of sampling localities and coordinates, day of sampling, and collector.

Stand	Species	Stand ID	Stand Size	Continent	Country/Region	Locality	Latitude (°)	Longitude (°)	Sampling Date	COLLECTOR(S)
1	*Cetraria aculeata*	Argentina, Calafate	11	South America	Argentina	Calafate	-50.635833	-71.375270	2009	F. Fernandez-Mendoza
2	*Cetraria aculeata*	Argentina, Rio Gallegos	12	South America	Argentina	Rio Gallegos	-51.614302	-69.301375	2010	F. Fernandez-Mendoza
3	*Cetraria aculeata*	Chile, Pali Aike	20	South America	Argentina	Pali Aike	-52.168880	-69.790830	2008	F. Fernandez-Mendoza
4	*Cetraria aculeata*	Chile, Punta Arenas	25	South America	Chile	Punta Arenas	-53.163830	-70.917060	November 23, 2009	F. Fernandez-Mendoza
5	*Cetraria aculeata*	Chile, Tierra del Fuego	11	South America	Chile	Tierra del Fuego	-54.569880	-69.135080	2009	F. Fernandez-Mendoza
6	*Cetraria aculeata*	Chile, Tierra del Fuego	12	South America	Chile	Tierra del Fuego	-54.675594	-69.440270	2009	F. Fernandez-Mendoza
7	*Cetraria aculeata*	Chile, Navarino	11	South America	Chile	Navarino Island	-54.970950	-67.633400	January 29, 2017	C. Printzen & I. Starke-Ottich
8	*Cetraria aculeata*	Chile, Navarino	12	South America	Chile	Navarino Island	-54.932500	-68.349720	2008	C. Printzen & I. Starke-Ottich
9	*Cetraria aculeata*	Falkland	19	South America	Falkland		-51.698166	-57.820416	2007	C. Printzen & I. Ottich
10	*Cetraria aculeata*	Elephant Island 1	19	Antarctica	Elephant Island	Stinker Point	-61.221517	-55.367550	January 21, 2016	M. Andreev
11	*Cetraria aculeata*	Elephant Island 2	20	Antarctica	Elephant Island	Stinker Point	-61.222228	-55.359683	February 8, 2016	M. Andreev
12	*Cetraria aculeata*	King George Island 1	13	Antarctica	King George Island	Carlini	-62.237000	-58.658200	December 18, 2015	E. Lagostina & B. Kanz
13	*Cetraria aculeata*	King George Island 2	17	Antarctica	King George Island	Carlini	-62.246389	-58.677750	2007	I. Ottich & P. Jordan
14	*Cetraria aculeata*	King George Island 3	21	Antarctica	King George Island	Fildes Peninsula	-62.190283	-58.926733	April 1, 2016	M. Andreev
15	*Cetraria aculeata*	Primavera Base 1	23	Antarctica	Antarctic Peninsula	Primavera base	-64.093430	-60.565630	December 1, 2016	M. Scur
16	*Cetraria aculeata*	Primavera Base 2	20	Antarctica	Antarctic Peninsula	Primavera base	-64.093430	-60.574260	December 3, 2016	M. Scur
1	*Usnea antarctica*	Elephant Island	19	Antarctica	Elephant Island	Stinker Point	-61.222283	-55.359683	February 8, 2016	M. Andreev
2	*Usnea antarctica*	King George Island 1	19	Antarctica	King George Island	Carlini	-62.237000	-58.658200	December 18, 2015	E. Lagostina & B. Kanz
3	*Usnea antarctica*	King George Island 2	17	Antarctica	King George Island	Glacial Point	-62.239383	-58.653360	December 27, 2015	E. Lagostina & B. Kanz
4	*Usnea antarctica*	King George Island 3	19	Antarctica	King George Island	Penguinera	-62.252900	-58.649516	December 22, 2015	E. Lagostina & B. Kanz
5	*Usnea antarctica*	King George Island 4	19	Antarctica	King George Island	BellingShausen	-62.190260	-58.926733	April 8, 2016	M. Andreev
6	*Usnea antarctica*	King George Island 5	7	Antarctica	King George Island	Field Pennsula	-62.205383	-58.954433	December 22, 2005	N. Wirtz
7	*Usnea antarctica*	King George Island 6	19	Antarctica	King George Island	BellingShausen	-62.232000	-59.010200	February 15, 2018	M. Andreev
8	*Usnea antarctica*	Livingston Island 1	18	Antarctica	Livingston Island	Caleta Argentina	-62.666900	-60.400900	February 23, 2018	C. Printzen
9	*Usnea antarctica*	Livingston Island 2	5	Antarctica	Livingston Island	Reyna Sofia	-62.669100	-60.381030	February 24, 2018	C. Printzen
10	*Usnea antarctica*	Livingston Island 3	20	Antarctica	Livingston Island	Sally Rock	-62.701390	-60.416390	February 27, 2018	C. Printzen
11	*Usnea antarctica*	Livingston Island 4	20	Antarctica	Livingston Island	Punta Hanna	-62.650470	-60.602890	March 1, 2018	C. Printzen
12	*Usnea antarctica*	Livingston Island 5	20	Antarctica	Livingston Island	Barnard Point	-62.751360	-60.330360	March 8, 2018	C. Printzen
13	*Usnea antarctica*	Deception Island	9	Antarctica	Deception Island		-62.983333	-60.683333	February, 2002	B. Schroeter
14	*Usnea antarctica*	Primavera Base 1	21	Antarctica	Antartic Peninsula	Primavera base	-64.095010	-60.565630	November 30, 2016	M. Scur
15	*Usnea antarctica*	Primavera Base 2	25	Antarctica	Antartic Peninsula	Primavera base	-64.092280	-60.371320	November 26&29, 2016	M. Scur
16	*Usnea antarctica*	Primavera Base 3	22	Antarctica	Antartic Peninsula	Primavera base	-64.092150	-60.571960	January 5, 2017	M. Scur
17	*Usnea antarctica*	Esperanza Base 1	27	Antarctica	Antartic Peninsula	Esperanza base	-63.401330	-56.990083	January 13, 2017	A. Lorenz
18	*Usnea antarctica*	Esperanza Base 2	24	Antarctica	Antartic Peninsula	Esperanza base	-63.409027	-57.013610	January 18, 2017	A. Lorenz
19	*Usnea antarctica*	Esperanza Base 3	21	Antarctica	Antartic Peninsula	Esperanza base	-63.407220	-57.018250	January 15, 2017	A. Lorenz
20	*Usnea antarctica*	Esperanza Base 4	19	Antarctica	Antartic Peninsula	Esperanza base	-63.413750	-57.04150	January 15, 2017	A. Lorenz
1	*Usnea aurantiacoatra*	Chile, Torres Del Paine	14	South America	Chile	Torres del Paine	-51.211300	-73.256700	January 30, 2018	C. Printzen & C. Ivanovich
2	*Usnea aurantiacoatra*	Chile, Monte Tarn	49	South America	Chile	Mount Tarn	-53.752000	-71.023500	February 1, 2017	C. Printzen
3	*Usnea aurantiacoatra*	Chile, Navarino 1	20	South America	Chile	Navarino Island	-54.970483	-67.635766	January 30, 2017	C. Printzen & I. Starke-Ottich
4	*Usnea aurantiacoatra*	Chile, Navarino 2	35	South America	Chile	Navarino Island	-54.977916	-67.649550	February 1, 2017	C. Printzen & I. Starke-Ottich
5	*Usnea aurantiacoatra*	Chile, Navarino 3	19	South America	Chile	Navarino Island	-54.975583	-67.630500	February 15, 2017	C. Printzen & I. Starke-Ottich
6	*Usnea aurantiacoatra*	Falkland 1	18	South America	Falkland	Gipsy point	-51.676282	-57.808785	January 28, 2018	U. Ruprecht & U. Søchting
7	*Usnea aurantiacoatra*	Falkland 2	18	South America	Falkland	Mt Usborne	-51.712790	-58.853037	January 30, 2018	U. Ruprecht & U. Søchting
8	*Usnea aurantiacoatra*	Falkland 3	17	South America	Falkland	Pebble Island	-51.306922	-59.615442	February 5, 2018	U. Ruprecht & U. Søchting
9	*Usnea aurantiacoatra*	Elephant Island	18	Antarctica	Elephant Island	Stinker Point	-61.222283	-55.359683	February 4, 2016	M. Andreev
10	*Usnea aurantiacoatra*	King George Island 1	19	Antarctica	King George Island	Carlini Station	-62.237000	-58.658200	December 18, 2015	E. Lagostina & B. Kanz
11	*Usnea aurantiacoatra*	King George Island 2	21	Antarctica	King George Island	Glacial Point	-62.239383	-58.653360	December 27, 2015	E. Lagostina & B. Kanz
12	*Usnea aurantiacoatra*	King George Island 3	18	Antarctica	King George Island	Penguinera	-62.252900	-58.649516	December 22, 2015	E. Lagostina & B. Kanz
13	*Usnea aurantiacoatra*	King George Island 4	19	Antarctica	King George Island	King Sejong Station	-62.216600	-58.783330	December 16, 2015	E. Lagostina & B. Kanz
14	*Usnea aurantiacoatra*	King George Island 5	15	Antarctica	King George Island	Bellingshausen	-62.203616	-58.992750	April 8, 2016	M. Andreev
15	*Usnea aurantiacoatra*	King George Island 6	15	Antarctica	King George Island	Bellingshausen	-62.203616	-58.963883	April 18, 2016	M. Andreev
16	*Usnea aurantiacoatra*	King George Island 7	8	Antarctica	King George Island	Fildes Peninsula	-62.205383	-58.954433	December 22, 2005	N. Wirtz
17	*Usnea aurantiacoatra*	King George Island 8	15	Antarctica	King George Island	Bellingshausen	-62.185230	-58.972610	January 27, 2018	M. Andreev
18	*Usnea aurantiacoatra*	Deception Island	26	Antarctica	Deception Island		-62.983333	-60.683333	February, 2002	B. Schroeter
19	*Usnea aurantiacoatra*	Livingston Island 1	21	Antarctica	Livingston Island	Nunatak	-62.681020	-60.344190	March 3, 2018	F. Grewe
20	*Usnea aurantiacoatra*	Livingston Island 2	18	Antarctica	Livingston Island	Punta Hesperides	-62.643260	-60.372500	March 6, 2018	F. Grewe
21	*Usnea aurantiacoatra*	Livingston Island 3	22	Antarctica	Livingston Island	Mt Reyna Sofia	-62.666988	-60.400966	February 24, 2018	C. Printzen
22	*Usnea aurantiacoatra*	Livingston Island 4	16	Antarctica	Livingston Island	Sally Rocks	-62.66910	-60.381030	February 27, 2018	C. Printzen

DNA was extracted with the same protocol reported in chapter 2 for the SSR analysis. From publication 1, 21 and 22 SSR markers to genotype *Usnea aurantiacoatra* and *U. antarctica* samples were chosen, respectively. The other two-three SSR markers were excluded due to the high number of null alleles reported in the South American and Falklands populations. Markers were amplified in three

different 10 µL multiplex reactions following the protocol of publication 1. Eight out of 15 microsatellite markers from Lutsak et al. (2016) were selected to analyse *Cetraria aculeata*. The other seven markers were excluded due to the high number of null alleles reported, or the absence of variability within populations. Markers were amplified in two reactions following the PCR protocol conditions reported by Lutsak et al. (2016). PCR amplicons were electrophoresed using an Applied Biosystems 3730 platform, with the LIZ 600 (for *Usnea* sp.) or LIZ 500 (for *C. aculeata*) size standard (Applied Biosystems, Waltham, Mass., USA), and allele sizes were manually scored using the Geneious 10 microsatellites tool (Kearse et al. 2012).

Methods to generate microsatellite datasets are reported in the previous chapter. Allele frequencies and genetic diversity (Shannon's information index) were calculated using the software GenAlEx 6.503 (Peakall & Smouse 2006, 2012) for the three species. Tests for clonal population structure were calculated with the software GenoDive 2.0b23 (Meirmans & Van Tienderen 2004). Clones in each population were detected using a stepwise mutation model, discarding null alleles, and assessed based on the number of genotypes, with 999 permutations randomizing alleles over individuals across all populations. Cluster analyses were run with Structure v.2.3.4 (Pritchard et al. 2000; Falush et al. 2003). To estimate the optimal number of admixture clusters, the summary likelihood statistic ΔK proposed by Evanno et al. (2005) was used through the website Pophelper v1.0.10 (Francis 2016, www.pophelper.com). The number of clusters was chosen as the value of K where ΔK reached its first minimum. Results of the ten runs for each species were summarized using CLUMPP (Jakobsson & Rosenberg 2007) and printed out through the web interface of Pophelper v1.0.10.

Intercontinental gene flow was estimated with the coalescent sampler Migrate-N only from South America to Antarctica for *Usnea aurantiacoatra* and *Cetraria aculeata*. In order to keep the number of parameters low, samples of *U. aurantiacoatra* were pooled into 5 regions: South America, Falkland, Elephant Island, King George Island, and Livingston + Deception Island. The dataset for *C. aculeata* was divided into: South America + Falkland, Elephant Island, King George Island,

and Primavera (Antarctic Peninsula). All South American samples presumed to represent *U. antarctica* were shown to belong to other species. Consequently, it was not possible to analyse intercontinental gene flow for this species. For the *U. aurantiacoatra* dataset, were used 0.0–10 priors on θ and 0.0-20 priors on M, divided into 1500 bins, and ran four chains with static heating (temperatures of 1.0, 1.2, 3.0 and 1×10^6) for 10 replicated long runs of 5×10^4 generations (sampling every 500th step) with a burn-in of 4×10^4. For the *C. aculeata* dataset, were used uniform priors (0.0–25) on both θ and M, divided into 1500 bins, and was run with four chains with static heating of 1×10^5 generations (sampling every 500th step) with a burn-in of 5×10^4. Convergence of Markov chains was monitored with Tracer (http:// beast.bio.ed.ac.uk). All effective sample sizes of the Markov chain Monte Carlo (MCMC) chain were larger than 10^5.

3.3. RESULTS

As mentioned before, results are extracted from publication 4. For *Cetraria aculeata* the final dataset was comprised of 2128 alleles including 19 null alleles, for *Usnea antarctica* the final dataset was comprised of 8140 alleles including 41 null alleles, and for *U. aurantiacoatra* the final dataset was comprised of 9261 alleles including 164 null alleles.

Usnea aurantiacoatra had the highest total number of alleles (232), with the highest mean number of observed (7.476) and effective (4.016) alleles recorded on Navarino Island in South America, followed by Livingston Island in the Antarctic (7.238; 2.725, table 8. The highest mean number of private alleles was observed on Livingston Island (0.857) followed by Navarino Island (0.762). The Shannon information index was highest on Navarino (1.490) with rather similar values around 1.0-1.1 on Livingston, King George, and Falkland Islands. None of the diversity metrics showed a clear latitudinal pattern (table 8). In *Cetraria aculeata* the highest observed number of alleles (4.750) was also found on Navarino and decreased to the north and south. The highest effective number of alleles (2.902) was detected in a stand in Chile and the observed (1.250) and effective number of alleles (ca. 1.0) was lowest on Elephant Island and near Primavera Base on the Antarctic Peninsula. Private alleles were detected in all South American populations (except Falklands) and on King George Island, but not on Elephant Island and on the Antarctic Peninsula. In *Usnea antarctica* the observed (effective) mean number of alleles ranged between 4.682 (1.954) on Livingston and 1.591 (1.238) on Deception Island. Private alleles were recorded in all the sampling areas except for Deception Island (table 8).

TABLE 8: Allele analysis for each species. In order are listed sampling locality, numbers of samples, numbers of alleles, numbers of effective alleles, numbers of private alleles, and Information Index (comparable to the Shannon-Weaver index of ecology).

Cetraria aculeata					
Locality	n° of Samples	n° of Alleles	Effective n° of Alleles	n° Private Alleles	information Index
Argentina, Calafate	11	2.250 ± 0.366	1.691 ± 0.263	0.125 ± 0.125	0.534 ± 0.149
Argentina, Rio Gallegos	12	3.250 ± 0.726	2.301 ± 0.580	0.125 ± 0.125	0.789 ± 0.224
Chile, Pali Aike	20	4.000 ± 1.069	2.902 ± 0.961	0.375 ± 0.183	0.907 ± 0.247
Chile, Punta Arenas	25	4.250 ± 0.996	2.409 ± 0.674	0.375 ± 0.263	0.899 ± 0.193
Chile, Tierra del Fuego	23	4.250 ± 0.773	2.638 ± 0.548	0.375 ± 0.183	0.981 ± 0.210
Chile, Navarino	23	4.750 ± 0.977	2.771 ± 0.555	0.250 ± 0.164	1.081 ± 0.203
Falkland	19	3.500 ± 0.756	2.426 ± 0.657	0 ± 0	0.841 ± 0.194
Elephant Island	39	1.250 ± 0.250	1.014 ± 0.014	0 ± 0	0.031 ± 0.031
King George Island	51	3.375 ± 1.449	2.483 ± 1.073	0.500 ± 0.378	0.541 ± 0.325
Primavera Base	43	1.250 ± 0.164	1.018 ± 0.013	0 ± 0	0.037 ± 0.026
Placopsis antarctica					
Locality	n° of Samples	n° of Alleles	Effective n° of Alleles	n° Private Alleles	information Index
Elephant Island	19	2.000 ± 0.293	1.423 ± 0.112	0.133 ± 0.133	0.392 ± 0.095
King George Island	43	3.267 ± 0.284	1.796 ± 0.159	0.733 ± 0.182	0.679 ± 0.081
Robert Island	20	2.067 ± 0.206	1.490 ± 0.101	0.067 ± 0.067	0.454 ± 0.083
Livingston Island	111	3.333 ± 0.433	1.777 ± 0.218	0.800 ± 0.175	0.611 ± 0.109
Deception Island	40	2.133 ± 0.256	1.537 ± 0.123	0.067 ± 0.067	0.451 ± 0.089
Placopsis contortuplicata					
Locality	n° of Samples	n° of Alleles	Effective n° of Alleles	n° Private Alleles	information Index
King George Island	101	3.800 ± 0.439	1.610 ± 0.113	1.933 ± 0.441	0.586 ± 0.084
Livingston Island	65	2.400 ± 0.515	1.446 ± 0.201	0.533 ± 0.291	0.352 ± 0.133
Usnea antarctica					
Locality	n° of Samples	n° of Alleles	Effective n° of Alleles	n° Private Alleles	information Index
Elephant Island	19	2.227 ± 0.246	1.547 ± 0.161	0.045 ± 0.045	0.43 ± 0.094
King George Island	100	4.409 ± 0.425	1.808 ± 0.216	0.818 ± 0.243	0.645 ± 0.103
Livingston Island	83	4.682 ± 0.485	1.954 ± 0.195	1.227 ± 0.394	0.765 ± 0.101
Deception Island	9	1.591 ± 0.107	1.238 ± 0.053	0 ± 0	0.262 ± 0.051
Primavera Base	68	3.591 ± 0.454	1.769 ± 0.187	0.318 ± 0.121	0.593 ± 0.111
Esperanza Base	91	3.136 ± 0.396	1.712 ± 0.215	0.227 ± 0.091	0.517 ± 0.115
Usnea aurantiacoatra					
Locality	n° of Samples	n° of Alleles	Effective n° of Alleles	n° Private Alleles	information Index
Chile, Torres del Paine	14	2.857 ± 0.210	1.899 ± 0.153	0 ± 0	0.722 ± 0.077
Chile, Monte Tarn	49	3.810 ± 0.496	2.141 ± 0.316	0 ± 0	0.718 ± 0.136
Chile, Navarino	74	7.476 ± 0.770	4.016 ± 0.415	0.762 ± 0.266	1.490 ± 0.104
Falkland 1	18	3.095 ± 0.337	1.970 ± 0.180	0.048 ± 0.048	0.742 ± 0.098
Falkland 2	18	4.00 ± 0.431	2.642 ± 0.294	0.190 ± 0.148	1.011 ± 0.106
Falkland 3	17	3.524 ± 0.394	2.280 ± 0.235	0.095 ± 0.066	0.847 ± 0.119
Elephant Island	18	3.238 ± 0.300	1.995 ± 0.213	0.095 ± 0.095	0.753 ± 0.098
King George Island	130	6.476 ± 0.635	2.449 ± 0.275	0.286 ± 0.101	1.037 ± 0.106
Livingston Island	77	7.238 ± 0.756	2.725 ± 0.349	0.857 ± 0.221	1.141 ± 0.117
Deception Island	26	3.381± 0.327	2.013 ± 0.165	0 ± 0	0.788 ± 0.089

Every individual of *U. aurantiacoatra* belonged to a different clone. Hence, there was no evidence for clonal structure of populations (table 9). In *C. aculeata* there was strong evidence for clonal reproduction. The program Genodive also

inferred significant clonal population structure in *U. antarctica*, although the number of clones was almost as high as expected.

TABLE 9: Tests for clonal population structure performed in GenoDive. Species, number of samples, expected and observed number of clones, percent of clones, and probability P of observing this number of clones under random mating.

Species	n° of Samples	n° of Expected Clones	n° of Observed Clones	%	P
Cetraria aculeata	266	210.734	130.000	51.128	0.001
Usnea antarctica	370	369.329	342.000	7.568	0.001
Usnea aurantiacoatra	441	441.000	441.000	0	**1.000**

Each dataset was clustered with a STRUCTURE analysis (fig. 9). Evanno's methods were performed to find out the best number of clusters to fit the datasets. For all datasets, the first lowest ΔK value from Evanno's test was K=4. Antarctic populations of *C. aculeata* display extreme regional genetic structure with different gene pools on the Antarctic Peninsula, King George, and Elephant Islands. The gene pool on Elephant Island is also relatively common in South America, where it co-occurs with a fourth gene pool that is absent from Antarctica. South American populations show no strong differences in gene pool composition. Populations of *U. aurantiacoatra* in Falkland and Navarino Island are dominated by local gene pools that are absent elsewhere. A third gene pool is largely restricted to Antarctica. About half of the samples from Livingston Island belong to a fourth gene pool that also predominates in populations from Mt Tarn and Torres del Paine in Chile. Populations of *U. antarctica* on Livingston and Deception Island are dominated by two gene pools that are virtually absent in other localities. Most samples from Elephant Island and Esperanza belong to a third gene pool that, together with a fourth one, also occur on King George Island and near Primavera.

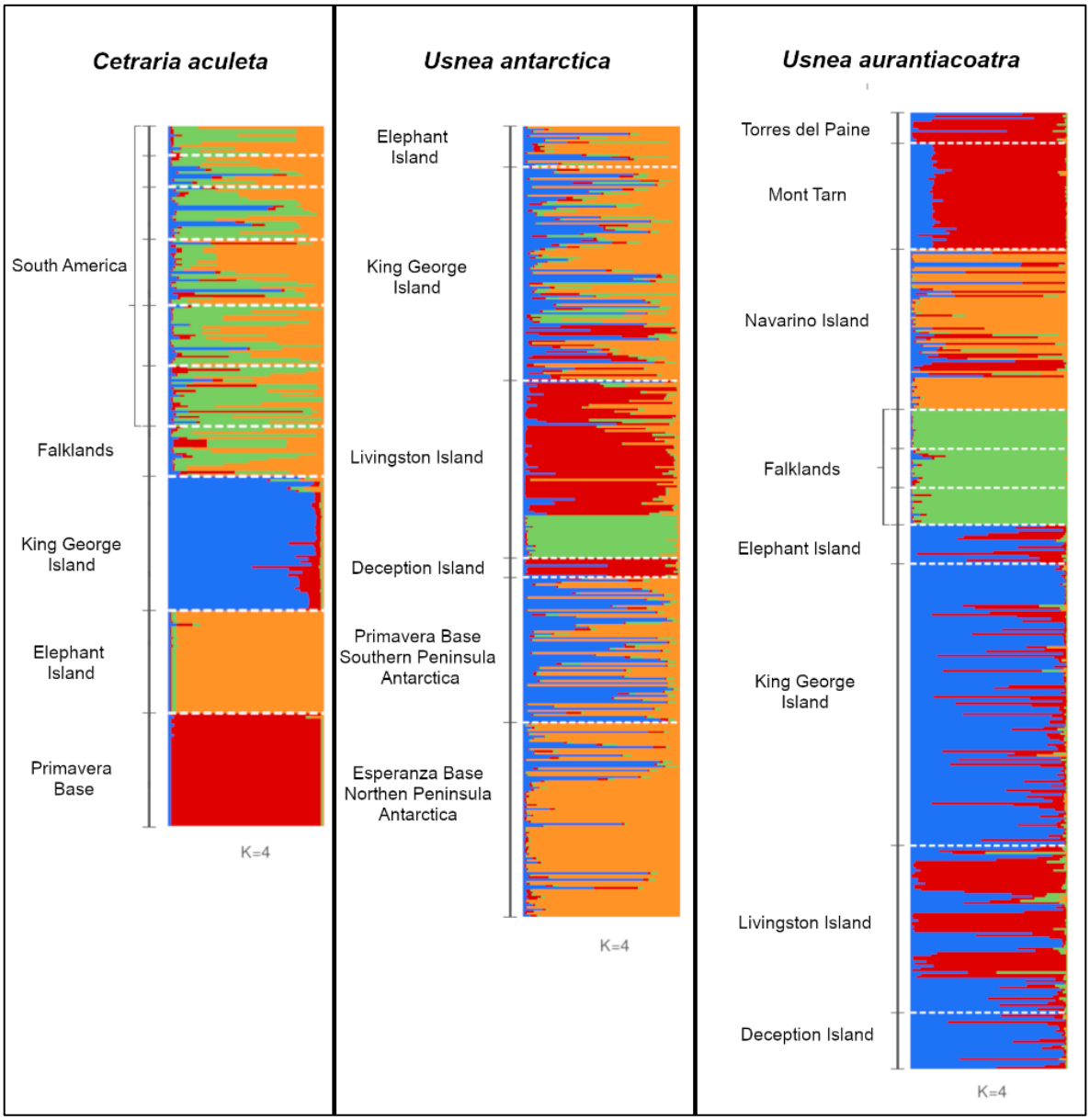

FIGURE 9: Assignment of individuals to gene pools as obtained by running Structure on the three species datasets. Populations are sorted geographically. Each population of *Usnea* sp. and *C. aculeata* are separated with a dashed line. The height of each color corresponds to the estimated probability with which the individual belongs to the respective gene pool.

Since *U. antarctica* was not found in southern South America, I studied intercontinental gene flow only for *Usnea aurantiacoatra* and *Cetraria aculeata* from South America and Falkland to three different localities of the Maritime Antarctic, divided according to the presence of human activities (fig. 10). *C. aculeata* showed

dispersal rates of 4.4 migrants per generation from South America to Elephant Island (low human impact). Gene flow was considerably lower towards the Antarctic Peninsula (1.5 migrants per generation) and absent towards King George Island. All Antarctic populations had comparably low effective population sizes (0.3 for Elephant Island and Primavera base and 0.5 for King George Island). In *U. aurantiacoatra*, the highest values of >5 migrants/generation were inferred from South America to Elephant Island (low human impact). Gene flow into Antarctica along the other routes was considerably lower and ranged between 1.4 (Falkland to King George Island – high human impact) and 3.5 (South America to King George Island – high human impact) migrants per generation. Dispersal between continental South America and Falkland was negligible (<1 migrants per generation in both directions). Effective population sizes in different Antarctic localities differed vastly. At the moment, there is no support for human-mediated intercontinental movement of propagules.

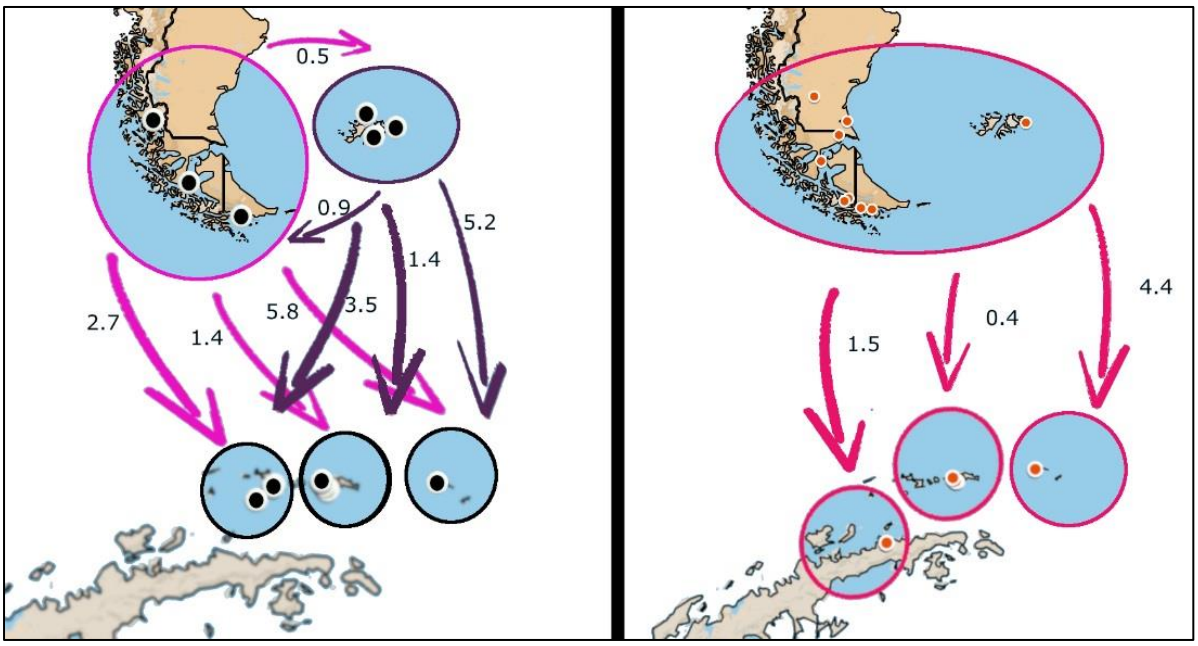

FIGURE 10: MigrateN analysis. The circle represents the region and the arrows quantify the migration. On the left is *Usnea aurantiacoatra*, migration was calculated from South America and Falkland to 3 Antarctic localities. On the right is *Cetraria aculeata*, migration was calculated from merged South America and Falkland to 3 Antarctic localities.

3.4. DISCUSSION

This thesis is the first work that systematically analyses and evaluates gene flow of lichens between several localities of South America and the Maritime Antarctic. Sampling in Antarctica is complicated by the difficulty in reaching the continent, the harsh climatic conditions, and the distance between different areas of ice-free plots. Cooperation partners were involved to reduce the travel costs and to extend the area of the study. This chapter focuses on three lichens species with different dispersal strategies and history. The two species of *Usnea* originated in the southern hemisphere (Walker 1985), but *U. aurantiacoatra* has a sexual dispersal strategy while *U. antarctica* is asexual. In contrast, *Cetraria aculeata* reaches the Antarctic from the Arctic passing through the Andes (Fernández-Mendoza & Printzen 2013), and it also has vegetative dispersal. Information about the spatial genetic structure of lichens is therefore urgently needed to understand the joint effects of local human activities and a global temperature increase on Antarctic terrestrial vegetation.

This chapter provide insights into a range of different topics:

1. The impact of reproductive mode on diversity and spatial structure of Antarctic lichen populations.
2. The impact of colonization and glacial history as well as stand disturbance on genetic patterns.
3. The dispersal capacities of lichens.
4. The detection of human impact on propagule dispersion.

3.4.1. IMPACT OF REPRODUCTIVE MODE ON GENETIC DIVERSITY AND STRUCTURE

As expected by population genetic theory (e.g. Bengtsson 2003), the sexually reproducing *U. aurantiacoatra* shows higher genetic diversity than the mostly asexual *C. aculeata* and *U. antarctica*, both in terms of allele richness and diversity (table 8). While diversity levels are difficult to compare among *Cetraria* and *U. aurantiacoatra* due to the different numbers of genotyped loci (eight vs. 21), results for the two closely related *Usnea*-species indicate that reproductive mode has an impact on overall and per-population genetic diversity (publication 3). This result is in line with observations based on DNA sequence data from *Degelia plumbea* and *D. atlantica* (Otálora et al. 2013) showing that the predominantly asexual reproduction in *D. atlantica* is associated with lower genetic diversity. The tendency for clonal population structure in the two asexual species (table 9) provides further evidence that the lower diversity is in fact due to differences in reproductive mode (see e. g. Bengtsson 2003).

The high genetic diversity observed in the two *Usnea* species contrasts starkly with the low levels of diversity observed in some Antarctic bryophytes based on DNA sequences (*Chorisodontium aciphyllum*, Biersma et al. 2018a) or AFLP data (*Sanionia uncinata*, Hebel et al. 2018). These low levels were explained by the fact that Antarctic populations of these mosses rarely show sexual reproduction but may also be due to the fact that relatively conservative markers were used in those studies, in contrast to the much more variable SSR markers used in this study. In a previous study on the genetic diversity of *C. aculeata* based on DNA sequences from three gene loci, Domaschke et al. (2012) found only two multigene haplotypes on King George Island, while in this chapter 14 clones were distinguished (table 9). It is also noteworthy that the largely asexual *U. antarctica* displays high allelic richness and genetic diversity in our samples (table 8).

3.4.2. IMPACT OF HISTORICAL FACTORS

Biersma and coauthors (2018b) also invoked recent colonization of Antarctic sites and Pleistocene population size bottlenecks as possible explanations for the reduced genetic diversity of bryophytes, thereby stressing the importance of historical factors for the explanation of spatial genetic patterns. The diversity metrics inferred in this chapter show pronounced regional differences and these patterns, as well as genetic structure, differ among species. South American populations of *Cetraria aculeata* are comprised of about twice as many alleles and have more than four times higher genetic diversity than Antarctic ones. This confirms previous studies based on DNA sequence data that also found lower levels of genetic variability in Antarctic populations of *C. aculeata* (Domaschke et al. 2012). In *U. aurantiacoatra* the number of alleles and private alleles is higher in the Antarctic than in South American populations, while genetic diversity is equal in both regions. Genetic differentiation among populations also shows opposite trends in both species. The results of Bayesian clustering (fig. 9) show that Antarctic populations of *C. aculeata* are strongly differentiated while *U. aurantiacoatra* shows strong differentiation in South America. These results conform rather well to the level of differentiation found between geographically isolated populations of *Buellia frigida* in the Queen Maud Mountains and other areas in the Ross Sea Region (Jones et al. 2015). In contrast, South American populations of *C. aculeata* and Antarctic populations of *U. aurantiacoatra* are considerably less well differentiated than their conspecific populations on the opposite side of the Drake passage. However, isolation by distance cannot explain these differences, because geographic distances among populations on both sides of the Drake Passage do not differ markedly. There is also no evidence that reproductive mode has anything to do with these differences, as the asexual *U. antarctica* and the sexual *U. aurantiacoatra* show similarly low values of differentiation within the Antarctic. It rather appears that phylogeographic history has had a major impact on patterns of genetic diversity and differentiation of the studied lichen populations.

Based on DNA sequence data, Fernández-Mendoza & Printzen (2013) previously demonstrated that *C. aculeata* originated in the Northern Hemisphere and dispersed into South America during the Pleistocene with later colonization of the Antarctic. The relatively long presence of *C. aculeata* in South America together with

low to moderate levels of gene flow prevented strong genetic differentiation between populations. The more recent colonization of geographically isolated islands in the Maritime Antarctic by independent long-distance dispersal events apparently was too recent to allow homogenization of gene pools by dispersal between these peripheral populations. The pattern of genetic diversity of *C. aculeata* is in fact similar to that displayed by the Mediterranean-Macaronesian *Parmelina carporrhizans* with diverse, poorly differentiated source populations in the Mediterranean and genetically less variable sink populations on the Canary Islands (Alors et al. 2017).

Species of the *Neuropogon* group of *Usnea*, on the other hand, have their center of diversity in the Southern Hemisphere (Walker 1985). Consequently, *U. antarctica* and *U. aurantiacoatra* can be assumed to have evolved either in the Antarctic or in southern South America. *Usnea aurantiacoatra* shows a pattern of population differentiation geographically inverted to that of *C. aculeata.* In the Structure analysis, most individuals were assigned to three different gene pools with a clear geographic pattern. Two of these gene pools are restricted to the Falklands and Navarino Island, respectively. Individuals from Torres del Paine and Mt Tarn were assigned to a third gene pool that was also common in the Antarctic, particularly on Livingston Island. If, as in *C. aculeata*, stronger differentiation among lichen populations also indicates a more recent colonization history, then postglacial recolonization in *U*. *aurantiacoatra* apparently took place from southern source populations.

3.4.3. GLACIAL POPULATION HISTORY

The effects of Pleistocene glacial cycles on the distribution ranges of species and their genetic diversity have frequently been studied in the Northern Hemisphere (Hewitt 2004). The effects of southern hemispheric glaciations on biota have received less attention, but the geographical isolation of Antarctica stands in sharp contrast to the situation in Arctic areas that are in direct land contact with southern refugia. Demographic processes, including range shifts, extinction of populations, and recolonization during glacials and interglacials will therefore likely differ between these regions (Fraser et al. 2012). The extension of ice caps and severe

environmental conditions during the last glacial maximum were once believed to have precluded survival of organisms in polar regions (e.g. Nordal 1987). Nowadays, the glacial persistence of organisms even in the Continental Antarctic is hardly questioned (Pugh & Convey 2008; Hills et al. 2010; Biersma et al. 2018). Nunataks, perhaps associated with geothermal activities, or debris covering glaciers may have acted as possible refugial habitats (Fraser et al. 2014). Population genetic studies on lichens covering glacial refugia and glaciated areas are generally scarce and so far Antarctic lichens have not been studied in this respect, but a positive impact of long glacial continuity of populations on genetic diversity, association of private alleles with glacial refugia, and a gradual decrease of genetic diversity at larger distances from refugial areas have been observed in a few Northern Hemispheric species (Printzen et al. 2003; Scheidegger et al. 2012; Allen et al. 2018).

Cetraria aculeata and *U. aurantiacoatra* show their highest genetic diversity on Navarino Island. *Usnea aurantiacoatra* has a second diversity center on Livingston and King George Island, where *U. antarctica* also displays its highest allelic richness and genetic diversity. As high levels of allelic richness, particularly of private alleles, generally indicate long persistence and often glacial survival of populations (Widmer & Lexer 2001), this result provides further evidence for the existence of a southern Patagonian refugium postulated for plant and fungal species (Sérsic et al. 2011; Baranzelli et al. 2018; Eizaguirre et al. 2018). The high diversity is also consistent with recent reconstructions of the Patagonian ice shield indicating that Navarino Island was at least partly ice-free during the last glacial maximum (LGM; Glasser & Jansson 2008; Darvill et al. 2014). With the current data it is impossible to say whether the gradually declining levels of genetic diversity in *C. aculeata* indicate postglacial recolonization of more northern localities from the southern refugium or persistence in smaller refugia. The more pronounced diversity gradient in *U. aurantiacoatra* combined with the lack of private alleles in Torres del Paine and Mt. Tarn suggest that these populations are either of more recent origin or have experienced more pronounced population size bottlenecks during the LGM, probably because, as a saxicolous upland species, *U. aurantiacoatra* had more restricted glacial habitats than the terricolous lowland *Cetraria.*

All three species display high allelic richness associated with relatively high average numbers of private alleles on Livingston and King George Island, indicating glacial survival in a refugium on the South Shetland Islands. This result is consistent

with the reconstruction of Nunataks in the South Shetland Islands, e.g. on Livingston, King George, and Deception Island (Simms et al. 2011; Ruiz-Fernández & Oliva 2016). Since it was not possible to confirm the presence of *U. antarctica* in South America using molecular genetic data, it could be that this species is an Antarctic endemic that never managed to establish itself in South America (which may be regarded as indirect evidence for glacial survival of this species as well). As outlined above, the subcosmopolitan *C. aculeata* colonized the Antarctic relatively recently. The high diversity on King George Island is comparable to that found in South American populations and, together with a relatively high number of private alleles, indicates a relatively high, probably pre-glacial age of this population.

In all three species, Antarctic populations on Elephant Island, Deception Island, and the Antarctic Peninsula show lower genetic diversity. In *C. aculeata* these differences are particularly pronounced and, together with high levels of population differentiation, suggest founder effects during independent colonization events. In fact, the clone assignment test (additional material in publication 4) ascribes the population of *C. aculeata* in Elephant Island as a clone from a South American stand.

The strongly diverging levels of genetic diversity of *U. antarctica* and *U. aurantiacoatra* populations on Livingston and Deception Island merit some attention as well. Both islands are close to each other and Deception Island, the most active volcano in the area, was probably not glaciated during the LGM (Simms et al. 2011; Guillemin et al. 2018). The low number of private alleles and low genetic diversity on Deception Island could be explained with volcanic activity; the last volcanic eruptions were reported in 1967, 1969, and 1970 (Lewis-Smith 1984). The eruptions may have reduced the size of lichen populations present at the time on the Island, resulting in either a bottleneck due to a strong reduction in population size, or a complete eradication of lichens and subsequent founder effects during recolonization.

3.4.4. INTERCONTINENTAL GENE FLOW

Due to its geographical distance from other continents and the strong effects of the Antarctic Circumpolar Current, Antarctica is considered the biologically most isolated continent. As judged from levels of endemism, the degree of isolation varies strongly with the taxonomic group considered (Barnes et al. 2006), and such data for terrestrial organisms is still very scarce. Distribution patterns of bryophytes and lichens on sub-Antarctic islands are indeed correlated with the prevailing wind patterns, indicating directional long-distance colonization (Muñoz et al. 2004). However, for some bryophytes with bipolar distributions, long-distance dispersal mediated by migratory birds has been demonstrated (Lewis et al. 2014a, 2014b). The wide geographical ranges of many lichens and genetic similarities among widely separated populations have sometimes been interpreted as evidence for ongoing long-range dispersal, even between continents (Geml et al. 2010). But although numerous lichen species occur in South America and Antarctica, our data does not confirm dispersal of lichens across the Drake Passage on short time scales. Since we could not confirm the presence of *U. antarctica* in South America, this species might be an Antarctic endemic that never managed to cross the Drake Passage. Neither the MigrateN analyses nor genetic differentiation among populations (fig. 10) indicate high levels of ongoing gene flow in *U. aurantiacoatra* or *C. aculeata*. *Usnea aurantiacoatra* apparently survived the Last Glacial Maximum in separate refugia north and south of the Drake passage, while the high genetic differentiation of peripheral Antarctic populations of *C. aculeata* suggest *in situ* survival in small populations or rare colonization events with founder effects.

The invasion of non-native species and propagule transfer into Antarctica has been a major concern of conservationists (Hughes & Convey 2010) and is regarded as “one of the most significant conservation problems in the Antarctic” (Chown & Convey 2007). The increasing risk of accidental introduction of invasive species and genetic homogenization of Antarctic gene pools is due to two interacting factors: global warming and human transfer.

While global warming is beginning to change the Antarctic Circumpolar Current and associated aerial currents (Chown et al. 2015; Fraser et al. 2018), exposes so far uninhabited, disturbed ground, and alleviates physiological stress, growing numbers of researchers and tourists in the region act as possible vectors for

propagules. Although this chapter results do not indicate any immediate threat to the genetic composition of lichen populations, they suggest that *C. aculeata* and *U. aurantiacoatra* are exposed to different risks. Conservation measures for Antarctic organisms should therefore consider the different phylogeographic histories and spatial genetic structure of the species. The genetically diverse and poorly differentiated Antarctic populations of the two *Usnea* species are apparently experiencing high natural levels of gene flow. On this background, additional human transfer of propagules will have comparatively little impact (and would be difficult if not impossible to detect). The genetically poor and highly differentiated populations of *C. aculeata*, on the other hand, require stronger conservation measures to avoid the introduction of non-native genotypes and the homogenization of gene pools. The different distributional patterns of both species in South America, a result of their different phylogeographic histories, exacerbate this problem. *U. aurantiacoatra* only occurs in small and isolated patches and prefers higher elevations, reducing the risk of accidental introduction into Antarctica, e. g. by tourists. In contrast, *C. aculeata* is much more widespread in South America and also grows at lower elevations. It therefore has a much higher chance to be transferred by Antarctic visitors.

CHAPTER 4: CONCLUSIONS

The aim of this thesis is to clarify the genetic structure among Antarctic lichen populations in order to evaluate the consequences for conservation strategies, and to investigate the role of human impact on possible transfer of propagules into the Antarctic and among Antarctic regions. Before studying the lichen populations, it was necessary to delimit species of lichens and to develop proper tools able to detect the genetic diversity within population and genetic differentiation among populations. For that reason, the second chapter is dedicated to the development of specific markers (microsatellites and SNPs) able to delimit species and genetic variability. This thesis is the first work to design microsatellites that cross amplify species pairs to successfully clarify their phylogenetic relationship. Markers have been developed for the species pair *Usnea antarctica* and *U. aurantiacoatra* because classic molecular methods based on recommended universal fungal barcode sequences failed to discriminate between the two morphotypes. Microsatellite markers were also developed for the well discriminated species pair of *Placopsis antarctica* and *P. contortuplicata*, and were included in this thesis to confirm the reliability of SSRs to discriminate sister taxa. Then, SNPs obtained with the modern technique RADseq (based on Next Generation Sequencing) were tested on a small number of *Usnea* samples, which gave equivalent results. The main conclusion of chapter two is that SSRs and SNPs are suitable methods to delimit species pairs and to evaluate the genetic structure of lichen populations. In addition, chapter two introduced two innovations: SSRs were applied to lichens to delimit closely related species for the first time, and second, the methods adopted to develop microsatellites and use BLAST to compare the genome of one species pair against the other reduced the

cost of sequencing and therefore increased the probability in developing markers for non-fungal genomes.

The third chapter of the thesis is dedicated to answering the work hypothesis. Chapter three uses microsatellites to characterize the genetic structure of Antarctic lichens, focusing on three species with different dispersal strategies and history. Two species of *Usnea* originated in the southern hemisphere (Walker 1985); *U. aurantiacoatra* has a sexual dispersal strategy while *U. antarctica* has a vegetative dispersal strategy. In contrast, *Cetraria aculeata* reaches the Antarctic from the Arctic passing through the Andes (Fernández-Mendoza & Printzen 2013), and also uses vegetative dispersal. Chapter three pinpoints that the propagation mode does not affect the populations' structure *per se*, but that the history of the species better explains the structured pattern of each population. Both the northern immigrant *C. aculeata* and the (sub)Antarctic *U. aurantiacoatra* show higher levels of genetic differentiation in marginal than central populations. Diversity hotspots for both species suggest the existence of glacial refugia on Navarino Island and Livingston or King George Islands, where *U. antarctica* also displays the highest diversity. Comparing the vegetative species *Cetraria aculeata* and *Usnea antarctica*, they show different population structure. *C. aculeata* in the Antarctic displays an elevated level of regionalism due to a founder effect and the difficulty in spreading from one island to the other. In contrast, the allele analyses of *Usnea antarctica* estimated an elevated number of effective alleles in all localities, confirming high genetic variability in the Antarctic. *Usnea antarctica* has not been detected outside of the Antarctic continent, leading to the conclusion that this species is endemic. Second, in comparing the *Usnea* species-pair (sexual and vegetative species), it is clear that their dispersal strategies do not affect the genetic variability of the species. Indeed, in the Antarctic populations, the genetic variability is high in both *Usnea*. A vegetative dispersal strategy allowed both symbionts to spread together and to quickly colonize new areas. Perhaps, the presence of the vegetative species could help the sexual species to encounter a photobiont and could favor its dispersal. However, without genetic confirmation of the photobionts' similarity, this idea remains as speculation. It has been described that different species of lichens with different dispersal strategies (*Umbilicaria spodochroa* and *Lasallia pustulata*) often share the same species of photobiont within a single site and across different localities (Hestmark et al. 2016). In addition, Beck and co-authors (2019) showed that *Placopsis antarctica* and *P.*

contortuplicata are clearly separated sister species with different reproductive strategies, but both share the same photobiont pools.

A positive result for Antarctic conservation is that, at the moment, lichens do not show any recent gene flow between Southern South America and the Maritime Antarctic. The isolation of the Maritime Antarctic is likely due to the Drake Passage and the Antarctic Circumpolar Current, the Sub-Antarctic Front, and Antarctic Polar Front, which appear to maintain biodiversity in the cold continent. Nevertheless, global climate change is influencing the composition of terrestrial ecosystems (Nolan et al. 2018), as the deglaciated area available for vegetation will be profoundly enlarged in the future (Turner et al. 2005; Meredith & King 2005), and is estimated to increase up to 25% before the end of this century (Lee et al. 2017). King George Island has experienced some of the most significant atmospheric temperature rises of the planet with an increase of around 3.0 °C in the last 60 years, and consequently lost 7% of its ice cover (Simões et al. 2015). Rodriguez and co-authors (2018) studied the Potter Peninsula in King George Island and found three assemblages of different lichen species' communities that are dependent on deglaciation and other variables, such as the altitude of the sampling points. The availability of new ice-free lands will play an important role in the structure of Antarctic communities, and may expand their habitat and connect isolate populations. The genetic exchange between isolated and locally adapted lineages could lead to a homogenization of the Antarctic species (Terauds et al. 2012) and inter-regional transfer of propagules will become more frequent, causing a loss of biodiversity as a consequence. Indeed, the strong genetic structure of *Cetraria aculeata* calls for protective measures to avoid gene flow between isolated populations. In addition, in order to preserve the Antarctic genetic structure of populations, it is important to avoid introducing propagules and non-native species into the Antarctic.

REFERENCES

Aguileta G., Marthey S., Chiapello H., Lebrun MH., Rodolphe F., Fournier E., Gendrault-Jacquemard A., Giraud T. (2008) Assessing the performance of single-copy genes for recovering robust phylogenies. Systematic Biology 57: 613–627.

Ahmadjian V. (1993) The Lichen Symbiosis. New York, NY: JohnWiley & Sons.

Allegrucci G., Carchini G., Convey P., Sbordoni V. (2012) Evolutionary geographic relationships among orthicladine chironomid midges from the maritime Antarctic and sub-Antarctic islands. Biological Journal of the Linnean Society 106: 258–274.

Allegrucci G., Carchini G., Todisco V., et al. (2006) A molecular phylogeny of antarctic chironomidae and its implications for biogeographical history. Polar Biology 29(4): 320–326.

Allen JL., McKenzie SK., Sleith RS., Alter SE. (2018) First genome-wide analysis of the endangered, endemic lichen *Cetradonia linearis* reveals isolation by distance and strong population structure. American Journal of Botany 105: 1556–1567.

Allendorf FW. (2017) Genetics and the conservation of natural populations: allozymes to genomes. Mol Ecol 26: 420–430.

Alors D., Dal Grande F., Cubas P., Crespo A., Schmitt I., Molina MC., Divakar PD. (2017) Panmixia and dispersal from the Mediterranean Basin to Macaronesian Islands of a macrolichen species. Sci Rep 7: 40879.

Altermann S., Leavitt SD., Goward T., Nelsen MP., Lumbsch HT. (2014) How do you solve a problem like *Letharia*? A new look at cryptic species in lichen-forming fungi using Bayesian clustering and SNPs from multilocus sequence data. PLoS One 9:e97556.

Articus K., Mattsson JE., Tibell L., Grube M., Wedin M. (2002) Ribosomal DNA and beta-tubulin data do not support the separation of the lichens *Usnea florida* and *U. subfloridana* as distinct species. Mycological Research 106: 412–418.

Baas Becking LGM. (1934) Geobiologie of Inleiding tot de Milieukunde. The Hague: Van Stockum & Zoon

Baird HP., Miller KJ., Stark JS. (2011) Evidence ofhidden biodiversity, ongoing speciation and diverse patterns of genetic structure in giant Antarctic amphipods. Mol. Ecol. 20: 3439–3454.

Baird NA., Etter PD., Atwood TS., Currey MC., Shiver AL., Lewis ZA., Selker EU., Cresko WA., Johnson EA. (2008) Rapid SNP discovery and genetic mapping using sequenced RAD markers. Plos One 3(10): e3376.

Baranzelli MC., Cosacov A., Espíndola A., del Rosario Iglesias M., Chan LM., Johnson LA., Sérsic AN. (2018) Echoes of the whispering land: interacting roles of vicariance and selection in shaping the evolutionary divergence of two *Calceolaria* (Calceolariaceae) species from Patagonia and Malvinas/Falkland Islands. Evolution and Ecology 32: 287–314.

Barnes DKA., Hodgson DA., Convey P., Allen CS., Clarke A. (2006) Incursion and excursion of Antarctic biota: past, present and future. Glob Ecol Biogeogr 15: 121–142.

Beck A., Bechteler J., Casanova-Katny A., Dzhilyanova I. (2019) The pioneer lichen *Placopsis* in maritime Antarctica: Genetic diversity of their mycobionts and green algal symbionts, and their correlation with deglaciation time. Symbiosis 79: 1–24.

Beier S., Thiel T., Münch T., Scholz U., Mascher M. (2017) MISA-web: a web server for microsatellite prediction, Bioinformatics 33(16): 2583–2585.

Belinchón R., Ellis CJ., Yahr R. (2014) Microsatellite loci in two epiphytic lichens with contrasting dispersal modes: *Nephroma laevigatum* and *N. parile* (Nephromataceae) App Plant Sci 2(11): 1400080.

Bengtsson BO. (2003) Genetic variation in organisms with sexual and asexual reproduction. J Evol Biol 16: 189–199.

Bergstrom DM. & Selkirk P. (1997) Distribution of bryophytes on subantarctic Heard Island. Bryologist 100: 349–355.

Biersma EM., Jackson JA., Bracegirdle TJ., Griffiths H., Linse K., Convey P. (2018a) Low genetic variation between South American and Antarctic populations of the

bank-forming moss *Chorisodontium aciphyllum* (Dicranaceae). Polar Biology 41: 599–610.

Biersma EM., Jackson JA., Stech M., Griffiths H., Linse K., Convey P. (2018b) Molecular data suggest long-term in situ Antarctic persistence within Antarctica's most speciose plant genus, *Schistidium*. Frontiers in Ecology and Evolution 6: 77.

Blacket MJ., Robin C., Good RT., Lee SF., Miller AD. (2012) Universal primers for fluorescent labelling of PCR fragments—An efficient and cost-effective approach to genotyping by fluorescence. Mol Ecol Resour.12: 456–463.

Bolger AM., Lohse M., Usadel B. (2014) Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30: 2114–2120.

Bradnam KR., Fass JN., Alexandrov A., et al. (2013) Assemblathon 2: evaluating de novo methods of genome assembly in three vertebrate species, GigaScience,2(10): 1-31.

Buschbom J. & Barker D. (2006) Evolutionary history of vegetative reproduction in *Porpidia* s.l. (lichen-forming ascomycota). Syst Biol 55: 471–484.

Buschbom J. & Mueller GM. (2006) Testing "species pair" hypotheses: evolutionary processes in the lichen-forming species complex *Porpidia flavocoerulescens* and *Porpidia melinodes*. Mol Biol Evol 23:574–586.

Chong CW., Pearce DA., Convey P. (2015) Emerging spatial patterns in Antarctic prokaryotes. Frontiers in Microbiology 6: 1058–1071.

Chown SL. & Convey P. (2007) Spatial and temporal variability across life's hierarchies in the terrestrial Antarctic. Phil Trans R Soc B 362: 2307–2331.

Chown SL., Clarke A., Fraser CI., Cary SC., Moon KL., McGeoch MA. (2015) The Changing Form of Antarctic Biodiversity. Nature 522(7557): 431–438.

Clement M., Snell Q., Walke P., Posada D., Crandall K. (2002) TCS: estimating gene genealogies. In: Proc 16th Int Parallel Distrib Process Symp., Fort Lauderdale, FL, Apr. 15–19, 2002. Vol. 2. Los Alamitos, CA: IEEE. p. 184.

Convey P. & Stevens MI. (2007) Antarctic Biodiversity. Science 317: 1877–1878.

Convey P., Chown SL., Clarke A. et al. (2014) The spatial structure of Antarctic biodiversity. Ecol Monogr 84: 203-244.

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

Cornejo C. & Scheidegger C. (2015) Multi-gene phylogeny of the genus *Lobaria*: Evidence of species pair and allopatric cryptic speciation in East Asia. Am J Bot 102(12): 2058–2073.

Cornejo C., Chabanenko S., Scheidegger C. (2009) Phylogenetic analysis indicates transitions from vegetative to sexual reproduction in the *Lobaria retigera* group (Lecanoromycetidae, Ascomycota). Lichenologist 41(3): 275–284.

Cornejo C., Chabanenko S., Scheidegger S. (2018) Are species pairs diverging lineages? A nine-locus analysis uncovers speciation among species pairs of the *Lobaria meridionalis*-Group (Ascomycota). Molecular Phylogenetics and Evolution 129: 48–59.

Courtright EM, Wall DH, Virginia RA, et al. (2000) Nuclear and mitochondrial DNA sequence diversity in the Antarctic nematode *Scottnema lindsayae*. J Nematol 32: 143–153.

Cowan DA., Chown SL., Convey P., Tuffin M., Hughes K., Pointing S., Vincent WS. (2011) Non-Indigenous Microorganisms in the Antarctic: Assessing the Risks. Trends in Microbiology 19(11): 540–548.

Crame JA. (1999) An evolutionary perspective on marine faunal connections between southernmost South America and Antarctica. Sci. Mar. 63: 1–14.

Crespo A. & Pérez-Ortega S. (2009) Cryptic species and species pairs in lichens: a discussion on the relationship between molecular phylogenies and morphological characters. Anales Del Jardín Botánico de Madrid 66(S1): 71–81.

Cubero OF. & Crespo A. (2002) Isolation of nucleic acids from lichens. In I. C. Kranner, R. P. Beckett, and A. K. Varma [eds.], Protocols in lichenology: Culturing, biochemistry, ecophysiology, and use in biomonitoring, 381–391. Springer, Berlin, Germany.

Dal Grande F., Widmer I., Beck A., Scheidegger C. (2009) Microsatellite markers for *Dictyochloropsis reticula*ta (Trebouxiophyceae), the symbiotic alga of the lichen *Lobaria pulmonaria* (L.). Conserv Genet 11: 1147– 1149.

Darvill CM., Stokes CR., Bentley MJ., Lovell H. (2014) A glacial geomorphological map of the southernmost ice lobes of Patagonia: the Bahía Inútil – San Sebastián, Magellan, Otway, Skyring and Río Gallegos lobes. Journal of Maps 10: 500–520.

Davey JW., Hohenlohe PA., Etter PD., Boone JQ., Catchen JM., Blaxter ML. (2011) Genome-wide genetic marker discovery and genotyping using next-generation sequencing. Nature Reviews Genetics 12: 499–510.

De Queiroz K. (2007) Species Concepts and Species Delimitation. Systematic Biology 56(6): 879–886.

Del-Prado R., Divakar P.K., Lumbsch H.T., Crespo A.M. (2016) Hidden Genetic Diversity in an Asexually Reproducing Lichen Forming Fungal Group. PloS one, 11(8), e0161031.

Devkota S., Cornejo C., Werth S., Chaudhary RP., Scheidegger C. (2014) Characterization of microsatellite loci in the Himalayan lichen fungus *Lobaria pindarensis* (Lobariaceae). Appl Plant Sci 2: 1300101.

Domaschke S., Fernández-Mendoza F., García MA., Printzen C. (2012) Low genetic diversity in Antarctic populations of the lichen *Cetraria aculeata* and its photobiont. Polar Research 31: 17353.

Du Rietz G.E. (1924) Die Soredien und Isidien der Flechten. Svensk Botanisk Tidskrift. 18: 371-396.

Duffy GA., Coetzee BWT., Latombe G., Akerman AH., McGeoch MA., Chown SL. (2017) Barriers to globally invasive species are weakening across the Antarctic. Diversity and Distributions 23(9): 982–96.

Eaton DAR. & Ree RH. (2013) Inferring phylogeny and introgression using RADseq data: An example from flowering plants (*Pedicularis*: Orobanchaceae). Systematic Biology 62: 689–706.

Eisenreich W., Knispel N., Beck A. (2011) Advanced methods for the study of the chemistry and the metabolism of lichens. Phytochemistry Reviews 10: 445–456.

Eizaguirre JI., Peris D., Rodríguez ME., Lopes CA., De Los Ríos P., Hittinger CT., Libkind D. (2018) Phylogeography of the wild Lager-brewing ancestor (*Saccharomyces eubayanus*) in Patagonia. Environmental Microbiology 20: 3732–3743.

Ellegren H. (2014) Genome sequencing and population genomics in non-model organisms. Trends in Ecology & Evolution 29: 51–63.

Evanno G. Regnaut S. Goudet J. (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. Mol Ecol 14: 2611-2620.

Falush D., Stephens M., Pritchard JK. (2003) Inference of population structure: Extensions to linked loci and correlated allele frequencies. Genetics 164: 1567–1587.

Farrell RL., Arenz BE., Duncan SM., Held BW., Jurgens JA., Blanchette RA. (2011) Introduced and indigenous fungi of the Ross Island historic huts and pristine areas of Antarctica. Polar Biol 34: 1669–1677.

Fenchel T. & Finlay BJ (2004) The ubiquity of small species: patterns of local and global diversity. Bioscience 54: 777–784.

Fernández-Mendoza F. & Printzen C. (2013) Pleistocene expansion of the bipolar lichen *Cetraria aculeata* into the Southern hemisphere. Mol Ecol 22: 1961–1983.

Fernández-Mendoza F., Domaschke S., García MA., Jordan P., MartínMP, Printzen C. (2011) Population structure of mycobionts and photobionts of the widespread lichen *Cetraria aculeata*. Mol Ecol 20: 1208–1232.

Feuerer T. & Hawksworth DL. (2007) Biodiversity of lichens, including a world-wide analysis of checklist data based on Takhtajan's floristic regions. Biodivers Conserv. 16(1): 85–98.

Finlay BJ. (2002) Global dispersal of free-living microbial eukaryote species. Science 296: 1061–1063.

Francis RM. (2016) POPHELPER: An R package and web app to analyze and visualize population structure. Mol Ecol Resour 17(1):27-32.

Fraser CI., Morrison AK., McC Hogg A., Macaya EC., van Sebille E., Ryan PG., Padovan A., Cameron J., Valdivia N., Waters LM. (2018) Antarctica's Ecological Isolation Will Be Broken by Storm-Driven Dispersal and Warming. Nature Climate Change 8(8):704–708.

Fraser CI., Nikula R., Ruzzante DE., Waters JM. (2012) Poleward bound: Biological impacts of Southern Hemisphere glaciation. Trends in Ecology & Evolution 27: 462–471

Fraser CI., Terauds A., Smellie J., et al. (2014) Geothermal activity helps life survive glacial cycles. PNAS 111: 5634–5639.

Frenot Y., Chown SL., Whinam J., Selkirk PM., Convey P., Skotnicki M., Bergstrom M. (2005) Biological invasions in the Antarctic: extent, impacts and implications. Biological Reviews, 80: 45–72.

Galloway DJ., Lewis-Smith RI., Quilhot W. (2005) A New Species of *Placopsis* (Agyriaceae: Ascomycota) from Antarctica. Lichenologist 37(4): 321–327.

Gardes M. & Bruns TD. (1993) Its primers with enhanced specificity for basidiomycetes-application to identification of mycorhizae and rusts. Mol Ecol 2: 113–118.

Geiser DM., Pitt JI., Taylor JW. (1998) Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. Proc Natl Acad Sci USA 95: 388–393.

Geml J., Kauff F., Brochmann C., Lutzoni F., Laursen GA., Redhead SA., Taylor DL. (2012) Frequent circumarctic and rare transequatorial dispersals in the lichenised agaric genus *Lichenomphalia* (Hygrophoraceae, Basidiomycota). Fungal Biol 116: 388–400.

Glasser N. & Jansson K. (2008) The glacial map of southern South America. Journal of Maps 4: 175–196.

Gonthier P., Sillo F., Lagostina E., Roccotelli A., Cacciola OS., Stenlid J., Garbelotto M. (2015) Selection processes in simple sequence repeats suggest a correlation with their genomic location: insights from a fungal model system. BMC Genomics 16(1): 1107.

Green TGA., Sancho LG., Türk R., et al. (2011) 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.

Green TGA., Schroeter B., and Sancho LG. (1999) Plant Life in Antarctica. In Handbook of Functional Plant Ecology (F. Pugnaire, and F. Valladares, eds), New York: Marcel Dekker Inc., pp 496-543.

Greenslade P. & Convey P. (2012) Exotic Collembola on subantarctic islands: pathways, origins and biology. Biol Inv 14: 405–417.

Grube M. & Winka K. (2002) Progress in understanding the evolution and classification of lichenized ascomycetes. Th e Mycologist 16: 67 – 76.

Guillemin ML., Dibrasquet H., Reyes J., Valero M. (2018) Comparative phylogeography of six red algae along the Antarctic Peninsula: extreme genetic depletion linked to historical bottlenecks and recent expansion. Polar Biology 41: 827–837.

Guzow-Krzeminska B. & Stocker-Wörgötter E. (2013) Development of microsatellite markers in *Protoparmeliopsis muralis* (lichenized Ascomycete)—a common lichen species. Lichenologist 45:791–798.

Hagen M., Kissling WD., Rasmussen C., De Aguiar MA., Brown LE., Carstensen DW., Alves-Dos-Santos I., Dupont YL., Edwards FK., Genini J., Guimaraes PR Jr (2012) Biodiversity, species interactions and ecological networks in a fragmented world. Adv Ecol Res 46: 89–120.

Halanych KM. & Mahon AR. (2018) Challenging Dogma Concerning Biogeographic Patterns of Antarctica and the Southern Ocean. Annual Review of Ecology, Evolution, and Systematics 49(1): 355–378.

Hansen J., Ruedy R., Glascoe J., Sato M. (1999) GISS analysis of surface temperature change. J Geophys Res. 104: 30997–31022.

Harder AM., Halanych KM., Mahon AR. (2016) Diversity and distribution within the sea spider genus *Pallenopsis* (Chelicerata: Pycnogonida) in the Western Antarctic as revealed by mitochondrial DNA. Polar Biol. 39: 677–688.

Hausdorf B. & Hennig C. (2010) Species Delimitation Using Dominant and Codominant Multilocus Markers, Systematic Biology 59(5): 491–503.

Havermans C., Nagy ZT., Sonet G., De Broyer C., Martin P. (2011) DNA barcoding reveals new insights into the diversity of Antarctic species of *Orchomene* sensu lato (Crustacea: Amphipoda: Lysianassoidea). Deep-Sea Res. Part II 58: 230–241.

Hebel I., Dacasa Rüdinger MC., Jaña RA., Bastias J. (2018) Genetic structure and gene flow of moss *Sanionia uncinata* (Hedw.) Loeske in Maritime Antarctica and Southern-Patagonia. Frontiers in Ecology and Evolution 6: 152.

Helms, G., Friedl, T., Rambold G., Mayrhofer H. (2001) Identification of photobionts from the lichen family *Physciaceae* using algal-specific ITS rDNA sequencing. Lichenologist 33: 74–86.

Hestmark G., Lutzoni F., Miadlikowska J. (2016) Photobiont Associations in Co-Occurring Umbilicate Lichens with Contrasting Modes of Reproduction in Coastal Norway. Lichenologist 48 (5): 545–57.

Hewitt GM. (2004) Genetic consequences of climatic oscillations in the Quaternary. Philosophical Transaction of the Royal Society London, Series B, 359: 183–195.

Hills SFK., Stevens MI., Gemmill CEC. (2010) Molecular support for Pleistocene persistence of the continental Antarctic moss *Bryum argenteum*. Antarctic Science 22: 721–726.

Hodel RGJ., Segovia-Salcedo MC., Landis JB., Crowl AA., et al. (2016) The Report of My Death Was an Exaggeration: A Review for Researchers Using Microsatellites in the 21st Century. Applications in Plant Sciences 4(6): 1600025.

Honegger R. (2000) Great Discoveries in bryology and lichenology – Simon Schwendener (1829-1919) and the Dual Hypothesis of Lichens. Bryologist. 103(2): 307–313.

Huges KA. (2003) Aerial dispersal and survival of sewage-derived faecal coliforms in Antarctica. Atmos Env 37: 3147–3155.

Hughes KA. & Convey P. (2010) The protection of Antarctic terrestrial ecosystems from inter- and intra-continental transfer of non-indigenous species by human activities: A review of current systems and practices. Global Env Change 20: 96–112.

Hughes KA. & Worland MR. (2010) Spatial distribution, habitat preference and colonization status of two alien terrestrial invertebrate species in Antarctica. Antarctic Sci 22: 221–231.

Huiskes AHL., Gremmen NJM., Bergstrom DM., Frenot Y., Hughes KA., Imura S., Kiefer K., Lebouvier M., Lee JF., Tsujimoto M., Ware C.,Van de Vijver B., Chown SL. (2014) Aliens in Antarctica: Assessing Transfer of Plant Propagules by Human Visitors to Reduce Invasion Risk. Biological Conservation 171: 278–284.

Jakobsson M. & Rosenberg NA. (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23(14): 1801–1806.

Jombart T. (2008) adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24: 1403–1405.

Jombart T. & Ahmed I. (2011) adegenet 1.3-1: new tools for the analysis of genome-wide SNP data. Bioinformatics 27: 3070–3071.

Jombart T., Devillard S., Balloux F. (2010) Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. Bmc Genetics 11(1): 94.

Jones TC, Hogg ID, Wilkins RJ, Green TGA. (2013) Photobiont selectivity for lichens and evidence for a possible glacial refugium in the Ross Sea region, Antarctica. Polar Biology 36: 767–774.

Jones TC., Green TGA., Hogg ID., Wilkins RJ. (2012) Isolation and characterization of microsatellites in the lichen *Buellia frigida* (Physciaceae), an Antarctic endemic. Am J Bot 99: 131–133.

Jones TC., Hogg ID., Wilkins RJ Green TGA. (2015) Microsatellite analyses of the Antarctic endemic lichen *Buellia frigida* Darb. (Physciaceae) suggest limited dispersal and the presence of glacial refugia in the Ross Sea region. Polar Biology 38(7). Springer Berlin Heidelberg: 941–949.

Kärnefelt I. (1979) The brown fruticose species of *Cetraria*. Opera Botanica 46: 1–150.

Kasuga T., Taylor JW., White T.J (1999) Phylogenetic relationships of varieties and geographical groups of the human pathogenic fungus *Histoplasma capsulatum* Darling. J Clin Microbiol 37: 653–663.

Kearse M., Moir R., Wilson A., Stones-Havas S., Cheung M., Sturrock S., Buxton S., Cooper A., Markowitz S., Duran C., Thierer T., Ashton B., Meintjes P., Drummond A. (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28: 1647–1649.

Koufopanou V., Burt A., Szaro T., Taylor JW. (2001) Gene genealogies, cryptic species, and molecular evolution in the human pathogen *Coccidioides immitis* and relatives (Ascomycota, Onygenales). Mol Biol Evol 18: 1246–1258.

Kraus RHS. & Wink M. (2015) Avian genomics: fledging into the wild! Journal of Ornithology 156: 851–865.

Kroken S. & Taylor J.W. (2000) Phylogenetic species, reproductive mode, and specificity of the green alga *Trebouxia* forming lichens with the fungal genus *Letharia*. Bryologist 103: 645–660.

Lawver LA. & Gahagan LM. (2003) Evolution of Cenozoic seaways in the circum-Antarctic region. Palaeogeogr. Palaeoclimatol. Palaeoecol 198: 11–37.

Leavitt SD., Grewe F., Widhelm T., Muggia L., Wray B., Lumbsch HT. (2016) Resolving evolutionary relationships in lichen-forming fungi using diverse phylogenomic data-sets and analytical approaches. Scientific Reports 6:22262

Lee JE. & Chown SL. (2011) Quantification of intra-regional propagule movements in the Antarctic. Antarctic Sci 23: 337–342.

Lee JR., Raymond B., Bracegirdle TJ., Chadès I., Fuller RA., Shaw JD., Terauds A. (2017) Climate change drives expansion of Antarctic ice-free habitat. Nature 547: 49–54.

Lendemer JC. & Harris RC. (2014) Studies in lichens and lichenicolous fungi—no. 18: resolution of three names introduced by Degelius and Magnusson based on material from the Great Smoky Mountains. Castanea 79: 106–117.

Lewis LR., Behling E., Gousse H., Qian E., Elphick,CS., Lamarre JF., Bêty J., Liebezeit J., Rozzi, R., Goffinet B,. (2014a) First evidence of bryophyte diaspores in the plumage of transequatorial migrant birds. PeerJ 2: e424.

Lewis LR., Rozzi R., Goffinet B,. (2014b) Direct long-distance dispersal shapes a New World amphitropical disjunction in the dispersal-limited dung moss *Tetraplodon* (Bryopsida: *Splachnaceae*). Journal of Biogeography, 41(12): 2385-2395.

Lewis-Smith RI. (1984) Colonization and recovery by cryptogams following recent volcanic activity on Deception Island, South Shetland Islands. British Antarctic Survey Bulletin, 62: 25-51.

Liebrand D., de Bakker AT., Beddow HM., Wilson PA., Bohaty AM., et al. (2017) Evolution of the early Antarctic ice ages. PNAS 114: 3867–72.

Lindgren H., Leavitt SD., Lumbsch HT. (2016) Characterization of microsatellite markers in the cosmopolitan lichen-forming fungus *Rhizoplaca melanophthalma* (Lecanoraceae). MycoKeys 14: 31-36.

Liu J., Wilson M., Hu G., Liu J., Wu J., Yu M. (2018) How Does Habitat Fragmentation Affect the Biodiversity and Ecosystem Functioning Relationship? Landscape Ecology 33 (3). Springer Netherlands: 341–352.

Lohtander K., Myllys L., Källersjö M., Moberg R., Stenroos S., Tehler A. (2009) New entities in Physcia aipolia–P. caesia group (Physciaceae, Ascomycetes): an analysis based on mtSSU, ITS, group I intron and betatubulin sequences. Annales Botanici Fennici 46:43–53. Lohtander

Lohtander K., Myllys L., Sundin R., Källersjö M., Tehler A. (1998) The species pair concept in the lichen *Dendrographa leucophaea* (Arthoniales): analyses based on ITS sequences. The Bryologist 101: 404–411.

Lumbsch H. & Wirtz N. (2011) Phylogenetic relationships of the neuropogonoid core group in the genus *Usnea* (Ascomycota: Parmeliaceae). The Lichenologist, 43(6), 553-559.

Lumbsch HT. & Leavitt SD. (2011) Goodbye morphology? A paradigm shift in the delimitation of species in lichenized fungi. Fungal Diversity 50: 59–72.

Lutsak T., Fernández-Mendoza F., Greshake B., Dal Grande F., Ebersberger I., Ott S., Pritzen C. (2016) Characterization of microsatellite loci in the lichen-forming fungus *Cetraria aculeata* (Parmeliaceae, Ascomycota). Applications in Plant Sciences 4(9): 1600047.

Lutsak T., Fernández-Mendoza F., Kirika P., Wondafrash M., Pritzen C. (in press 2020) Coalescence-based species delimitation using genome-wide data reveals hidden diversity in a cosmopolitan group of lichens. Organisms diversity & evolution.

Magain N., Forrest LL., Serusiaux E., Goffinet B. (2010) Microsatellite primers in the *Peltigera dolichorhiza* complex (lichenized ascomycete, Peltigerales). Am J Bot 97: e102–e104

Magrach A., Laurance WF., Larrinaga AR., Santamaria L. (2014) Meta-analysis of the effects of forest fragmentation on interspecific interactions. Conserv Biol 28: 1342–1348.

Mark K., Saag L., Leavitt SD., Will-Wolf S., Nelsen MP., Tõrra T., Saag A., Randlane T., Lumbsch HT. (2016) Evaluation of traditionally circumscribed species in the lichen-forming genus *Usnea*, section *Usnea* (*Parmeliaceae*, Ascomycota) using a six-locus dataset. Organisms Diversity & Evolution 16: 497–524.

Mattsson JE. & Lumbsch HT. (1989) The use of the species pair concept in lichen taxonomy. Taxon 38: 238–241.

McGaughran A., Torricelli G., Carapelli A., et al. (2010) Contrasting phylogeographical paterns for springtails reflect different evolutionary histories between the Antarctic Peninsula and continental Antarctica. Journal of Biogeography 37: 103–119.

Meirmans PG. & Van Tienderen P.H. (2004) GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms, Molecular Ecology Notes, 4(4): 792-794.

Meredith MP. & King JC. (2005) Rapid climate change in the ocean west of the Antarctic Peninsula during the second half of the 20th century. Geophys Res Lett 32: L19604.

Messuti MI., Passo A., Scervino JM., Vidal-Russell R. (2016) The species pair *Pseudocyphellaria pilosella-piloselloides* (lichenized Ascomycota: Lobariaceae) is a single species. Lichenologist 48: 141–146.

Miadlikowska J., Schoch CL., Kageyama SA., Molnar K., Lutzoni F., McCune B. (2011) *Hypogymnia* phylogeny, including *Cavernularia*, reveals biogeographic structure. Bryologist 114: 392–400.

Millanes AM., Diederich P., Wedin M. (2016) *Cyphobasidium* gen. nov., a new licheninhabiting lineage in the *Cystobasidiomycetes* (*Pucciniomycotina, Basidiomycota*, Fungi). Fungal Biol. 120(11): 1468-1477.

Mortimer E., Jansen van Vuuren B., Lee JE., et al. (2011) Mite dispersal among the Southern Ocean islands and Antarctica before the last glacial maximum. Proceedings of the Royal Society B 278: 1247–1255.

Muggia L., Kraker S., Gößler T., Grube M. (2018) Enforced fungal-algal symbioses in alginate spheres, FEMS Microbiology Letters. 365: 14.

Muñoz J., Felicisimo M., Cabezas F., et al. (2004) Wind as a long-distance dispersal vehicle in the southern hemisphere. Science 304: 1144–1147.

Myllys L., Lohtander K., Tehler A. (2001) Beta-tubulin, ITS and group I intron sequences challenge the species pair concept in *Physcia aipolia* and *P. caesia*. Mycologia 93: 335–343.

Nadyeina O., Cornejo C., Boluda CG., Myllys L., Rico VJ., Crespo A., Scheidegger C. (2014) Characterization of microsatellite loci in lichen-forming fungi of *Bryoria* Section *Implexae* (Parmeliaceae). Appl Plant Sci 2: 1400037.

Nash III TH. (2008) Lichen biology, 2nd ed., pp. 486. Cambridge University Press, Cambridge, UK.

Nolan C., Overpeck JO., Allen JRM., Anderson PM., Betancourt JL., Binney HA., Brewer S., Bush MB., Chase BM., Cheddadi R., et al (2018) Past and future global

transformation of terrestrial ecosystems under climate change. Science 361: 920–923.

Nolan L., Hogg ID., Stevens MI., Haase M. (2006) Fine scale distribution of mtDNA haplotypes for the springtail *Gomphiocephalus hodgsoni* (Collembola) corresponds to an ancient shoreline in Taylor Valley, continental Antarctica. Polar Biol 29: 813–819.

Nordal I. (1987) Tabula rasa after all? Botanical evidence for ice-free refugia in Scandinavia reviewed. Journal of Biogeography 14: 377–388.

Nurk S., Bankevich A., Antipov D., et al. (2013) Assembling genomes and mini-metagenomes from highly chimeric reads. In M. Deng, R. Jiang, F. Sun, and X. Zhang [eds.], Research in computational molecular biology, vol. 7821, 158–170. Springer, Berlin, Germany.

Ogilvie HA., Heled J., Xie D., Drummond AJ. (2016) Computational performance and statistical accuracy of *BEAST and comparisons with other methods, Systematic Biology 65:381–396.

Olech M. (1996) Human impact on terrestrial ecosystems in West Antarctica. Proc NIPR Symp Polar Biol 9: 299–306.

Osyczka P. (2010) Alien lichens unintentionally transported to the “Arctowski” station (South Shetlands, Antarctica). Polar Biol 33: 1067–1073.

Otálora MAG., Salvador C., Martínez I., Aragón G. (2013) Does the reproductive strategy affect the transmission and genetic diversity of bionts in cyanolichens? A case study using two closely related species. Microb Ecol 65: 517–530.

Øvstedal DO. & Lewis Smith RI. (2001) Lichens of Antarctica and South Georgia. A guide to their identification and ecology. Cambridge University Press 424 pp.

Ozsolak F. & Milos PM. (2011) RNA sequencing: advances, challenges and opportunities. Nature Reviews Genetics 12: 87–98.

Papke RT., Ramsing NB., Bateson MM., Ward DM. (2003) Geographical isolation in hot spring cyanobacteria. Environ Microbiol 5: 650–659.

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

Peakall R. & Smouse PE. (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics 28: 2537-2539.

Peat HJ., Clarke A., Convey, P. (2007) Diversity and biogeography of the Antarctic flora. J. Biogeogr 34: 132–146.

Peh KS, Lin Y, Luke SH. (2014) Forest fragmentation and ecosystem function. In: Kettle CJ, Koh LP (eds) Global forest fragmentation. CABI, Wallingford 96–114.

Poelt J. (1970) Das Konzept der Artenpaare bei den Flechten. Vorträge aus dem Gesamtgebiet der Botanik 4: 187-198.

Printzen C., Domaschke S., Fernández-Mendoza F., Pérez-Ortega S. (2013) Biogeography and ecology of *Cetraria aculeata*, a widely distributed lichen with a bipolar distribution. MycoKeys 6: 33-53.

Printzen C., Ekman S., Tønsberg T. (2003) Phylogeography of *Cavernularia hultenii*: Evidence for slow genetic drift in a widely disjunct lichen. Mol Ecol 12: 1473–1486.

Pritchard JK., Stephens M., Donnelly, P. (2000) Inference of population structure using multilocus genotype data. Genetics 155: 945–959.

Pugh PJA. & Convey P. (2008) Surviving out in the cold: Antarctic endemic invertebrates and their refugia. Journal of Biogeography 35: 2176–2186.

Rodriguez, JM., Passo A., Chiapella JO. (2018) Lichen Species Assemblage Gradient in South Shetlands Islands, Antarctica: Relationship to Deglaciation and Microsite Conditions. Polar Biology, no. 0123456789. Springer Berlin Heidelberg.

Rogers AD. (2007) Evolution and Biodiversity of Antarctic Organisms: A Molecular Perspective. Philosophical Transactions of the Royal Society B: Biological Sciences 362 (1488): 2191–2214.

Romeike J., Friedl T., Helms G., Ott S. (2002) Genetic Diversity of Algal and Fungal Partners in Four Species of *Umbilicaria* along a Transect of the Antarctic Peninsula. 1209–1217.

Rubin BER. & Moreau CS. (2016) Comparative genomics reveals convergent rates of evolution in ant- plant mutualisms. Nature Communications 7: 12679.

Ruiz-Fernández J. & Oliva M. (2016) Relative paleoenvironmental adjustments following deglaciation of the Byers Peninsula (Livingston Island, Antarctica). Arctic, Antarctic, and Alpine Research 48: 345–359.

Ruprecht U., Brunauer G, Printzen C. (2012) Genetic Diversity of Photobionts in Antarctic Lecideoid Lichens from an Ecological View Point. The Lichenologist 44(05): 661–678.

Saag L., Mark K., Saag A., Randlane T. (2014) Species delimitation in the lichenized fungal genus *Vulpicida* (Parmeliaceae, Ascomycota) using gene concatenation and coalescent-based species tree approaches. Am J Bot 101: 2169–2182.

Scheidegger C., Bilovitz PO., Werth S., Widmer I., Mayrhofer H. (2012) Hitchhiking with forests: population genetics of the epiphytic lichen *Lobaria pulmonaria* in primeval and managed forests in southeastern Europe. Ecology and Evolution 2: 2223–2240.

Scher HD. & Martin EE. (2006) Timing and climatic consequences of the opening of Drake Passage. Science 312: 428–430.

Sérsic AN., Cosacov A., Cocucci AA., Johnson LA., Pozner R., Avila LJ., Sites JW jr., Morando M. (2011) Emerging phylogeographical patterns of plants and terrestrial vertebrates from Patagonia. Biological Journal of the Linnean Society 103: 475–494.

Seymour F., Crittenden P., Wirtz N., Øvstedal D., Dyer P., Lumbsch HT. (2007) Phylogenetic and morphological analysis of Antarctic lichen-forming *Usnea* species in the group *Neuropogon*. Antarctic Science 19: 71–82.

Shepherd A., Fricker HA., Farrell SL. (2018) Trends and connections across the Antarctic cryosphere. Nature 588: 223–232.

Simms AR., Milliken KT., Anderson JB., Wellner JS. (2011) The marine record of deglaciation of the South Shetland Islands, Antarctica since the Last Glacial Maximum. Quaternary Science Reviews 30: 1583–1601.

Simões CL., da Rosa KK., Czapela FF., Vieira R., Simões JC. (2015) Collins glacier retreat process and regional climatic variations, King George Island, Antarctica. Geogr Rev 105: 462–471.

Singh SM., Olech M., Cannone N., Convey P. (2015) Contrasting patterns in lichen diversity in the continental and maritime Antarctic. Polar Science 9(3): 311–318.

Skotnicki ML., Mackenzie AM., Ninham JA., Selkirk PM. (2004) High levels of genetic variability in the moss *Ceratodon purpureus* from continental Antarctica, subantarctic Heard and Macquarie Islands, and Australasia. Polar Biology 27: 687–698.

Smith MD. & Knapp, A. K. (2003) Dominant species maintain ecosystem function with non-random species loss. Ecology Letters 6: 509-517.

Soler-Membrives A., Linse K., Miller KJ., Arango CP. (2017) Genetic signature of Last Glacial Maximum regional refugia in a circum-Antarctic sea spider. Open Sci. 4: 170615.

Solhaug KA. & Gauslaa Y. (2012) Secondary lichen compounds as protection against excess solar radiation and herbivores. Progress in Botany 73 (eds Lüttge U., Beyschlag W., Büdel B., Francis D.), pp. 283–304. Springer, Berlin, Heidelberg.

Spribille T., Tuovinen V., Resl P., Vanderpool D., Wolinski H., Aime MC., Schneider K., Stabentheiner E., Toome-Heller M., Thor G., et al. (2016) Basidiomycete yeasts in the cortex of ascomycete macrolichens. Science.;353(6298): 488–492.

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). Zool. Scripta 36: 201–212.

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. Mol. Ecol. 12: 2357–69.

Stratton M. (2008) Genome resequencing and genetic variation. Nature Biotechnology 26: 65–66.

Strous M., Kraft B., Bisdorf R., Tegetmeyer HT. (2012) The binning of metagenomic contigs for microbial physiology of mixed cultures. Frontiers in Microbiology 3: 410.

Tehler A. (1982) The species pair concept in lichenology. Taxon 31: 708–714.

Tehler A., Damien E., Irestedt M. (2013) The genus *Dirina* (Roccellaceae, Arthoniales) revisited. Lichenologist 45: 427–476.

Terauds A., Chown SL., Morgan F., Peat HJ., Watts DJ., Keys H., Convey P., Bergstrom DM. (2012) Conservation biogeography of the Antarctic. Diversity and Distributions 18 (7): 726–41.

Thatje S., Hillenbrand CD., Larter R. (2005) On the origin of Antarctic marine benthic community structure. Trends Ecol. Evol. 20: 534–540.

Thomas ER., Hosking JS., Tuckwell RR., WarrenRA., Ludlow EC. (2015) Twentieth century increase in snowfall in coastal West Antarctica. Geophys. Res. Lett. 42: 9387–9393.

Tibell LB. (1994) Distribution patterns and dispersal strategies of *Caliciales*. Bot J Linn Soc 116: 159–202.

Tõrra T., Cornejo C., Cheenacharoen S., Grande FD., Marmor L., Scheidegger C. (2014) Characterization of fungus-specific microsatellite markers in the lichen fungus *Usnea subfloridana* (Parmeliaceae). Appl Plant Sci 2:1400034.

Tripp EA. (2016) Is asexual reproduction an evolutionary dead end in lichens? The Lichenologist 48: 559–580.

Turner J., Colwell SR., Marshall GJ., Lachlan-Cope TA., Carleton AM., Jones PD., Lagun V., Reid PA., Iagovkina S. (2005) Antarctic climate change during the last 50 years. Int J Climatol 25: 279–294.

Turner J., Lu H., White I., King JC., Phillips T., Hosking JS., Bracegirdle TJ., Marshall GJ., Mulvaney R., Deb P. (2016) Absence of 21st century warming on Antarctic Peninsula consistent with natural variability. Nature 535: 411–415.

van de Wouw M., van Dijk P., Huiskes AHL. (2008) Regional genetic diversity patterns in Antarctic hairgrass (*Deschampsia antarctica* Desv.). Journal of Biogeography 35: 365–376.

Vaughan DG., Marshall GJ., Connolley WM., King JC., Mulvaney R. (2001) Devil in the detail. Science. 293: 1777–1779.

Vera ML. (2011) Colonization and demographic structure of *Deschampsia antarctica* and *Colobanthus quitensis* along an altitudinal gradient on Livingston Island, South Shetland Islands, Antarctica. Polar Res 30: 7146.

Volonterio O., Ponce de León R., Convey P., Krzemińska E. (2013) First record of *Trichoceridae* (Diptera) in the maritime Antarctic. Polar Biol 36: 1125–1131.

Wachi N., Matsubayashi KW., Maeto K. (2018) Application of next-generation sequencing to the study of non-model insects. Entomological Science 21: 3–11.

Walker FJ. (1985) The lichen genus *Usnea* subgenus *Neuropogon*. Bulletin of the British Museum (Natural History), Botany 13: 1–130.

Walser JC., Gugerli F., Holderegger R., Kuonen D., Scheidegger C. (2004) Recombination and clonal propagation in different populations of the lichen *Lobaria pulmonaria*. Heredity 93: 322–329.

Walser JC., 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(1): 72-82.

Walther GR., Post E., Convey P., Menzel A., Parmesan C., Beebee TJC., Fromentin JM., Hoegh-Guldberg O., Bairlein F. (2002) Ecological responses to recent climate change. Nature, 416: 389–395.

Wei XL., McCune B., Lumbsch HT., Li H., Leavitt S., Yamamoto Y., Tchabanenko S., Wei JC. (2016) Limitations of species delimitation based on phylogenetic analyses: A Case study in the *Hypogymnia hypotrypa* group. (Parmeliaceae, Ascomycota). PLOS ONE 11(11): e0163664

Werth S., Gugerli F., Holderegger R., Wagner HH., Csencsics D., Scheidegger C. (2007) Landscape-level gene flow in *Lobaria pulmonaria*, an epiphytic lichen. Mol Ecol 16: 2807–2815.

Werth S., Miao VPW., Jónsson ZO., Andrésson OS. (2015) High-throughput sequencing in studies of lichen population biology. In: Upreti DK., Divakar PK., Shukla V., Bajpai R. (Eds.), Recent Advances in Lichenology. Modern Methods and Approaches in Lichen Systematics and Culture Techniques, vol. 2. Springer India, New Delhi, pp. 61-94.

Whitaker RJ., Grogan DW., Taylor JW. (2003) Geographic barriers isolate endemic populations of hyperthermophilic archaea. Science 301: 976–978.

White TJ., Bruns T., Lee S., Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Taylor J, and Sninsky JJ, Eds., PCR protocols: A guide to methods and applications. New York: Academic Press Inc. pp. 315–322.

Wickett NJ., Mirarab S., Nam N., et al. (2014) Phylotranscriptomic analysis of the origin and early diversification of land plants. Proceedings of the National Academy of Sciences of the United States of America 111: 4859–4868.

Widhelm TJ., Egan RS., Bertoletti FR., Asztalos MJ., Kraichak E., Leavitt SD., Lumbsch HT. (2016) Picking holes in traditional species delimitations: an integrative taxonomic reassessment of the *Parmotrema perforatum* group (Parmeliaceae, Ascomycota). Bot. J. Linnean Soc. 182: 868–884.

Widmer A. & Lexer C. (2001) Glacial refugia: sanctuaries for allelic richness, but not for gene diversity. Trends in Ecology and Evolution 16: 267–269.

Widmer I., Dal Grande F., Excoffier L., Holderegger R., Keller C., Mikryukov VS., Scheidegger C. (2012) European phylogeography of the epiphytic lichen fungus *Lobaria pulmonaria* and its green algal symbiont. Mol Ecol 21: 5827–5844.

Wilkinson DM. (2018) What is a lichen? Changing ideas on the lichen symbiosis. British Wildlife Volume 29(5): 351-357.

Wilson MC., Chen XY., Corlett RT., et al. (2016) Habitat Fragmentation and Biodiversity Conservation: Key Findings and Future Challenges. Landscape Ecology 31 (2). Springer Netherlands: 229–227.

Wilson NG., Hunter RL., Lockhart SJ., Halanych KM. (2007) Absence ofpanmixia in the Antarctic ‘circumpolar’ crinoid *Promachocrinus kerguelensis* Carpenter, 1888. Mar. Biol. 152: 895–904.

Winkelmann R., Levermann A., Martin MA., Frieler K. (2012) Increased future ice discharge from Antarctica owing to higher snowfall. Nature 492: 239–242.

Wirtz N., Printzen C., Lumbsch HT. (2008) The delimitation of Antarctic and bipolar species of Neuropogonoid *Usnea* (*Ascomycota, Lecanorales*): A Cohesion Approach of Species Recognition for the *Usnea Perpusilla* Complex. Mycological Research 112(4): 472–484.

Wirtz N., Printzen C., Lumbsch HT. (2012) Using haplotype networks, estimation of gene flow and phenotypic characters to understand species delimitation in fungi of a predominantly Antarctic *Usnea* Group (Ascomycota, Parmeliaceae). Organisms Diversity and Evolution 12(1): 17–37.

Wittmann MJ., Metzler D., Gabriel W., Jeschke JM. (2014) Decomposing propagule pressure: the effects of propagule size and propagule frequency on invasion success. Oikos 123: 441–450.

Wu JG. (2009) Ecological dynamics in fragmented landscapes. In: Levin SA (ed) The Princeton guide to ecology. Princeton University Press, Princeton, New Jersey, pp 438–444.

Zhang, J., Kobert K., Flouri T., Stamatakis A. (2014) PEAR: A fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30: 614–620.

Zimmer EA. & Wen J. (2015) Using nuclear gene data for plant phylogenetics: Progress and prospects II. Next-gen approaches. Journal of Systematics and Evolution 53: 371–379.

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ANNEX 1: REPRINTED PUBLICATIONS

PUBLICATION 1: Elisa Lagostina, Francesco Dal Grande, Sieglinde Ott, and Christian Printzen. 2017. **Fungus-Specific SSR Markers in the Antarctic Lichens *Usnea antarctica* and *U. aurantiacoatra* (*Parmeliaceae*, Ascomycota).** Applications in Plant Sciences 5 (9).

PUBLICATION 2: Elisa Lagostina, Francesco Dal Grande, Mikhail Andreev, and Christian Printzen. 2018. **The use of microsatellite markers for species delimitation in Antarctic *Usnea* subgenus *Neuropogon*.** Mycologia.

PUBLICATION 3: Felix Grewe, Elisa Lagostina, Huini Wu, Christian Printzen, and H. Thorsten Lumbsch. 2018. **Population genomic analyses of RAD sequences resolves the phylogenetic relationship of the lichen-forming fungal species *Usnea antarctica* and *Usnea aurantiacoatra*.** MycoKeys.

MANUSCRIPT 4: Elisa Lagostina, Mikhail Andreev, Francesco Dal Grande, Felix Grewe, Aline Lorenz, H. Thorsten Lumbsch, Ricardo Rozzi, Ulrike Ruprecht, Leopoldo García Sancho, Ulrik Søchting, Mayara Scur, Nora Wirtz, Christian Printzen. **Effects of dispersal strategy and migration history on genetic diversity and population structure of Antarctic lichens.**

Applications in Plant Sciences 2017 5(9): 1700054

PRIMER NOTE

FUNGUS-SPECIFIC SSR MARKERS IN THE ANTARCTIC LICHENS *USNEA ANTARCTICA* AND *U. AURANTIACOATRA* (PARMELIACEAE, ASCOMYCOTA)[1]

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- *Premise of the study: Usnea antarctica* and *U. aurantiacoatra* (Parmeliaceae) are common lichens in the maritime Antarctic. These species share the same habitats on King George Island (South Shetland Islands, Antarctica) and are distinguishable based on reproductive strategies.
- *Methods and Results:* We developed 23 fungus-specific simple sequence repeat (SSR) markers that cross-amplify between the two species. We used a low-coverage genome-skimming approach on one sample of each species to identify SSR repeats in the two species. Primers were designed for 3–4-bp repeats, and only the loci common to both species were selected for further analyses. Seventy-seven samples of the two species were selected to assess fungal specificity, genetic variability, and linkage of the markers. In addition, we tested cross-amplification in other *Usnea* species.
- *Conclusions:* The 23 newly designed SSR markers are suitable for population genetic and phylogeographic studies of *Usnea* species.

Key words: Antarctic lichens; microsatellites; Parmeliaceae; *Usnea antarctica*; *Usnea aurantiacoatra*.

Lichens constitute an important component of Antarctic terrestrial biota. The most common lichen species in the maritime Antarctic are *Usnea aurantiacoatra* (Jacq.) Bory and *U. antarctica* Du Rietz (Lecanoromycetes, Parmeliaceae). The two species occupy similar habitats but show different dispersal strategies, morphology, and distribution. *Usnea antarctica* usually propagates asexually by so-called soredia, while *U. aurantiacoatra* has apothecia and its thalli are usually larger. *Usnea antarctica* is reported to be circumpolar and to have the widest ecological amplitude and distribution of any Antarctic macrolichen (Øvstedal & Lewis Smith, 2001), while *U. aurantiacoatra* is absent from the continental Antarctic. Phylogenetic analysis, performed on several Southern Hemisphere *Usnea* Dill. ex Adans. species, showed that the two species are closely related, and they are considered a single group. Some authors have suggested that the two species might constitute a species pair, in which *U. aurantiacoatra* represents the fertile and *U. antarctica* the sterile counterpart, but phylogenetic studies indicated that they could be conspecific (Wirtz et al., 2012). Here, we developed highly variable microsatellite loci to better understand the genetic differences between the two lichen species and to investigate gene flow between maritime Antarctica and South America.

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METHODS AND RESULTS

Total genomic DNA was extracted from a thallus of *U. antarctica* from population C1 and from one thallus of *U. aurantiacoatra* from a population that was not included in the later primer design (Appendix 1) from King George Island, Antarctica, in the austral summer of 2015/2016. Twenty milligrams of thallus were pretreated with acetone to remove secondary metabolites and ground with liquid nitrogen using a sterilized mortar and pestle. DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Cubero and Crespo, 2002). Paired-end libraries (300 bp) were prepared and sequenced on a MiSeq version 3 (2 × 300-bp) Illumina sequencer (LGC Genomics GmbH, Berlin, Germany).

We obtained 3,098,758 raw reads for *U. antarctica* and 1,755,882 for *U. aurantiacoatra*. Raw data were adapter-, quality- (PHREAD = 26), and length- (>150 bp) filtered using Trimmomatic version 0.35 (Bolger et al., 2014). Forward and reverse reads were assembled with Paired-End reAd mergeR (PEAR) software (Zhang et al., 2014). The resulting overlapping, paired, and singleton reads were then assembled with SPAdes version 3.9 (Nurk et al., 2013). Scaffolds were taxonomically binned using Metawatt (Strous et al., 2012). Scaffolds assigned to the phylum Ascomycota were used to search for mycobiont-specific microsatellite motifs. Simple sequence repeat (SSR) motifs repeated at least six times were searched in both genomes with the MIcroSAtellite Identification Tool (MISA) (Thiel et al., 2003), and primers were designed for 3–4-bp repeats using the Primer3 plugin in Geneious 10 (Kearse et al., 2012). A total of 150 SSRs were identified in the two species. Each repeat from one genome was BLASTed against the other genome. Due to the partial coverage of both

genomes, only 30 SSRs were found to share the same flanking regions in silico. We designed primers for these 30 markers and tested them on four samples of *U. antarctica* and *U. aurantiacoatra* in single touchdown PCR reactions. After sequencing we selected 23 markers (Table 1) with perfectly matching flanking regions to be tested in multiplex reactions and on algal pure cultures to confirm that they were fungal specific. The remaining seven markers were excluded because of insertions and/or deletions in the flanking regions.

A fluorescent dye–associated tag was attached to the forward primers. Four different tails were selected to multiplex the reactions (FAM: GCCTCCCTCGCGCCA, VIC: GCCTTGCCAGCCCGC, NED: CAGGACCAGGCTACCGTG, PET: CGGAGAGCCGAGAGGTG) (Blacket et al., 2012). PCRs were performed in 25-µL reactions using illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, United Kingdom) according to the manufacturer's protocol with the following touchdown conditions: initial hot start at 94°C for 15 min; five cycles of 94°C for 45 s, 65°C for 45 s, 72°C for 45 s; five cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 45 s; 10 cycles of 94°C for 45 s, 57°C for 45 s, 72°C for 45 s; 20 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 45 s; and final elongation at 72°C for 15 min. The PCR products were sent to Macrogen Europe (Amsterdam, The Netherlands) for sequencing to check the stability of the flanking regions in both species. To confirm that the chosen SSRs are indeed fungal loci, the primers were also tested on four axenic cultures of *Trebouxia jamesii* (Hildreth and Ahmadjian) Gärtner isolated from *U. antarctica*, *U. aurantiacoatra*, *U. lambii* (Imshaug) Wirtz & Lumbsch, and *U. trachycarpa* (Stirt.) Müll. Arg. (Appendix 1). The PCRs were performed under the same conditions as above, and no amplifications were observed.

After fungal specificity of the primers was confirmed, 77 samples from two populations of *U. antarctica* and *U. aurantiacoatra* (sampled in the same areas

Table 1. Characteristics of 23 fungus-specific SSR primers developed for *Usnea antarctica* and *U. aurantiacoatra*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Tail[b]	Fluorescent dye	Allele size range (bp)[c]	No. of SSR repeats	*A*	Multiplex	GenBank accession no.
Ua1	F: GCATCTGGGCTCTTGGACTT	$(CTT)_n$	A	FAM	235–271	3–15	9	1	KY940723
	R: CATTTGCAGGCAGTCCATCG								
Ua2	F: GGGATAACTCGCTATGGCCC	$(CTT)_n$	B	VIC	256–283	6–15	9	1	KY940724
	R: ACACCCTGATCGATCAAACCA								
Ua3	F: AAGCACACGCAAAGCTTCAG	$(CTG)_n$	C	NED	248–275	4–13	6	1	KY940725
	R: CGGAGGTCTGAATGTCGGAG								
Ua4	F: CTTTCACTGTCCTGCCCTGT	$(CCT)_n$	D	PET	277–304	2–13	10	1	KY940726
	R: GAGACCCCGTGTCCAATCAG								
Ua5	F: GGAAGGGGAAGGGGAGAGAT	$(CTT)_n$	A	FAM	555–573	8–14	4	1	KY940727
	R: GGTGGGCAACTGGAATGGTA								
Ua6	F: TTGAGCCGCCACAAGAGATT	$(AAG)_n$	B	VIC	337–349	4–8	5	1	KY940728
	R: ATCGGCCAATTGATACCCCG								
Ua7	F: AAGACGGACATTCCACCACC	$(GGA)_n$	D	PET	552–573	2–9	5	1	KY940729
	R: ACCGCTCTGGCTACCTCTTA								
Ua8	F: AAGAAGCCAGCTTTGACGGA	$(TGT)_n$	A	FAM	393–402	6–9	4	1	KY940730
	R: GCTTGTCTCAGGCAGGATGA								
Ua9	F: AACGGAGCTTCCTTCCATTGA	$(CATC)_n$	A	FAM	285–297	8–11	4	2	KY940731
	R: ACAACACAGACAACCCCGAA								
Ua10	F: GACTTTACGGCCCACATCCA	$(AGA)_n$	B	VIC	284–314	4–14	8	2	KY940732
	R: TTTCCATGTGGCTTGGAGGG								
Ua11	F: TCGCATTATTCGTGCAAGCG	$(TATG)_n$	C	NED	254–274	5–10	6	2	KY940733
	R: GTAATATCCGCTCGCCCACA								
Ua12	F: AGGCGCTGTGTGAGAACC	$(CGAA)_n$	D	PET	232–256	3–9	6	2	KY940734
	R: AGCAAGACCAAGAAGGCGAG								
Ua13	F: CCAAGCCAACCTCAGACCAT	$(CAG)_n$	A	FAM	393–408	4–9	6	2	KY940735
	R: CGACGTCTCCTTCCATAGCC								
Ua14	F: GTCAGCCCATCTACCGTACG	$(GAA)_n$	B	VIC	386–419	5–16	8	2	KY940736
	R: TGGGTTGGGAAAGGAAGTGT								
Ua15	F: CGCAAACAGTACAACCGGAA	$(GCT)_n$	C	NED	341–347	5–7	3	2	KY940737
	R: GCCACAACAAAGGTGACGAC								
Ua16	F: GTTTGGAAGACCACCGGCTA	$(AGT)_n$	D	PET	334–355	10–17	6	2	KY940738
	R: CCAAGCACACCCTGACATCT								
Ua17	F: ATGACGTGCTGTAGGTGTGG	$(CTGGTA)_n$	A	FAM	403–415	4–6	3	3	KY940739
	R: GTGTCAAGTGTCGAGCAGGA								
Ua18	F: AGGGAGTTCTGCAGGGGATA	$(GAA)_n$	B	VIC	355–388	6–17	6	3	KY940740
	R: AGTGATTGATGCTCCGGTGG								
Ua19	F: AGCCATTTTTCCGAGGTCGT	$(GAC)_n$	C	NED	349–367	6–12	4	3	KY940741
	R: GCTTTGTTGCGCTTCACTGA								
Ua20	F: GATCACTCTTCGAGCTCCCG	$(AAGC)_n$	D	PET	405–421	6–10	5	3	KY940742
	R: CCAGAGTACCTTCCGTTGCA								
Ua21	F: TTCCCGAGCTCCAATCACAC	$(TCC)_n$	A	FAM	260–272	7–11	5	3	KY940743
	R: CCATATCCCGTCCTCGCAAA								
Ua22	F: TGGTCCACTTTAGCCAGTCAT	$(ATG)_n$	C	NED	272–290	8–14	6	3	KY940744
	R: TCTGCCCTTGACATCTTTGACA								
Ua23	F: TAGTGCGAGGCCTGATGTTC	$(CTT)_n$	D	PET	244–253	6–9	3	3	KY940745
	R: ACCGAAAAGGCTTGGACGAT								

Note: *A* = number of alleles.

[a]Melting temperature (T_m) for all primers ranged from 59.1°C to 60.3°C.

[b]Tails attached to the forward primers: A = GCCTCCCTCGCGCCA, B = GCCTTGCCAGCCCGC, C = CAGGACCAGGCTACCGTG, D = CGGAGAGCCGAGAGGTG.

[c]The range includes the size of the tail.

TABLE 2. Allelic richness and genetic diversity of SSR loci in four populations of *Usnea* spp. collected on King George Island.[a]

	Total (n = 77)			*U. antarctica* (C1; n = 20)			*U. antarctica* (P3; n = 19)			*U. aurantiacoatra* (C1; n = 20)			*U. aurantiacoatra* (P3; n = 18)		
Locus	n	mean A	H_e	n	A	H_e	n	A	H_e	n	A	H_e	n	A	H_e
Ua1	76	5.3	0.66	20	3	0.36	19	4	0.61	19	6	0.61	18	8	0.90
Ua2	77	5.3	0.75	20	4	0.71	19	5	0.76	20	6	0.76	18	6	0.78
Ua3	77	2.5	0.38	20	2	0.10	19	3	0.37	20	3	0.37	18	2	0.50
Ua4	74	5.5	0.73	20	5	0.72	19	5	0.78	17	5	0.78	18	7	0.77
Ua5	77	1.8	0.08	20	1	0.00	19	2	0.11	20	1	0.11	18	3	0.22
Ua6	77	2.5	0.22	20	2	0.19	19	2	0.28	20	3	0.28	18	3	0.22
Ua7	70	2.3	0.19	20	2	0.19	19	3	0.20	14	2	0.20	17	2	0.22
Ua8	77	2.3	0.16	20	1	0.00	19	1	0.00	20	4	0.00	18	3	0.22
Ua9	76	2.5	0.53	20	2	0.44	19	2	0.46	19	3	0.46	18	3	0.66
Ua10	76	4.5	0.45	19	4	0.30	19	2	0.11	20	5	0.11	18	7	0.75
Ua11	77	4.3	0.68	20	6	0.79	19	5	0.75	20	3	0.75	18	3	0.62
Ua12	76	2.8	0.32	19	1	0.00	19	1	0.00	20	4	0.00	18	5	0.61
Ua13	76	2.8	0.31	20	1	0.00	19	1	0.00	19	4	0.00	18	5	0.56
Ua14	76	4.8	0.73	19	4	0.73	19	6	0.82	20	5	0.82	18	4	0.73
Ua15	77	1.5	0.05	20	1	0.00	19	1	0.00	20	1	0.00	18	3	0.22
Ua16	77	2.5	0.32	20	2	0.10	19	1	0.00	20	4	0.00	18	3	0.66
Ua17	75	3.0	0.54	19	3	0.29	18	3	0.65	20	3	0.65	18	3	0.57
Ua18	77	3.0	0.57	20	4	0.57	19	2	0.49	20	3	0.49	18	3	0.60
Ua19	77	1.5	0.05	20	2	0.10	19	1	0.00	20	1	0.00	18	2	0.11
Ua20	77	2.5	0.32	20	3	0.59	19	3	0.37	20	2	0.37	18	2	0.11
Ua21	77	3.8	0.63	20	4	0.59	19	4	0.59	20	3	0.59	18	4	0.70
Ua22	77	2.0	0.22	20	2	0.19	19	2	0.20	20	1	0.20	18	3	0.50
Ua23	77	1.8	0.21	20	1	0.00	19	1	0.00	20	2	0.00	18	3	0.46
Mean		3.05	0.40		2.61	0.30		2.61	0.33		3.22	0.33		3.78	0.51

Note: A = number of alleles; H_e = Nei's unbiased gene diversity; n = total number of samples.
[a]Locality data for populations can be found in Appendix 1.

on King George Island) were chosen to evaluate the variability of the markers. All samples were tested with multiplexed PCRs of seven to eight markers with the Type-it Multiplex Kit (QIAGEN, Hilden, Germany). PCR reactions were conducted in 10-µL volumes containing 1.5 µg of genomic DNA, 5 µL of PCR Master Mix, and 3 µL of primer multiplex (0.25 µM of each forward primer and fluorescent dye, 0.125 µM of each reverse primer). PCR products were amplified using the same touchdown program as above. PCR amplicons were electrophoresed using an Applied Biosystems 3730 platform, with the LIZ 600 Size Standard (Applied Biosystems, Foster City, California, USA), and allele sizes were manually scored using the Geneious 10 microsatellite tool (Kearse et al., 2012).

The variability of each microsatellite locus was measured by counting the number of alleles and calculating Nei's unbiased gene diversity using GenAlEx 6.5 (Peakall and Smouse, 2012). All of the 23 markers amplified in both species. In *U. aurantiacoatra*, all of the SSR markers were polymorphic, while five were monomorphic in *U. antarctica* (Table 2).

Linkage disequilibrium was tested with GENEPOP (Raymond and Rousset, 1995; Rousset, 2008) twice: separately for each species to estimate P values and with the two species together to perform a global test (Fisher's method) for each pair of loci across populations and species. Holm-Bonferroni correction (Holm, 1979) of the P values resulting from the last test using the implementation by Gaetano (2013) revealed significant evidence for linkage disequilibrium between loci Ua3, Ua4, and Ua6 (Ua3–Ua4: P = 0.001, Ua3–Ua6: P = 0.006, Ua4–Ua6: P = 0.0).

Cross-species amplification was tested on DNA extracts of *U. sphacelata* R. Br. and *U. trachycarpa*, two related species from *Usnea* subgenus *Neuropogon* (Nees & Flotow) Jatta (Truong et al., 2013). First, we tested a population of eight samples of *U. sphacelata* with the multiplex protocol. To verify the identity and stability of the flanking regions, we then selected two samples with different peaks for single PCR amplification and sequencing. The multiplex analysis was omitted for *U. trachycarpa*. Thirteen markers showed the same flanking regions in all three species, five markers had deletions and/or insertions in the flanking regions in *U. sphacelata* and *U. trachycarpa*, and for four markers the PCRs failed. Marker Ua18 shared the flanking region only with *U. sphacelata*, while it had a deletion in *U. trachycarpa* (Table 3).

CONCLUSIONS

The markers developed here are suitable to study population structure and gene flow in *U. antarctica* and *U. aurantiacoatra*. The markers shared the same flanking regions in the two species and showed high variability even within a small geographic area. Nineteen of these microsatellite primers also cross-amplify in related species from *Usnea* subgenus *Neuropogon*; however, the flanking regions of some markers are less stable. The usability of the newly developed SSR markers for cross-amplification in other *Usnea* species therefore requires further validation using a broader taxon sampling and a higher number of replicates per species. We conclude that the newly developed SSR markers presented here can be used to infer gene flow within Southern Hemisphere lichen populations and resolve connectivity patterns among populations of *U. antarctica* and *U. aurantiacoatra*.

TABLE 3. Cross-amplification of SSR markers developed for *Usnea antarctica* and *U. aurantiacoatra* with related species of *Usnea* subgenus *Neuropogon*.

Species	Ua1	Ua2	Ua3	Ua4	Ua5	Ua6	Ua7	Ua8	Ua9	Ua10	Ua11	Ua12	Ua13	Ua14	Ua15	Ua16	Ua17	Ua18	Ua19	Ua20	Ua21	Ua22	Ua23
U. sphacelata	+	—	+	+	D	D	+	—	—	+	+	+	+	—	+	D	+	+	+	D	+	D	+
U. trachycarpa	+	—	+	+	D	D	+	—	—	+	+	+	+	—	+	D	+	D	+	D	+	D	+

Note: + = SSRs present; — = PCR failed; D = deletion/insertion within the flanking region.

LITERATURE CITED

Blacket, M. J., C. Robin, R. T. Good, S. F. Lee, and A. D. Miller. 2012. Universal primers for fluorescent labelling of PCR fragments—An efficient and cost-effective approach to genotyping by fluorescence. *Molecular Ecology Resources* 12: 456–463.

Bolger, A. M., M. Lohse, and B. Usadel. 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* 30: 2114–2120.

Cubero, O. F., and A. Crespo. 2002. Isolation of nucleic acids from lichens. *In* I. C. Kranner, R. P. Beckett, and A. K. Varma [eds.], Protocols in lichenology: Culturing, biochemistry, ecophysiology, and use in biomonitoring, 381–391. Springer, Berlin, Germany.

Gaetano, J. 2013. Holm-Bonferroni sequential correction: An Excel calculator (1.2). Website https://www.researchgate.net/publication/242331583_Holm-Bonferroni_Sequential_Correction_An_EXCEL_Calculator_-_Ver._1.2 [accessed 8 June 2017].

Holm, S. 1979. A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics* 6: 65–70.

Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, et al. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28: 1647–1649.

Nurk, S., A. Bankevich, D. Antipov, A. A. Gurevich, A. Korobeynikov, A. Lapidus, A. D. Prjibelski, et al. 2013. Assembling genomes and mini-metagenomes from highly chimeric reads. *In* M. Deng, R. Jiang, F. Sun, and X. Zhang [eds.], Research in computational molecular biology, vol. 7821, 158–170. Springer, Berlin, Germany.

Øvstedal, D. O., and R. I. Lewis Smith. 2001. Lichens of Antarctica and South Georgia. Studies in Polar Research. Cambridge University Press, Cambridge, United Kingdom.

Peakall, R., and P. E. Smouse. 2012. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research–An update. *Bioinformatics* 28: 2537–2539.

Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86: 248–249.

Rousset, F. 2008. GENEPOP'007: A complete reimplementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

Strous, M., B. Kraft, R. Bisdorf, and H. E. Tegetmeyer. 2012. The binning of metagenomic contigs for microbial physiology of mixed cultures. *Frontiers in Microbiology* 3: 410.

Thiel, T., W. Michalek, R. K. Varshney, and A. Graner. 2003. Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics* 106: 411–422.

Truong, C., P. K. Divakar, R. Yahr, A. Crespo, and P. Clerc. 2013. Testing the use of ITS rDNA and protein-coding genes in the generic and species delimitation of the lichen genus *Usnea* (Parmeliaceae, Ascomycota). *Molecular Phylogenetics and Evolution* 68: 357–372.

Wirtz, N., C. Printzen, and H. T. Lumbsch. 2012. Using haplotype networks, estimation of gene flow and phenotypic characters to understand species delimitation in fungi of a predominantly Antarctic *Usnea* group (Ascomycota, Parmeliaceae). *Organisms Diversity and Evolution* 12: 17–37.

Zhang, J., K. Kobert, T. Flouri, and A. Stamatakis. 2014. PEAR: A fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* 30: 614–620.

APPENDIX 1. Locality and collection data for *Usnea* samples used in this study.

Species (Population)	*n*	Geographic coordinates	Locality data	Collector (Collection year)	Voucher no. (Herbarium)[a]
U. antarctica Du Rietz (C1)	20	62°14.220′S, 58°39.492′W	King George Island, Antarctica	Lagostina, Kanz (2015)	FR-0264579 (FR)
U. antarctica (P3)	19	62°15.174′S, 58°38.971′W	King George Island, Antarctica	Lagostina, Kanz (2015)	FR-0264581 (FR)
U. aurantiacoatra (Jacq.) Bory (C1)	20	62°14.220′S, 58°39.492′W	King George Island, Antarctica	Lagostina, Kanz (2015)	FR-0264584 (FR)
U. aurantiacoatra (P3)	18	62°15.174′S, 58°38.971′W	King George Island, Antarctica	Lagostina, Kanz (2015)	FR-0264586 (FR)
U. aurantiacoatra	1	62°14.363′S, 58°39.202′W	King George Island, Antarctica	Lagostina, Kanz (2015)	FR-0264585 (FR)
U. sphacelata R. Br.	4	79°17′N, 16°04′W	Spitsbergen, Svalbard	Tønsberg (2002)	BG-L94105 (BG)
U. sphacelata	4	64°45′N, 18°15′W	Central Highland, Iceland	Heidmarsson (2002)	Heidmarsson 28916 (F)
U. trachycarpa (Stirt.) Müll. Arg.	2	54°58.257′S, 67°38.004′W	Isla Navarino, Chile	Printzen (2017)	FR-0264590 (FR)

Note: *n* = number of individuals tested.

[a]Vouchers are deposited in the Herbarium Senckenbergianum Frankfurt (FR), Frankfurt am Main, Germany; University Museum of Bergen (BG), Bergen, Norway; and the Field Museum of Natural History (F), Chicago, Illinois, USA.

MYCOLOGIA
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The use of microsatellite markers for species delimitation in Antarctic *Usnea* subgenus *Neuropogon*

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ABSTRACT

Lichens are symbiotic associations consisting of a fungal (mycobiont) and one or more photosynthetic (photobionts) partners and are the dominant component, and most important primary producers, of Antarctic terrestrial ecosystems. The most common lichens in the maritime Antarctic are *Usnea antarctica* and *U. aurantiacoatra*, a so-called "species pair" in which *U. antarctica* shows asexual reproduction and propagation via soredia and *U. aurantiacoatra* forms ascospores in apothecia. Previous molecular analyses were not able to unambiguously distinguish the two morphotypes as species. Therefore, the goal of this study was to find out whether fast-evolving SSR (single sequence repeat) markers are able to separate morphotypes more clearly and help to clarify their taxonomy. We investigate 190 individuals from five mixed stands of both morphotypes collected in King George Island and Elephant Island (South Shetland Islands, Antarctica). Based on 23 microsatellite markers designed from sequenced genomes, discriminant analysis of principal components (DAPC), Bayesian clustering analysis, and coalescent-based estimation of gene flow show clear evidence for the existence of two different species distinguishable by reproductive mode. We did not detect any statistical association between genetic clusters and three previously reported chemical races of each species.

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DAPC; gene flow; haplotype network; ITS; *Parmeliaceae*

INTRODUCTION

Lichens are symbiotic associations consisting of a fungal partner (mycobiont) and one or more photosynthetic (photobionts) partners living together to form a coherent structure called a thallus (Ahmadjian 1993). Lichens are present in virtually all terrestrial habitats and cover a considerable part of the earth's surface but are often overlooked by biologists. Because of this, the diversity of lichens is still very poorly known. Many regions outside Europe and North America even lack preliminary checklists. But the situation is far from satisfactory in better-studied regions of the world. For example, the number of lichens reported from North America has increased linearly over the last 20 years, from ca. 4000 in 1997 to almost 5500 reported species today (Esslinger 2015 and previous versions). Although this increase is largely due to range extensions of already described species, the number of newly described species has increased in a similar manner, particularly within inconspicuous crustose groups (e.g., Lumbsch et al. 2011; Lücking et al. 2014b). As in practically all other groups of organisms, most lichen taxa have originally been delimited using a strictly morphological species concept, although morphological characters can be notoriously variable in lichens. Besides poor exploration, unclear species delimitation due to phenotypic plasticity (Pérez-Ortega et al. 2012; Wirtz et al. 2012) is probably the most important reason for our current knowledge gaps.

The use of molecular data offers additional insights into species limits of lichens. Phylogenetic analyses indicate that the number of lichen species may have been grossly underestimated, not only in small crustose groups occurring in underexplored regions (Lücking et al. 2014a). Even seemingly well-known macrolichens from Europe and North America were shown to comprise several phylogenetic species (e.g., Blom and Lindblom 2009; Leavitt et al. 2011; Magain et al. 2015). On the one hand, revised species delimitations based on molecular data have reinforced the impression that the true diversity of lichens may be cryptic and perhaps only recognizable with molecular tools (Crespo

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and Pérez-Ortega 2009; Crespo and Lumbsch 2010; Lumbsch and Leavitt 2011). In contrast, several recent studies confirmed that molecular differences between species may often be correlated with distinctive morphological or chemical characters (Spribille et al. 2011; Lücking et al. 2014a; Magain et al. 2015).

Hence, although molecular data offer additional evidence, species delimitation in lichens is still not straightforward. The published studies have so far almost exclusively relied on DNA sequence data from relatively few loci. Incomplete lineage sorting (Printzen et al. 2003) may lead to incongruence between gene and species trees (Aguileta et al. 2008) and between morphological and molecular species concepts (e.g., Myllys et al. 2001; Lohtander et al. 2009). These problems could be overcome by increasing the number of studied gene loci or by using faster evolving markers. However, even coalescent-based species delimitation studies utilizing several gene loci and additional data were often inconclusive (e.g., Leavitt et al. 2011; Kanz et al. 2015, Singh et al. 2015; reviewed in Leavitt et al. 2015). For example, attempts to recover six previously identified phylogenetic groups within *Letharia columbiana* and *L. vulpina* (Kroken and Taylor 2001) using 15 gene loci (Altermann et al. 2014) could not unambiguously resolve all lineages. Based on a genome-wide data set, Leavitt et al. (2016) recently recovered consistent species phylogenies for the *Rhizoplaca melanophthalma* complex using data sets comprising between one hundred 1000-kb loci and a total of 16.8 Mb, whereas reconstructions using twenty-five 1000-kb loci differed among each other. This supports results from simulation studies (Ogilvie et al. 2016) showing that genome-wide data sets might be necessary to delimit recently diverged taxa.

Here, we use recently developed microsatellites or single sequence repeat (SSR) markers to see whether two morphological species of lichens form distinct genetic clusters or whether there is evidence for ongoing gene flow between them justifying their synonymization. As an example, we take a so-called "species pair" (Poelt 1970) consisting of presumed sister taxa that differ solely in reproductive mode. Lichen-forming fungi have developed reproductive strategies involving both sexual and vegetative propagules that comprise both symbionts in a single propagule. Asexual, vegetative dispersal has the advantage of spreading both symbionts together, whereas sexual reproduction (in lichens restricted to the fungal partner) requires the germinating spore to encounter a compatible photobiont partner. The species within such a pair have presumably diverged only recently, exacerbating problems with incomplete lineage sorting. Indeed, several sequence-based studies have recently failed to find supporting evidence for the existence of species pairs (e.g., Lohtander et al. 1998; Myllys et al. 2001; Articus et al. 2002).

We reinvestigate the hypothesized species pair *Usnea antarctica* and *U. aurantiacoatra*, using microsatellites in addition to DNA sequence data. In order to exclude the confounding effects of geographical population structure, we restrict our sampling to maritime Antarctic populations of the two morphotypes or species. *Usnea* is one of the largest genera in the family Parmeliaceae (Lecanorales, Ascomycota), comprising ca. 350 species and more than 770 published names (Clerc 1998; Thell et al. 2012). Its members are characterized by beard-like, finely branched pendent or erect thalli with a stiff central axis, are widely distributed in polar, temperate, and tropical regions (Walker 1985; Clerc and Herrera-Campos 1997), and are notoriously difficult to identify due to morphological plasticity. Many authors have recently tried to clarify the taxonomy of this genus using multigene data sets (Seymour et al. 2007; Lumbsch and Wirtz 2011; Wirtz et al. 2008, 2012; Truong et al. 2013; Mark et al. 2016). *Usnea antarctica* and *U. aurantiacoatra* are the most common lichen species in the maritime Antarctic and have been separated based on their reproductive strategy: *U. antarctica* disperses by soredia, whereas *U. aurantiacoatra* lacks soredia and forms apothecia. Previous molecular analyses based on the nuc rDNA internal transcribed spacer region ITS1-5.8S-ITS2 (ITS) and the gene for the largest subunit of RNA polymerase II (*RPB1*) separated these species from *U. sphacelata* and *U. subantarctica* but did not find support to reliably distinguish them from each other. It has thus been suggested to synonymize the two species (Lumbsch and Wirtz 2011; Wirtz et al. 2012). In many places of the maritime Antarctic, the two species occupy the same habitats, making them ideal study objects because genetic differentiation of the two species can be studied at the local scale.

MATERIALS AND METHODS

Sample collection and site description.—In order to exclude the confounding effects of geographical population structure, we sampled four mixed stands of sorediate (*Usnea antarctica*) and esorediate morphotypes (*U. aurantiacoatra*) in King George Island (South Shetland Islands, Antarctica) during December 2015 and January 2016: three in km2 (area) around the Argentinian research station Carlini on Potter Peninsula and one close to the Russian station Bellingshausen on Fildes Peninsula. One additional stand was sampled in Elephant Island (TABLE 1). The two *Usnea* species were morphologically identified by

Table 1. List of stands sampled for this study, with abbreviations, sampling locations, geographic coordinates, sample sizes, collectors, and herbarium voucher numbers.

Species	Abbreviation	Sampling location	Coordinates	Sample size	Collector	Voucher no.
Usnea antarctica	UantKGI1	King George Island	62°14.220′N, 58°39.492′W	20	E. Lagostina, B. Kanz	FR-0264579
Usnea antarctica	UantKGI2	King George Island	62°14.363′N, 58°39.202′W	18	E. Lagostina, B. Kanz	FR-0264580
Usnea antarctica	UantKGI3	King George Island	62°15.174′N, 57°38.971′W	19	E. Lagostina, B. Kanz	FR-0264581
Usnea antarctica	UantKGI4	King George Island	62°11.416′N, 58°55.604′W	19	M. Andreev	FR-0264583
Usnea antarctica	UantEF	Elephant Island	61°13.337′N, 55°21.581′W	19	M. Andreev	FR-0264582
Usnea aurantiacoatra	UaurKGI1	King George Island	62°14.220′N, 58°39.492′W	20	E. Lagostina, B. Kanz	FR-0264584
Usnea aurantiacoatra	UaurKGI2	King George Island	62°14.363′N, 58°39.202′W	20	E. Lagostina, B. Kanz	FR-0264585
Usnea aurantiacoatra	UaurKGI3	King George Island	62°15.174′N, 57°38.971′W	18	E. Lagostina, B. Kanz	FR-0264586
Usnea aurantiacoatra	UaurKGI4	King George Island	61°12.217′N, 58°57.833′W	19	M. Andreev	FR-0264589
Usnea aurantiacoatra	UaurEF	Elephant Island	60°12.992′N, 55°21.527′W	20	M. Andreev	FR-0264587

their reproductive strategies following Olech (2004). Samples with soredia were identified as *Usnea antarctica*, whereas samples without soredia (and with apothecia) were named *U. aurantiacoatra*. In total, we sampled 95 thalli identified as *U. antarctica* and 95 assigned to *U. aurantiacoatra*.

DNA extraction, amplification, and sequencing.—Total DNA was extracted from young terminal branches without signs of infection by parasitic fungi. Branches were collected in 2-mL reinforced tubes with metal beads inside and ground with the Bead Ruptor 24 (Omni International, Kennesaw, Georgia) in three cycles of 25 s at speed 4.20. Before each cycle, tubes were dipped in liquid nitrogen for 15 s. DNA was extracted with the Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The nuc rDNA ITS1-5.8S-ITS2 region (ITS) was amplified using primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). Polymerase chain reactions (PCRs) were performed in 25 µL volume using Illustra PureTaq ready-to-Go PCR beads (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) containing 5 µL of DNA extract and 0.4 nM of each primer. Cycling conditions included initial denaturation at 94 C for 5 min; five cycles of 94 C for 30 s, 54 C for 45 s, and 72 C for 60 s; 33 cycles of 94 C for 30 s, 48 C for 30 s, and 72 C for 60 s; and a final elongation step at 72 C for 10 min. The PCR products were sequenced by Macrogen Europe (Amsterdam, The Netherlands), and the sequences were assembled, edited, and manually aligned with Geneious 10 (Kearse et al. 2012). We used the TCS algorithm implemented in popART (Clement et al. 2002) to generate a haplotype network.

Microsatellite analysis.—We used the 23 fungal-specific microsatellite markers published by Lagostina et al. (2017) to genotype our samples. The data set has been deposit in PANGEA (https://doi.pangaea.de/10.1594/PANGAEA.892967). Because it has recently been shown that Basidiomycetes may be obligate partners of ascomycetous lichen symbioses (Spribille et al. 2016), we checked whether our markers were specific to Ascomycetes by comparing the contigs from which they were developed against the National Center for Biotechnology Information (NCBI) database using the BLASTn approach. Twenty-one out of the 23 contigs (ca. 3000–35000 bp in length) contained genes with sequences most similar to Ascomycota. Two of the five shortest contigs did not show closer similarity to any sequences deposited in GenBank.

Markers were amplified in three different 10-µL multiplex reactions following the protocol by Lagostina et al. (2017). PCR amplicons were electrophoresed using an Applied Biosystems 3730 platform, with the LIZ 600 size standard (Applied Biosystems, Waltham, Massachusetts), and allele sizes were manually scored using the Geneious 10 microsatellites tool (Kearse et al. 2012). To partition the observed genetic variance within and among stands for each species and between species, we performed an analysis of molecular variance (AMOVA) on the full data sets and on the two subsets containing only one of the two species *Usnea antarctica* and *U. aurantiacoatra* using Arlequin 3.5 with default parameters (Excoffier and Lischer 2010).

We inferred the genetic structure within our sample with discriminant analysis of principal components (DAPC) using the R package ADEGENET 2.1.0 (Jombart 2008; Jombart and Ahmed 2011). DAPC was chosen over classical multivariate analyses (principal component analysis [PCA], discriminant analysis [DA]) because the purpose of our study was to identify groups, and, in contrast to other multivariate methods, DAPC attempts to maximize among-group variation (Jombart et al. 2010). Analyses were run with 40, 50, and 60 retained principal components to check results for consistency. Models with different numbers of k (genetic groups) were compared using Bayes factors. In order to explore the impact of the number of loci on our results, we also ran DAPC analyses using only loci from one of the three multiplex reactions (first multiplex with eight primers, Ua1–Ua8; second

multiplex with eight primers, Ua9–Ua18; and third multiplex with seven primers, Ua19–Ua23; Lagostina et al. 2017).

To confirm the clustering of samples, we ran a STRUCTURE (2.3.4) analysis (Pritchard et al. 2000; Falush et al. 2003) using the same matrix as for the DAPC analysis. The analyses were based on 10 serial runs for each number of clusters (k) between 2 and 10. Admixture models used a uniform alpha prior, independent allele frequencies, and no prior population information. All analyses were run for 5×10^5 generations after a burn-in of 25×10^4 generations. To estimate the optimal number of admixture clusters, we used the summary likelihood statistics ΔK proposed by Evanno et al. (2005) at the Web site http://taylor0.biology.ucla.edu/structureHarvester.

The coalescent-based method Migrate-n 3.6.11 was used to test for the presence of gene flow between the two morphotypes. Relative mutation rate among loci was estimated from the data. Bayesian estimates of mutation-scaled immigration rates and θ were obtained for three migration (gene flow) models: a full-migration model with two "population" sizes (one for each morphotype) and gene flow into both populations/morphotypes, and two models with two "population" sizes and the migration rate for one of the populations/morphotypes set to zero to model absence of gene flow between morphotypes. We used uniform priors (0.0–25) on both θ and M divided into 1500 bins and ran four chains with static heating (temperatures of 1, 1.5, 3, and 10 000) for 10 replicated long runs of 1 × 105 generations (sampling every 500th step) with a burn-in of 5 × 104. We used the Bezier approximation score to calculate the Bayes factor and selected the most probable among the three models. Convergence of Markov chains was monitored with Tracer (http://beast.bio.ed.ac.uk). All effective sample sizes of the Markov chain Monte Carlo (MCMC) chain were larger than 10^5.

Chemical analysis.—*Usnea* is a genus rich in secondary metabolites, some of which have been used to discriminate species or chemotypes. Usnic acid is found in all species of *Usnea*. Walker (1985) distinguished the same three chemical "races" in *U. antarctica* and *U. aurantiacoatra*: containing (i) fumarprotocetraric acid, (ii) norstictic and salazinic acids, and (iii) only usnic acid. In order to check whether the genetic variability in our sample was associated with chemical differences, we identified secondary metabolites of our samples using thin-layer chromatography (TLC) in solvent C (toluene: acetic acid, 20:3) according to Arup et al. (1993). Correlations between secondary metabolite patterns and morphological and genetic groupings were tested by Fisher's exact test using an online calculator at http://www.physics.csbsju.edu/stats/. We tested the association of three chemotypes found in our data set with two and five genetic clusters (corresponding to two potential species and the optimal clustering solution found by DAPC).

RESULTS

ITS analysis.—We generated nuc rDNA ITS1-5.8S-ITS2 region (ITS) sequences from 179 samples of *Usnea antarctica* and *U. aurantiacoatra* selected from five mixed stands of both morphotypes; sequences of 33 haplotypes (14 for *U. antarctica* and 19 for *U. aurantiacoatra*) were deposited in GenBank (accession numbers MG200275–MG200307; see SUPPLEMENTARY MATERIAL 1) and also in PANGEA (https://doi.pangaea.de/10.1594/PANGAEA.892969). The total alignment was 666 bp long and included an intron of 216 bp length 226 bp from the 3′ end of the small subunit of the ribosomal RNA. Our alignment included 33 polymorphic sites, 15 of them in the intron. The haplotype network (FIG. 1) shows 33 haplotypes. *Usnea aurantiacoatra* was genetically more variable (20 haplotypes) than *U. antarctica* (15 haplotypes). Sorediate and esorediate morphs do not form reciprocally monophyletic groups on the haplotype network, and even share two haplotypes. Nevertheless, they appear relatively well clustered.

Microsatellite analysis.—The success of polymerase chain reaction (PCR) amplification was 100% for 19 SSRs. Three markers reported one to three null alleles in four different stands. One marker had six missing peaks in one stand. We generated information for 95 samples of each *Usnea* morphotype, with 6 missing alleles in 4 samples of sorediate (*U. antarctica*) and 18 missing alleles in 16 samples of esorediate samples (*U. aurantiacoatra*). To summarize, the data set lacked 22 from an expected total of 4370 peaks.

The results of the AMOVA (TABLE 2) highlighted that the largest portion of the total genetic variation (55.80%) was found between the two morphotypes (fixation index [FST] = 0.58), followed by variation within stands ("populations") (41.78%). Variation among stands but within morphotypes was low (2.42%). AMOVA on data sets for the single morphotypes displayed similarly low levels of variation among stands and most of the variation within stands (*U. antarctica* 96.25%, *U. aurantiacoatra* 93.04%). Nonsorediate morphotypes (*Usnea aurantiacoatra*)

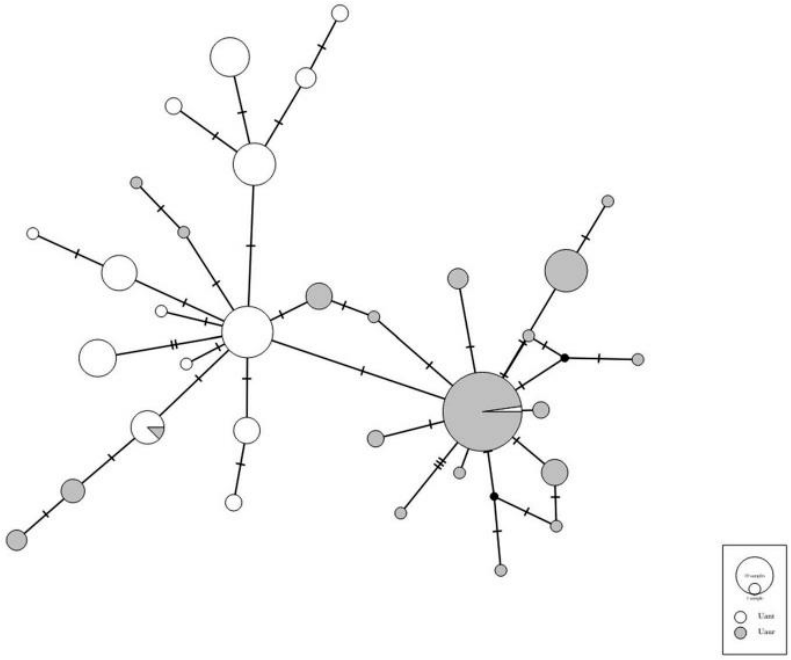

Figure 1. Haplotype network of *Usnea antarctica* (87 samples) and *U. aurantiacoatra* (92 samples) based on an alignment of 666 bp of partial 18S-ITS1-5.8S-partial ITS2 sequences. White circles represent *U. antarctica* and gray circles are *U. aurantiacoatra*; long lines connect linked haplotypes; small horizontal lines represent single mutational steps (substitutions) between haplotypes. The size of the circles is correlated with the number of samples belonging to the haplotype.

Table 2. AMOVA for five mixed stands of sorediate *Usnea antarctica* and esorediate *U. aurantiacoatra* sampled on King George Island and Elephant Island and for the single morphotypes.

Source of variation	*df*	Sum of squares	Variance components	Percentage of variance
AMOVA: five stands of *Usnea antarctica* and five stands of *U. aurantiacoatra*				
Among species	1	553 816	5735	55.80*
Among stands within species	8	72 089	0.24837	2.42
Within stands	180	772 932	4294	41.78
Total	189	1399	10 277	
AMOVA: five stands of *Usnea antarctica*				
Among stands	4	24.55	0.137	3.75
Within stands	90	317 482	3528	96.25
Total	94	1399	10 277	
AMOVA: five stands of *Usnea aurantiacoatra*				
Among stands	4	45 556	0.352	6.96
Within stands	90	423 413	4705	93.04
Total	94	468 968	5057	

*$P = 0.000 \pm 0.0009$.

showed almost twice as much variation between stands than the sorediate ones.

DAPC pointed out a clear genetic separation of the two morphotypes (FIG. 2). The Bayesian information criterion (BIC) for models with different numbers of clusters k indicated a sharp drop between $k = 1$ and $k = 2$ and a minimum at $k = 5$ ($k = 1$: 510.00; $k = 2$: 411.52; $k = 3$: 407.57; $k = 4$: 405.57; $k = 5$: 404.21; $k = 6$: 404.60). In all of the models from $k = 2$ to $k = 6$, sorediate and nonsorediate samples were assigned to different genetic clusters with the exception of a single individual from stand KGI2 originally identified as *U. antarctica* due to the apparent presence of soralia. No geographically restricted genetic clusters were observed for $k = 2$ to $k = 6$ (see SUPPLEMENTARY MATERIAL 2). Analyses based on 40, 50, and 60 retained principal components did not change the assignment of samples to clusters (data not shown). Likewise, DAPC on data sets containing only seven or eight SSR loci separated the two morphotypes in a similar manner (SUPPLEMENTARY

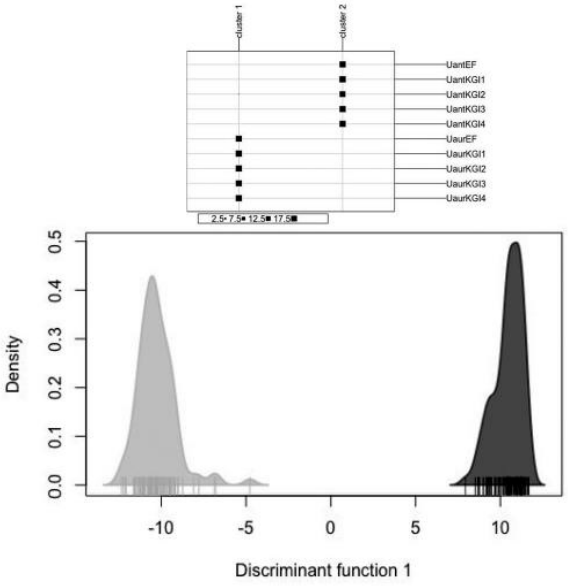

Figure 2. Results of DAPC based on *Usnea* morphotypes; number of principle components = 50, number of groups k = 2. Upper panel: assignment of samples to clusters. Names on the right indicate morphotype and stand. With one exception (in UantKGI2), sorediate and esorediate samples are assigned to different clusters. Lower panel: density distribution of esorediate (gray = *Usnea aurantiacoatra*) and sorediate (black = *Usnea antarctica*) morphs along the first discriminant function. Vertical strokes on the *x*-axis indicate individuals.

MATERIAL 3). Reinvestigation of the single wrongly assigned thallus revealed that the "soralia" were in fact protuberances presumably caused by a lichenicolous fungus and that the specimen was likely a damaged individual of *U. aurantiacoatra*.

DAPC was also run to test the clustering of samples according to chemical races (see below) retaining 50 principal components and using k = 2 and k = 5 groups (see SUPPLEMENTARY MATERIAL 4). In both analyses, chemical races and genetic clusters were incongruent. For the case of k = 2, this is evident from FIG. 2. For k = 5, clusters 1 and 4 comprised samples of *U. antarctica* whereas all samples from clusters 2, 3, and 5 belonged to *U. aurantiacoatra* but chemotypes were scattered across all clusters. Nevertheless, Fisher's exact test suggested that the associations of chemical races with k = 2 and k = 5 genetic clusters were not random ($P < 0.001$ in both cases).

STRUCTURE analyses were run for numbers of clusters between k = 2 and k = 10. FIG. 3 displays the result for k = 2 (the optimal solution following the approach by Evanno et al. 2005). The analysis confirmed that sorediate and nonsorediate morphotypes belong to different clusters.

Models with one migration rate set to zero for one of the morphotypes received higher support than the model assuming bidirectional migration, with the best model being the one assuming no migration from *Usnea aurantiacoatra* (TABLE 3) and low levels of gene flow from *U. antarctica* (M = 0.32). The inferred effective population size of *U. aurantiacoatra* (θ = 3.38) was slightly larger than that of *U. antarctica* (θ = 2.39).

Chemical analysis.—Secondary metabolite profiles were generated for the total data set of 190 samples (TABLE 4). Usnic acid was found in all samples. Fumarprotocetraric acid and usnic acid ("race 1") were found in nine sorediate (*U. antarctica*) and 38 nonsorediate (*U. aurantiacoatra*) thalli. Salazinic acid and usnic acid occurred only in one sorediate specimen collected in Elephant Island; this chemotype is like "race 2" of Walker (1985) but lacks norstictic acid. Eighty-five sorediate and 57 nonsorediate samples had no metabolites other than usnic acid ("race 3" according to Walker 1985).

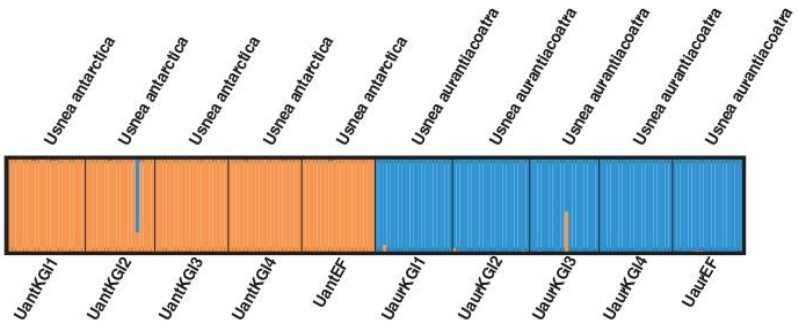

Figure 3. Bar plot of the STRUCTURE analysis for k = 2 clusters. All sorediate individuals are clearly assigned to cluster 1 (orange), the esorediate ones to cluster 2 (blue). Only one sorediate individual from stand UantKGI2 and one esorediate from UaurKGI3 show some evidence of admixture.

Table 3. Log marginal likelihoods (lmL), log Bayes factors, and model probabilities for three migration models based on 23 SSRs and stands of *U. antarctica* and *U. aurantiacoatra*.

Model	Bezier lmL	Log Bayes factor	Model probability
1	−17728.83	−3578.94	0
2	−17052.66	−2226.6	0
3	−15939.36	0	1

Note. Model 1: bidirectional gene flow; Model 2: no gene flow from *U. antarctica*; Model 3: no gene flow from *U. aurantiacoatra*.

Table 4. Chemical analysis of *Usnea* morphotypes divided for populations.

Morphotype	Abbreviation	No.	Race 1	Race 2	Race 3
Sorediate "*U. antarctica*"	UantKGI1	20	—	—	20
Sorediate "*U. antarctica*"	UantKGI2	18	2	—	16
Sorediate "*U. antarctica*"	UantKGI3	19	3	—	16
Sorediate "*U. antarctica*"	UantKGI4	19	2	—	17
Sorediate "*U. antarctica*"	UantEF	19	2	1	16
Total sorediate "*U. antarctica*"		95	9	1	85
Nonsorediate "*U. aurantiacoatra*"	UaurKGI1	20	9	—	11
Nonsorediate "*U. aurantiacoatra*"	UaurKGI2	20	8	—	12
Nonsorediate "*U. aurantiacoatra*"	UaurKGI3	18	6	—	12
Nonsorediate "*U. aurantiacoatra*"	UaurKGI4	19	8	—	11
Nonsorediate "*U. aurantiacoatra*"	UaurEF	18	7	—	11
Total nonsorediate "*U. aurantiacoatra*"		95	38	0	57

Note. Race 1: usnic and fumarprotocetraric acids; race 2: usnic and salazinic acids; race 3: usnic acid only.

DISCUSSION

The delimitation of species is of basic importance because species are the fundamental units in many fields of biology. Depending on the organismal group and the specific focus of researchers, different species concepts have focused on phenotypic and ecological differences, reproductive features, limitations of gene flow, or phylogenetic or genealogical relatedness of individuals (morphological, ecological, biological, phylogenetic, etc., species concepts). De Queiroz (2007) recently highlighted that, despite their apparent differences, more or less all species concepts agree in defining species as separately evolving population-level lineages and summarized their commonalities in the general lineage concept of species. As in most other organismal groups, most lichens were originally described as morphospecies, but traditional characters used to differentiate species are nowadays increasingly supplemented by approaches based on molecular data (Hausdorf and Hennig 2010). However, multilocus DNA sequence data sets were often not able to resolve hypothesized morphospecies in several groups of lichens (Lohtander et al. 1998; Myllys et al. 2001; Articus et al. 2002; Altermann et al. 2014). In this study, we tested whether highly variable microsatellite markers may complement other data sets for species delimitation studies. SSRs have previously been used to discriminate species in plants (e.g., Cycadaceae, Feng et al. 2016) and animals (e.g., ants, Ross et al. 2010; Lepidoptera, Lumley and Sperling 2011).

In lichen fungi, SSRs have been used to evaluate population structure and gene flow in different species (e.g., *Lobaria pulmonaria*, Walser et al. 2005; *Parmelina carporrhizans*, Alors et al. 2017), but so far no studies have used SSRs for species delimitation. One of the reasons may be that microsatellites frequently do not amplify across species. The SSR markers used in this study were designed based on draft genomes of both *Usnea* species and amplify sorediate as well as esorediate samples (Lagostina et al. 2017). This is evident from our data set with less than 0.5% null alleles.

Our analyses demonstrate that *U. antarctica* and *U. aurantiacoatra* form two separate genetic lineages. Although our samples were taken from five mixed stands (10 "populations"), DAPC and STRUCTURE divided our sample into two well-separated clusters that correlate with the two reproductive types. Accordingly, the AMOVA allocated most of the genetic variation between the putative species and the migration analysis found no support for ongoing gene flow between them. These results also hold when only 7 to 8 out of 23 SSR loci are used.

Walker (1985) detected three chemical "races" within *U. antarctica* and *U. aurantiacoatra*. Our data set contained two of these chemotypes for either of the two species and one thallus, with an additional so far unreported chemotype with salazinic in addition to usnic acid, similar to race 2 of Walker (1985). These chemotypes are not completely associated with any of the genetic clusters detected by DAPC. However, Fisher's exact test revealed that they are not randomly distributed either among the two morphotypes or among the genetic clusters. This adds evidence for the existence of two independently evolving lineages but provides no support for a further taxonomic subdivision of each species based on chemical characters.

The recent debate around the species status of *U. antarctica* and *U. aurantiacoatra* arose because phylogenetic trees and haplotype networks did not resolve the two as mutually exclusive monophyletic lineages (Lumbsch and Wirtz 2011; Wirtz et al. 2012). Our haplotype network, based on a data set including a polymorphic type I intron near the end of the ribosomal small subunit (SSR) that has not previously been studied in this group, confirms this finding. Similar to previous studies, it shows the two morphotypes as nonmonophyletic lineages sharing two haplotypes with each other. The two morphotypes, however, are clustered in the network, a pattern supporting the DAPC and STRUCTURE results. Although this is somewhat speculative, we think that previous studies may also have suffered from the inclusion of misidentified samples. In

our sample, one individual was initially apparently wrongly assigned to *Usnea antarctica* due to soredia-like protuberans on the thallus surface probably caused by a lichenicolous fungus. A reinvestigation of samples from previous studies could resolve this question but is beyond the scope of the present study.

The clear separation between reproductive types in the DAPC analysis could be due to either the higher number of loci studied by us or the higher evolutionary rates of SSR loci. In our example, evolutionary rate apparently plays a more important role because seven to eight SSRs still recover the clear separation between the two morphotypes. This finding may have important implications for the use of DNA barcoding. The nuc rDNA ITS1-5.8S-ITS2 region (ITS), the suggested universal barcode for fungi (Schoch et al. 2012), appears to evolve too slowly to allow reliable separation of genetically independent lineages. This result is in line with the observation of slow genetic drift in lichens by Printzen et al. (2003) who used ITS and parts of the intergenic spacer region (IGS) to study population structure of *Hypogymnia hultenii* and failed to find clear haplotype groups in geographically widely disjunct and presumably long isolated populations. Similarly, ITS and mitochondrial Small Subunit Ribosomal DNA repeat (mtSSU) regions failed to separate species of the genus *Endocena* (*Icmadophilaceae*; Fryday et al. 2017).

Our data suggest that the presence and absence of soredia can be used to safely discriminate between *U. antarctica* (with soredia) and *U. aurantiacoatra* (without soredia). The presence of apothecia, on the other hand, is a more unreliable character, because young thalli of *U. aurantiacoatra* often lack apothecia. Care must also be taken not to mistake galls on parasitized thalli of *U. aurantiacoatra* for soralia, as the single apparently wrongly assigned sample shows. Species pairs have previously been reported from a wide variety of lichen genera, although the idea of asexual lineages arising from sexually reproducing lichen species has always remained contentious (see Tripp 2016 for a recent review) and many early studies failed to reconstruct presumed species pairs as reciprocally monophyletic (e.g., Lohtander et al. 1998; Myllys et al. 2001). More recent molecular analyses have found increasingly more evidence that species pairs do exist (e.g., in *Lobaria*; Cornejo and Scheidegger 2015), and our study confirms this. Although we detected lower genetic variability in *U. antarctica* than in *U. aurantiacoatra*, probably reflecting the higher effective population size of sexually reproducing species, *U. antarctica* was surprising variable, contradicting the old idea that asexually reproducing, sorediate lichens constitute "clones or groups of clones" (Tehler 1982).

To summarize, by confirming the species status and distinction of *U. antarctica* and *U. aurantiacoatra*, our study provides further evidence for the existence of species pairs in lichens distinguished chiefly or exclusively by their mode of reproduction (sexual vs. vegetative). The two species studied here can safely be distinguished by the presence of soralia in the asexual counterpart. The presence or absence of apothecia, on the other hand, is not a useful distinguishing character. This is in line with the general observation that, with few exceptions, vegetatively reproducing lichens may also form apothecia under favorable ecological conditions. Although DNA sequence data from the suggested barcoding marker for fungi (ITS) do not resolve both taxa as reciprocally monophyletic lineages, SSR data leave no doubt that they are genetically isolated. We therefore recommend caution in lumping closely related lichen taxa just because DNA sequence data show them to be nonmonophyletic.

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LITERATURE CITED

Aguileta G, Marthey S, Chiapello H, Lebrun MH, Rodolphe F, Fournier E, Gendrault-Jacquemard A, Giraud T. 2008. Assessing the performance of single-copy genes for recovering robust phylogenies. Systematic Biology 57:613–627.

Ahmadjian V. 1993. The lichen symbiosis. New York: John Wiley & Sons. 250 p.

Alors D, Dal Grande F, Cubas P, Crespo A, Schmitt I, Molina MC, Divakar PK. 2017. Panmixia and dispersal from the Mediterranean Basin to Macaronesian Islands of a macrolichen species. Scientific Reports 7:40879.

Altermann S, Leavitt SD, Goward T, Nelsen MP, Lumbsch HT. 2014. How do you solve a problem like *Letharia*? A new look at cryptic species in lichen-forming fungi using Bayesian clustering and SNPs from multilocus sequence data. PLoS ONE 9:e97556.

Articus K, Mattsson JE, Tibell L, Grube M, Wedin M. 2002. Ribosomal DNA and beta-tubulin data do not support the separation of the lichens *Usnea florida* and *U. subfloridana* as distinct species. Mycological Research 106:412–418.

Arup U, Ekman S, Lindblom L, Mattsson J-E. 1993. High performance thin layer chromatography (HPTLC), an improved technique for screening lichen substances. The Lichenologist 25:61–71.

Blom H, Lindblom L. 2009. *Degelia cyanoloma* (Schaer.) H.H. Blom & L. Lindblom comb. et stat. nov., a distinct species from western Europe. The Lichenologist 42:23–27.

Clement M, Snell Q, Walke P, Posada D, Crandall K. 2002. TCS: estimating gene genealogies. In: Proc 16th Int Parallel Distrib Process Symp., Fort Lauderdale, FL, Apr. 15–19, 2002. Vol. 2. Los Alamitos, CA: IEEE. p. 184.

Clerc P. 1998. Species concepts in the genus *Usnea* (lichenized Ascomycetes). The Lichenologist 30:321–340.

Clerc P, Herrera-Campos MA. 1997. Saxicolous species of *Usnea* subgenus *Usnea* (lichenized Ascomycetes) in North America. The Bryologist 100:281–301.

Cornejo C, Scheidegger C. 2015. Multi-gene phylogeny of the genus *Lobaria*: evidence of species-pair and allopatric cryptic speciation in East Asia. American Journal of Botany 102:2058–2073.

Crespo A, Lumbsch HT. 2010. Cryptic species in lichen-forming fungi. IMA Fungus 1:167–170.

Crespo A, Pérez-Ortega S. 2009. Cryptic species and species pairs in lichens: a discussion on the relationship between molecular phylogenies and morphological characters. Anales del Jardín Botánico Madrid 66:71–81.

De Queiroz K. 2007. Species concepts and species delimitation. Systematic Biology 56:879–886.

Esslinger TL. 2015. A cumulative checklist for the lichen-forming, lichenicolous and allied fungi of the continental United States and Canada. North Dakota State University, Fargo, North Dakota. [cited 2015 Apr 19]. Available from: http://www.ndsu.edu/pubweb/~esslinge/chcklst/chcklst7.htm (first posted 1 December 1997; Version 20, 19 April 2015)

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software structure: a simulation study. Molecular Ecology 14:2611–2620.

Excoffier L, Lischer HEL. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10:564–567.

Falush D, Stephens M, Pritchard JK. 2003. Inference of population structure: extensions to linked loci and correlated allele frequencies. Genetics 164:1567–1587.

Feng X, Liu J, Gong X. 2016. Species delimitation of the *Cycas segmentifida* complex (Cycadaceae) resolved by phylogenetic and distance analyses of molecular data. Frontiers in Plant Science 7:1–11.

Fryday A, Schmitt I, Pèrez-Ortega S. 2017. The genus *Endocena* (*Icmadophilaceae*): DNA evidence suggests the same fungus forms different morphologies. The Lichenologist 49:347–363.

Gardes M, Bruns TD. 1993. Its primers with enhanced specificity for basidiomycetes-application to identification of mycorhizae and rusts. Molecular Ecology 2:113–118.

Hausdorf B, Hennig C. 2010. Species delimitation using dominant and codominant multilocus markers. Systematic Biology 59:491–503.

Jombart T. 2008. adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24:1403–1405

Jombart T, Ahmed I. 2011. adegenet 1.3-1: new tools for the analysis of genome-wide SNP data. Bioinformatics 27:3070–3071.

Jombart T, Devillard S, Balloux F. 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. Bmc Genetics 11 (1):94. doi:10.1186/1471-2156-11-94.

Kanz B, von Brackel W, Cezanne R, Eichler M, Hohmann ML, Teuber D, Printzen C. 2015. DNA barcodes for the distinction of reindeer lichens: a case study using *Cladonia rangiferina* and *C. stygia*. Herzogia 28:445–464.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649.

Kroken S, Taylor JW. 2001. A gene genealogical approach to recognize phylogenetic species boundaries in the lichenized fungus *Letharia*. Mycologia 93:38–53.

Lagostina E, Dal Grande F, Ott S, Printzen C. 2017. Fungus-specific SSR markers in the Antarctic lichens *Usnea antarctica* and *U. aurantiacoatra* (Parmeliaceae, Ascomycota). Applications in Plant Sciences 5(9):1700054.

Leavitt SD, Fankhauser J, Leavitt DH, Porter L, Johnson L, St. Clair L. 2011. Complex patterns of speciation in cosmopolitan "rock posy" lichens—discovering and delimiting cryptic fungal species in the lichen-forming *Rhizoplaca melanophthalma* species-complex (Lecanoraceae, Ascomycota). Molecular Phylogenetics and Evolution 59:587–602.

Leavitt SD, Grewe F, Widhelm T, Muggia L, Wray B, Lumbsch HT. 2016. Resolving evolutionary relationships in lichen-forming fungi using diverse phylogenomic datasets and analytical approaches. Scientific Reports 6:22262

Leavitt SD, Moreau C, Lumbsch HT. 2015. The dynamic discipline of species delimitation: progress toward effectively recognizing species boundaries in natural populations. In: Upreti DK, Divakar PK, Shukla V, Bajpai R, eds. Recent advances in lichenology. New Delhi, India: Springer India. p. 11–44.

Lohtander K, Myllys L, Källersjö M, moberg R, Stenroos S, Tehler A. 2009. New entities in Physcia aipolia–P. caesia group (Physciaceae, Ascomycetes): an analysis based on mtSSU, ITS, group I intron and betatubulin sequences. Annales Botanici Fennici 46:43–53.

Lohtander K, Myllys L, Sundin R, Källersjö M, Tehler A. 1998. The species pair concept in the lichen *Dendrographa leucophaea* (Arthoniales): analyses based on ITS sequences. The Bryologist 101:404–411.

Lücking R, Dal-Forno M, Sikaroodi M, Gillevet PM, Bungartz FB, Moncada B, Yánez-Ayabaca A, Chaves JL, Coca LF, Lawrey JD. 2014a. A single macrolichen constitutes hundreds of unrecognized species. Proceedings of the National

Academy of Sciences of the United States of America 111:11091–11096.

Lücking R, Johnston MK, Aptroot A. 2014b. One hundred and seventy-five new species of Graphidaceae: closing the gap or a drop in the bucket? Phytotaxa 189:7–38.

Lumbsch HT, Ahti T, Altermann S, Amo De Paz G, Aptroot A, Arup U, Bárcenas Peña A, Bawingan PA, Benatti MN, Betancourt L, Björk CR, Boonpragob K, Brand M, Bungartz F, Cáceres MES, Candan M, Luis Chaves J, Clerc P, Common R, Coppins BJ, Crespo A, Dal-Forno M, Divakar PK, Duya MV, Elix JA, Elvebakk A, Fankhauser JD, Farkas E, Ferraro LI, Fischer E, Galloway DJ, Gaya E, Giralt M, Goward T, Grube M, Hafellner J, Hernández M, Jesús E, Herrera Campos MDL, Kalb K, Kärnefelt I, Kantvilas G, Killmann D, Kirika P, Knudsen K, Komposch H, Kondratyuk S, Lawrey JD, Mangold A, Marcelli MP, McCune B, Messuti MI, Michlig A, Gonzälez RM, Moncada B, Naikatini A, Nelsen MP, Øvstedal DO, Palice Z, Papong K, Parnmen S, Pérez-Ortega S, Printzen C, Rico VJ, Plata ER, Robayo J,; Rosabal D, Ruprecht U, Allen NS, Sancho L, De Jesus LS, Vieira TS, Schultz M, Seaward MRD, Sérusiaux E, Schmitt I, Sipman HJM, Sohrabi M, Søchting U, Søgaard MZ, Sparrius LB, Spielmann A, Spribille T, Sutjaritturakan J, Thammathaworn A, Thell A, Thor G, Thüs H, Timdal E, Truong C, Türk R, Umaña Tenorio L, Upreti DK, Van den Boom P, Vivas Rebuelta M, Wedin M, Will-Wolf S, Wirth V, Wirtz N, Yahr R, Yeshitela K, Ziemmeck F, Wheeler T, Lücking R. 2011. One hundred new species of lichenized fungi: a signature of undiscovered global diversity. Phytotaxa 18:1–127.

Lumbsch HT, Leavitt SD. 2011. Goodbye morphology? A paradigm shift in the delimitation of species in lichenized fungi. Fungal Diversity 50:59–72.

Lumbsch HT, Wirtz N. 2011. Phylogenetic relationships of the neuropogonoid core group in the genus *Usnea* (Ascomycota: *Parmeliaceae*). The Lichenologist 43:553–559.

Lumley LM, Sperling FAH. 2011. Utility of microsatellites and mitochondrial DNA for species delimitation in the spruce budworm (*Choristoneura fumiferana*) species complex (Lepidoptera: Tortricidae). Molecular Phylogenetics and Evolution 58:232–243.

Magain N, Sérusiaux E. 2015. Dismantling the treasured flagship lichen *Sticta fuliginosa* (Peltigerales) into four species in Western Europe. Mycological Progress 14:97.

Mark K, Saag L, Leavitt SD, Will-Wolf S, Nelsen MP, Tõrra T, Saag A, Randlane T, Lumbsch HT. 2016. Evaluation of traditionally circumscribed species in the lichen-forming genus *Usnea*, section *Usnea* (Parmeliaceae, Ascomycota) using a six-locus dataset. Organisms Diversity & Evolution 16:497–524.

Myllys L, Lohtander K, Tehler A. 2001. Beta-tubulin, ITS and group I intron sequences challenge the species pair concept in *Physcia aipolia* and *P. caesia*. Mycologia 93:335–343.

Ogilvie HA, Heled J, Xie D, Drummond AJ. 2016. Computational performance and statistical accuracy of *BEAST and comparisons with other methods, Systematic Biology 65:381–396.

Olech M. 2004. Lichens of King George Island, Antarctica. Kraków, Poland: Institute of Botany of the Jagiellonian University. 391 p.

Pérez-Ortega S, Fernández-Mendoza F, Raggio J, Vivas M, Ascaso C, Sancho LG, Printzen C, de Los Ríos A. 2012. Extreme phenotypic variation in *Cetraria aculeata* (lichenized Ascomycota): adaptation or incidental modification? Annals of Botany 109:1133–1148.

Poelt, J. 1970. Das Konzept der Artenpaare bei den Flechten. Vorträge aus dem Gesamtgebiet der Botanik 4:187–198.

Printzen C, Ekman S, Tønsberg T. 2003. Phylogeography of *Cavernularia hultenii*: evidence for slow genetic drift in a widely disjunct lichen. Molecular Ecology 12:1473–1486.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

Ross KG, Gotzek D, Ascunce MS, Shoemaker DD. 2010. Species delimitation: a case study in a problematic ant taxon. Systematic Biology 59:162–184.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences of the United States of America 109:6241–6246.

Seymour F, Crittenden P, Wirtz N, Øvstedal D, Dyer P, Lumbsch HT. 2007. Phylogenetic and morphological analysis of Antarctic lichen-forming *Usnea* species in the group *Neuropogon*. Antarctic Science 19:71–82.

Singh G, Dal Grande F, Divakar PK, Otte J, Leavitt SD, Szczepanska K, Crespo A, Rico VJ, Aptroot A, de Silva Càceres ME, Lumbsch HT, Schmitt I. 2015. Coalescent-based species delimitation approach uncovers high cryptic diversity in the cosmopolitan lichen-forming fungal genus *Protoparmelia* (Lecanorales, Ascomycota). PLoS ONE 10: e0124625

Spribille T, Klug B, Mayrhofer H. 2011. A phylogenetic analysis of the boreal lichen *Mycoblastus sanguinarius* (Mycoblastaceae, lichenized Ascomycota) reveals cryptic clades correlated with fatty acid profiles. Molecular Phylogenetics and Evolution 59:603–614.

Spribille T, Tuovinen V, Resl P, Vanderpool D, Wolinski H, Aime MC, Schneider K, Stabentheiner E, Toome-Heller M, Thor G, Mayrhofer H, Johannesson H, McCutcheon JP. 2016. Basidiomycete yeasts in the cortex of ascomycete macrolichens. Science 353:488–492.

Tehler A. 1982. The species pair concept in lichenology. Taxon 31:708–714.

Thell A, Crespo A, Divakar PK, Kärnefelt I, Leavitt SD, Lumbsch HT, Seaward MRD. 2012. A review of the lichen family Parmeliaceae—history, phylogeny and current taxonomy. Nordic Journal of Botany 30:641–664.

Tripp EA. 2016. Is asexual reproduction an evolutionary dead end in lichens? The Lichenologist 48:559–580.

Truong C, Divakar PK, Yahr R, Crespo A, Clerc P. 2013. Testing the use of ITS rDNA and protein-coding genes in the generic and species delimitation of the lichen genus *Usnea* (Parmeliaceae, Ascomycota). Molecular Phylogenetics and Evolution 68:357–372.

Walker FJ. 1985. The lichen genus *Usnea* subgenus *Neuropogon*. Bulletin of the British Museum (Natural History), Botany 13:1–130.

Walser JC, 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.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Taylor J, and Sninsky JJ, Eds., PCR protocols: A guide to methods and applications. New York: Academic Press Inc. p. 315–322. doi:10.1016/B978-0-12-372180-8.50042-1

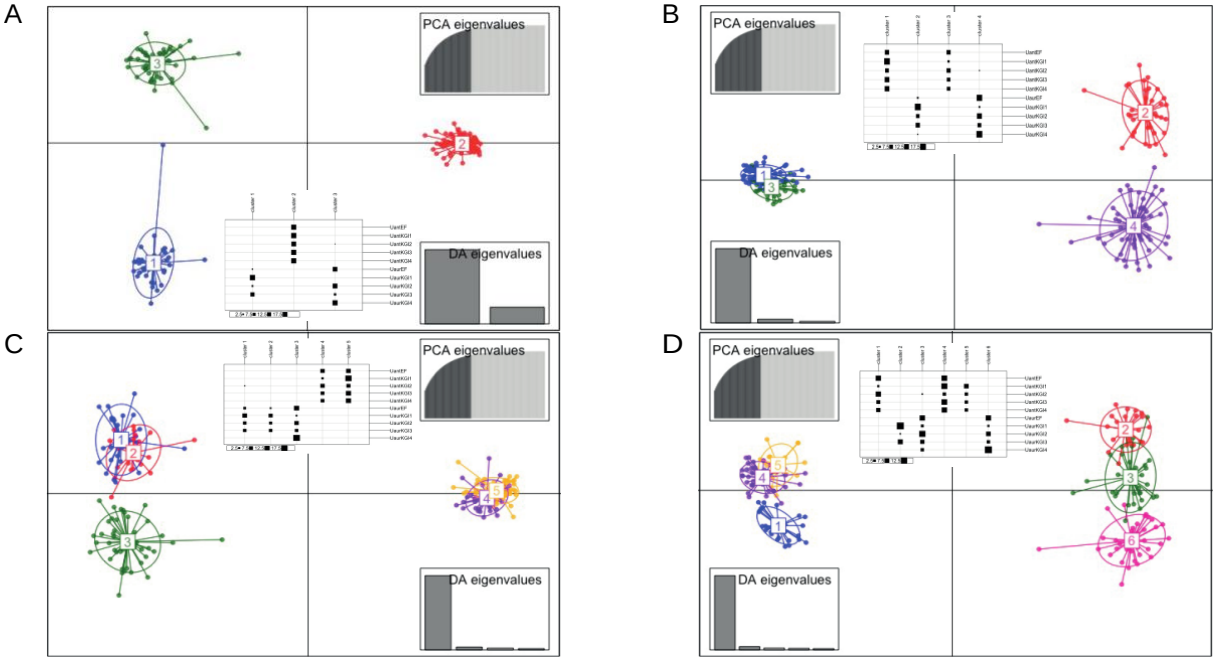
Wirtz N, Printzen C, Lumbsch HT. 2008. The delimitation of Antarctic and bipolar species of neuropogonoid *Usnea* (Ascomycota, Lecanorales): a cohesion approach of species recognition for the *Usnea perpusilla* complex. Mycological Research 112:472–484.

Wirtz N, Printzen C, Lumbsch HT. 2012. Using haplotype networks, estimation of gene flow and phenotypic characters to understand species delimitation in fungi of a predominantly Antarctic *Usnea* group (Ascomycota, Parmeliaceae). Organisms Diversity & Evolution 12:17–37.

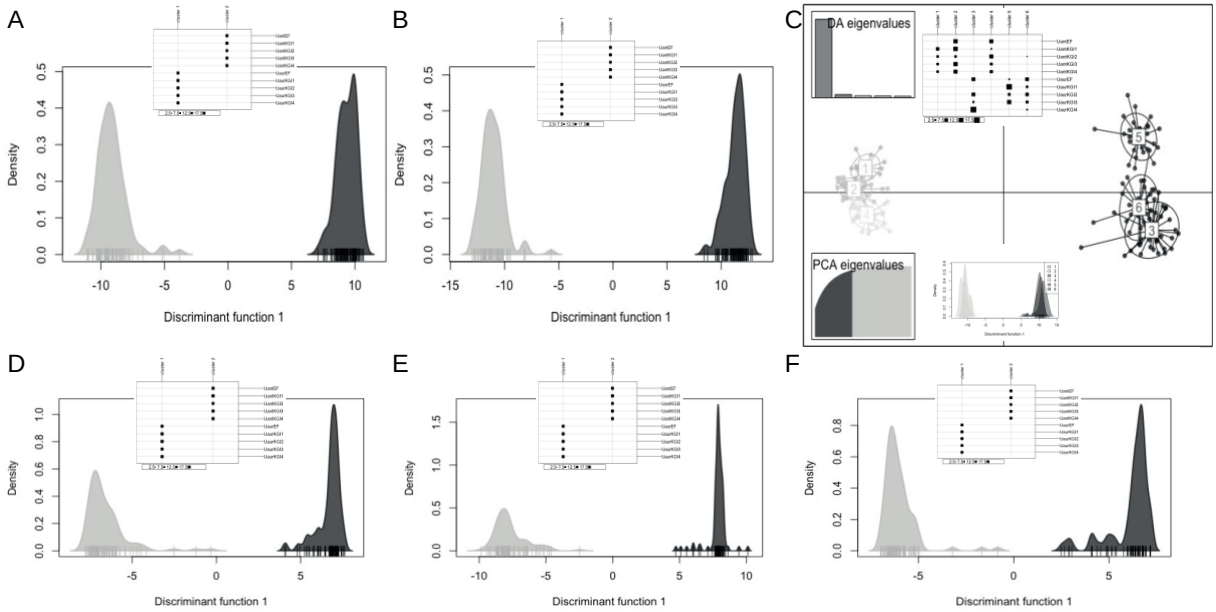
Additional material 1. List of sequences deposited in Genbank.

Species	Population	Sample	ITS Genbank code
Usnea antarctica	UantKGI1	Uant_001	MG200275
Usnea antarctica	UantKGI1	Uant_002	MG200276
Usnea antarctica	UantKGI1	Uant_004	MG200277
Usnea antarctica	UantKGI1	Uant_009	MG200278
Usnea antarctica	UantKGI1	Uant_010	MG200279
Usnea antarctica	UantKGI1	Uant_020	MG200280
Usnea antarctica	UantKGI2	Uant_022	MG200281
Usnea antarctica	UantKGI2	Uant_033	MG200282
Usnea antarctica	UantKGI2	Uant_037	MG200283
Usnea antarctica	UantKGI2	Uant_038	MG200284
Usnea antarctica	UantKGI3	Uant_043	MG200285
Usnea antarctica	UantEF	Uant_384_	MG200286
Usnea antarctica	UantKGI4	Uant_402	MG200287
Usnea antarctica	UantKGI4	Uant_407	MG200288
Usnea aurantiacoatra	UaurKGI1	Uaur_064	MG200289
Usnea aurantiacoatra	UaurKGI1	Uaur_065	MG200290
Usnea aurantiacoatra	UaurKGI1	Uaur_067	MG200291
Usnea aurantiacoatra	UaurKGI1	Uaur_069	MG200292
Usnea aurantiacoatra	UaurKGI1	Uaur_070	MG200293
Usnea aurantiacoatra	UaurKGI1	Uaur_071	MG200294
Usnea aurantiacoatra	UaurKGI1	Uaur_072	MG200295
Usnea aurantiacoatra	UaurKGI1	Uaur_073	MG200296
Usnea aurantiacoatra	UaurKGI2	Uaur_091	MG200297
Usnea aurantiacoatra	UaurKGI2	Uaur_096	MG200298
Usnea aurantiacoatra	UaurKGI2	Uaur_101b	MG200299
Usnea aurantiacoatra	UaurKGI3	Uaur_114	MG200300
Usnea aurantiacoatra	UaurKGI3	Uaur_418	MG200301
Usnea aurantiacoatra	UaurEF	Uaur_419	MG200302
Usnea aurantiacoatra	UaurEF	Uaur_421	MG200303
Usnea aurantiacoatra	UaurEF	Uaur_433	MG200304
Usnea aurantiacoatra	UaurKGI4	Uaur_440	MG200305
Usnea aurantiacoatra	UaurKGI4	Uaur_449	MG200306
Usnea aurantiacoatra	UaurKGI4	Uaur_450	MG200307

Additional material 2. Results of DAPC based on *Usnea* morphotypes; number of principle components = 50, number of groups, a. *k*=3, b. *k*=4, c. K=5, d. K=6. Insert graphs: assignment of samples to clusters. Names on the right indicate morphotype and stand. With one exception (in UantKGI2), sorediate and esorediate samples are always assigned to different clusters while no geographically restricted genetic clusters are observed.

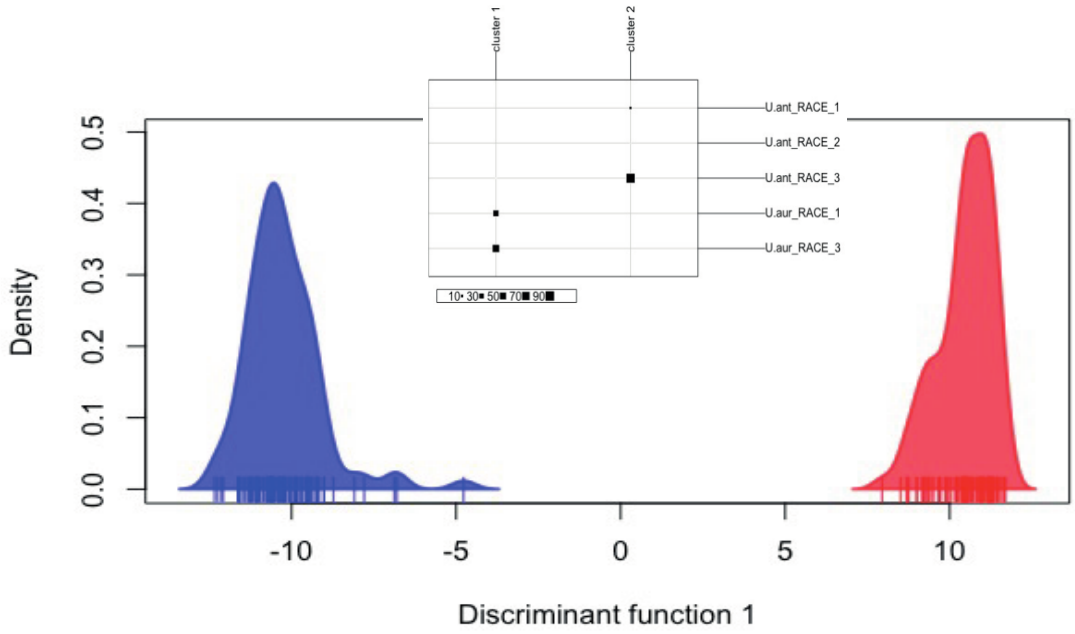


Additional material 3. DAPC based on *Usnea* morphotypes;, a. PCs 40 *k*=2, b. PCs 60 *k*=2, c. PCs 50 *k*=6, d. 8 SSRs markers (Ua1-Ua8) from the first multiplex reaction, e. 8 SSRs markers (Ua9-Ua16) from the second multiplex reaction, f. 7 SSRs markers (Ua17-Ua23) from the third multiplex reaction.

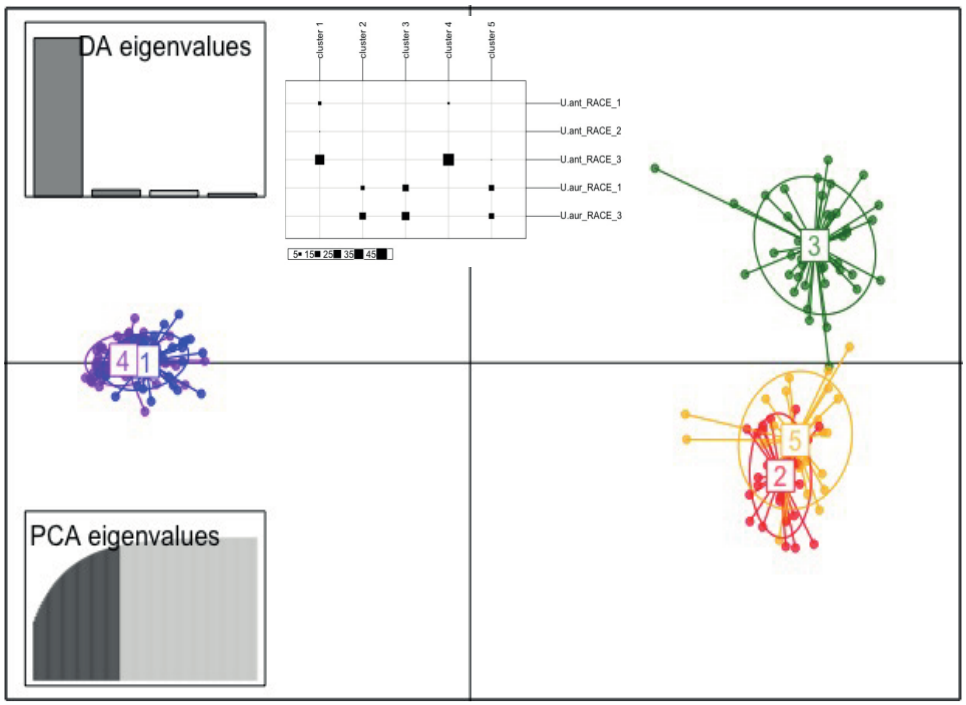


Additional material 4. Results of DAPC based on *Usnea* morphotypes and chemical races, a. DAPC based on 50 PCs and k=2 clusters. Density distribution of esorediate (blue = *Usnea aurantiacoatra*) and sorediate (red = *Usnea antarctica*) morphs along the first discriminant function. Insert graphic: assignment of chemoraces to clusters, b. Same for k=5 groups. Genetic clusters discriminate between morphotypes but not between chemical races.

A



B



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RESEARCH ARTICLE

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Population genomic analyses of RAD sequences resolves the phylogenetic relationship of the lichen-forming fungal species *Usnea antarctica* and *Usnea aurantiacoatra*

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Abstract

Neuropogonoid species in the lichen-forming fungal genus *Usnea* exhibit great morphological variation that can be misleading for delimitation of species. We specifically focused on the species delimitation of two closely-related, predominantly Antarctic species differing in the reproductive mode and representing a so-called species pair: the asexual *U. antarctica* and the sexual *U. aurantiacoatra*. Previous studies have revealed contradicting results. While multi-locus studies based on DNA sequence data provided evidence that these two taxa might be conspecific, microsatellite data suggested they represent distinct lineages. By using RADseq, we generated thousands of homologous markers to build a robust phylogeny of the two species. Furthermore, we successfully implemented these data in fine-scale population genomic analyses such as DAPC and fineRADstructure. Both *Usnea* species are readily delimited in phylogenetic inferences and, therefore, the hypothesis that both species are conspecific was rejected. Population genomic analyses also strongly confirmed separated genomes and, additionally, showed different levels of co-ancestry and substructure within each species. Lower co-ancestry in the asexual *U. antarctica* than in the sexual *U. aurantiacoatra* may be derived from a wider distributional range of the former species. Our results demonstrate the utility of this RADseq method in tracing population dynamics of lichens in future analyses.

Keywords
Antarctica, Ascomycota, lichens, Parmeliaceae, phylogeny, RADseq

Introduction

Over the last decades, the use of DNA sequence data to delimit species and reconstruct phylogenetic relationships has become standard (Barraclough and Nee 2001; de Queiroz 2007; Holder and Lewis 2003; Huelsenbeck et al. 2001; Taylor et al. 2000; Wiens and Penkrot 2002). In groups with high morphological plasticity and homoplasy in phenotypical data sets, such as fungi, molecular data have dramatically changed our understanding of evolution and coinciding taxonomic interpretations (Hibbett et al. 2007; James et al. 2006; Lutzoni et al. 2004; McLaughlin et al. 2009; Robbertse et al. 2006; Schoch et al. 2009; Spatafora et al. 2017; Spatafora and Robbertse 2010; Stajich et al. 2009).

The general lineage species concept (de Queiroz 2007) allows researchers to use different empirical data to test the hypothesis of lineage separation, including phenotypical characters and molecular data. The latter dataset often provides strong evidence if analysed within a rigorous statistical framework (Rannala 2015). With regards to species delimitation, numerous studies of lichen-forming fungi detected distinct lineages lacking obvious distinguishing phenotypical characters, the so-called cryptic species (Bickford et al. 2007; Crespo and Lumbsch 2010; Crespo and Pérez-Ortega 2009; Lumbsch and Leavitt 2011). However, some studies also demonstrated that morphologically distinct populations could not be separated using single- or multi-locus genetic data. These results have been interpreted either as an indication of recent diversification and incomplete lineage sorting (Leavitt et al. 2016a; Zhao et al. 2017) or that the phenotypes represented populations of the same species (Articus et al. 2002; Buschbom and Mueller 2006; Kotelko and Piercey-Normore 2010; Lohtander et al. 1998; Myllys et al. 2001; Velmala et al. 2009). The latter result was often found in so-called species pairs. These are lichens that differ in forming either ascomata and reproducing sexually or forming asexual diaspores (soredia), which propagate the fungal and photosynthetic partner simultaneously (Mattsson and Lumbsch 1989; Poelt 1970; Tehler 1982). Otherwise, these species are morphologically identical, but were traditionally regarded as distinct species due to their different reproductive modes (Poelt 1972). The *Parmotrema perforatum* group was used as a model system of species delimitation based on the reproductive mode and secondary metabolites (Culberson and Culberson 1973). However, a recent study suggests that the phylogenetic relationships between sexual and asexual populations might be more complex (Widhelm et al. 2016).

We here focus on a complex of two morphologically similar species that differ in their reproductive mode and are considered a species pair in the genus *Usnea*: *U. aurantiacoatra* and *U. antarctica*, the latter reproducing by asexual soredia (Walker 1985). Within the genus, there is group of species predominantly occurring in Antarctica and adjacent cool-temperate to polar regions with a thallus that consists of yellow (containing usnic acid) and blackish areas (caused by melanins). Species in this group, which is also called neuropogonoid (Lumbsch and Wirtz 2011; Wirtz et al. 2008), can be difficult to

distinguish by their general appearance and hence, molecular data, such as DNA marker sequencing, can be helpful in delimiting lineages (Articus 2004; Lumbsch and Wirtz 2011; Seymour et al. 2007; Wirtz et al. 2008; Wirtz et al. 2012). Earlier studies based on morphological and chemical data considered the neuropogonoid species as a subgenus *Neuropogon* in *Usnea* (Lamb 1964; Walker 1985) or as a distinct genus *Neuropogon* (Krog 1976; 1982; Lamb 1939). Molecular studies confirmed *Usnea* (including *Neuropogon*) as a monophyletic genus within Parmeliaceae (Crespo et al. 2007); however, the relationship of *Neuropogon* and *Usnea* remained ambiguous. A two-marker DNA analysis of *Usnea* elevated *Neuropogon* to a generic rank (Articus 2004), but subsequent studies provided evidence that *Neuropogon* is polyphyletic with a core group nested within *Usnea* (Seymour et al. 2007; Wirtz et al. 2008; Wirtz et al. 2012; Wirtz et al. 2006). Multilocus DNA sequence data could not delimit individuals of the species *U. antarctica* and *U. aurantiacoatra* (Seymour et al. 2007; Wirtz et al. 2012) suggesting that they might be conspecific. In contrast, a recent microsatellite study provided evidence that the two species represent isolated lineages (Lagostina et al. 2018). Given the contradicting results of multi-locus and microsatellite data, we decided to employ a reduced genomic dataset to revisit the species delimitation of *U. antarctica* and *U. aurantiacoatra*.

The advent of next-generation sequencing (NGS), also referred to as high-throughput sequencing, drastically changed the scale of molecular datasets for systematic analyses and revolutionised our ability to assess evolutionary histories of organisms (Kraus and Wink 2015; Wachi et al. 2018; Zimmer and Wen 2015). Many molecular studies, such as the former species delimitation efforts for neuropogonoid *Usnea* spp., were limited to, at most, a dozen markers because their production would require tedious lab work and costly Sanger-sequencing (Hoffman and Lendemer 2018; Wilkinson et al. 2017). Population genomics of closely related organisms often relied on the descriptive power of microsatellite markers (Hodel et al. 2016). Compared to these traditional lab methods, NGS techniques allow a relatively straight-forward production of genome-scale datasets. Direct sequencing NGS methods, such as de-novo genome sequencing (Ellegren 2014), re-sequencing (Stratton 2008) or RNAseq of expressed genes (Ozsolak and Milos 2011; Wickett et al. 2014), can provide whole genome-scale data but may still be limited by high sequencing costs. Therefore, these methods are rarely applied to population studies which require the sequencing of many individuals. However, often a subset of the genome entails sufficient polymorphisms to answer questions of phylogenetic or population genomic studies. Hence, many NGS methods for systematic analyses are designed to be economical and generate reduced genome representation datasets (Allendorf 2017; Davey et al. 2011). One of these reduced representation methods is genotype-by-sequencing and its altered approach, which is known as restriction associated DNA sequencing (RADseq) (Baird et al. 2008). We recently designed a RADseq approach for metagenomic data derived from symbiotic lichen genomes, which allows reduced representation genomic analyses of numerous individuals for population-scale studies (Grewe et al. 2017).

By using genome-wide single nucleotide polymorphisms (SNPs) produced by restriction site-associated DNA sequencing (RADseq) of predominantly Antarctic li-

chen-forming fungi, our main aim in this study was to clarify the taxonomy of asexual *Usnea antarctica* and sexual *Usnea aurantiacoatra* and test their hypothesised species pair relationship. To further support our findings, we applied population genomic methods to measure the degree of genomic divergence and infer the levels of co-ancestry for each species.

Methods

Sample collection and site description

Samples were collected in Antarctica between December 2015 and January 2017. From these collected specimens, we chose to compare 105 representative specimens of the species *U. antarctica* and *U. aurantiacoatra* for this study (see details of specimens in Suppl. material 1). All selected specimens were either collected on King George Island (65) and Elephant Island (19) of the South Shetland Islands or in the Northern part of the Antarctic Peninsula (21) near the research bases Esperanza and Primavera. Fifty-eight of the 105 selected specimens were identified as *U. antarctica* and 47 specimens were identified as *U. aurantiacoatra* based on their phenotypical characters (Walker 1985). As a reference sequence to filter for lichen-fungal loci of *U. antarctica* and *U. aurantiacoatra* during the RADseq processing, we sequenced a specimen of *U. strigosa* that was collected in Arkansas, U.S.A. (Suppl. material 1).

DNA extraction

Total metagenomic DNA was extracted either by following a cetyltrimethylammonium bromide (CTAB) protocol as modified by Cubero et al. (1999) or by using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) as recommended by the manufacturer. We used the whole lichen thalli for DNA extraction from *U. antarctica* and *U. aurantiacoatra*, but only the central axis in *U. strigosa* to preferentially extract DNA from the lichen fungus (to avoid the photobiont). DNA concentrations of all samples were quantified with a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

Reference Sequencing and Assembly

We first deep-sequenced and assembled a reference sequence of an *Usnea strigosa* specimen to aid in mapping lichen-fungal loci during the processing of metagenomic RADseq data. A paired-end Illumina sequencing library of 150 bp read length was constructed from total DNA with the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA) and sequenced on a NextSeq platform at the University of Il-

linois Chicago's Sequencing Core Facility (Chicago, IL, USA). The resulting reads were quality trimmed using the programme Trimmomatic v0.36 (Bolger et al. 2014). Bases were trimmed when the average quality of 4-base sliding windows was below 15 and bases at the start and end of reads had a quality below 10. Subsequently, all trimmed reads, shorter than 25 bp, were filtered out (LEADING:10 TRAILING:10 SLIDINGWINDOW:4:15 MINLEN:25). The trimmed reads were used for a genome assembly with the programme SPAdes v3.5 (Bankevich et al. 2012) with default parameter settings. The assembled metagenomic scaffolds were loaded into the programme MetaWatt v3.5.3 (Strous et al. 2012) for a binning based on tetranucleotide frequencies. Scaffolds of fungal origin that clustered together were separated from the remaining scaffolds. All selected scaffolds that were larger than 10 kb were then included into the final reference sequence of *U. strigosa*. We used the Core Eukaryotic Gene Mapping Approach (CEGMA) to estimate the genomic completeness of the assembly (Parra et al. 2009). Finally, we created a Bowtie2 (Langmead and Salzberg 2012) database from the selected scaffolds for the mapping approach to filter for fungal RAD loci.

RADseq Library Preparation and Sequencing

RADseq libraries for *Usnea antarctica* and *U. aurantiacoatra* were prepared as described previously (Grewe et al. 2017). In short, for the RADseq library production, DNA isolations were pooled with sequence adapters (Rubin and Moreau 2016), subsequently digested with the restriction enzyme ApeKI (New England Biolabs, Ipswich, MA, USA) and ligated using T4 ligase (New England Biolabs). Up to 48 samples with compatible barcodes were pooled and selected for fragments of sizes between 300 and 500 bp using the BluePippin DNA size selection system (Sage Science, Beverly, MA, USA). The pooled libraries were amplified using the REDTaq ReadyMix (Sigma-Aldrich, St. Louis, MO, USA) prior to sequencing on an Illumina MiSeq using the MiSeq Reagent Kit v3 for 150 cycles (Illumina, San Diego, CA, USA) to produce single-end sequences with a length of 150 bp.

Assembly of RADseq datasets

The raw reads of *U. antarctica* and *U. aurantiacoatra* from the MiSeq sequencing were processed and assembled as described earlier for metagenomic datasets of lichens (Grewe et al. 2017). This process used a combination of the ipyRAD (https://github.com/dereneaton/ipyrad/blob/master/docs/index.rst) and pyRAD (Eaton and Ree 2013) pipelines with an additional mapping step that filtered for lichen-fungal loci with a reference sequence. Subsequently, we refer to the raw Illumina RAD sequences as 'read' and name the clustered reads per individual sample 'loci'; the final matrices are alignments of homologous loci from multiple samples with nucleotide substitutions referred to as 'SNP'. In pyRAD, we set the datatype to genotype-by-sequencing

(gbs), ploidy to haploid (1), a similarity threshold for the clustering of reads within and between individuals to 90% (.90) and a minimum coverage of four individuals per locus (4). For the reference-based filtering of RAD loci, we used Bowtie2 with adjusted parameters to allow one permitted mismatch (–N 1), a seed length of 20 (–L 20), up to 20 seed extension attempts (–D 20) and a maximum "re-seeding" of 3 (–R 3). Following an initial round with all sequenced samples, we re-ran step 7 of pyRAD and excluded samples with less than 1000 recovered loci. We used the filtered pyRAD output files, such as unlinked_snps, alleles and vcf, for further analyses.

Phylogenetic reconstructions

Phylogenetic trees were calculated from all unlinked SNPs of the filtered RADseq dataset, i.e. a matrix that was limited to one SNP per RAD locus. This matrix was used for a RAxML v7.2.8 (Stamatakis 2006) maximum likelihood analysis using the GTR + G model. For each analysis, 100 bootstrap replicates were calculated using the fast bootstrapping option implemented in RAxML (Stamatakis et al. 2008). The resulting phylogenetic tree was midpoint rooted and drawn to scale with FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Analysis of population structure

To calculate differences in the population structure between *U. antarctica* and *U. aurantiacoatra*, we created a reduced dataset that included all sites with a minor allele frequency (MAF) greater than or equal to 0.05 and greater than 50% coverage using vcftools v0.1.15 (Danecek et al. 2011). This reduced vcf file was converted into a genind object from the R package adegenet v2.0.2 (Jombart and Ahmed 2011; Jombart et al. 2010). The genind object was appended with additional information settings for haploid genomes and the population memberships for samples according to their initial identification based on morphological characters. With all information enclosed, the genind object became subject to population genetics analyses encoded in R.

To determine the degree to which both populations are subdivided, we estimated Gst (Nei 1973) and Hedrick's standardised genetic differentiation measures G'st (Hedrick 2005) and Jost's D (Jost 2008) by using the R package mmod v1.3.3 (Winter 2012). Gst is a good measure when the mutation rate is small relative to migration rate; contrarily, G'st and D fit to data with high mutation rates and two populations (Whitlock 2011). We used these multiple statistics to get a comprehensive measure of genomic differentiation. In a population pairwise comparison, we calculated these indices per site and plotted the values by frequency in separate histograms for Gst, G'st and D.

The genetic structure of samples of *U. antarctica* and *U. aurantiacoatra* was evaluated with the Discriminant Analysis of Principal Components (DAPC) implemented in the R package adegenet v2.02. This non-parametric method first transforms the data

using a principle components analysis (PCA) and subsequently distinguishes between two or more groups using a discriminant analysis (DA). The DAPC was conducted by using the first 60 principal components and all (two) DA-eigenvalues. In addition to the display of the genetic variation in genomic space, the DAPC allows a prediction of the group membership probability for each sample which is visualised in a STRUCTURE-like plot.

In addition to the nonparametric approach with DAPC, we used a model-based method to detect population subdivision using the programme fineRADstructure (Malinsky et al. 2018). This software is specifically designed to measure population structure amongst haplotypes inferred from RADseq datasets. We used the script finerad_input.py included in fineRADstructure-tools (https://github.com/edgardomortiz/fineRADstructure-tools) to convert the pyRAD alleles output into the input format for fineRADstructure. During the conversion, we also reduced the dataset to only unlinked loci (default parameter) with a minimum sample number of 4 (--minsample 4). As recommended by the authors, we then re-ordered the unsorted RAD loci with the script sampleLD.R which is part of the fineRADstructure package. Next, we used the scripts, RADpainter and fineSTRUCTURE, which are both implemented in fineRADstructure, to measure the population structure. First, we calculated the co-ancestry matrix with RADpainter for haploid datasets (-p 1). We then used fineSTRUCTURE for the Markov chain Monte Carlo (MCMC) clustering algorithm with the following arguments: -x 100,000, -z 100,000 and -y 1,000. We also started fineSTRUCTURE with the arguments -m T and -x 10,000 to run a simple tree-building algorithm on the data of the co-ancestry matrix. Finally, the co-ancestry matrix, MCMC output and the coalescence tree were loaded into the programme 'Finestructure GUI' for visualisation.

Reproducibility

The *U. strigosa* reference sequence and all scripts that were used in this study are available online (https://github.com/felixgrewe/Usnea). All RAD sequences were deposited in the NCBI Sequence Read Archive (SRA) with accession number PRJNA505526.

Results

Reference assembly and RADseq results

We assembled a draft reference genome of *U. strigosa* to filter for fungal RAD loci from *U. antarctica* and *U. aurantiacoatra*. The Illumina NextSeq sequencing of the whole *U. strigosa* lichen resulted in 8,552,530 metagenomic paired-end reads. First, we trimmed these raw data which reduced the paired-end reads to 8,366,962 (97.78% of raw data). The trimmed read pairs were then assembled into 16,932 scaffolds (N50 = 12,750 bp) with a total size of 40.9 Mbp (including 1,187 scaffolds of sizes larger than 10 kb).

Metagenomic binning identified 28.92 Mbp of the assembly as fungal derived from which we selected 1,100 scaffolds (N50 = 23,562 bp) with sizes larger than 10 kb; all but two of these scaffolds were continuous assemblies (contigs). The sorted draft genome of *U. strigosa* had a total size of 24.1 Mbp and an estimated completeness of 72.18%.

We included 105 specimens of *U. antarctica* and *U. aurantiacoatra* that were collected in Antarctica in four RADseq libraries (Suppl. material 2). The sequence read number of each sample varied widely from 13,659 for sample EL0059 to 1,942,819 for sample EL0074 with an average sequence read number of 488,468 (sd = 313,604). The number of loci (within sample clusters) that pyRAD generated from these sequences directly correlated with the initial number of sequences (R2 = 0.8017, Suppl. material 3). An average of 21.8% (sd = 2.9%) of all loci mapped to the lichen fungus reference genome and, of these, an average of 85.4% (sd = 5.5%) were included into the final pyRAD dataset. The numbers of loci before and after the mapping were directly correlated (R2 = 0.7598, Suppl. material 3); however, the number of mapped loci reached saturation at an average of 6,496 (sd = 801) for samples with more than 40,000 initial loci. In addition, the number of mapped loci were strongly correlated to the number of loci included in the final dataset (R2 = 0.9869, Suppl. material 3). Two samples of *U. antarctica* (EL0059, EL0281) and two samples of *U. aurantiacoatra* (EL0415, EL0437) had less than 1,000 loci in the final dataset and were removed from the analysis. All remaining 101 samples in the final dataset had on average of 4,143 (sd = 1,316) loci (Suppl. material 2).

Phylogenetic analysis of RADseq data

The phylogenetic analysis of the RADseq data showed two distinct and highly supported clades corresponding to the phenotypically circumscribed species *U. antarctica* and *U. aurantiacoatra* (Figure 1). The phylogenetic tree was calculated from a matrix with 7,087 positions and 53.24% gaps. Most internal relationships within each clade remained unresolved; however, the *U. antarctica* clade showed higher internal support values than the *U. aurantiacoatra* clade. Within the *U. antarctica* clade, three sister relationships of *U. antarctica* (EL0001 and EL0409, EL0382 and EL0390, EL0713 and EL0743) had a 100% bootstrap support and short branches, indicating low genomic divergence.

Population genomic analyses of RADseq data

We determined the degree to which both species complexes are subdivided by Gst, G'st and D measurements. For these analyses, we included only SNPs with a MAF greater than 0.05 and more than 50% coverage. This reduced the RAD dataset to a total of 4,132 SNPs. We plotted the frequency of Gst, G'st and D measures for each SNP (Figure 2). A strong tendency towards 1 for most SNPs in all three measures strongly indicated that genomes of both species were completely isolated. This was also supported by the average measures of Gst, G'st and D of 0.70, 0.93 and 0.60, respectively.

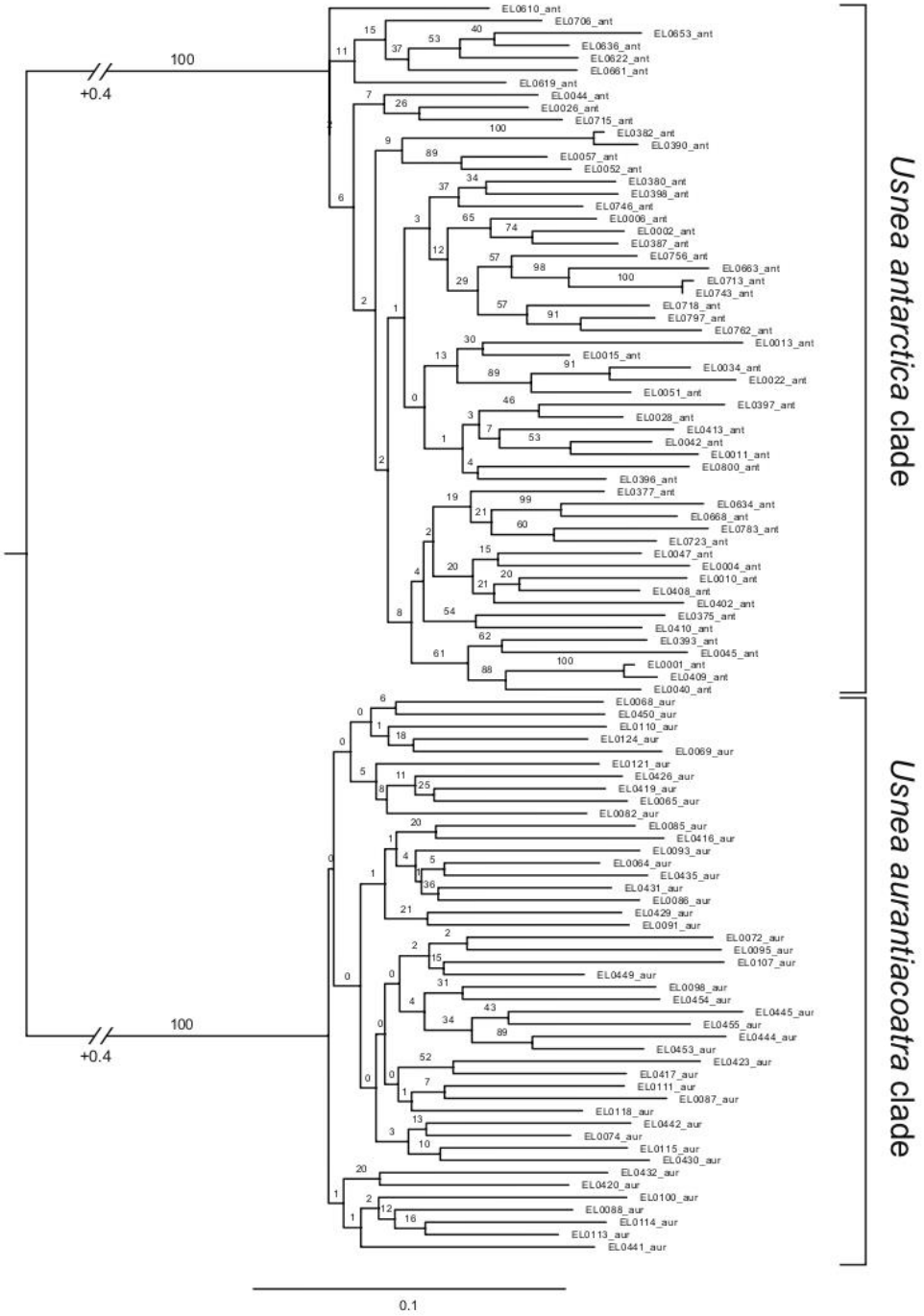

Figure 1. Phylogenetic tree inferred from the *U. antarctica* and *U. aurantiacoatra* RADseq data. The clades of each species are highlighted by brackets. Bootstrap values are indicated at the branches. The unit of branch length is substitutions per site. Note that branches leading to both major clades were abbreviated by 0.4 substitutions per site.

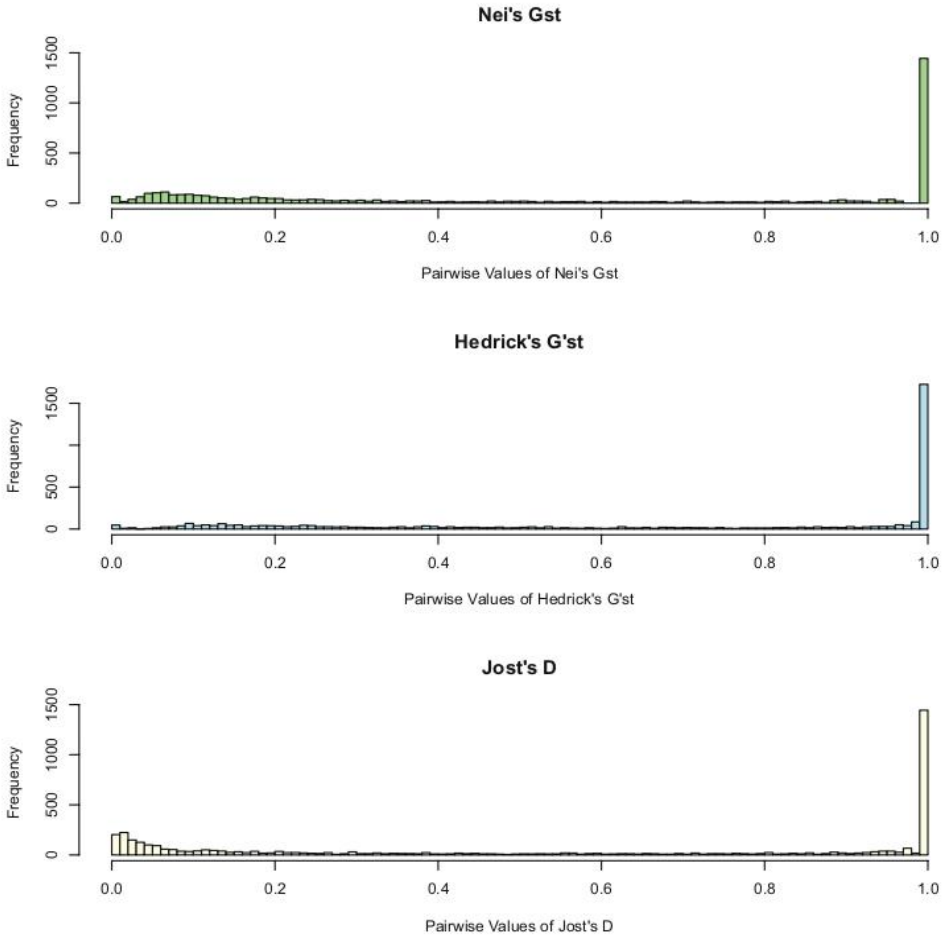

Figure 2. Pairwise Gst, G'st and D distribution. Pairwise values of Nei's Gst (green), Hedrick's G'st (blue) and Jost's D (yellow) are plotted by their frequency.

The same reduced dataset of 4,132 SNPs was used to differentiate the genomes by their variation in a non-parametric approach with a DAPC (Figure 3A). The DAPC combines a PCA with a DA for a separation of genomes based on their variance between groups rather than the total variance of the sample. The resulting clusters of both species were clearly separated in genomic space and showed no evidence for admixture. In addition, the group membership probabilities indicated absolute discrimination of the two species by the DAPC assigning each individual with 100% probability to their respective species (Figure 3B).

The separation of *U. antarctica* and *U. aurantiacoatra* was further supported by the results of a Bayesian model-based approach with the programme fineRADstructure. By converting the pyRAD allele output for fineRADstructure, we reduced the dataset to 3,803 unlinked SNPs with a minimum coverage of 4 samples. The resulting clustered

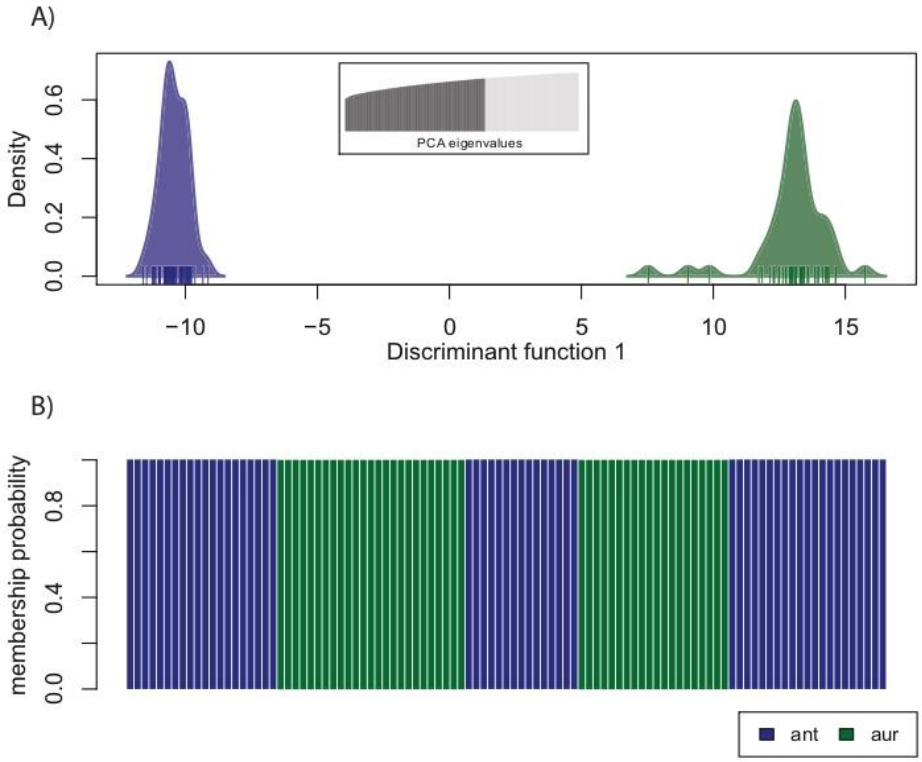

Figure 3. Genomic variation by non-parametric DAPC. **A** DAPC plot of the densities of *U. antarctica* (blue) and *U. aurantiacoatra* (green) on the first retained discriminant function **B** Bar plot of group membership probabilities.

co-ancestry matrix showed that both species shared more co-ancestry within each other than between species (Figure 4). By comparing both species clusters, *U. aurantiacoatra* showed a higher estimated co-ancestry than *U. antarctica* (Figure 4A). To avoid a sampling bias, we reduced the dataset for the fineRADstructure analysis to include only samples collected on King George Island and Elephant Island. This reduced the dataset to 80 samples and 3,652 unlinked SNPs with a minimum coverage of 4 samples. The resulting plot of the reduced dataset also showed higher shared co-ancestry within each species compared to that between species, but estimated higher co-ancestry of *U. antarctica* than *U. aurantiacoatra* (Figure 4B), opposite to the entire dataset. In addition, both matrices visualised different degrees of intraspecific co-ancestry and suggested substructure for a group of three specimens of *U. antarctica* from Potter Peninsula, King George Island (EL0022, EL0034 and EL0051) and for six specimens of *U. aurantiacoatra* from Fildes Peninsula, King George Island (EL0444, EL0435, EL0445, EL450, EL0455 and EL0453). Moreover, two *U. antarctica* specimen pairs collected on King George Island (EL0001 and EL0409, EL0382 and EL390) and one pair col-

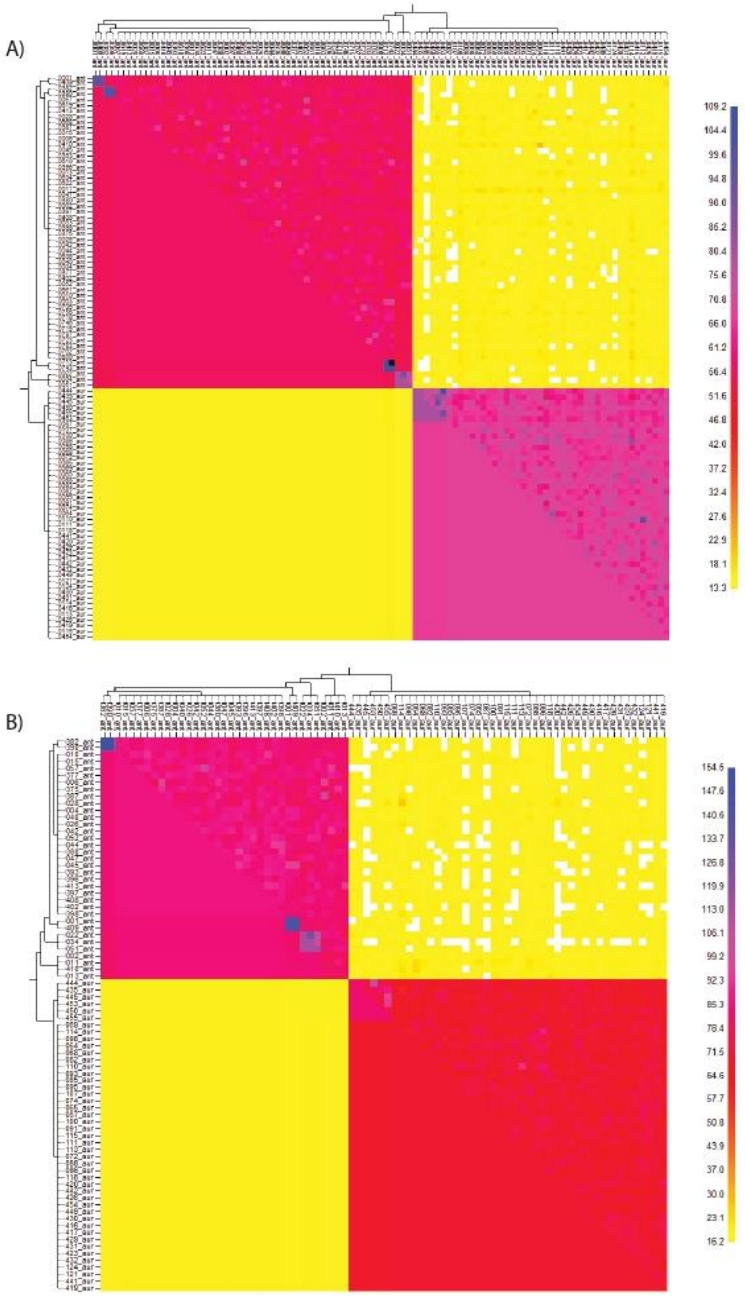

Figure 4. Clustered fineRADstructure co-ancestry matrix. **A** Full dataset including *U. antarctica* collected on the Antarctic Peninsula in addition to *U. antarctica* and *U. aurantiacoatra* collected on King George Island and Elephant Island **B** Reduced dataset with all *U. antarctica* and *U. aurantiacoatra* collected on King George Island and Elephant Island. Two major clades are corresponding to the two species *U. antarctica* (top-left) and *U. aurantiacoatra* (bottom-right). The top and left trees were calculated from the co-ancestry matrix to sort the individuals by their population structure. The matrix is diagonally split into the top-right half showing raw data and the bottom-left half displaying aggregated data.

lected on the Antarctic Peninsula (EL0713 and EL0743) showed the highest degrees of co-ancestry demonstrating very close relatedness, such as sister or clonal relationships. These results agreed with the phylogenetic inference (see above) in which the same *U. antarctica* specimens were close sister taxa.

Discussion

In this study, we used RAD sequencing for evaluating the delimitation of two predominantly Antarctic *Usnea* species. Phylogenetic evidence and population genomic analyses of the RADseq data strongly supported that the two species represent independent lineages. Although both species showed no overlapping genomic structure in a DAPC, we could compare levels of co-ancestry and detect genomic substructure within each species in a fineRADstructure plot.

In previous studies using multi-locus approaches, including the ITS barcoding marker (Schoch et al. 2012), the relationship of *U. antarctica* and *U. aurantiacoatra* remained unresolved and, since specimens of both species did not separate as different clades, conspecificity of the species was not ruled out (Seymour et al. 2007; Wirtz et al. 2012). Our study using RADseq supports the results obtained using microsatellite data that suggested the two species are distinct lineages (Lagostina et al. 2018). In *U. antarctica* and *U. aurantiacoatra*, the taxonomic interpretation of species pairs as separate species (Poelt 1972) is supported.

We developed a RADseq method for lineages involved in intimate symbiotic associations (Grewe et al. 2017), which we here successfully implemented for the use of delimiting two species. Different to the previously described RADseq method that used a reference genome from a lichen-fungal culture, we successfully generated a reference genome from a metagenomic *de-novo* assembly of *U. strigosa*. The filtering of the metagenomic assembly for fungal derived content reduced the size and completeness of the fungal reference (28.92 Mbp, CEGMA: 72.18%) compared to the reference genome assembly from a lichen-fungal culture which was used in earlier studies (31.6 Mbp, CEGMA: 96.77%) (Grewe et al. 2017; Leavitt et al. 2016b). However, the saturation of successfully mapped loci to the reference (Suppl. material 3) suggested that the maximum number of possible mapped loci was reached for samples with many initial loci. Therefore, although using a smaller reference and less fungal derived loci than in our initial study (Grewe et al. 2017), this RADseq approach still was successful in mapping a large number of fungal loci sufficient for phylogenetic and population genomic methods. This widens the potential application of RADseq for intimate symbiotic organisms and includes studies where cultures of one symbiotic partner are not readily available.

RADseq data are extremely powerful, since the method generates a matrix of thousands of homologous loci derived from randomly distributed regions across the genome. Many studies have successfully used large RADseq datasets for phylogenetic analysis which were difficult to resolve due to insufficient signals in available markers

(Eaton and Ree 2013; Escudero et al. 2014; Hipp et al. 2014; Vargas et al. 2017; Wagner et al. 2018). Our phylogenetic and population genomic results from the RADseq dataset clearly delimited *U. antarctica* and *U. aurantiacoatra* into two lineages (Figures 1–4) supporting the acceptance of two species. This confirms that closely related species are difficult to separate using sequence-based multi-locus approaches and great care should be taken when interpreting results from molecular studies when it comes to testing for conspecificity. On the other hand, the microsatellite-based multi-locus approach by Lagostina et al. (2018) rendered almost identical results, including nearly 100 % correct assignment of samples to their species.

The fineRADstructure matrix estimated lower co-ancestry (and hence higher genotypic variation) for the sexually-reproducing *U. aurantiacoatra*, compared to the asexually-reproducing *U. antarctica* when comparing samples that were collected in the same geographic range (Figure 4B). This result agrees with earlier observations that asexual populations have lower genotypic variation than sexual populations in modelling approaches (Balloux et al. 2003) and empirical measures (Delmotte et al. 2002). Moreover, Lagostina et al. (2018) inferred lower genetic variability for *U. antarctica* than *U. aurantiacoatra* using 23 microsatellite loci. These authors also used samples collected in mixed stands of both species from King George and Elephant Island. When we increased our sampling of *U. antarctica* to include a much wider geographical range (Antarctic Peninsula in addition to King George and Elephant Island) compared to the sampling of *U. aurantiacoatra* (King George and Elephant Island only), the matrix indicated increased levels of co-ancestry and a lower genotypic variation (Figure 4A). Although this comparative analysis is lacking collections of *U. aurantiacoatra* from the Antarctic Peninsula for a direct comparison, it should be noted that *U. antarctica* covers a wider geographical range than *U. aurantiacoatra* (Walker 1985) and this wider species distribution might increase genetic variability. The difference in distribution may result from the main form of reproductive units of both *Usnea*. The exclusively sexual *U. aurantiacoatra* reproduces via the dispersal of fungal spores which are required to meet with an appropriate photobiont after germination. The asexual *U. antarctica* on the other hand is in majority vegetatively reproducing via soredia, which already include the photobiont. Therefore, even if both reproductive units are dispersed over similar distances, the success rate of colonisation may be higher for soredia and explain the overall wider distribution and therefore genetic variability of *U. antarctica*. Finally, it was predicted that a small number of sexual individuals per generation — and *U. antarctica* rarely can be found with apothecia — is sufficient to make an apparently asexual population highly variable (Bengtsson 2003).

Despite the lower co-ancestry of *U. antarctica* compared to *U. aurantiacoatra*, we detected three pairs of very close relatives with high co-ancestry of *U. antarctica* (Figure 4). The three pairs were collected on Elephant Island, King George Island and on the Antarctic Peninsula, respectively and may indicate almost immediately related clones. On Elephant Island and the Antarctic Peninsula, the pairs were collected in the same locations with a greater chance to pick up clones. However, the clonal pair from King George Island must have dispersed between Fildes and Potter Peninsula over ice or water bounda-

ries prior to our collection. Contrarily, none of the individuals of *U. aurantiacoatra* expressed similarly close relationships. However, we could detect substructure for a group of six individuals of *U. aurantiacoatra* collected at the same location and three specimens of *U. antarctica* collected at different locations on King George Island, which indicates the potential of this analysis to identify (sub)population structure. Using this detailed method to measure co-ancestry on a deeper sampling of individuals of *Usnea* may, in future, provide a comprehensive picture of population structure and diversification.

Conclusion

We successfully used RADseq for phylogenetic and population genomic studies on two species of the lichen-fungal genus *Usnea*. Phylogenetic inference using RAD data clearly delimited the species *U. antarctica* and *U. aurantiacoatra* into two lineages, which were irresolvable using multi-locus DNA sequence markers. Furthermore, the RADseq approach offered sufficient genotyping data for conclusive population genomic analyses. We used RADseq to measure lower co-ancestry in the asexual *U. antarctica* than in the sexual *U. aurantiacoatra*, potentially derived from a wider geographical distribution of *U. antarctica* in our sample. These results show that RADseq has much potential for future phylogenetic and population genomic studies on lichens, particularly for groups of organisms which remained unresolved by multi-locus markers.

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References

Allendorf FW (2017) Genetics and the conservation of natural populations: allozymes to genomes. Molecular Ecology 26: 420–430. https://doi.org/10.1111/mec.13948

Articus K (2004) *Neuropogon* and the phylogeny of *Usnea* s.l. (Parmeliaceae, lichenized Ascomycetes). Taxon 53: 925–934. https://doi.org/10.2307/4135560

Articus K, Mattsson JE, Tibell L, Grube M, Wedin M (2002) Ribosomal DNA and beta-tubulin data do not support the separation of the lichens *Usnea florida* and *U. subfloridana* as distinct species. Mycological Research 106: 412–418. https://doi.org/10.1017/S0953756202005786

Baird NA, Etter PD, Atwood TS, Currey MC, Shiver AL, Lewis ZA, Selker EU, Cresko WA, Johnson EA (2008) Rapid SNP discovery and genetic mapping using sequenced RAD markers. Plos One 3(10): e3376. https://doi.org/10.1371/journal.pone.0003376

Balloux F, Lehmann L, de Meeûs T (2003) The Population Genetics of Clonal and Partially Clonal Diploids. Genetics 164: 1635–1644.

Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Son P, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA (2012) SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. Journal of Computational Biology 19: 455–477. https://doi.org/10.1089/cmb.2012.0021

Barraclough TG, Nee S (2001) Phylogenetics and speciation. Trends in Ecology & Evolution 16: 391–399. https://doi.org/10.1016/S0169-5347(01)02161-9

Bengtsson BO (2003) Genetic variation in organisms with sexual and asexual reproduction. Journal of Evolutionary Biology 16: 189–199. https://doi.org/10.1046/j.1420-9101.2003.00523.x

Bickford D, Lohman DJ, Sodhi NS, Ng PKL, Meier R, Winker K, Ingram KK, Das I (2007) Cryptic species as a window on diversity and conservation. Trends in Ecology & Evolution 22: 148–155. https://doi.org/10.1016/j.tree.2006.11.004

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30: 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Buschbom J, Mueller GM (2006) Testing "species pair" hypotheses: Evolutionary processes in the lichen-forming species complex *Porpidia flavocoerulescens* and *Porpidia melinodes*. Molecular Biology and Evolution 23: 574–586. https://doi.org/10.1093/molbev/msj063

Crespo A, Lumbsch HT (2010) Cryptic species in lichen-forming fungi. IMA Fungus 1: 167–170. https://doi.org/10.5598/imafungus.2010.01.02.09

Crespo A, Lumbsch HT, Mattsson JE, Blanco O, Divakar PK, Articus K, Wiklund E, Bawingan PA, Wedin M (2007) Testing morphology-based hypotheses of phylogenetic relationships in Parmeliaceae (Ascomycota) using three ribosomal markers and the nuclear RPB1 gene. Molecular Phylogenetics and Evolution 44: 812–824. https://doi.org/10.1016/j.ympev.2006.11.029

Crespo A, Pérez-Ortega S (2009) Cryptic species and species pairs in lichens: A discussion on the relationship between molecular phylogenies and morphological characters. Anales Del Jardin Botanico De Madrid 66: 71–81. https://doi.org/10.3989/ajbm.2225

Cubero OF, Crespo A, Fatehi J, Bridge PD (1999) DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized, and other fungi. Plant Systematics and Evolution 216: 243–249. https://doi.org/10.1007/BF01084401

Culberson WL, Culberson CF (1973) Parallel evolution in the lichen-forming fungi. Science 180: 196–198. https://doi.org/10.1126/science.180.4082.196

Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, Handsaker RE, Lunter G, Marth GT, Sherry ST, McVean G, Durbin R, 1000 Genomes Project Analysis Group (2011) The variant call format and VCFtools. Bioinformatics 27: 2156–2158. https://doi.org/10.1093/bioinformatics/btr330

Davey JW, Hohenlohe PA, Etter PD, Boone JQ, Catchen JM, Blaxter ML (2011) Genome-wide genetic marker discovery and genotyping using next-generation sequencing. Nature Reviews Genetics 12: 499–510. https://doi.org/10.1038/nrg3012

de Queiroz K (2007) Species concepts and species delimitation. Systematic Biology 56: 879–886. https://doi.org/10.1080/10635150701701083

Delmotte F, Leterme N, Gauthier JP, Rispe C, Simon JC (2002) Genetic architecture of sexual and asexual populations of the aphid *Rhopalosiphum padi* based on allozyme and microsatellite markers. Molecular Ecology 11: 711–723. https://doi.org/10.1046/j.1365-294X.2002.01478.x

Eaton DAR, Ree RH (2013) Inferring phylogeny and introgression using RADseq data: An example from flowering plants (*Pedicularis*: Orobanchaceae). Systematic Biology 62: 689–706. https://doi.org/10.1093/sysbio/syt032

Ellegren H (2014) Genome sequencing and population genomics in non-model organisms. Trends in Ecology & Evolution 29: 51–63. https://doi.org/10.1016/j.tree.2013.09.008

Escudero M, Eaton DAR, Hahn M, Hipp AL (2014) Genotyping-by-sequencing as a tool to infer phylogeny and ancestral hybridization: A case study in *Carex* (Cyperaceae). Molecular Phylogenetics and Evolution 79: 359–367. https://doi.org/10.1016/j.ympev.2014.06.026

Grewe F, Huang JP, Leavitt SD, Lumbsch HT (2017) Reference-based RADseq resolves robust relationships among closely related species of lichen-forming fungi using metagenomic DNA. Scientific Reports 7: 9884. https://doi.org/10.1038/s41598-017-09906-7

Hedrick PW (2005) A standardized genetic differentiation measure. Evolution 59: 1633–1638. https://doi.org/10.1111/j.0014-3820.2005.tb01814.x

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lücking R, Lumbsch HT, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai YC, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Koljalg U, Kurtzman CP, Larsson KH, Lichtwardt R, Longcore J, Miadlikowska J, Miller A, Moncalvo JM, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schussler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao YJ, Zhang N (2007) A higher-level phylogenetic classification of the Fungi. Mycological Research 111: 509–547. https://doi.org/10.1016/j.mycres.2007.03.004

Hipp AL, Eaton DAR, Cavender-Bares J, Fitzek E, Nipper R, Manos PS (2014) A framework phylogeny of the American Oak clade based on sequenced RAD data. Plos One 9(4): e102272. https://doi.org/10.1371/journal.pone.0093975

Hodel RDGJ, Segovia-Salcedo MC, Landis JB, Crowl AA, Sun M, Liu X, Gitzendanner MA, Douglas NNA, Germain-Aubrey CC, Chen S, Soltis DE, Soltis PS (2016) The report of my death was an exaggeration: a review for researchers using microsatellites in the 21st century. Applications in Plant Sciences 4. https://doi.org/10.3732/apps.1600025

Hoffman JR, Lendemer JC (2018) A meta-analysis of trends in the application of Sanger and next-generation sequencing data in lichenology. Bryologist 121: 133–147. https://doi.org/10.1639/0007-2745-121.2.133

Holder M, Lewis PO (2003) Phylogeny estimation: Traditional and Bayesian approaches. Nature Reviews Genetics 4: 275–284. https://doi.org/10.1038/nrg1044

Huelsenbeck JP, Ronquist F, Nielsen R, Bollback JP (2001) Evolution – Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294: 2310–2314. https://doi.org/10.1126/science.1065889

James TY, Kauff F, Schoch CL, Matheny PB, Hofstetter V, Cox CJ, Celio G, Gueidan C, Fraker E, Miadlikowska J, Lumbsch HT, Rauhut A, Reeb V, Arnold AE, Amtoft A, Stajich JE, Hosaka K, Sung GH, Johnson D, O'Rourke B, Crockett M, Binder M, Curtis JM, Slot JC, Wang Z, Wilson AW, Schussler A, Longcore JE, O'Donnell K, Mozley-Standridge S, Porter D, Letcher PM, Powell MJ, Taylor JW, White MM, Griffith GW, Davies DR, Humber RA, Morton JB, Sugiyama J, Rossman AY, Rogers JD, Pfister DH, Hewitt D, Hansen K, Hambleton S, Shoemaker RA, Kohlmeyer J, Volkmann-Kohlmeyer B, Spotts RA, Serdani M, Crous PW, Hughes KW, Matsuura K, Langer E, Langer G, Untereiner WA, Lücking R, Büdel B, Geiser DM, Aptroot A, Diederich P, Schmitt I, Schultz M, Yahr R, Hibbett DS, Lutzoni F, McLaughlin DJ, Spatafora JW, Vilgalys R (2006) Reconstructing the early evolution of Fungi using a six-gene phylogeny. Nature 443: 818–822. https://doi.org/10.1038/nature05110

Jombart T, Ahmed I (2011) adegenet 1.3–1: new tools for the analysis of genome-wide SNP data. Bioinformatics 27: 3070–3071. https://doi.org/10.1093/bioinformatics/btr521

Jombart T, Balloux F, Dray S (2010) adephylo: new tools for investigating the phylogenetic signal in biological traits. Bioinformatics 26: 1907–1909. https://doi.org/10.1093/bioinformatics/btq292

Jost L (2008) GST and its relatives do not measure differentiation. Molecular Ecology 17: 4015–4026. https://doi.org/10.1111/j.1365-294X.2008.03887.x

Kotelko R, Piercey-Normore MD (2010) *Cladonia pyxidata* and *C. pocillum*; genetic evidence to regard them as conspecific. Mycologia 102: 534–545. https://doi.org/10.3852/09-030

Kraus RHS, Wink M (2015) Avian genomics: fledging into the wild! Journal of Ornithology 156: 851–865. https://doi.org/10.1007/s10336-015-1253-y

Krog H (1976) *Lethariella* and *Protousnea*, two new lichen genera in the Parmeliaceae. Norwegian Journal of Botany 23: 83–106.

Krog H (1982) Evolutionay trends in foliose and fruticose lichens of the Parmeliaceae. Journal of the Hattori Botanical Laboratory 52: 303–311.

Lagostina E, Dal Grande F, Andreev M, Printzen C (2018) The use of microsatellite markers for species delimitation in Antarctic *Usnea* subgenus *Neuropogon*. Mycologia. https://doi.org/10.1080/00275514.2018.1512304

Lamb IM (1939) A review of the genus *Neuropogon* (Nees.) Nyl., with special reference to the Antarctic species. Journal of the Linnean Society, Botany 52: 199–237. https://doi.org/10.1111/j.1095-8339.1939.tb01602.x

Lamb IM (1964) Antarctic lichens. I. The genera *Usnea*, *Ramalina*, *Himantormia*, *Alectoria*, *Cornicularia*. 38: 1–34.

Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nature Methods 9: 357–354. https://doi.org/10.1038/nmeth.1923

Leavitt SD, Divakar PK, Crespo A, Lumbsch HT (2016a) A matter of time – understanding the limits of the power of molecular data for delimiting species boundaries. Herzogia 29: 479–492. https://doi.org/10.13158/heia.29.2.2016.479

Leavitt SD, Grewe F, Widhelm T, Muggia L, Wray B, Lumbsch HT (2016b) Resolving evolutionary relationships in lichen-forming fungi using diverse phylogenomic datasets and analytical apporaches. Scientific Reports 6: 22262. https://doi.org/10.1038/srep22262

Lohtander K, Källersjö M, Tehler A (1998) Dispersal strategies in *Roccellina capensis* (Arthoniales). Lichenologist 30: 341–350. https://doi.org/10.1006/lich.1998.0154

Lumbsch HT, Leavitt SD (2011) Goodbye morphology? A paradigm shift in the delimitation of species in lichenized fungi. Fungal Diversity 50: 59–72. https://doi.org/10.1007/s13225-011-0123-z

Lumbsch HT, Wirtz N (2011) Phylogenetic relationships of the neuropogonoid core group in the genus *Usnea* (Ascomycota: Parmeliaceae). Lichenologist 43: 553–559. https://doi.org/10.1017/S0024282911000417

Lutzoni F, Kauff F, Cox C, McLaughlin D, Celio G, Dentinger B, Padamsee M, Hibbett D, James TY, Baloch E, Grube M, Reeb V, Hofstetter V, Schoch C, Arnold AE, Miadlikowska J, Spatafora J, Johnson D, Hambleton S, Crockett M, Shoemaker R, Sung G-H, Lücking R, Lumbsch HT, O'Donnell K, Binder M, Diederich P, Ertz D, Gueidan C, Hansen K, Harris RC, Hosaka K, Lim Y-W, Matheny B, Nishida H, Pfister D, Rogers J, Rossman A, Schmitt I, Sipman H, Stone J, Sugiyama J, Yahr R, Vilgalys R (2004) Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits. American Journal of Botany 91: 1446–1480. https://doi.org/10.3732/ajb.91.10.1446

Malinsky M, Trucchi E, Lawson DJ, Falush D (2018) RADpainter and fineRADstructure Population Inference from RADseq Data. Molecular Biology and Evolution 35: 1284–1290. https://doi.org/10.1093/molbev/msy023

Mattsson J-E, Lumbsch HT (1989) The use of the species pair concept in lichen taxonomy. Taxon 38: 238–241. https://doi.org/10.2307/1220840

McLaughlin DJ, Hibbett DS, Lutzoni F, Spatafora JW, Vilgalys R (2009) The search for the fungal tree of life. Trends in Microbiology 17: 488–497. https://doi.org/10.1016/j.tim.2009.08.001

Myllys L, Lohtander K, Tehler A (2001) beta-tubulin, ITS and group I intron sequences challenge the species pair concept in *Physcia aipolia* and *P. caesia*. Mycologia 93: 335–343. https://doi.org/10.2307/3761655

Nei M (1973) Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Sciences 70: 3321–3323. https://doi.org/10.1073/pnas.70.12.3321

Ozsolak F, Milos PM (2011) RNA sequencing: advances, challenges and opportunities. Nature Reviews Genetics 12: 87–98. https://doi.org/10.1038/nrg2934

Parra G, Bradnam K, Ning Z, Keane T, Korf I (2009) Assessing the gene space in draft genomes. Nucleic Acids Research 37: 289–297. https://doi.org/10.1093/nar/gkn916

Poelt J (1970) Das Konzept der Artenpaare bei den Flechten. Vorträge aus dem Gesamtgebiet der Botanik. Neue Folge 4: 187–198.

Poelt J (1972) Die taxonomische Behandlung von Artenpaaren bei den Flechten. Botaniska Notiser 125: 77–81.

Rannala B (2015) The art and science of species delimitation. Current Zoology 61: 846–853. https://doi.org/10.1093/czoolo/61.5.846

Robbertse B, Reeves JB, Schoch CL, Spatafora JW (2006) A phylogenomic analysis of the Ascomycota. Fungal Genetics and Biology 43: 715–725. https://doi.org/10.1016/j.fgb.2006.05.001

Rubin BER, Moreau CS (2016) Comparative genomics reveals convergent rates of evolution in ant-plant mutualisms. Nature Communications 7: 12679. https://doi.org/10.1038/ncomms12679

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Bolchacova E, Voigt K, Crous PW, Miller AN, Wingfield MJ, Aime MC, An KD, Bai FY, Barreto RW, Begerow D, Bergeron MJ, Blackwell M, Boekhout T, Bogale M, Boonyuen N, Burgaz AR, Buyck B, Cai L, Cai Q, Cardinali G, Chaverri P, Coppins BJ, Crespo A, Cubas P, Cummings C, Damm U, de Beer ZW, de Hoog GS, Del-Prado R, Dentinger B, Dieguez-Uribeondo J, Divakar PK, Douglas B, Duenas M, Duong TA, Eberhardt U, Edwards JE, Elshahed MS, Fliegerova K, Furtado M, Garcia MA, Ge ZW, Griffith GW, Griffiths K, Groenewald JZ, Groenewald M, Grube M, Gryzenhout M, Guo LD, Hagen F, Hambleton S, Hamelin RC, Hansen K, Harrold P, Heller G, Herrera G, Hirayama K, Hirooka Y, Ho HM, Hoffmann K, Hofstetter V, Högnabba F, Hollingsworth PM, Hong SB, Hosaka K, Houbraken J, Hughes K, Huhtinen S, Hyde KD, James T, Johnson EM, Johnson JE, Johnston PR, Jones EB, Kelly LJ, Kirk PM, Knapp DG, Koljalg U, Kovacs GM, Kurtzman CP, Landvik S, Leavitt SD, Liggenstoffer AS, Liimatainen K, Lombard L, Luangsa-Ard JJ, Lumbsch HT, Maganti H, Maharachchikumbura SS, Martin MP, May TW, McTaggart AR, Methven AS, Meyer W, Moncalvo JM, Mongkolsamrit S, Nagy LG, Nilsson RH, Niskanen T, Nyilasi I, Okada G, Okane I, Olariaga I, Otte J, Papp T, Park D, Petkovits T, Pino-Bodas R, Quaedvlieg W, Raja HA, Redecker D, Rintoul T, Ruibal C, Sarmiento-Ramirez JM, Schmitt I, Schussler A, Shearer C, Sotome K, Stefani FO, Stenroos S, Stielow B, Stockinger H, Suetrong S, Suh SO, Sung GH, Suzuki M, Tanaka K, Tedersoo L, Telleria MT, Tretter E, Untereiner WA, Urbina H, Vagvolgyi C, Vialle A, Vu TD, Walther G, Wang QM, Wang Y, Weir BS, Weiss M, White MM, Xu J, Yahr R, Yang ZL, Yurkov A, Zamora JC, Zhang N, Zhuang WY, Schindel D, Fungal Barcoding C (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences of the United States of America 109: 6241–6246. https://doi.org/10.1073/pnas.1117018109

Schoch CL, Sung GH, Lopez-Giraldez F, Townsend JP, Miadlikowska J, Hofstetter V, Robbertse B, Matheny PB, Kauff F, Wang Z, Gueidan C, Andrie RM, Trippe K, Ciufetti LM, Wynns A, Fraker E, Hodkinson BP, Bonito G, Groenewald JZ, Arzanlou M, de Hoog GS, Crous PW, Hewitt D, Pfister DH, Peterson K, Gryzenhout M, Wingfield MJ, Aptroot A, Suh SO, Blackwell M, Hillis DM, Griffith GW, Castlebury LA, Rossman AY, Lumbsch HT, Lücking R, Büdel B, Rauhut A, Diederich P, Ertz D, Geiser DM, Hosaka K, Inderbitzin P, Kohlmeyer J, Volkmann-Kohlmeyer B, Mostert L, O'Donnell K, Sipman H, Rogers JD, Shoemaker RA, Sugiyama J, Summerbell RC, Untereiner W, Johnston PR, Stenroos S, Zuccaro A, Dyer PS, Crittenden PD, Cole MS, Hansen K, Trappe JM, Yahr R, Lutzoni F, Spatafora JW (2009) The Ascomycota tree of life: A phylum-wide phylogeny clarifies the origin and evolution of fundamental reproductive and ecological traits. Systematic Biology 58: 224–239. https://doi.org/10.1093/sysbio/syp020

Seymour FA, Crittenden PD, Wirtz N, Øvstedal DO, Dyer PS, Lumbsch HT (2007) Phylogenetic and morphological analysis of Antarctic lichen-forming *Usnea* species in the group *Neuropogon*. Antarctic Science 19: 71–82. https://doi.org/10.1017/S0954102007000107

Spatafora JW, Aime MC, Grigoriev IV, Martin F, Stajich JE, Blackwell M (2017) The fungal tree of life: from molecular systematics to genome-scale phylogenies. Microbiology Spectrum 5(5): FUNK-0053-2016.

Spatafora JW, Robbertse B (2010) Chapter 4 : phylogenetics and phylogenomics of the Fungal Tree of Life. In: Borkovich KA, Ebbole DJ (Eds) Cellular and Molecular Biology of Filamentous Fungi. ASM Press, 36–49. http://dx.doi.org/10.1128/9781555816636.ch4

Stajich JE, Berbee ML, Blackwell M, Hibbett DS, James TY, Spatafora JW, Taylor JW (2009) The Fungi. Current Biology 19: 840–845. https://doi.org/10.1016/j.cub.2009.07.004

Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688–2690. https://doi.org/10.1093/bioinformatics/btl446

Stamatakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the RAxML web servers. Systematic Biology 57: 758–771. https://doi.org/10.1080/10635150802429642

Stratton M (2008) Genome resequencing and genetic variation. Nature Biotechnology 26: 65–66. https://doi.org/10.1038/nbt0108-65

Strous M, Kraft B, Bisdorf R, Tegetmeyer HE (2012) The binning of metagenomic contigs for microbial physiology of mixed cultures. Frontiers in Microbiology 3: 410. https://doi.org/10.3389/fmicb.2012.00410

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC (2000) Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology 31: 21–32. https://doi.org/10.1006/fgbi.2000.1228

Tehler A (1982) The species pair concept in lichenology. Taxon 31: 708–714. https://doi.org/10.2307/1219689

Vargas OM, Ortiz EM, Simpson BB (2017) Conflicting phylogenomic signals reveal a pattern of reticulate evolution in a recent high-Andean diversification (Asteraceae: Astereae: *Diplostephium*). New Phytologist 214: 1736–1750. https://doi.org/10.1111/nph.14530

Velmala S, Myllys L, Halonen P, Goward T, Ahti T (2009) Molecular data show that *Bryoria fremontii* and *B. tortuosa* (Parmeliaceae) are conspecific. Lichenologist 41: 231–242. https://doi.org/10.1017/S0024282909008573

Wachi N, Matsubayashi KW, Maeto K (2018) Application of next-generation sequencing to the study of non-model insects. Entomological Science 21: 3–11. https://doi.org/10.1111/ens.12281

Wagner ND, Gramlich S, Hörandl E (2018) RAD sequencing resolved phylogenetic relationships in European shrub willows (*Salix* L. subg. *Chamaetia* and subg. *Vetrix*) and revealed multiple evolution of dwarf shrubs. Ecology and Evolution 2018(00): 1–13. https://doi.org/10.1002/ece3.4360

Walker FJ (1985) The lichen genus *Usnea* subgenus *Neuropogon*. Bulletin of the British Museum (Natural History), Botany Series 13: 1–130.

Whitlock MC (2011) G ‘(ST) and D do not replace F-ST. Molecular Ecology 20: 1083–1091. https://doi.org/10.1111/j.1365-294X.2010.04996.x

Wickett NJ, Mirarab S, Nam N, Warnow T, Carpenter E, Matasci N, Ayyampalayam S, Barker MS, Burleigh JG, Gitzendanner MA, Ruhfel BR, Wafula E, Der JP, Graham SW, Mathews S, Melkonian M, Soltis DE, Soltis PS, Miles NW, Rothfels CJ, Pokorny L, Shaw AJ, DeGironimo L, Stevenson DW, Surek B, Villarreal JC, Roure B, Philippe H, dePamphilis CW, Chen T, Deyholos MK, Baucom RS, Kutchan TM, Augustin MM, Wang J, Zhang Y, Tian Z, Yan Z, Wu X, Sun X, Wong GK-S, Leebens-Mack J (2014) Phylotranscriptomic analysis of the origin

and early diversification of land plants. Proceedings of the National Academy of Sciences of the United States of America 111: 4859–4868. https://doi.org/10.1073/pnas.1323926111

Widhelm TJ, Egan RS, Bertoletti FR, Asztalos MJ, Kraichak E, Leavitt SD, Lumbsch HT (2016) Picking holes in traditional species delimitations: an integrative taxonomic reassessment of the *Parmotrema perforatum* group (Parmeliaceae, Ascomycota). Botanical Journal of the Linnean Society 182: 868–884. https://doi.org/10.1111/boj.12483

Wiens JJ, Penkrot TA (2002) Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). Systematic Biology 51: 69–91. https://doi.org/10.1080/106351502753475880

Wilkinson MJ, Szabo C, Ford CS, Yarom Y, Croxford AE, Camp A, Gooding P (2017) Replacing Sanger with Next Generation Sequencing to improve coverage and quality of reference DNA barcodes for plants. Scientific Reports 7. https://doi.org/10.1038/srep46040

Winter DJ (2012) MMOD: an R library for the calculation of population differentiation statistics. Molecular Ecology Resources 12: 1158–1160. https://doi.org/10.1111/j.1755-0998.2012.03174.x

Wirtz N, Printzen C, Lumbsch HT (2008) The delimitation of Antarctic and bipolar species of neuropogonoid *Usnea* (Ascomycota, Lecanorales): a cohesion approach of species recognition for the *Usnea perpusilla* complex. Mycological Research 112: 472–484. https://doi.org/10.1016/j.mycres.2007.05.006

Wirtz N, Printzen C, Lumbsch HT (2012) Using haplotype networks, estimation of gene flow and phenotypic characters to understand species delimitation in fungi of a predominantly Antarctic *Usnea* group (Ascomycota, Parmeliaceae). Organisms Diversity & Evolution 12: 17–37. https://doi.org/10.1007/s13127-011-0066-y

Wirtz N, Printzen C, Sancho LG, Lumbsch HT (2006) The phylogeny and classification of *Neuropogon* and *Usnea* (Parmeliaceae, Ascomycota) revisited. Taxon 55: 367–376. https://doi.org/10.2307/25065584

Zhao X, Fernández-Brime S, Wedin M, Locke M, Leavitt SD, Lumbsch HT (2017) Using multi-locus sequence data for addressing species boundaries in commonly accepted lichen-forming fungal species. Organisms Diversity & Evolution 17: 351–363. https://doi.org/10.1007/s13127-016-0320-4

Zimmer EA, Wen J (2015) Using nuclear gene data for plant phylogenetics: Progress and prospects II. Next-gen approaches. Journal of Systematics and Evolution 53: 371–379. https://doi.org/10.1111/jse.12174

Supplementary material 1

Origin of samples used for this study

Authors: Felix Grewe, Elisa Lagostina, Huini Wu, Christian Printzen, H. Thorsten Lumbsch

Data type: species data

Link: https://doi.org/10.3897/mycokeys.43.29093.suppl1

Specimen Number	Collector	Species Name	Continent	Location	Coordinates	sampling date
TW2605	Todd Widhelm	Usnea strigosa	North America	USA, Arkansas, Queen Wilhelmina State Park	34° 41' N 94° 19' W	19.05.16
EL0001	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 14.220' S 58° 39.492' W	18.12.15
EL0002	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 14.220' S 58° 39.492' W	18.12.15
EL0004	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 14.220' S 58° 39.492' W	18.12.15
EL0006	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 14.220' S 58° 39.492' W	18.12.15
EL0010	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 14.220' S 58° 39.492' W	18.12.15
EL0011	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 14.220' S 58° 39.492' W	18.12.15
EL0013	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 14.220' S 58° 39.492' W	18.12.15
EL0015	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 14.220' S 58° 39.492' W	18.12.15
EL0022	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 14.363' S 58° 39.202' W	27.12.15
EL0026	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 14.363' S 58° 39.202' W	27.12.15
EL0028	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 14.363' S 58° 39.202' W	27.12.15
EL0034	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 14.363' S 58° 39.202' W	27.12.15
EL0040	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 14.363' S 58° 39.202' W	27.12.15
EL0042	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0044	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0045	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0047	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0051	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0052	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0057	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0059	Elisa Lagostina	Usnea antarctica	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0064	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 14.220' S 58° 39.492' W	18.12.15
EL0065	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 14.220' S 58° 39.492' W	18.12.15
EL0068	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 14.220' S 58° 39.492' W	18.12.15
EL0069	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 14.220' S 58° 39.492' W	18.12.15
EL0072	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 14.220' S 58° 39.492' W	18.12.15
EL0074	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 14.220' S 58° 39.492' W	18.12.15
EL0082	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 14.220' S 58° 39.492' W	18.12.15
EL0085	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 14.363' S 58° 39.202' W	27.12.15
EL0086	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 14.363' S 58° 39.202' W	27.12.15
EL0087	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 14.363' S 58° 39.202' W	27.12.16
EL0088	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 14.363' S 58° 39.202' W	27.12.15
EL0091	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 14.363' S 58° 39.202' W	27.12.15
EL0093	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 14.363' S 58° 39.202' W	27.12.15
EL0095	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 14.363' S 58° 39.202' W	27.12.15
EL0098	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 14.363' S 58° 39.202' W	27.12.15
EL0100	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 14.363' S 58° 39.202' W	27.12.15
EL0107	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0110	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0111	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0113	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0114	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0115	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0118	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0121	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0124	Elisa Lagostina	Usnea aurantiacoatra	Antarctica	King George Island, Potter Peninsula	62° 15.174' S 58° 38.971' W	22.12.15
EL0375	Mikhael Andreev	Usnea antarctica	Antarctica	Elephant Island	61° 13.337' S 55° 21.581' W	08.02.16
EL0377	Mikhael Andreev	Usnea antarctica	Antarctica	Elephant Island	61° 13.337' S 55° 21.581' W	08.02.16
EL0380	Mikhael Andreev	Usnea antarctica	Antarctica	Elephant Island	61° 13.337' S 55° 21.581' W	08.02.16
EL0381	Mikhael Andreev	Usnea antarctica	Antarctica	Elephant Island	61° 13.337' S 55° 21.581' W	08.02.16
EL0382	Mikhael Andreev	Usnea antarctica	Antarctica	Elephant Island	61° 13.337' S 55° 21.581' W	08.02.16
EL0387	Mikhael Andreev	Usnea antarctica	Antarctica	Elephant Island	61° 13.337' S 55° 21.581' W	08.02.16
EL0390	Mikhael Andreev	Usnea antarctica	Antarctica	Elephant Island	61° 13.337' S 55° 21.581' W	08.02.16
EL0393	Mikhael Andreev	Usnea antarctica	Antarctica	Elephant Island	61° 13.337' S 55° 21.581' W	08.02.16
EL0396	Mikhael Andreev	Usnea antarctica	Antarctica	King George Island, Fildes Peninsula	62° 11.416' S 58° 55.604' W	08.04.16
EL0397	Mikhael Andreev	Usnea antarctica	Antarctica	King George Island, Fildes Peninsula	62° 11.416' S 58° 55.604' W	08.04.16
EL0398	Mikhael Andreev	Usnea antarctica	Antarctica	King George Island, Fildes Peninsula	62° 11.416' S 58° 55.604' W	08.04.16
EL0402	Mikhael Andreev	Usnea antarctica	Antarctica	King George Island, Fildes Peninsula	62° 11.416' S 58° 55.604' W	08.04.16
EL0408	Mikhael Andreev	Usnea antarctica	Antarctica	King George Island, Fildes Peninsula	62° 11.416' S 58° 55.604' W	08.04.16
EL0409	Mikhael Andreev	Usnea antarctica	Antarctica	King George Island, Fildes Peninsula	62° 11.416' S 58° 55.604' W	08.04.16
EL0410	Mikhael Andreev	Usnea antarctica	Antarctica	King George Island, Fildes Peninsula	62° 11.416' S 58° 55.604' W	08.04.16
EL0413	Mikhael Andreev	Usnea antarctica	Antarctica	King George Island, Fildes Peninsula	62° 11.416' S 58° 55.604' W	08.04.16
EL0415	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	Elephant Island	61° 12.992' S 55° 21.527' W	04.02.16
EL0416	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	Elephant Island	61° 12.992' S 55° 21.527' W	04.02.16
EL0417	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	Elephant Island	61° 12.992' S 55° 21.527' W	04.02.16
EL0419	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	Elephant Island	61° 12.992' S 55° 21.527' W	04.02.16
EL0420	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	Elephant Island	61° 12.992' S 55° 21.527' W	04.02.16
EL0423	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	Elephant Island	61° 12.992' S 55° 21.527' W	04.02.16
EL0426	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	Elephant Island	61° 12.992' S 55° 21.527' W	04.02.16

EL0429	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	Elephant Island	61° 12.992' S 55° 21.527' W	04.02.16
EL0430	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	Elephant Island	61° 12.992' S 55° 21.527' W	04.02.16
EL0431	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	Elephant Island	61° 12.992' S 55° 21.527' W	04.02.16
EL0432	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	Elephant Island	61° 12.992' S 55° 21.527' W	04.02.16
EL0435	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	King George Island, Fildes Peninsula	62° 12.172' S 58° 59.565' W	08.04.16
EL0437	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	King George Island, Fildes Peninsula	62° 12.172' S 58° 59.565' W	08.04.16
EL0441	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	King George Island, Fildes Peninsula	62° 12.172' S 58° 59.565' W	08.04.16
EL0442	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	King George Island, Fildes Peninsula	62° 12.172' S 58° 59.565' W	08.04.16
EL0444	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	King George Island, Fildes Peninsula	62° 12.172' S 58° 59.565' W	08.04.16
EL0445	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	King George Island, Fildes Peninsula	62° 12.172' S 58° 59.565' W	08.04.16
EL0449	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	King George Island, Fildes Peninsula	62° 12.172' S 58° 59.565' W	08.04.16
EL0450	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	King George Island, Fildes Peninsula	62° 12.172' S 58° 59.565' W	08.04.16
EL0453	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	King George Island, Fildes Peninsula	62° 12.172' S 58° 59.565' W	08.04.16
EL0454	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	King George Island, Fildes Peninsula	62° 12.172' S 58° 59.565' W	08.04.16
EL0455	Mikhael Andreev	Usnea aurantiacoatra	Antarctica	King George Island, Fildes Peninsula	62° 12.172' S 58° 59.565' W	08.04.16
EL0610	Mayara Scur	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Primavera	64° 5.322' S 60° 22.167' W	30.11.16
EL0619	Mayara Scur	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Primavera	64° 5.322' S 60° 22.167' W	30.11.16
EL0622	Mayara Scur	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Primavera	64° 5.322' S 60° 22.167' W	30.11.16
EL0634	Mayara Scur	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Primavera	64° 5.317' S 60° 34.190' W	26.11.16
EL0636	Mayara Scur	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Primavera	64° 5.317' S 60° 34.190' W	26.11.16
EL0653	Mayara Scur	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Primavera	64° 5.363' S 60° 34.229' W	29.11.16
EL0661	Mayara Scur	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Primavera	64° 10.350' S 61° 2.485' W	05.01.17
EL0663	Mayara Scur	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Primavera	64° 10.350' S 61° 2.485' W	05.01.17
EL0668	Mayara Scur	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Primavera	64° 10.350' S 61° 2.485' W	05.01.17
EL0706	Aline Lorenz	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Esperanza	63° 24.048' S 56° 59.243' W	13.01.17
EL0713	Aline Lorenz	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Esperanza	63° 24.048' S 56° 59.243' W	13.01.17
EL0715	Aline Lorenz	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Esperanza	63° 24.048' S 56° 59.243' W	13.01.17
EL0718	Aline Lorenz	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Esperanza	63° 24.325' S 57° 00.490' W	18.01.17
EL0723	Aline Lorenz	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Esperanza	63° 24.325' S 57° 00.490' W	18.01.17
EL0743	Aline Lorenz	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Esperanza	63° 24.325' S 57° 00.490' W	18.01.17
EL0746	Aline Lorenz	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Esperanza	63° 24.260' S 57° 01.057' W	15.01.17
EL0756	Aline Lorenz	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Esperanza	63° 24.260' S 57° 01.057' W	15.01.17
EL0762	Aline Lorenz	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Esperanza	63° 24.260' S 57° 01.057' W	15.01.17
EL0783	Aline Lorenz	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Esperanza	63° 24.495' S 57° 02.294' W	15.01.17
EL0797	Aline Lorenz	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Esperanza	63° 24.495' S 57° 02.294' W	15.01.17
EL0800	Aline Lorenz	Usnea antarctica	Antarctica	Antarctic Peninsula, near base Esperanza	63° 24.495' S 57° 02.294' W	15.01.17

Supplementary material 2

Overview of RADseq results after individual steps of RAD analyses

Authors: Felix Grewe, Elisa Lagostina, Huini Wu, Christian Printzen, H. Thorsten Lumbsch

Data type: molecular data

Link: https://doi.org/10.3897/mycokeys.43.29093.suppl2

Specimen Number	Species Name	MiSeq sequencing	Within sample cluster (loci)	Loci mapped to refernce	Percentage mapped	Loci in final dataset	Loci in fin dataset (>1000)
EL0001	Usnea antartica	556,958	22,561	4,915	21.8%	4,106	4,108
EL0002	Usnea antartica	156,335	11,131	2,559	23.0%	2,393	2,393
EL0004	Usnea antartica	428,463	20,713	4,934	23.8%	4,081	4,083
EL0006	Usnea antartica	294,306	19,364	4,424	22.8%	4,133	4,133
EL0010	Usnea antartica	347,330	16,384	3,718	22.7%	3,006	3,008
EL0011	Usnea antartica	307,277	16,342	3,681	22.5%	3,029	3,026
EL0013	Usnea antartica	376,494	19,259	4,570	23.7%	3,772	3,774
EL0015	Usnea antartica	406,654	22,695	5,103	22.5%	4,665	4,666
EL0022	Usnea antartica	210,615	12,658	3,029	23.9%	2,380	2,382
EL0026	Usnea antartica	1,639,547	36,253	8,246	22.7%	7,169	7,170
EL0028	Usnea antartica	201,408	13,476	3,070	22.8%	2,852	2,852
EL0034	Usnea antartica	188,669	13,088	3,006	23.0%	2,799	2,799
EL0040	Usnea antartica	532,132	21,068	4,405	20.9%	3,671	3,673
EL0042	Usnea antartica	626,833	23,759	5,558	23.4%	4,720	4,722
EL0044	Usnea antartica	799,833	28,368	6,640	23.4%	5,750	5,752
EL0045	Usnea antartica	174,992	9,665	2,290	23.7%	1,733	1,735
EL0047	Usnea antartica	453,480	24,895	5,778	23.2%	5,210	5,211
EL0051	Usnea antartica	803,397	28,199	6,695	23.7%	5,798	5,800
EL0052	Usnea antartica	126,118	8,419	1,920	22.8%	1,777	1,777
EL0057	Usnea antartica	557,873	27,533	5,783	21.0%	5,200	5,202
EL0059	Usnea antartica	13,659	63	10	15.9%	10	
EL0064	Usnea aurantiaco-atra	116,561	7,525	1,747	23.2%	1,571	1,572
EL0065	Usnea aurantiaco-atra	202,688	12,670	2,898	22.9%	2,443	2,442
EL0068	Usnea aurantiaco-atra	1,023,871	47,973	5,786	12.1%	4,883	4,887
EL0069	Usnea aurantiaco-atra	638,013	26,113	6,105	23.4%	5,257	5,261
EL0072	Usnea aurantiaco-atra	329,200	17,358	4,181	24.1%	3,425	3,429
EL0074	Usnea aurantiaco-atra	1,942,819	55,459	7,531	13.6%	6,381	6,385
EL0082	Usnea aurantiaco-atra	313,415	16,611	3,839	23.1%	3,098	3,103
EL0085	Usnea aurantiaco-atra	589,806	29,379	4,360	14.8%	3,562	3,566
EL0086	Usnea aurantiaco-atra	643,322	25,389	6,099	24.0%	5,289	5,293
EL0087	Usnea aurantiaco-atra	538,540	22,856	4,833	21.1%	3,997	4,001
EL0088	Usnea aurantiaco-atra	1,002,560	30,095	7,087	23.5%	6,214	6,218
EL0091	Usnea aurantiaco-atra	578,617	26,430	5,721	21.6%	4,786	4,790
EL0093	Usnea aurantiaco-atra	711,192	29,078	5,441	18.7%	4,520	4,525
EL0095	Usnea aurantiaco-atra	383,863	18,990	4,589	24.2%	3,757	3,761
EL0098	Usnea aurantiaco-atra	304,105	15,797	3,633	23.0%	2,890	2,894
EL0100	Usnea aurantiaco-atra	167,610	9,691	2,228	23.0%	1,967	1,965
EL0107	Usnea aurantiaco-atra	244,936	14,771	3,617	24.5%	2,857	2,861
EL0110	Usnea aurantiaco-atra	88,501	5,732	1,379	24.1%	1,262	1,260
EL0111	Usnea aurantiaco-atra	755,220	31,945	5,190	16.2%	4,313	4,317
EL0113	Usnea aurantiaco-atra	837,008	36,040	5,563	15.4%	4,641	4,645
EL0114	Usnea aurantiaco-atra	128,508	8,310	1,917	23.1%	1,696	1,696
EL0115	Usnea aurantiaco-atra	401,037	19,826	4,814	24.3%	3,999	4,003

EL0118	Usnea aurantiaco-atra	623,414	29,417	5,001	17.0%	4,111	4,115
EL0121	Usnea aurantiaco-atra	1,256,791	34,172	8,018	23.5%	6,936	6,941
EL0124	Usnea aurantiaco-atra	287,888	15,019	3,635	24.2%	2,849	2,852
EL0375	Usnea antartica	608,102	24,878	5,924	23.8%	5,017	5,020
EL0377	Usnea antartica	457,290	25,467	5,385	21.1%	4,879	4,880
EL0380	Usnea antartica	650,500	34,318	6,348	18.5%	5,688	5,690
EL0381	Usnea antartica	57,322	1,624	312	19.2%	297	
EL0382	Usnea antartica	602,744	25,718	5,907	23.0%	4,994	4,996
EL0387	Usnea antartica	509,889	27,727	6,022	21.7%	5,389	5,390
EL0390	Usnea antartica	316,809	16,815	3,953	23.5%	3,178	3,180
EL0393	Usnea antartica	415,861	19,531	4,546	23.3%	3,787	3,789
EL0396	Usnea antartica	1,033,299	45,350	7,179	15.8%	6,280	6,282
EL0397	Usnea antartica	277,565	16,040	3,815	23.8%	3,069	3,071
EL0398	Usnea antartica	675,252	30,271	6,841	22.6%	5,994	5,996
EL0402	Usnea antartica	401,543	19,930	4,822	24.2%	3,948	3,950
EL0408	Usnea antartica	825,308	35,339	6,633	18.8%	5,881	5,883
EL0409	Usnea antartica	391,229	18,947	4,396	23.2%	3,582	3,584
EL0410	Usnea antartica	777,413	37,711	6,564	17.4%	5,819	5,820
EL0413	Usnea antartica	205,831	12,412	2,897	23.3%	2,265	2,267
EL0415	Usnea aurantiaco-atra	42,389	1,887	443	23.5%	408	
EL0416	Usnea aurantiaco-atra	751,019	25,530	5,761	22.6%	4,823	4,827
EL0417	Usnea aurantiaco-atra	623,045	26,611	6,309	23.7%	5,368	5,372
EL0419	Usnea aurantiaco-atra	1,288,183	49,256	5,905	12.0%	4,945	4,949
EL0420	Usnea aurantiaco-atra	622,806	31,966	5,148	16.1%	4,254	4,258
EL0423	Usnea aurantiaco-atra	453,929	18,105	4,071	22.5%	3,294	3,297
EL0426	Usnea aurantiaco-atra	348,399	18,815	4,644	24.7%	3,790	3,794
EL0429	Usnea aurantiaco-atra	573,360	22,980	5,163	22.5%	4,301	4,305
EL0430	Usnea aurantiaco-atra	344,277	18,128	4,423	24.4%	3,667	3,671
EL0431	Usnea aurantiaco-atra	735,439	27,213	5,587	20.5%	4,697	4,701
EL0432	Usnea aurantiaco-atra	656,918	26,007	6,354	24.4%	5,475	5,479
EL0435	Usnea aurantiaco-atra	174,671	7,725	1,880	24.3%	1,303	1,306
EL0437	Usnea aurantiaco-atra	77,221	3,090	746	24.1%	469	
EL0441	Usnea aurantiaco-atra	820,039	28,747	6,563	22.8%	5,699	5,703
EL0442	Usnea aurantiaco-atra	363,275	18,901	3,432	18.2%	2,607	2,612
EL0444	Usnea aurantiaco-atra	404,154	18,331	4,392	24.0%	3,551	3,554
EL0445	Usnea aurantiaco-atra	206,372	10,974	2,222	20.2%	1,583	1,586
EL0449	Usnea aurantiaco-atra	253,377	13,104	2,967	22.6%	2,211	2,214
EL0450	Usnea aurantiaco-atra	385,470	21,179	5,208	24.6%	4,356	4,360
EL0453	Usnea aurantiaco-atra	464,018	22,385	4,584	20.5%	3,643	3,647
EL0454	Usnea aurantiaco-atra	817,117	28,409	6,900	24.3%	5,901	5,905
EL0455	Usnea aurantiaco-atra	460,912	22,076	5,370	24.3%	4,488	4,492
EL0610	Usnea antartica	789,315	33,454	6,929	20.7%	6,087	6,089
EL0619	Usnea antartica	357,139	22,189	5,154	23.2%	4,607	4,608
EL0622	Usnea antartica	274,638	18,005	4,172	23.2%	3,734	3,735
EL0634	Usnea antartica	388,312	20,748	4,806	23.2%	4,274	4,275
EL0636	Usnea antartica	496,502	23,176	4,114	17.8%	3,708	3,708
EL0653	Usnea antartica	244,311	15,567	3,682	23.7%	3,324	3,325

EL0661	Usnea antartica	740,280	48,830	6,075	12.4%	5,333	5,333
EL0663	Usnea antartica	382,187	22,285	4,814	21.6%	4,253	4,254
EL0668	Usnea antartica	450,918	25,702	5,615	21.8%	4,913	4,913
EL0706	Usnea antartica	330,495	18,424	3,987	21.6%	3,607	3,607
EL0713	Usnea antartica	286,634	17,397	3,799	21.8%	3,465	3,466
EL0715	Usnea antartica	226,991	14,425	3,290	22.8%	3,054	3,054
EL0718	Usnea antartica	537,289	25,927	5,999	23.1%	5,361	5,362
EL0723	Usnea antartica	688,297	29,979	6,517	21.7%	5,802	5,804
EL0743	Usnea antartica	672,100	31,321	6,351	20.3%	5,621	5,623
EL0746	Usnea antartica	361,687	22,251	5,186	23.3%	4,663	4,665
EL0756	Usnea antartica	257,965	18,131	4,316	23.8%	3,936	3,937
EL0762	Usnea antartica	339,665	20,967	4,808	22.9%	4,344	4,345
EL0783	Usnea antartica	335,663	21,587	5,159	23.9%	4,623	4,623
EL0797	Usnea antartica	306,453	19,557	4,485	22.9%	4,002	4,001
EL0800	Usnea antartica	438,075	24,271	5,491	22.6%	4,837	4,836

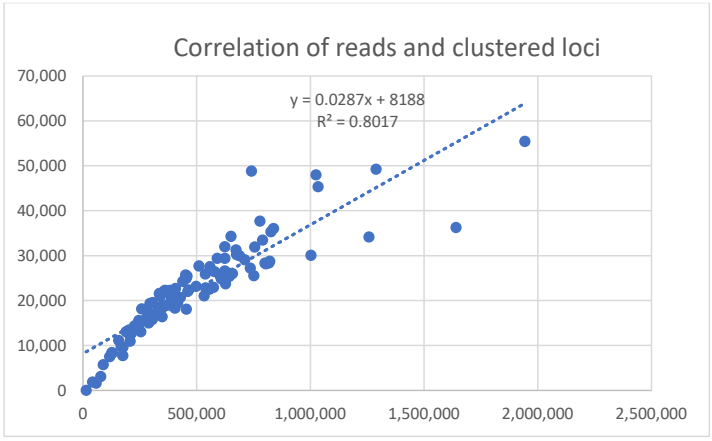
Supplementary material 3

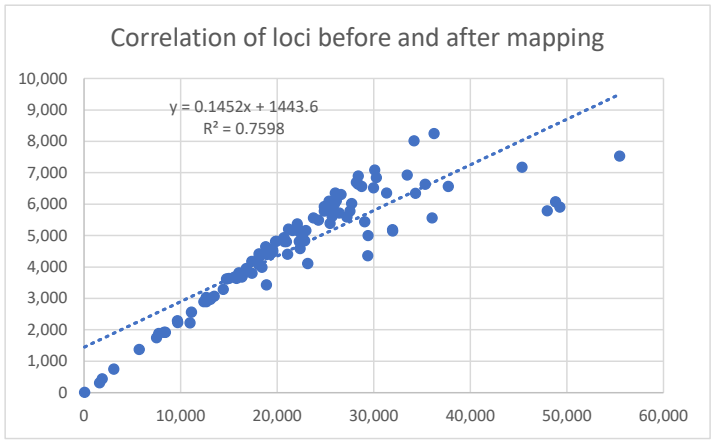
Correlation of RADseq results after individual steps of RAD analyses

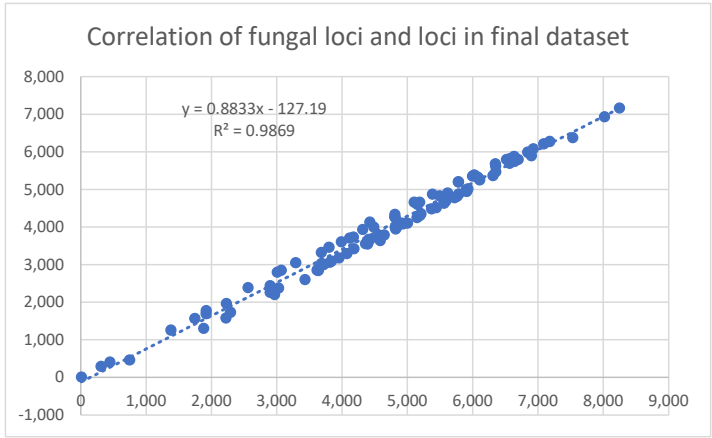
Authors: Felix Grewe, Elisa Lagostina, Huini Wu, Christian Printzen, H. Thorsten Lumbsch

Data type: molecular data

Link: https://doi.org/10.3897/mycokeys.43.29093.suppl3







Effects of dispersal strategy and migration history on genetic diversity and population structure of Antarctic lichens

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ABSTRACT

Aim To study the effects of dispersal strategy and phylogeographic history on the population genetic structure of Antarctic lichens.

Location Maritime Antarctic and southern South America.

Methods Populations of three fruticose lichen species, *Usnea aurantiacoatra*, *U. antarctica* and *Cetraria aculeata*, were collected in different localities in the Maritime Antarctic and southern South America. *Usnea aurantiacoatra* reproduces sexually by ascospores, whereas the other two species disperse asexually by symbiotic diaspores. Samples were genotyped at 8–22 microsatellite loci. Different diversity and variance metrics and Bayesian cluster analyses were used to study population genetic structure. Gene flow between southern South America and different locations in the Antarctic was investigated for *U. aurantiacoatra* and *C. aculeata* by coalescent sampling using MIGRATE-N.

Results The two asexual species display lower levels of genetic diversity than *U. aurantiacoatra*. Low levels of genetic differentiation within the Antarctic and higher levels in Patagonia indicate a long-lasting presence of *U. aurantiacoatra* in Antarctica and dispersal to South America from there. Genetic differentiation between populations of *U. antarctica* were comparable to the ones found in *U. aurantiacoatra* but the species was not found in South America. Low diversity and strong genetic differentiation of *C. aculeata* in the Antarctic confirms that the species colonized the Antarctic from Patagonia. Glacial refugia were identified on Navarino Island and in the South Shetland Islands. We found no evidence of migration or ongoing gene flow between the two continents.

Main Conclusions Phylogeographic history better explains the population genetic structure of each species than mode of propagation. Contrasting patterns of genetic differentiation provide evidence for glacial *in situ* survival of *Usnea antarctica* and *U. aurantiacoatra* in the Antarctic.

Keywords

Biodiversity, climate change, conservation, microsatellites, MigrateN, *Usnea antarctica, U. aurantiacoatra, Cetraria aculeata*, *Parmeliaceae*.

1. INTRODUCTION

Antarctica separated from South America over 40 million years ago (Scher & Martin 2006) and today is 900 kilometres distant from the southernmost tip of South America. Its strong spatial isolation is reinforced by the Antarctic Circumpolar Current (ACC) and atmospheric circulation patterns that both act as effective barriers against colonization from the north (Fraser et al. 2018). Consequently, levels of endemism are high (between 35 and 100% in different organismal groups, Rogers 2007). The Antarctic biota is restricted to widely separated and small ice-free areas that cover only 0.3 % of the continent (Convey & Stevens 2007) and show distinct biogeographical structure (Chown & Convey 2007, Terauds et al. 2012). Patterns of genetic diversity in Antarctica organisms have been shaped by isolation and recolonization, allopatric divergence among populations, founder events and the occasional occurrence of secondary contact zones (Domaschke et al. 2012, Nolan et al. 2006, Rogers 2007), but above all by limited migration and gene flow due to the strong fragmentation of habitable areas and reduced dispersal abilities of many organisms. Therefore, strong local and regional genetic differentiation has been observed in most Antarctic terrestrial organisms (Chong et al. 2015, Courtright et al. 2000, McGaughran et al. 2010, Skotnicki et al. 2004, van de Wouw et al. 2008). Together with high levels of endemism this is evidence for long-standing survival of terrestrial and lacustrine organisms in glacial refugia (Convey & Stevens 2007, de Wever et al. 2009, Green et al. 2011, Jones et al. 2013) perhaps concentrated around areas of geothermal activity (Fraser et al. 2014). From a biological perspective, the Antarctic thus presents an assemblage of widely spaced “habitat islands” (Bergstrom & Selkirk 1997) with sufficiently long continuity to support considerable genetic diversity (Convey et al. 2014).

The Western Antarctic region (south of the Pacific Ocean), particularly the Antarctic Peninsula and the Bellingshausen Sea, have until recently been subject to rapid regional warming (Turner et al. 2005). The ensuing glacial retreat exposes so far uninhabited disturbed ground, potentially favouring the establishment of invasive species (Chown et al. 2012). Moreover, higher temperatures alleviate physiological stress, and the increase in available habitat leads to larger population sizes and reduced competition as witnessed by 5-25-fold increases in local abundance of indigenous plants over a few decades (Fowbert & Lewis Smith 1994). Simultaneously, human impact on Antarctic ecosystems is growing, either because of increased scientific activities (>100 research facilities in the Antarctic

Treaty area) and rising numbers of tourists with multiple landings in different Antarctic regions. Both activities facilitate propagule movement into Antarctica and among different habitats and bioregions. Together with an expansion of habitable terrain this facilitates the breakdown of dispersal barriers and the merging of genetically isolated populations (Chown et al. 2015). The potential genetic homogenization of gene pools that are now highly differentiated has been identified as a serious threat to Antarctic biodiversity (Hughes & Convey 2010, Terauds et al. 2012) and "one of the most significant conservation problems in the Antarctic" (Chown & Convey 2007). Consequently, there is a growing need to reassess and monitor the extent of Antarctica's biological isolation and the genetic structure of its biota (Fraser et al. 2018).

Lichens, symbioses of heterotrophic fungi (mycobionts) and autotrophic green algae and/or cyanobacteria (photobionts), play a dominant role in the Antarctic terrestrial vegetation. Of the more than 400 reported species, 34% are endemics, indicating isolation of lichen biota over geological timescales. The other species are mostly cosmopolitan or bipolar, many are found in southern South America. Global distribution patterns and molecular phylogenetic analyses suggest that some of the more widespread species evolved in the Antarctic and colonized South America and the Arctic from there (Søchting & Castello 2012) while others migrated from the Northern Hemisphere southwards into Patagonia and Antarctica (Fernández-Mendoza & Printzen 2013). Lichens display different reproductive and dispersal strategies that may affect their dispersal abilities and gene flow between isolated populations. Small-sized meiotic and mitotic fungal spores are generally considered ideal vehicles for long-distance dispersal by wind (Tibell 1994) while asexual propagules (soredia, isidia or thallus fragments) containing both symbionts may facilitate the establishment on newly exposed substrata. Human-induced gene flow between Antarctic lichen populations and increased migration rates between South America and Antarctica would be of immediate conservation concern, because both would change the genetic composition of Antarctic lichen populations and endanger the survival of genetically isolated and locally adapted lineages.

Information about the spatial genetic structure of lichens is therefore urgently needed to understand the joint effects of local human activities and global temperature increase on Antarctic terrestrial vegetation. We present here population genetic data on three fruticose lichens species reported from South America and the Maritime Antarctic: *Usnea*

aurantiacoatra reproducing sexually via ascospores, *U. antarctica* with asexual propagation via soredia and *Cetraria aculeata* dispersing by thallus fragments. *Usnea antarctica* and *U. aurantiacoatra* belong to the *Neuropogon* group of *Usnea*. Most species of this group occur in southernmost South America, Australasia and Antarctica and have likely evolved there (Jørgensen 1983, Wirtz et al. 2008, 2012). *C. aculeata* is a bipolar lichen species that colonized Antarctica from Patagonia during the Pleistocene (Fernández-Mendoza & Printzen 2013). Therefore, these this three species are representative taxa to study the effects of dispersal strategy and phylogeographic history on the population genetic structure of Antarctic lichens and assess the likely effects of climate change and human impact on them. Our main research questions can be summarized as follows:

- Are lichen populations genetically isolated, or exists gene flow, particularly between southern South America and the Antarctic?
- How does the dispersal strategy influence the genetic structure of the mycobionts?
- What impact does the phylogeographic history have on the population genetic structure of the Antarctic lichens?

2. MATERIAL AND METHODS:

2.1 Sample collection and DNA extraction

Sampling covered a wide range of localities in the Maritime Antarctic (61-64^{o}S) and southern South America (50-55^{o}S) (see localities in S1), including the Falkland Islands (hereafter "Falkland"). Most samples were collected between 2015 and 2018. A few populations sampled between 2007 and 2014 and cryo-conserved at Herbarium Senckenbergianum (FR) were added to the dataset. For most analyses, samples from different nearby stands (e.g. on the same island) were pooled into "localities". The data sets comprised: 10 localities/22 stands/441 individuals for *U. aurantiacoatra*, 6 localities/20 stands/370 individuals for *U. antarctica* and 10 localities/16 stands/266 individuals for *C. aculeata*. For further details on sampling locations see Supplementary Table 1.

Total DNA was extracted from young terminal branches. Branches were ground with the Bead Ruptor 24 (Omni International Inc., Kennesaw, Ga., USA) and DNA was extracted with the GeneOn BioTech Plant Kit (BGgreen Biotech, Ratingen, Germany) according to the manufacturer's instructions. The identification of the two *Usnea* species was confirmed with

a Discriminant Analysis of Principal Components (DAPC) based on microsatellite markers as reported in Lagostina et al. (2018).

2.2 Microsatellite analyses and genetic diversity

Samples *of Usnea aurantiacoatra* and *U. antarctica* were genotyped using 21 and 22 microsatellites markers, respectively. Eight consistently amplifying markers were used for *Cetraria aculeata*. Detailed information on primers and PCR amplification can be found in Lagostina et al. (2017) and Lutsak et al. (2016). PCR amplicons were electrophoresed using an Applied Biosystems 3730 sequencer, with the LIZ 600 (*Usnea* sp.) or LIZ 500 (*C. aculeata*) size standards (Applied Biosystems, Waltham, Mass., USA). Allele sizes were manually scored using the Geneious 10 microsatellites tool (Kearse et al. 2012).

Allele frequencies and genetic diversity (Shannon's information index) were calculated using the software GenAlEx 6.503 (Peakall & Smouse 2006, 2012) for the three species. Tests for clonal population structure and differentiation among populations using Jost's D were calculated with the software GenoDive 2.0b23 (Meirmans & Van Tienderen 2004). Clones in each population were detected using a stepwise mutation model, discarding null alleles and assessed based on the number of genotypes, with 999 permutations randomizing alleles over individuals over all populations.

2.3 Clustering analysis

Individuals of each species were clustered into gene pools using STRUCTURE v.2.3.4 (Pritchard et al. 2000, Falush et al. 2003). The analyses were based on ten serial runs for each number of clusters (K) between one and ten. Admixture models used a uniform alpha prior, independent allele frequencies and no prior population information. All analyses were run for $5*10^5$ generations after a burn-in of $25*10^4$ generations. To estimate the optimal number of admixture clusters we used the summary likelihood statistics ΔK proposed by Evanno et al. (2005) through the website Pophelper v1.0.10 (Francis 2016, www.pophelper.com). The number of clusters was chosen as the value of K where ΔK reached its first minimum. Results of the ten runs for each species were summarized using CLUMPP (Jakobsson & Rosenberg 2007) and printed out through the web interface of Pophelper v1.0.10.

2.4 Estimation of gene flow

We used the coalescent sampler Migrate-N estimate gene flow from South America and Falkland to different areas of the Maritime Antarctic. In order to keep the number of parameters low, samples of *U. aurantiacoatra* were pooled into 5 regions: South America, Falkland, Elephant Island, King George Island, and Livingston + Deception Island. The data set for *C. aculeata* was divided into: South America + Falkland, Elephant Island, King George Island and Primavera (Antarctic Peninsula). For this species, stands from South America and Falkland were pooled, because there was no evidence of population differentiation in the Structure analysis (Fig. 2). All South American samples presumed to represent *U. antarctica* proved to belong to other species. Consequently, we could not analyse intercontinental gene flow for this species. For the *U. aurantiacoatra* dataset, we used 0.0–10 priors on θ and 0.0-20 on M, divided into 1500 bins, and ran four chains with static heating (temperatures of 1.0, 1.2, 3.0 and 1×10^6) for 10 replicated long runs of 5×10^4 generations (sampling every 500th step) with a burn-in of 4×10^4. For the *C. aculeata* dataset, we used uniform priors (0.0–25) on both θ and M divided into 1500 bins and ran four chains with static heating of 1×10^5 generations (sampling every 500th step) with a burn- in of 5×10^4. Convergence of Markov chains was monitored with Tracer (http:// beast.bio.ed.ac.uk). All effective sample sizes of the Markov chain Monte Carlo (MCMC) chain were larger than 10^5.

3. RESULTS

3.1 Genetic diversity

We sampled 22 stands of *Usnea aurantiacoatra* and 16 stands of *Cetraria aculeata* in southern South America, Falkland, and the Maritime Antarctic as well as 20 stands of *U. antarctica* in the South Shetland Islands and the Antarctic Peninsula. We confirmed identification of *Usnea antarctica* and *U. aurantiacoatra* with a DAPC analysis (Fig. S2 in supplementary material). The analysis showed that all of the supposed samples of *U. antarctica* from South America were identified as U*. aurantiacoatra*.

For *Cetraria aculeata* the final dataset comprised 2128 alleles including 19 null alleles. For *U. antarctica* we analysed 8140 alleles including 41 null alleles and for *Usnea aurantiacoatra* we scored 9261 alleles including 164 null alleles. *Usnea aurantiacoatra* had the highest total number of alleles (232), with the highest mean number of observed (7.476) and effective alleles (4.016) recorded on Navarino Island in South America followed by

Livingston Island in the Antarctic (7.238; 2.725, Table 1). The highest mean number of private alleles was observed on Livingston Island (0.857) followed by Navarino Island (0.762). The Shannon information index was highest on Navarino (1.490) with rather similar values around 1.0-1.1 on Livingston, King George and Falkland. None of the diversity metrics showed a clear latitudinal pattern. In *Cetraria aculeata* the highest observed number of alleles (4.750) was also found on Navarino and decreased to the north and south. The highest effective number of alleles (2.902) was detected in a stand in Chile and the observed (1.250) and effective number of alleles (ca. 1.0) was lowest on Elephant Island and near Primavera Base on the Antarctic Peninsula. Private alleles were detected in all South American populations (except Falkland) and on King George Island, but not on Elephant Island and on the Antarctic Peninsula. In *Usnea antarctica* the observed (effective) mean number of alleles ranged between 4.682 (1.954) on Livingston and 1.591 (1.238) on Deception Island. Private alleles were recorded in all the sampling areas except for Deception Island.

Every individual of *U. aurantiacoatra* belonged to a different clone. Hence there was no evidence for clonal structure of populations (Table 2). In *C. aculeata* there was strong evidence for clonal reproduction. The 133 samples from South America and Falkland belonged to 113 different clones (Supplementary Table 3), while all individuals from Elephant Island and the Antarctic Peninsula belonged to the same multilocus genotype and samples from King George Island and Primavera on the Antarctic Peninsula were dominated by a single clone. Genodive also inferred significant clonal population structure in *U. antarctica* although the number of clones was almost as high as expected.

3.2 Genetic structure

Antarctic populations of *C. aculeata* were strongly differentiated from each other and from South American localities. The highest value of Jost's D (0.502) was observed between Primavera base and Tierra del Fuego (Table 3). South American localities were poorly differentiated (D values ranging between 0.003 and 0.131). The highest differentiation in *U. aurantiacoatra* was observed between localities in South America and Falkland (0.495 between Navarino and Falkland 1). Antarctic localities of *U. aurantiacoatra* were poorly differentiated (Jost's D 0.021-0.075). Antarctic localities of *U. antarctica* showed similarly low differentiation (between 0.007 and 0.095), only for Deception Island D exceeded 0.2.

The STRUCTURE analysis showed different geographic structure in all three species (Figure 2). For all datasets, the optimal number of clusters was inferred as K=4. Antarctic populations of *C. aculeata* display extreme regional genetic structure with different gene pools on the Antarctic Peninsula, King George and Elephant Islands. The gene pool on Elephant Island is also relatively common in South America, where it co-occurs with a fourth gene pool that is absent from Antarctica. South American populations show no strong differences in gene pool composition. Populations of *U. aurantiacoatra* in Falkland and Navarino Island are dominated by local gene pools that are absent elsewhere. A third gene pool is largely restricted to Antarctica. About half of the samples from Livingston Island belong to a fourth gene pool that also predominates in populations from Mt Tarn and Torres del Paine in Chile. Populations of *U. antarctica* on Livingston and Deception Island are dominated by two gene pools that are virtually absent in other localities. Most samples from Elephant Island and Esperanza belong to a third gene pool that, together with a fourth one, also occurs on King George Island and near Primavera.

3.3 Migration and intercontinental gene flow from South America

Since *U. antarctica* was not found by us in southern South America, we studied intercontinental gene flow only in *U. aurantiacoatra* and *C. aculeata* (Figure 3). *C. aculeata* showed dispersal rates of 4.4 migrants per generation from South America to Elephant Island. Gene flow was considerably lower towards the Antarctic Peninsula (1.5 migrants/generation) and absent towards King George Island. All Antarctic populations had comparably low effective population sizes (0.3 for Elephant Island and Primavera base and 0.5 for King George Island). In *U. aurantiacoatra*, the highest values of >5 migrants per generation were inferred from South America to Elephant Island. Gene flow into Antarctica along the other routes was considerably lower and ranged between 1.4 (Falkland to King George Island) and 3.5 (South America to King George Island) migrants per generation. Dispersal between continental South America and Falkland was negligible (<1 migrant per generation in both directions). Effective population sizes in different Antarctic localities differed vastly. Populations on Livingston + Deception island (6.1) were at least three times larger than those of the remaining stands (0.9 Elephant Island and 2.0 King George Island) and comparable to the South American populations (8.1) and Falkland (5.2).

4. DISCUSSION

Fine-scale population genetic data on Antarctic lichens, the most important primary producers of Antarctic terrestrial ecosystems, is still largely lacking. This lack of data is mostly due to the logistic challenges of sampling over large areas and has made it difficult to assess the present and future human impact on Antarctic vegetation. Our study provides a first insight into levels of genetic diversity, connectivity and isolation among populations of three common Antarctic lichens. By including populations from southern South America we were also able to study levels of intercontinental gene flow in two of the three species. Our results allow us to assess the impact of different factors – reproductive mode, colonization and glacial history – on the diversity and spatial structure of Antarctic lichen populations. They also provide further insight into dispersal capacities and conservation of Antarctic lichens.

4.1 Impact of reproductive mode on genetic diversity

As expected by population genetic theory (e.g. Bengtsson 2003), the sexually reproducing *U. aurantiacoatra* shows higher genetic diversity than the mostly asexual *C. aculeata* and *U. antarctica*. While diversity levels are difficult to compare among *Cetraria* and *U. aurantiacoatra* due to the different numbers of genotyped loci, results for the two closely related *Usnea* species are based on the same set of loci and confirm that asexual reproduction reduces genetic diversity in lichens (Grewe et al. 2018; Otálora et al. 2013). The observed clonal population structure in the two asexual species (Tab. 2) further supports this interpretation. However, SSR data discovers much higher genetic diversity in the two asexual species than was previously found based on DNA sequences (Domaschke et al. 2012). In *C. aculeata* we found 130 clones and a total of 67 SSR alleles (data not shown). *U. antarctica* displays even higher allelic richness and genetic diversity in our sample. The extremely high genetic diversity in *U. aurantiacoatra* corresponds well with the genotypic richness found in the Mediterranean *Parmelina carporrhizans* (Alors et al. 2017) indicating that this might be a general trend among sexually reproducing lichens.

4.2 Impact of historical factors

Biersma, Jackson, Bracegirdle et al. (2018) explained the reduced genetic diversity of Antarctic bryophytes with colonization events and Pleistocene population size bottlenecks.

The observed differences in diversity and genetic structure among the species studied by us exemplify the important impact of historical factors on the spatial genetic structure of lichens, particularly at the range margins (Eckert et al. 2008). South American populations of *C. aculeata* comprise two to four times higher genetic diversity than Antarctic ones confirming similar results by Domaschke et al. (2012) based on DNA sequence data. In contrast, *U. aurantiacoatra* displays higher numbers of alleles and private alleles in Antarctic than in South American populations, while genetic diversity is equal in both regions. Genetic differentiation among populations also shows opposite trends in both species (Fig. 2, Tab. 3). Antarctic populations of *C. aculeata* are strongly differentiated while *U. aurantiacoatra* shows strong differentiation in South America. The D-values for both species in these regions resemble the level of differentiation found between geographically isolated populations of *Buellia frigida* in the Queen Maud Mts and other areas in the Ross Sea Region (Jones et al. 2015). In contrast, South American populations of *C. aculeata* and Antarctic populations of *U. aurantiacoatra* are considerably less well differentiated. These pronounced differences are hardly explained by geographic distances among populations on both sides of the Drake Passage (Fig. 1), but rather reflect range centres and margins of the two species and, hence, their different phylogeographic histories.

While *C. aculeata* originated in the Northern Hemisphere, dispersed into South America during the Pleistocene and colonized the Antarctic recently (Fernández-Mendoza & Printzen 2013), the two *Usnea* species are assumed to have evolved either in the Antarctic or in southern South America (Jørgensen 1983). The longer presence of *C. aculeata* in South America together with moderate levels of gene flow apparently prevented strong genetic differentiation between populations, while long-distance dispersal into the Maritime Antarctic was too recent to allow homogenization of gene pools between these geographically isolated, marginal populations. A similar pattern of genetic diversity has been reported from *Parmelina carporrhizans* with diverse, poorly differentiated source populations in the Mediterranean and sink populations on the Canary Islands (Alors et al. 2017). In contrast, populations of *U. aurantiacoatra* from Falkland, Navarino Island and more northern sites in Patagonia are assigned to three distinct gene pools, whereas Antarctic populations are poorly differentiated (Fig. 2, Tab. 3). If, as in *C. aculeata*, stronger differentiation among lichen populations indicates a more recent colonization history, then

postglacial recolonization in *U*. *aurantiacoatra* apparently took place from southern source populations.

4.3 Glacial population history

The effects of Pleistocene glacial cycles on the distribution ranges of species and their genetic diversity have frequently been studied in the Northern Hemisphere (Hewitt 2004). The effects of southern hemispheric glaciations on biota have received less attention, but due to the stronger geographical isolation of Antarctica, demographic processes, including range shifts, extinction of populations and recolonization during glacials and interglacials are likely to differ between these regions (Fraser et al. 2012). The extension of ice caps and severe environmental conditions during the last glacial maximum were once believed to have precluded survival of organisms in polar regions (e.g. Nordal 1987). Nowadays, the glacial persistence of organisms in the Antarctic is hardly questioned (Pugh & Convey 2008, Biersma, Jackson, Stech et al. 2018). Nunataks, perhaps associated with geothermal activities, or debris covering glaciers may have provided refugial habitats (Fickert et al. 2007, Fraser et al. 2014). Comparative population genetic data on lichens from glacial refugia and formerly glaciated areas are scarce and entirely lacking for Antarctic lichens, but higher genetic diversity and numbers of private alleles in glacial refugia with gradual decrease of diversity with increasing distance from these areas have been observed in some Northern Hemispheric species (Printzen et al. 2003, Scheidegger et al. 2012, Allen et al. 2018).

The high genetic diversity of *C. aculeata* and *U. aurantiacoatra* on Navarino Island therefore supports the existence of a southern Patagonian refugium postulated for plant and fungal species (Sérsic et al. 2011, Eizaguirre et al. 2018) and is consistent with reconstructions of the Patagonian ice shield indicating that Navarino Island was at least partly ice-free during the Last Glacial Maximum (LGM, Glasser & Jansson 2008, Darvill et al. 2014). Our data does not indicate whether the gradually declining levels of genetic diversity in *C. aculeata* resulted from postglacial recolonization of northern localities from the Navarino refugium or persistence in smaller local refugia. The more pronounced diversity gradient in *U. aurantiacoatra* and the lack of private alleles in Torres del Paine and Mt. Tarn suggest more pronounced population size bottlenecks during the LGM either because of more recent origin or because, as a saxicolous subalpine species, *U. aurantiacoatra* had more restricted glacial habitats than the terricolous lowland *Cetraria.*

Another refugium, in Antarctica, is indicated by the higher allelic richness and numbers of private alleles on Livingston and King George Island as compared to Elephant Island, Deception Island or the Antarctic Peninsula in all three species. Such a refugium would be consistent with the reconstruction of Nunataks in the region, e.g. on Livingston, King George and Deception Islands (Simms et al. 2011, Ruiz-Fernández & Oliva 2016). In *C. aculeata*, the diversity on King George Island is comparable to that found in South American populations. This and the presence of private alleles indicate a relatively high, probably pre-glacial age of this population. The extremely low diversity and effective population sizes on Elephant Island and the Antarctic Peninsula contrast with the higher diversity found in the moss *Chorisodontium aciphyllum* (Biersma, Jackson, Bracegirdle et al. 2018) and speak against Elephant Island as a glacial refugium for *C. aculeata*. Instead, although postglacial recolonization from lower latitudes appear to have been extremely rare among terrestrial Antarctic taxa (Fraser et al. 2012) the apparent absence of migration between Antarctic populations and the fact that all individuals of *C. aculeata* on Elephant Island belong to a single clone also present near Calafate in Argentina suggest founder effects during independent colonization events from South America. The strongly diverging levels of genetic diversity of *U. antarctica* and *U. aurantiacoatra* populations on Livingston and Deception Island merit some attention. Both islands are close to each other and Deception Island, the most active volcano in the area, was probably not glaciated during the LGM (Simms et al. 2011, Guillemin et al. 2018). The absence of private alleles and low diversity on Deception Island could result from recent volcanic eruptions in 1967, 1969 and 1970 that strongly reduced the size of lichen populations (Lewis-Smith 1984), but are more likely an artefact resulting from low sample sizes.

4.4 Intercontinental gene flow and consequences for conservation

Due to its geographical distance from other continents and the strong effects of the ACC Antarctica is considered the biologically most isolated continent. As judged from levels of endemism, the degree of isolation varies strongly with the taxonomic group considered (Barnes et al. 2006), and such data for terrestrial organisms is still very scarce. Distribution patterns of bryophytes and lichens on sub-Antarctic islands are indeed correlated with the prevailing wind patterns indicating directional long-distance colonization (Muñoz et al. 2004). However, for some bryophytes with bipolar distribution, long-distance dispersal

mediated by migratory birds has been demonstrated (Lewis, Behlin et al. 2014, Lewis, Rozzi et al. 2014). The wide geographical ranges of many lichens and genetic similarities among widely separated populations have sometimes been interpreted as evidence for ongoing long-range dispersal, even between continents (Geml et al. 2010). But although numerous lichen species occur in South America and Antarctica our data does not confirm dispersal of lichens across the Drake Passage on short time scales. Since we could not confirm the presence of *U. antarctica* in South America this species might be an Antarctic endemic that never managed to cross the Drake Passage. Neither do the MigrateN analyses and genetic differentiation among populations (Fig. 3) indicate high levels of ongoing gene flow in *U. aurantiacoatra* or *C. aculeata*. *U. aurantiacoatra* apparently survived the LGM in separate refugia north and south of the Drake passage, while the high genetic differentiation of peripheral Antarctic populations of *C. aculeata* suggest *in situ* survival in small populations or rare colonization events with founder effects.

The invasion of alien species and propagule transfer into Antarctica has been a major concern of conservationists (Hughes & Convey 2010) and is regarded as "one of the most significant conservation problems in the Antarctic" (Chown & Convey 2007). The increasing risk of accidental introduction of invasive species and genetic homogenization of Antarctic gene pools is due to two interacting factors. While global warming is beginning to change the ACC and associated aerial currents (Chown et al. 2015, Fraser et al. 2018), exposes so far uninhabited, disturbed ground and alleviates physiological stress, growing numbers of researchers and tourists in the region act as possible vectors for propagules. Although our results do not indicate any immediate threat to the genetic composition of lichen populations, they suggest that *C. aculeata* and *U. aurantiacoatra* are exposed to different risks. Conservation measures for Antarctic organisms should therefore consider the different phylogeographic histories and spatial genetic structure of the species. The genetically diverse and poorly differentiated Antarctic populations of the two *Usnea* species are apparently experiencing high natural levels of gene flow. On this background, additional human transfer of propagules will have comparatively little impact (and would be difficult if not impossible to detect). The genetically poor and highly differentiated populations of *C. aculeata*, on the other hand, require stronger conservation measures to avoid the introduction of alien genotypes and homogenization of gene pools. The different distributional patterns of both species in South America, a result of their different

phylogeographic histories, exacerbate this problem. *U. aurantiacoatra* only occurs in small and isolated patches and prefers higher elevations, reducing the risk of accidental introduction into Antarctica, e. g. by tourists. In contrast, *C. aculeata* is much more widespread in South America and also grows at lower elevations, e.g. around the airport of Rio Gallegos (Fernández-Mendoza, pers. comm.). It therefore has a much higher chance to be transferred by Antarctic visitors.

5. CONCLUSIONS

This is the first study to evaluate the effects of dispersal strategy and migration history on genetic diversity and population structure of Antarctic lichens. As expected, levels of genetic diversity are lower in the two asexual species but patterns of differentiation are affected by phylogeographic history rather than reproductive mode. Both the northern immigrant *C. aculeata* and the (sub)Antarctic *U. aurantiacoatra* show higher levels of genetic differentiation in marginal than central populations. Diversity hotspots for both species suggest the existence of glacial refugia on Navarino Island and Livingston or King George Island, where also *U. antarctica* displays highest diversity. Although we found no convincing evidence for ongoing gene flow from southern South America into the Maritime Antarctic, the strong genetic structure of *C. aculeata* calls for protective measures to avoid gene flow between isolated populations.

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LITERATURE CITED

Allen, J. L., McKenzie, S. K., Sleith, R. S., Alter, S. E. (2018). First genome-wide analysis of the endangered, endemic lichen *Cetradonia linearis* reveals isolation by distance and strong population structure. *American Journal of Botany,* 105(9):1556-1567.

Alors, D., Dal Grande, F., Cubas, P., Crespo, A., Schmitt, I., Molina, M. C., Divakar, P. D. (2017). Panmixia and dispersal from the Mediterranean Basin to Macaronesian Islands of a macrolichen species. *Scientific Reports* 7: 40879.

Barnes, D. K. A., Hodgson, D. A., Convey, P., Alle, C. S., Clarke, A. (2006). Incursion and excursion of Antarctic biota: past, present and future. *Global Ecology and Biogeography* 15: 121–142.

Bengtsson, B. O. (2003). Genetic variation in organisms with sexual and asexual reproduction. *Journal of Evolutionary Biology,* 16(2):189-99.

Bergstrom, D. M., & Selkirk, P. (1997). Distribution of bryophytes on subantarctic Heard Island. *Bryologist,* 100(3): 349–355.

Biersma, E. M., Jackson, J. A., Bracegirdle, T. J., Griffiths, H., Linse, K., Convey, P. (2018). Low genetic variation between South American and Antarctic populations of the bank-forming moss *Chorisodontium aciphyllum* (Dicranaceae). *Polar Biology,* 41(4): 599–610.

Biersma, E. M., Jackson, J. A., Stech, M., Griffiths, H., Linse, K., Convey, P. (2018). Molecular data suggest long-term *in situ* Antarctic persistence within Antarctica's most speciose plant genus, *Schistidium*. *Frontiers in Ecology and Evolution,* 6: 77.

Chong, C. W., Pearce, D. A., Convey, P. (2015). Emerging spatial patterns in Antarctic prokaryotes. *Frontiers in Microbiology,* 6: 1058–1071.

Chown, S. L., & Convey, P. (2007). Spatial and temporal variability across life's hierarchies in the terrestrial Antarctic. *Philosophical Transactions of the Royal Society* 362(1488):2307-31.

Chown, S. L., Huiskes, A. H. L., Gremmen, N. J. M., Lee, J. E., Terauds, A., Crosbie, K., Yves Frenot, … Bergstrom, D. A. (2012). Continent-wide risk assessment for the establishment of nonindigenous species in Antarctica. *PNAS* 109(13): 4938–4943.

Chown, S. L., Clarke, A., Fraser, C. I., Cary, S. C., Moon, K. L., McGeoch, M. A. (2015). The changing form of Antarctic biodiversity. *Nature,* 522(7557):431-8.

Convey, P., Chown, S. L., Clarke, A., Barnes, D. K. A., Bokhorst, S., Cummings, V., Ducklow, H. W., … Wall, D. H. (2014). The spatial structure of Antarctic biodiversity. *Ecological Monographs*, 84(2): 203-244.

Convey, P., & Stevens, M.I. (2007). Antarctic Biodiversity. *Science,* 317(5846): 1877–1878.

Courtright, E. M., Wall, D. H., Virginia, R. A., et al. 2000. Nuclear and mitochondrial DNA sequence diversity in the Antarctic nematode *Scottnema lindsayae*. *Journal of Nematology,* 32(2): 143–153.

Darvill, C. M., Stokes, C. R., Bentley, M. J., Lovell, H. (2014). A glacial geomorphological map of the southernmost ice lobes of Patagonia: the Bahía Inútil – San Sebastián, Magellan, Otway, Skyring and Río Gallegos lobes. *Journal of Maps,* 10: 500–520.

De Wever, A., Leliaert, F., Verleyen, E., Vanormelingen, P., Van der Gucht, K., Hodgson, D. A., 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*, 276(1673): 3591–3599.

Domaschke, S., Fernández-Mendoza, F., García, M. A., Martín, M. P. Printzen, C. (2012). Low genetic diversity in Antarctic populations of the lichen *Cetraria aculeata* and its photobiont. *Polar Research,* 31: 17353.

Eckert, C. G., Samis, K. E., Lougheed, S. C. (2008). Genetic variation across species' ranges: the central-marginal hypothesis and beyond. *Molecular Ecology,* 17(5): 1170–1188.

Eizaguirre, J. I., Peris, D., Rodríguez, M. E., Lopes, C. A., De Los Ríos, P., Hittinger, C. T., Libkind, D. (2018). Phylogeography of the wild Lager-brewing ancestor (*Saccharomyces eubayanus*) in Patagonia. *Environmental Microbiology,* 20(10):3732-3743.

Evanno, G., Regnaut, S., Goudet ,J. (2005). Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology*, 14: 2611-2620.

Falush, D., Stephens, M., Pritchard, J. K. (2003). Inference of population structure: Extensions to linked loci and correlated allele frequencies. *Genetics*, 164: 1567–1587.

Fernández-Mendoza, F., & Printzen, C. (2013). Pleistocene expansion of the bipolar lichen Cetraria aculeata into the Southern hemisphere. *Molecular Ecology*, 22(7):1961-83.

Fickert, T., Friend, D., Grüninger, F., Molnia, B., Richter, M. (2007). Did debris-covered glaciers serve as Pleistocene refugia for plants? A new hypothesis derived from observations of recent plant growth on glacier surfaces. *Arctic, Antarctic, and Alpine Research,* 39(2): 245–257.

Fowbert, J. A., & Lewis Smith, R. I. (1994). Rapid population increases in native vascular plants in the Argentine Islands, Antarctic Peninsula. *Arctic and Alpine Research*, 26(3): 290–296.

Francis, R. M. (2016). POPHELPER: An R package and web app to analyze and visualize population structure. *Molecular Ecology Resources*, 17(1):27-32.

Fraser, C. I., Terauds, A., Smellie, J., et al. (2014). Geothermal activity helps life survive glacial cycles. *PNAS,* 111(15): 5634–5639.

Fraser, C. I., Morrison, A. K., McC Hogg, A., Macaya, E. C., van Sebille, E., Ryan, P. G., Padovan, A., Cameron, J., Valdivia, N., Waters, L. M. (2018). Antarctica's ecological isolation will be broken by storm-driven dispersal and warming. *Nature Climate Change*, 8: 704–708.

Fraser, C. I., Nikula, R., Ruzzante, D. E., Waters, J. M. (2012). Poleward bound: Biological impacts of Southern Hemisphere glaciation. *Trends in Ecology & Evolution,* 27(8): 462–471.

Geml, J., Kauff, F., Brochmann, C., Taylor, D. L. (2010). Surviving climate changes: high genetic diversity and transoceanic gene flow in two arctic–alpine lichens, *Flavocetraria cucullata* and *F. nivalis* (Parmeliaceae, Ascomycota). *Journal of Biogeography,* 37(8): 1529–1542.

Glasser, N., & Jansson, K. (2008). The glacial map of southern South America. Journal of Maps 4(1): 175–196.

Green, T. G. A., Sancho, L. G., Türk, R., SeppeltI, R. D., Hogg D. (2011). High diversity of lichens at 84°S, Queen Maud Mountains, suggests preglacial survival of species in the Ross Sea region, Antarctica. Polar Biology 34(8): 1211–1220.

Grewe, F., Lagostina, E., Wu, H., Printzen, C., Lumbsch, H. T. (2018) Population genomic analyses of RAD sequences resolves the phylogenetic relationship of the lichen-forming fungal species *Usnea antarctica* and *Usnea aurantiacoatra*. *MycoKeys*, 113: 91–113.

Guillemin, M. L., Dibrasquet, H., Reyes, J., Valero, M. (2018). Comparative phylogeography of six red algae along the Antarctic Peninsula: extreme genetic depletion linked to historical bottlenecks and recent expansion. *Polar Biology.* 41(5): 827–837.

Hewitt, G. M. (2004). Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transaction of the Royal Society London, Series B,* 359(1442): 183–195..

Hughes, K. A. & Convey, P. (2010). The protection of Antarctic terrestrial ecosystems from inter- and intra-continental transfer of non-indigenous species by human activities: A review of current systems and practices. *Global Environmental C*hange, 20(1): 96-112.

Jakobsson, M., & Rosenberg, N. A. (2007). CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23(14): 1801–1806.

Jørgensen, P. M. (1983). Distribution patterns of lichens in the Pacific Region. *Australian Journal of Botany, Supplementary Series*, 13(10): 43–66.

Jones, T. C., Hogg, I. D., Wilkins, R. J., Green, T. G. A. (2013). Photobiont selectivity for lichens and evidence for a possible glacial refugium in the Ross Sea region, Antarctica. *Polar Biology,* 36(6): 767–774.

Jones, T. C., Hogg, I. D., Wilkins, R. J., Green, T. G. A. (2015). Microsatellite analyses of the Antarctic endemic lichen *Buellia frigida* Darb. (Physciaceae) suggest limited dispersal and the presence of glacial refugia in the Ross Sea Region. *Polar Biology*, 38(7): 941–949.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics,* 28(12): 1647-1649.

Lagostina, E., Dal Grande, F., Andreev, M., Printzen, C. (2018). The use of microsatellite markers for species delimitation in Antarctic *Usnea* subgenus *Neuropogon*. *Mycologia,* 110(6): 1047-1057.

Lagostina, E., Dal Grande, F., Ott, S., Printzen, C. (2017). Fungus-specific SSR markers in the Antarctic lichens *Usnea antarctica* and *U. aurantiacoatra* (*Parmeliaceae*, Ascomycota). *Applications in Plant Sciences,* 5(9): 1700054.

Lewis, L. R., Behling, E., Gousse, H., Qian, E., Elphick, C. S., Lamarre, J. F., Bêty, J., Liebezeit, J., Rozzi, R., Goffinet, B,. (2014). First evidence of bryophyte diaspores in the plumage of transequatorial migrant birds. *PeerJ* 2:e424.

Lewis, L. R., Rozzi, R., Goffinet, B,. (2014). Direct long-distance dispersal shapes a New World amphitropical disjunction in the dispersal-limited dung moss *Tetraplodon* (Bryopsida: Splachnaceae). *Journal of Biogeography*, 41(12): 2385-2395.

Lewis Smith, R. I. (1984). Colonization and recovery by cryptogams following recent volcanic activity on Deception Island, South Shetland Islands. *British Antarctic Survey Bulletin*, 62: 25-51.

Lutsak, T., Fernández-Mendoza, F., Greshake, B., Dal Grande, F., Ebersberger, I., Ott, S., Printzen, C. (2016). Characterization of Microsatellite Loci in the Lichen-Forming Fungus *Cetraria aculeata* (*Parmeliaceae*, Ascomycota). *Applications in Plant Sciences,* 4 (9): 1600047.

McGaughran, A., Torricelli, G., Carapelli, A., et al. (2010). Contrasting phylogeographical paterns for springtails reflect different evolutionary histories between the Antarctic Peninsula and continental Antarctica. *Journal of Biogeography,* 37(1): 103–119.

Meirmans, P. G., & Van Tienderen, P.H. (2004). GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms, *Molecular Ecology Notes,* 4(4): 792-794.

Muñoz, J., Felicisimo, M., Cabezas, F., et al. (2004). Wind as a long-distance dispersal vehicle in the southern hemisphere. *Science*, 304(5674):1144-1147.

Nolan, L., Hogg, I. D., Stevens, M. I., Haase, M. (2006). Fine scale distribution of mtDNA haplotypes for the springtail *Gomphiocephalus hodgsoni* (Collembola) corresponds to an ancient shoreline in Taylor Valley, continental Antarctica. *Polar Biology*, 29(10):813-819.

Nordal, I. (1987). Tabula rasa after all? Botanical evidence for ice-free refugia in Scandinavia reviewed. *Journal of Biogeography* ,14(4): 377–388.

Otálora, M. A. G., Salvador, C., Martínez, I., Aragón, G. (2013). Does the reproductive strategy affect the transmission and genetic diversity of bionts in cyanolichens? A case study using two closely related species. *Microbial Ecology,* 65(2): 517–530.

Peakall, R. & Smouse P.E. (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6(1): 288-295.

Peakall, R. & Smouse, P. E. (2012). GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. *Bioinformatics,* 28(19): 2537-2539.

Printzen, C., Ekman, S., Tønsberg, T. (2003). Phylogeography of *Cavernularia hultenii*: evidence of slow genetic drift in a widely disjunct lichen. *Molecular Ecology,* 12(6): 1473–1486.

Pritchard, J. K., Stephens, M., Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics,* 155(2): 945–959.

Pugh, P. J. A., & Convey, P. (2008). Surviving out in the cold: Antarctic endemic invertebrates and their refugia. Journal of Biogeography 35(12): 2176–2186.

Rogers, A. D. (2007). Evolution and biodiversity of Antarctic organisms: a molecular perspective. *Phil Trans R Soc B,* 362(1488): 2191–2214.

Ruiz-Fernández, J., & Oliva, M. (2016). Relative paleoenvironmental adjustments following deglaciation of the Byers Peninsula (Livingston Island, Antarctica). *Arctic, Antarctic, and Alpine Research,* 48(2): 345–359.

Scheidegger, C., Bilovitz, P. O., Werth, S., Widmer, I., Mayrhofer, H. (2012). Hitchhiking with forests: population genetics of the epiphytic lichen *Lobaria pulmonaria* in primeval and managed forests in southeastern Europe. *Ecology and Evolution,* 2(9): 2223–2240.

Scher, H. D., & Martin, E. E. (2006). Timing and climatic consequences of the opening of Drake Passage. *Science,* 312(5772): 428–430.

Sérsic, A. N., Cosacov, A., Cocucci, A. A., Johnson, L. A., Pozner, R., Avila, L. J., Sites, J. W. Jr., Morando, M. (2011). Emerging phylogeographical patterns of plants and terrestrial vertebrates from Patagonia. *Biological Journal of the Linnean Society,* 103(2): 475–494.

Simms, A. R., Milliken, K. T., Anderson, J. B., Wellner, J. S. (2011). The marine record of deglaciation of the South Shetland Islands, Antarctica since the Last Glacial Maximum. *Quaternary Science Reviews,* 30(13-14): 1583–1601.

Skotnicki, M. L., Mackenzie, A. M., Ninham, J. A., Selkirk, P. M. (2004). High levels of genetic variability in the moss *Ceratodon purpureus* from continental Antarctica, subantarctic Heard and Macquarie Islands, and Australasia. *Polar Biology,* 27(11): 687–698.

Søchting, U., & Castello, M. (2012). The polar lichens *Caloplaca darbishirei* and *C. soropelta* highlight the direction of bipolar migration. *Polar Biology,* 35(8): 1143–1149.

Terauds, A., Chown, S. L., Morgan, F., et al. (2012). Conservation biogeography of the Antarctic. *Diversity Distributions*, 18(7): 726–741.

Tibell, L. B. (1994) Distribution patterns and dispersal strategies of Caliciales. *Bot J Linn Soc* 116(3): 159–202.

Turner, J., Colwell, S. R., Marshall, G. J., Lachlan-Cope, T. A., Carleton, A. M., Jones, P. D., Lagun, V., Reid, P. A., Iagovkina, S. (2005). Antarctic climate change during the last 50 years. *International Journal of Climatology*, 25(3): 279–294.

van de Wouw ,M., van Dijk, P., Huiskes, A. H. L. (2008). Regional genetic diversity patterns in Antarctic hairgrass (*Deschampsia antarctica* Desv.). *Journal of Biogeography*, 35(2): 365–376.

Wirtz, N., Printzen, C., Lumbsch, H. T. (2008). The Delimitation of Antarctic and Bipolar Species of Neuropogonoid *Usnea* (*Ascomycota, Lecanorales*): A Cohesion Approach of Species Recognition for the *Usnea Perpusilla* Complex. *Mycological Research*, 112(4):472–84.

Wirtz, N., Printzen, C., Lumbsch, H. T. (2012). Using Haplotype Networks, Estimation of Gene Flow and Phenotypic Characters to Understand Species Delimitation in Fungi of a Predominantly Antarctic *Usnea* Group (Ascomycota, Parmeliaceae*). Organisms Diversity and Evolution,* 12(1):17–37.

DATA ACCESSIBILITY

Three microsatellites datasets are deposited in Pangaea (https://issues.pangaea.de/browse/PDI-21600).

BIOSKETCH

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Author contributions: C.P. designed and coordinated the project. E.L. carried out the laboratory experiment and data analysis. F.D.G. carried out the MigrateN analysis. All the authors collected the samples. E.L. and C.P. wrote the manuscript. All authors read and approved the final version.

TABLES AND FIGURES

Table 1: Localities of *Cetraria aculeata*, *Usnea antarctica* and *U. aurantiacoatra* investigated in this study, number of individuals N, mean number of alleles Na, effective mean number of alleles Ne, mean number of private alleles P and Shannon information index H.

Cetraria aculeata					
Locality	N	Na	Ne	P	H
Argentina, Calafate	11	2.250 ± 0.366	1.691 ± 0.263	0.125 ± 0.125	0.534 ± 0.149
Argentina, Rio Gallegos	12	3.250 ± 0.726	2.301 ± 0.580	0.125 ± 0.125	0.789 ± 0.224
Chile, Pali Aike	20	4.000 ± 1.069	2.902 ± 0.961	0.375 ± 0.183	0.907± 0.247
Chile, Punta Arenas	25	4.250 ± 0.996	2.409 ± 0.674	0.375 ± 0.263	0.899 ± 0.193
Chile, Tierra del Fuego	23	4.250 ± 0.773	2.638 ± 0.548	0.375 ± 0.183	0.981 ± 0.210
Chile, Navarino	23	4.750 ± 0.977	2.771 ± 0.555	0.250 ± 0.164	1.081 ± 0.203
Falkland	19	3.500 ± 0.756	2.426 ± 0.657	0 ± 0	0.841 ± 0.194
Elephant Island	39	1.250 ± 0.250	1.014 ± 0.014	0 ± 0	0.031 ± 0.031
King George Island	51	3.375 ± 1.449	2.483 ± 1.073	0.500 ± 0.378	0.541 ± 0.325
Primavera Base	43	1.250 ± 0.164	1.018 ± 0.013	0 ± 0	0.037 ± 0.026
Usnea antarctica					
Locality	N	Na	Ne	P	H
Elephant Island	19	2.227 ± 0.246	1.547 ± 0.161	0.045 ± 0.045	0.43 ± 0.094
King George Island	100	4.409 ± 0.425	1.808 ± 0.216	0.818 ± 0.243	0.645 ± 0.103
Livingston Island	83	4.682 ± 0.485	1.954 ± 0.195	1.227 ± 0.394	0.765 ± 0.101
Deception Island	9	1.591 ± 0.107	1.238 ± 0.053	0 ± 0	0.262 ± 0.051
Primavera Base	68	3.591 ± 0.454	1.769 ± 0.187	0.318 ± 0.121	0.593 ± 0.111
Esperanza Base	91	3.136 ± 0.396	1.712 ± 0.215	0.227 ± 0.091	0.517 ± 0.115
Usnea aurantiacoatra					
Locality	N	Na	Ne	P	H
Chile, Torres del Paine	14	2.857 ± 0.210	1.899 ± 0.153	0 ± 0	0.722 ± 0.077
Chile, Monte Tarn	49	3.810 ± 0.496	2.141 ± 0.316	0 ± 0	0.718 ± 0.136
Chile, Navarino	74	7.476 ± 0.770	4.016 ± 0.415	0.762 ± 0.266	1.490 ± 0.104
Falkland 1	18	3.095 ± 0.337	1.970 ± 0.180	0.048 ± 0.048	0.742 ± 0.098
Falkland 2	18	4.00 ± 0.431	2.642 ± 0.294	0.190 ± 0.148	1.011± 0.106
Falkland 3	17	3.524 ± 0.394	2.280 ± 0.235	0.095 ± 0.066	0.847 ± 0.119
Elephant Island	18	3.238 ± 0.300	1.995 ± 0.213	0.095 ± 0.095	0.753 ± 0.098
King George Island	130	6.476 ± 0.635	2.449 ± 0.275	0.286 ± 0.101	1.037 ± 0.106
Livingston Island	77	7.238 ± 0.756	2.725 ± 0.349	0.857 ± 0.221	1.141 ± 0.117
Deception Island	26	3.381± 0.327	2.013 ± 0.165	0 ± 0	0.788 ± 0.089

Table 2: Test for clonal population structure performed in GenoDive. Species, number of samples N, expected (CE) and observed (CO) number of clones, percentual % of clones, probability P of observing this number of clones under random mating.

Species	N	C_E	C_O	%	P
Cetraria aculeata	266	210.734	130.000	51.128	0.001
Usnea antarctica	370	369.329	342.000	7.568	0.001
Usnea aurantiacoatra	441	441.000	441.000	0	**1.000**

Table 3: Symmetrical matrix of Jost's D index of genetic differentiation for each species.

Cetraria aculeata	Argentina, Calafate	Argentina, Rio Gallegos	Chile, Pali Aike	Chile, Punta Arenas	Chile, Tierra del Fuego	Chile, Navarino	Falkland	Elephant Island	King George Island	Primavera Base
Argentina, Calafate	0	0.115	0.08	0.18	0.106	0.091	0.084	0.136	0.415	0.447
Argentina, Rio Gallegos	0.115	0	0.052	0.012	0.039	0.094	0.003	0.107	0.277	0.392
Chile, Pali Aike	0.08	0.052	0	0.129	0.081	0.122	0.012	0.227	0.291	0.433
Chile, Punta Arenas	0.18	0.012	0.129	0	0.086	0.131	0.064	0.143	0.344	0.408
Chile, Tierra del Fuego	0.106	0.039	0.081	0.086	0	0.104	0.068	0.223	0.412	0.502
Chile, Navarino	0.091	0.094	0.122	0.131	0.104	0	0.068	0.254	0.359	0.356
Falkland	0.084	0.003	0.012	0.064	0.068	0.068	0	0.164	0.282	0.338
Elephant Island	0.136	0.107	0.227	0.143	0.223	0.254	0.164	0	0.419	0.486
King George Island	0.415	0.277	0.291	0.344	0.412	0.359	0.282	0.419	0	0.244
Primavera Base	0.447	0.392	0.433	0.408	0.502	0.356	0.338	0.486	0.244	0

Usnea antarctica	Elephant Island	King George Island	Livingston Island	Deception Island	Primavera Base	Esperanza Base
Elephant Island	0	0.007	0.069	0.263	0.04	0.034
King George Island	0.007	0	0.054	0.242	0.03	0.027
Livingston Island	0.069	0.054	0	0.206	0.095	0.074
Deception Island	0.263	0.242	0.206	0	0.241	0.259
Primavera Base	0.04	0.03	0.095	0.241	0	0.034
Esperanza Base	0.034	0.027	0.074	0.259	0.034	0

Usnea aurantiacoatra	Chile_Torres Del Paine	Chile_Mont Tarn	Chile_Navarino	Falkland 1	Falkland 2	Falkland 3	Elephant Island	King George Island	Livingston Island	Deception Island
Chile_Torres Del Paine	0	0.24	0.434	0.383	0.291	0.328	0.158	0.163	0.093	0.226
Chile_Mont Tarn	0.24	0	0.359	0.462	0.378	0.406	0.118	0.166	0.15	0.168
Chile_Navarino	0.434	0.359	0	0.495	0.436	0.477	0.375	0.356	0.372	0.373
Falkland 1	0.383	0.462	0.495	0	0.208	0.173	0.366	0.374	0.332	0.403
Falkland 2	0.291	0.378	0.436	0.208	0	0.104	0.261	0.313	0.215	0.312
Falkland 3	0.328	0.406	0.477	0.173	0.104	0	0.307	0.353	0.292	0.379
Elephant Island	0.158	0.118	0.375	0.366	0.261	0.307	0	0.021	0.059	0.065
King George Island	0.163	0.166	0.356	0.374	0.313	0.353	0.021	0	0.064	0.069
Livingston Island	0.093	0.15	0.372	0.332	0.215	0.292	0.059	0.064	0	0.075
Deception Island	0.226	0.168	0.373	0.403	0.312	0.379	0.065	0.069	0.075	0

Figure 1: Sampling localities of a. *Cetraria aculeata* (purple), b. *U. antarctica* (blue) and c. *Usnea aurantiacoatra* (black).

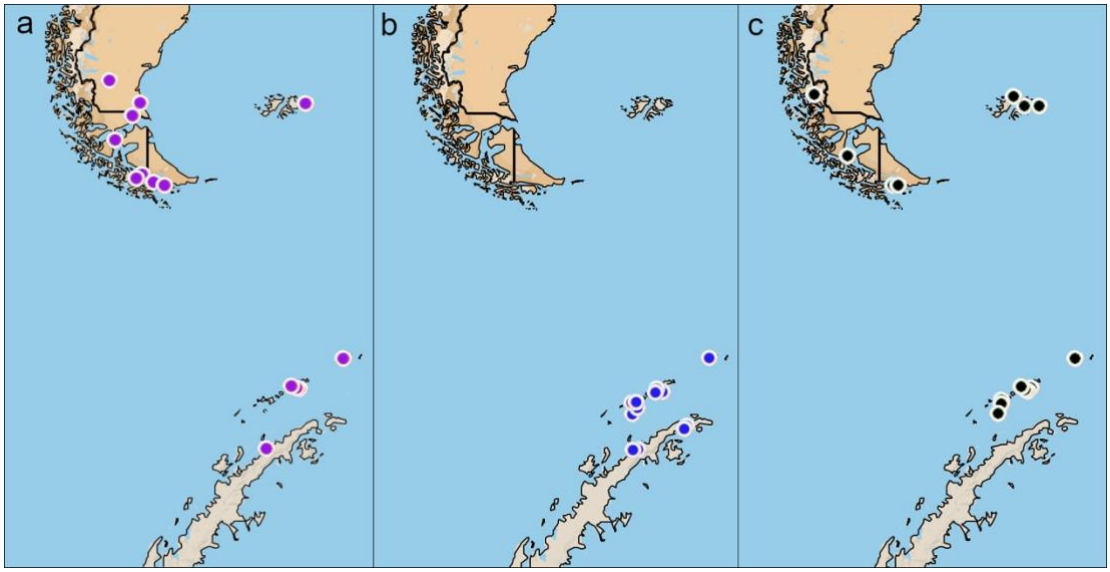


Figure 2: Assignment of individuals of the three species to gene pools obtained by Structure. Populations are sorted from North to South and separated with white dotted lines. The height of each colour in a bar corresponds to the estimated probability with which the individual belongs to the respective gene pool.

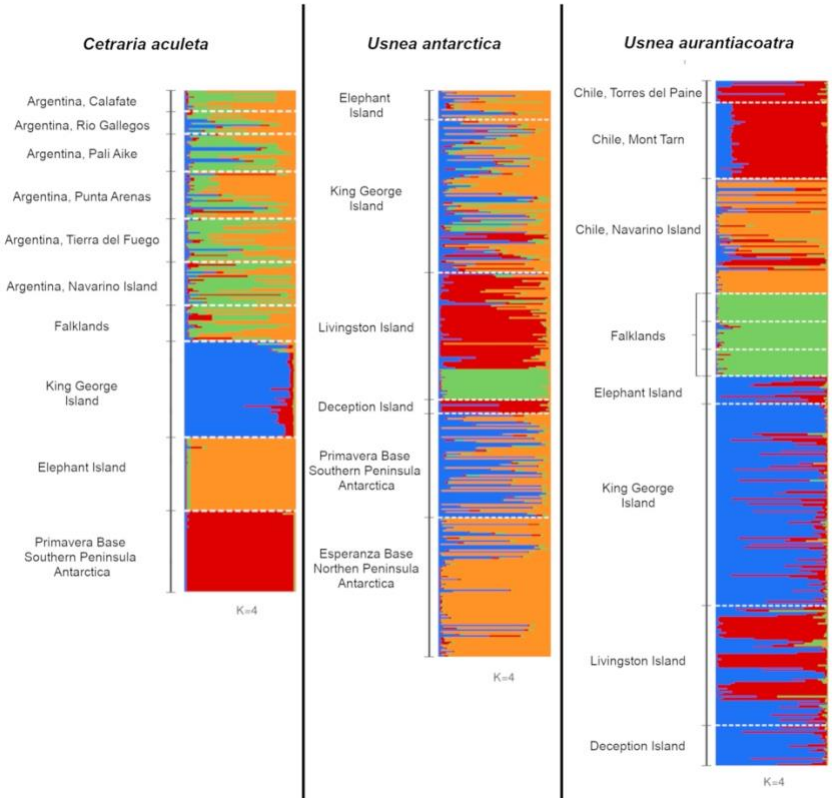
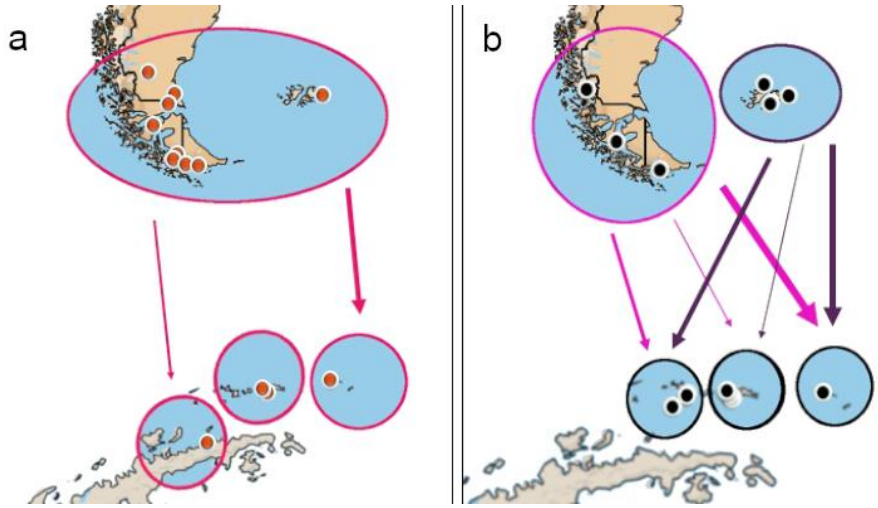


Figure 3: Schematic maps summarizing results from the MigrateN analysis. Circles represent regions between which migration was inferred, the width of the arrows is proportional to gene flow levels. a. *Cetraria aculeata*, migration calculated between South America/Falkland and three Antarctic regions. b. *Usnea aurantiacoatra*, migration calculated between South America, Falkland and three Antarctic regions.



Supporting information

Table S1: List of stands used in this study with the number of samples, collection localities, coordinates and date, the name of collector and the herbarium code.

Stand	Locality	Region (Migrate-N)	Species	Stand ID	Stand Size	Continent	Country/Region	Locality	Latitude (°)	Longitude (°)	Sampling Date	COLLECTOR(S)	Herbarium code
1	1	1	*Cetraria aculeata*	Argentina, Calafate	11	South America	Argentina	Calafate	-50.635833	-71.375270	2009	F. Fernandez-Mendoza	
2	2		*Cetraria aculeata*	Argentina, Rio Gallegos	12	South America	Argentina	Rio Gallegos	-51.614302	-69.301375	2010	F. Fernandez-Mendoza	
3	3		*Cetraria aculeata*	Chile, Pali Aike	20	South America	Argentina	Pali Aike	-52.168880	-69.790830	2008	F. Fernandez-Mendoza	
4	4		*Cetraria aculeata*	Chile, Punta Arenas	25	South America	Chile	Punta Arenas	-53.163830	-70.917060	November 23, 2009	F. Fernandez-Mendoza	
5	5		*Cetraria aculeata*	Chile, Tierra del Fuego	11	South America	Chile	Tierra del Fuego	-54.569880	-69.135080	2009	F. Fernandez-Mendoza	
6	6		*Cetraria aculeata*	Chile, Tierra del Fuego	12	South America	Chile	Tierra del Fuego	-54.675594	-69.440270	2009	F. Fernandez-Mendoza	
7			*Cetraria aculeata*	Chile, Navarino	11	South America	Chile	Navarino Island	-54.970950	-67.633400	January 29, 2017	C. Printzen & I. Starke-Ottich	FR-0264929
8			*Cetraria aculeata*	Chile, Navarino	12	South America	Chile	Navarino Island	-54.932500	-68.349720	2008	C. Printzen & I. Starke-Ottich	
9	7		*Cetraria aculeata*	Falkland	19	South America	Falkland	East of Stanley	-51.698166	-57.820416	2007	C. Printzen & I. Ottich	
10	8	2	*Cetraria aculeata*	Elephant Island 1	19	Antarctica	Elephant Island	Stinker Point	-61.221517	-55.367550	January 21, 2016	M. Andreev	FR-0264923
11			*Cetraria aculeata*	Elephant Island 2	20	Antarctica	Elephant Island	Stinker Point	-61.222228	-55.359683	February 8, 2016	M. Andreev	FR-0264924
12			*Cetraria aculeata*	King George Island 1	13	Antarctica	King George Island	Carlini	-62.237000	-58.658200	December 18, 2015	E. Lagostina & B. Kanz	FR-0264925
13	9	3	*Cetraria aculeata*	King George Island 2	17	Antarctica	King George Island	Carlini	-62.246389	-58.677750	2007	I. Ottich & P. Jordan Antarctica	
14			*Cetraria aculeata*	King George Island 3	21	Antarctica	King George Island	Fildes Peninsula	-62.190283	-58.926733	April 1, 2016	M. Andreev	FR-0264926
15	10	4	*Cetraria aculeata*	Primavera Base 1	23	Antarctica	Antarctic Peninsula	Primavera base	-64.093430	-60.565630	December 1, 2016	M. Scur	FR-0264927
16			*Cetraria aculeata*	Primavera Base 2	20	Antarctica	Antarctic Peninsula	Primavera base	-64.093430	-60.574260	December 3, 2016	M. Scur	FR-0264928
1	1		*Usnea antarctica*	Elephant Island	19	Antarctica	Elephant Island	Stinker Point	-61.222283	-55.359883	February 8, 2016	M. Andreev	FR-0264582
2			*Usnea antarctica*	King George Island 1	19	Antarctica	King George Island	Carlini	-62.237000	-58.658200	December 18, 2015	E. Lagostina & B. Kanz	FR-0264579
3			*Usnea antarctica*	King George Island 2	17	Antarctica	King George Island	Glacial Point	-62.239383	-58.653360	December 27, 2015	E. Lagostina & B. Kanz	FR-0264580
4	2		*Usnea antarctica*	King George Island 3	19	Antarctica	King George Island	Penguinera	-62.252900	-58.649516	December 22, 2015	E. Lagostina & B. Kanz	FR-0264581
5			*Usnea antarctica*	King George Island 4	19	Antarctica	King George Island	BellingShausen	-62.190260	-58.926733	April 8, 2016	M. Andreev	FR-0264583
6			*Usnea antarctica*	King George Island 5	7	Antarctica	King George Island	Field Pennsula	-62.205383	-58.954433	December 22, 2005	N. Wirtz	
7			*Usnea antarctica*	King George Island 6	19	Antarctica	King George Island	BellingShausen	-62.232000	-59.010200	February 15, 2018	M. Andreev	FR-0264896
8			*Usnea antarctica*	Livingston Island 1	18	Antarctica	Livingston Island	Caleta Argentina	-62.666900	-60.400900	February 23, 2018	C. Printzen	FR-0264897
9	3		*Usnea antarctica*	Livingston Island 2	5	Antarctica	Livingston Island	Reyna Sofia	-62.669100	-60.381030	February 24, 2018	C. Printzen	FR-0264898
10			*Usnea antarctica*	Livingston Island 3	20	Antarctica	Livingston Island	Sally Rock	-62.701390	-60.416390	February 27, 2018	C. Printzen	FR-0264899
11			*Usnea antarctica*	Livingston Island 4	20	Antarctica	Livingston Island	Punta Hanna	-62.650470	-60.602890	March 1, 2018	C. Printzen	FR-0264900
12			*Usnea antarctica*	Livingston Island 5	20	Antarctica	Livingston Island	Barnard Point	-62.751360	-60.330360	March 8, 2018	C. Printzen	FR-0264901
13	4		*Usnea antarctica*	Deception Island	9	Antarctica	Deception Island		-62.983333	-60.683333	February, 2002	B. Schroeter	
14	5		*Usnea antarctica*	Primavera Base 1	21	Antarctica	Antartic Peninsula	Primavera base	-64.095010	-60.565630	November 30, 2016	M. Scur	FR-0264902
15			*Usnea antarctica*	Primavera Base 2	25	Antarctica	Antartic Peninsula	Primavera base	-64.092280	-60.371320	November 26&29, 2016	M. Scur	FR-0264903
16			*Usnea antarctica*	Primavera Base 3	22	Antarctica	Antartic Peninsula	Primavera base	-64.092150	-60.571960	January 5, 2017	M. Scur	FR-0264904
17	6		*Usnea antarctica*	Esperanza Base 1	27	Antarctica	Antartic Peninsula	Esperanza base	-63.401330	-56.990083	January 13, 2017	A. Lorenz	FR-0264905
18			*Usnea antarctica*	Esperanza Base 2	24	Antarctica	Antartic Peninsula	Esperanza base	-63.409027	-57.013610	January 18, 2017	A. Lorenz	FR-0264906
19			*Usnea antarctica*	Esperanza Base 3	21	Antarctica	Antartic Peninsula	Esperanza base	-63.407220	-57.018250	January 15, 2017	A. Lorenz	FR-0264907
20			*Usnea antarctica*	Esperanza Base 4	19	Antarctica	Antartic Peninsula	Esperanza base	-63.413750	-57.04150	January 15, 2017	A. Lorenz	FR-0264908
1	1		*Usnea aurantiacoatra*	Chile, Torres Del Paine	14	South America	Chile	Torres del Paine	-51.211300	-73.256700	January 30, 2018	C. Printzen & C. Ivanovich	FR-0264919
2	2		*Usnea aurantiacoatra*	Chile, Monte Tarn	49	South America	Chile	Mount Tarn	-53.752000	-71.023500	February 1, 2017	C. Printzen	FR-0264917
3		1	*Usnea aurantiacoatra*	Chile, Navarino 1	20	South America	Chile	Navarino Island	-54.970483	-67.635766	January 30, 2017	C. Printzen & I. Starke-Ottich	FR-0264915
4	3		*Usnea aurantiacoatra*	Chile, Navarino 2	35	South America	Chile	Navarino Island	-54.977916	-67.649550	February 1, 2017	C. Printzen & I. Starke-Ottich	FR-0264916
5			*Usnea aurantiacoatra*	Chile, Navarino 3	19	South America	Chile	Navarino Island	-54.975583	-67.630500	February 15, 2017	C. Printzen & I. Starke-Ottich	FR-0264918
6	4		*Usnea aurantiacoatra*	Falkland 1	18	South America	Falkland	Gipsy point	-51.676282	-57.808785	January 28, 2018	U. Ruprecht & U. Sachting	FR-0264920
7	5	2	*Usnea aurantiacoatra*	Falkland 2	18	South America	Falkland	Mt Usborne	-51.712790	-58.853037	January 30, 2018	U. Ruprecht & U. Sachting	FR-0264921
8	6		*Usnea aurantiacoatra*	Falkland 3	17	South America	Falkland	Pebble Island	-51.306922	-59.615442	February 5, 2018	U. Ruprecht & U. Sachting	FR-0264922
9	7	3	*Usnea aurantiacoatra*	Elephant Island	18	Antarctica	Elephant Island	Stinker Point	-61.222283	-55.359683	February 4, 2016	M. Andreev	FR-0264587
10			*Usnea aurantiacoatra*	King George Island 1	19	Antarctica	King George Island	Carlini Station	-62.237000	-58.658200	December 18, 2015	E. Lagostina & B. Kanz	FR-0264584
11			*Usnea aurantiacoatra*	King George Island 2	21	Antarctica	King George Island	Glacial point	-62.239383	-58.653360	December 27, 2015	E. Lagostina & B. Kanz	FR-0264585
12			*Usnea aurantiacoatra*	King George Island 3	18	Antarctica	King George Island	Penguinera	-62.252900	-58.649516	December 22, 2015	E. Lagostina & B. Kanz	FR-0264586
13	8	4	*Usnea aurantiacoatra*	King George Island 4	19	Antarctica	King George Island	King Sejong Station	-62.216600	-58.783330	December 16, 2015	E. Lagostina & B. Kanz	FR-0264909
14			*Usnea aurantiacoatra*	King George Island 5	15	Antarctica	King George Island	Bellingshausen	-62.203616	-58.992750	April 8, 2016	M. Andreev	FR-0264588
15			*Usnea aurantiacoatra*	King George Island 6	15	Antarctica	King George Island	Bellingshausen	-62.203616	-58.963883	April 18, 2016	M. Andreev	FR-0264589
16			*Usnea aurantiacoatra*	King George Island 7	8	Antarctica	King George Island	Fildes Peninsula	-62.205383	-58.954433	December 22, 2005	N. Wirtz	
17			*Usnea aurantiacoatra*	King George Island 8	15	Antarctica	King George Island	Bellingshausen	-62.185230	-58.972610	January 27, 2018	M. Andreev	FR-0264910
18	9		*Usnea aurantiacoatra*	Deception Island	26	Antarctica	Deception Island		-62.983333	-60.683333	February, 2002	B. Schroeter	
19			*Usnea aurantiacoatra*	Livingston Island 1	21	Antarctica	Livingston Island	Nunatak	-62.681020	-60.344190	March 3, 2018	F. Grewe	FR-0264911
20	10	5	*Usnea aurantiacoatra*	Livingston Island 2	18	Antarctica	Livingston Island	Punta Hesperedes	-62.643260	-60.372500	March 6, 2018	F. Grewe	FR-0264912
21			*Usnea aurantiacoatra*	Livingston Island 3	22	Antarctica	Livingston Island	Mt Reyna Sofia	-62.666988	-60.400966	February 24, 2018	C. Printzen	FR-0264913
22			*Usnea aurantiacoatra*	Livingston Island 4	16	Antarctica	Livingston Island	Sally Rocks	-62.66910	-60.381030	February 27, 2018	C. Printzen	FR-0264914

Figure S2: Results of DAPC based on merged *Usnea antarctica* and *U. aurantiacoatra* datasets to check species delimitation. Number of retained principal components = 50 and number of groups K = 2. Left: DAPC density graph on discriminant function 1. The blue cluster comprises samples of *U. aurantiacoatra*, the red one samples of *U. antarctica*. Right: Assignment of samples to clusters for each population.

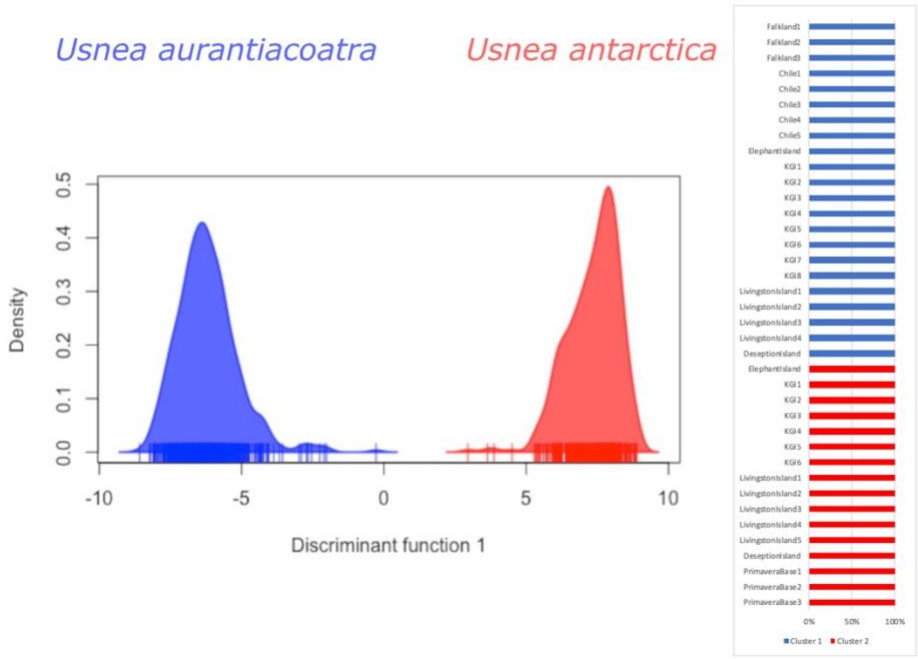


Table S3: Assigned clone for every individual, testing the probability of finding the observed clonal diversity under random mating.

Cetraria aculeata				*Usnea antarctica*				*Usnea aurantiacoatra*			
n°	Samples	Population	Clone	n°	Samples	Population	Clone	n°	Samples	Population	Clone
1	1857	Argentina, Calafate	1	1	EL375	Elephant IslandIsland	1	1	EL1162	Chile, Torres Del Paine	1
2	1858	Argentina, Calafate	2	2	EL376	Elephant IslandIsland	2	2	EL1163	Chile, Torres Del Paine	2
3	1859	Argentina, Calafate	2	3	EL377	Elephant IslandIsland	3	3	EL1165	Chile, Torres Del Paine	3
4	1860	Argentina, Calafate	3	4	EL378	Elephant IslandIsland	4	4	EL1167	Chile, Torres Del Paine	4
5	1861	Argentina, Calafate	2	5	EL379	Elephant IslandIsland	5	5	EL1170	Chile, Torres Del Paine	5
6	1862	Argentina, Calafate	2	6	EL380	Elephant IslandIsland	6	6	EL1172	Chile, Torres Del Paine	6
7	1863	Argentina, Calafate	4	7	EL381	Elephant IslandIsland	7	7	EL1174	Chile, Torres Del Paine	7
8	1866	Argentina, Calafate	5	8	EL382	Elephant IslandIsland	8	8	EL1176	Chile, Torres Del Paine	8
9	1868	Argentina, Calafate	6	9	EL383	Elephant IslandIsland	9	9	EL1178	Chile, Torres Del Paine	9
10	1870	Argentina, Calafate	7	10	EL384	Elephant IslandIsland	10	10	EL1182	Chile, Torres Del Paine	10
11	1872	Argentina, Calafate	8	11	EL385	Elephant IslandIsland	11	11	EL1183	Chile, Torres Del Paine	11
12	2186	Argentina, Rio Gallegos	9	12	EL387	Elephant IslandIsland	10	12	EL1188	Chile, Torres Del Paine	12
13	2187	Argentina, Rio Gallegos	10	13	EL388	Elephant IslandIsland	1	13	EL1196	Chile, Torres Del Paine	13
14	2188	Argentina, Rio Gallegos	11	14	EL389	Elephant IslandIsland	12	14	EL1197	Chile, Torres Del Paine	14
15	2189	Argentina, Rio Gallegos	12	15	EL390	Elephant IslandIsland	13	15	EL0889	Chile, Mont Tarn	15
16	2190	Argentina, Rio Gallegos	13	16	EL391	Elephant IslandIsland	14	16	EL0890	Chile, Mont Tarn	16
17	2191	Argentina, Rio Gallegos	14	17	EL392	Elephant IslandIsland	1	17	EL0891	Chile, Mont Tarn	17
18	2192	Argentina, Rio Gallegos	15	18	EL393	Elephant IslandIsland	15	18	EL0892	Chile, Mont Tarn	18
19	2193	Argentina, Rio Gallegos	16	19	EL394	Elephant IslandIsland	16	19	EL0893	Chile, Mont Tarn	19
20	2194	Argentina, Rio Gallegos	17	20	EL01	King George Island	17	20	EL0894	Chile, Mont Tarn	20
21	2195	Argentina, Rio Gallegos	18	21	EL02	King George Island	18	21	EL0896	Chile, Mont Tarn	21
22	2196	Argentina, Rio Gallegos	19	22	EL03	King George Island	19	22	EL0897	Chile, Mont Tarn	22
23	2197	Argentina, Rio Gallegos	20	23	EL04	King George Island	20	23	EL0898	Chile, Mont Tarn	23
24	1374	Chile, Pali Aike	21	24	EL05	King George Island	21	24	EL0899	Chile, Mont Tarn	24
25	1375	Chile, Pali Aike	22	25	EL06	King George Island	22	25	EL0900	Chile, Mont Tarn	25
26	1376	Chile, Pali Aike	23	26	EL07	King George Island	23	26	EL0901	Chile, Mont Tarn	26
27	1377	Chile, Pali Aike	24	27	EL08	King George Island	24	27	EL0902	Chile, Mont Tarn	27
28	1378	Chile, Pali Aike	25	28	EL09	King George Island	25	28	EL0903	Chile, Mont Tarn	28
29	1379	Chile, Pali Aike	26	29	EL10	King George Island	26	29	EL0904	Chile, Mont Tarn	29
30	1380	Chile, Pali Aike	27	30	EL11	King George Island	27	30	EL0905	Chile, Mont Tarn	30
31	1381	Chile, Pali Aike	28	31	EL12	King George Island	28	31	EL0906	Chile, Mont Tarn	31
32	1382	Chile, Pali Aike	29	32	EL13	King George Island	29	32	EL0907	Chile, Mont Tarn	32
33	1383	Chile, Pali Aike	30	33	EL14	King George Island	30	33	EL0908	Chile, Mont Tarn	33
34	1384	Chile, Pali Aike	31	34	EL15	King George Island	31	34	EL0909	Chile, Mont Tarn	34
35	1385	Chile, Pali Aike	25	35	EL17	King George Island	32	35	EL0910	Chile, Mont Tarn	35
36	1386	Chile, Pali Aike	22	36	EL18	King George Island	33	36	EL0911	Chile, Mont Tarn	36
37	1387	Chile, Pali Aike	21	37	EL19	King George Island	34	37	EL0912	Chile, Mont Tarn	37
38	1388	Chile, Pali Aike	32	38	EL20	King George Island	35	38	EL0913	Chile, Mont Tarn	38
39	1389	Chile, Pali Aike	33	39	EL22	King George Island	36	39	EL0914	Chile, Mont Tarn	39
40	1390	Chile, Pali Aike	34	40	EL23	King George Island	37	40	EL0915	Chile, Mont Tarn	40
41	1391	Chile, Pali Aike	35	41	EL24	King George Island	38	41	EL0916	Chile, Mont Tarn	41
42	1392	Chile, Pali Aike	36	42	EL25	King George Island	39	42	EL0917	Chile, Mont Tarn	42
43	1393	Chile, Pali Aike	37	43	EL26	King George Island	40	43	EL0918	Chile, Mont Tarn	43
44	171-2463	Chile, Punta Arenas	38	44	EL27	King George Island	41	44	EL0919	Chile, Mont Tarn	44
45	171-2465	Chile, Punta Arenas	39	45	EL28	King George Island	42	45	EL0920	Chile, Mont Tarn	45
46	171-2467	Chile, Punta Arenas	40	46	EL29	King George Island	43	46	EL0921	Chile, Mont Tarn	46
47	171-2468	Chile, Punta Arenas	41	47	EL30	King George Island	44	47	EL0922	Chile, Mont Tarn	47
48	171-2469	Chile, Punta Arenas	41	48	EL32	King George Island	45	48	EL0923	Chile, Mont Tarn	48
49	171-2470	Chile, Punta Arenas	40	49	EL33	King George Island	46	49	EL0924	Chile, Mont Tarn	49
50	171-2471	Chile, Punta Arenas	42	50	EL34	King George Island	47	50	EL0925	Chile, Mont Tarn	50
51	171-2472	Chile, Punta Arenas	43	51	EL35	King George Island	48	51	EL0926	Chile, Mont Tarn	51
52	171-2473	Chile, Punta Arenas	44	52	EL37	King George Island	49	52	EL0927	Chile, Mont Tarn	52
53	171-2474	Chile, Punta Arenas	43	53	EL38	King George Island	50	53	EL0928	Chile, Mont Tarn	53
54	171-2476	Chile, Punta Arenas	45	54	EL39	King George Island	51	54	EL0929	Chile, Mont Tarn	54
55	171-2477	Chile, Punta Arenas	38	55	EL40	King George Island	52	55	EL0930	Chile, Mont Tarn	55
56	171-2478	Chile, Punta Arenas	46	56	EL42	King George Island	53	56	EL0931	Chile, Mont Tarn	56
57	171-2479	Chile, Punta Arenas	47	57	EL43	King George Island	54	57	EL0932	Chile, Mont Tarn	57
58	173-2483	Chile, Punta Arenas	48	58	EL44	King George Island	55	58	EL0933	Chile, Mont Tarn	58
59	173-2484	Chile, Punta Arenas	49	59	EL45	King George Island	56	59	EL0934	Chile, Mont Tarn	59
60	173-2485	Chile, Punta Arenas	50	60	EL46	King George Island	57	60	EL0935	Chile, Mont Tarn	60
61	173-2486	Chile, Punta Arenas	51	61	EL47	King George Island	58	61	EL0936	Chile, Mont Tarn	61
62	173-2487	Chile, Punta Arenas	52	62	EL48	King George Island	59	62	EL0937	Chile, Mont Tarn	62
63	173-2488	Chile, Punta Arenas	53	63	EL49	King George Island	60	63	EL0938	Chile, Mont Tarn	63
64	173-2489	Chile, Punta Arenas	54	64	EL50	King George Island	61	64	EL0829	Chile, Navarino	64
65	173-2490	Chile, Punta Arenas	55	65	EL51	King George Island	62	65	EL0830	Chile, Navarino	65
66	173-2490b	Chile, Punta Arenas	56	66	EL52	King George Island	63	66	EL0831	Chile, Navarino	66
67	173-2490c	Chile, Punta Arenas	57	67	EL53	King George Island	64	67	EL0832	Chile, Navarino	67
68	173-2490d	Chile, Punta Arenas	58	68	EL54	King George Island	65	68	EL0833	Chile, Navarino	68
69	2169	Chile, Tierra del Fuego	59	69	EL56	King George Island	66	69	EL0834	Chile, Navarino	69
70	2170	Chile, Tierra del Fuego	60	70	EL57	King George Island	67	70	EL0835	Chile, Navarino	70
71	2171	Chile, Tierra del Fuego	61	71	EL59	King George Island	68	71	EL0836	Chile, Navarino	71
72	2172	Chile, Tierra del Fuego	62	72	EL60	King George Island	69	72	EL0837	Chile, Navarino	72
73	2173	Chile, Tierra del Fuego	63	73	EL61	King George Island	70	73	EL0838	Chile, Navarino	73
74	2174	Chile, Tierra del Fuego	63	74	EL62	King George Island	71	74	EL0839	Chile, Navarino	74
75	2175	Chile, Tierra del Fuego	64	75	EL395	King George Island	72	75	EL0840	Chile, Navarino	75
76	2177	Chile, Tierra del Fuego	65	76	EL396	King George Island	73	76	EL0841	Chile, Navarino	76
77	2178	Chile, Tierra del Fuego	66	77	EL397	King George Island	74	77	EL0842	Chile, Navarino	77
78	2179	Chile, Tierra del Fuego	67	78	EL398	King George Island	75	78	EL0843	Chile, Navarino	78
79	2180	Chile, Tierra del Fuego	66	79	EL399	King George Island	76	79	EL0844	Chile, Navarino	79
80	2142	Chile, Tierra del Fuego	68	80	EL400	King George Island	77	80	EL0845	Chile, Navarino	80
81	2143	Chile, Tierra del Fuego	69	81	EL401	King George Island	78	81	EL0846	Chile, Navarino	81
82	2144	Chile, Tierra del Fuego	70	82	EL402	King George Island	79	82	EL0847	Chile, Navarino	82
83	2145	Chile, Tierra del Fuego	71	83	EL403	King George Island	47	83	EL0848	Chile, Navarino	83
84	2146	Chile, Tierra del Fuego	72	84	EL404	King George Island	80	84	EL0859	Chile, Navarino	84
85	2147	Chile, Tierra del Fuego	73	85	EL405	King George Island	81	85	EL0860	Chile, Navarino	85
86	2148	Chile, Tierra del Fuego	74	86	EL406	King George Island	82	86	EL0861	Chile, Navarino	86
87	2149	Chile, Tierra del Fuego	75	87	EL407	King George Island	83	87	EL0862	Chile, Navarino	87
88	2150	Chile, Tierra del Fuego	76	88	EL408	King George Island	84	88	EL0863	Chile, Navarino	88
89	2151	Chile, Tierra del Fuego	77	89	EL409	King George Island	85	89	EL0864	Chile, Navarino	89
90	2152	Chile, Tierra del Fuego	78	90	EL410	King George Island	86	90	EL0865	Chile, Navarino	90
91	2153	Chile, Tierra del Fuego	79	91	EL411	King George Island	87	91	EL0866	Chile, Navarino	91
92	EL1323	Chile, Navarino	80	92	EL412	King George Island	88	92	EL0867	Chile, Navarino	92
93	EL1324	Chile, Navarino	81	93	EL413	King George Island	89	93	EL0869	Chile, Navarino	93
94	EL1327	Chile, Navarino	82	94	S09	King George Island	90	94	EL0870	Chile, Navarino	94
95	EL1329	Chile, Navarino	83	95	S10	King George Island	91	95	EL0871	Chile, Navarino	95
96	EL1330	Chile, Navarino	84	96	S11	King George Island	92	96	EL0872	Chile, Navarino	96
97	EL1332	Chile, Navarino	85	97	S13	King George Island	93	97	EL0873	Chile, Navarino	97
98	EL1333	Chile, Navarino	84	98	S15	King George Island	94	98	EL0874	Chile, Navarino	98
99	EL1336	Chile, Navarino	86	99	s18	King George Island	95	99	EL0876	Chile, Navarino	99
100	EL1337	Chile, Navarino	87	100	s19	King George Island	96	100	EL0879	Chile, Navarino	100
101	EL1338	Chile, Navarino	68	101	EL1721	King George Island	97	101	EL0939	Chile, Navarino	101
102	EL1341	Chile, Navarino	88	102	EL1722	King George Island	98	102	EL0940	Chile, Navarino	102
103	1877	Chile, Navarino	89	103	EL1723	King George Island	99	103	EL0941	Chile, Navarino	103
104	1878	Chile, Navarino	90	104	EL1724	King George Island	100	104	EL0942	Chile, Navarino	104
105	1879	Chile, Navarino	90	105	EL1725	King George Island	101	105	EL0943	Chile, Navarino	105
106	1880	Chile, Navarino	91	106	EL1726	King George Island	102	106	EL0944	Chile, Navarino	106
107	1881	Chile, Navarino	92	107	EL1727	King George Island	103	107	EL0945	Chile, Navarino	107
108	1882	Chile, Navarino	93	108	EL1728	King George Island	104	108	EL0946	Chile, Navarino	108
109	1883	Chile, Navarino	94	109	EL1729	King George Island	105	109	EL0947	Chile, Navarino	109
110	1884	Chile, Navarino	95	110	EL1731	King George Island	106	110	EL0948	Chile, Navarino	110
111	1885	Chile, Navarino	96	111	EL1732	King George Island	107	111	EL0949	Chile, Navarino	111
112	1886	Chile, Navarino	97	112	EL1733	King George Island	108	112	EL0950	Chile, Navarino	112
113	1887	Chile, Navarino	98	113	EL1734	King George Island	109	113	EL0951	Chile, Navarino	113
114	1888	Chile, Navarino	99	114	EL1735	King George Island	110	114	EL0952	Chile, Navarino	114
115	1091	Falklands	100	115	EL1736	King George Island	111	115	EL0953	Chile, Navarino	115
116	1092	Falklands	101	116	EL1737	King George Island	112	116	EL0954	Chile, Navarino	116
117	1093	Falklands	102	117	EL1738	King George Island	113	117	EL0957	Chile, Navarino	117
118	1094	Falklands	103	118	EL1739	King George Island	100	118	EL0958	Chile, Navarino	118
119	1095	Falklands	104	119	EL1740	King George Island	114	119	EL0804	Chile, Navarino	119
120	1121	Falklands	105	120	EL1424	Livingston Island	115	120	EL0805	Chile, Navarino	120
121	1122	Falklands	105	121	EL1427	Livingston Island	116	121	EL0806	Chile, Navarino	121
122	1123	Falklands	105	122	EL1428	Livingston Island	117	122	EL0807	Chile, Navarino	122
123	1124	Falklands	102	123	EL1429	Livingston Island	118	123	EL0808	Chile, Navarino	123
124	1125	Falklands	106	124	EL1430	Livingston Island	118	124	EL0809	Chile, Navarino	124
125	1127	Falklands	107	125	EL1431	Livingston Island	119	125	EL0810	Chile, Navarino	125
126	1128	Falklands	108	126	EL1432	Livingston Island	120	126	EL0811	Chile, Navarino	126
127	1129	Falklands	109	127	EL1433	Livingston Island	121	127	EL0812	Chile, Navarino	127
128	1130	Falklands	110	128	EL1434	Livingston Island	122	128	EL0813	Chile, Navarino	128
129	1131	Falklands	111	129	EL1435	Livingston Island	123	129	EL0814	Chile, Navarino	129
130	1132	Falklands	112	130	EL1436	Livingston Island	124	130	EL0815	Chile, Navarino	130
131	1133	Falklands	112	131	EL1437	Livingston Island	125	131	EL0816	Chile, Navarino	131
132	1134	Falklands	113	132	EL1438	Livingston Island	126	132	EL0817	Chile, Navarino	132
133	1135	Falklands	104	133	EL1439	Livingston Island	127	133	EL0818	Chile, Navarino	133
134	EL1233	Elephant Island	5	134	EL1440	Livingston Island	128	134	EL0819	Chile, Navarino	134
135	EL1234	Elephant Island	5	135	EL1441	Livingston Island	129	135	EL0820	Chile, Navarino	135
136	EL1236	Elephant Island	5	136	EL1442	Livingston Island	130	136	EL0821	Chile, Navarino	136
137	EL1237	Elephant Island	5	137	EL1443	Livingston Island	131	137	EL0823	Chile, Navarino	137
138	EL1238	Elephant Island	5	138	EL1453	Livingston Island	132	138	EL1017	Falkland 1	138
139	EL1239	Elephant Island	5	139	EL1460	Livingston Island	133	139	EL1018	Falkland 1	139
140	EL1240	Elephant Island	5	140	EL1461	Livingston Island	134	140	EL1019	Falkland 1	140
141	EL1241	Elephant Island	5	141	EL1462	Livingston Island	135	141	EL1020	Falkland 1	141
142	EL1242	Elephant Island	5	142	EL1463	Livingston Island	136	142	EL1021	Falkland 1	142
143	EL1243	Elephant Island	5	143	EL1464	Livingston Island	137	143	EL1023	Falkland 1	143
144	EL1244	Elephant Island	5	144	EL1465	Livingston Island	138	144	EL1024	Falkland 1	144
145	EL1245	Elephant Island	5	145	EL1466	Livingston Island	139	145	EL1025	Falkland 1	145
146	EL1246	Elephant Island	5	146	EL1467	Livingston Island	140	146	EL1026	Falkland 1	146
147	EL1247	Elephant Island	5	147	EL1468	Livingston Island	141	147	EL1027	Falkland 1	147
148	EL1248	Elephant Island	5	148	EL1469	Livingston Island	142	148	EL1028	Falkland 1	148
149	EL1249	Elephant Island	5	149	EL1470	Livingston Island	143	149	EL1029	Falkland 1	149
150	EL1250	Elephant Island	5	150	EL1471	Livingston Island	144	150	EL1030	Falkland 1	150
151	EL1251	Elephant Island	5	151	EL1472	Livingston Island	145	151	EL1031	Falkland 1	151
152	EL1252	Elephant Island	5	152	EL1473	Livingston Island	146	152	EL1032	Falkland 1	152
153	EL1253	Elephant Island	5	153	EL1474	Livingston Island	146	153	EL1033	Falkland 1	153
154	EL1254	Elephant Island	5	154	EL1475	Livingston Island	147	154	EL1035	Falkland 1	154
155	EL1255	Elephant Island	5	155	EL1476	Livingston Island	148	155	EL1036	Falkland 1	155
156	EL1256	Elephant Island	5	156	EL1477	Livingston Island	149	156	EL1040	Falkland 2	156
157	EL1257	Elephant Island	5	157	EL1478	Livingston Island	150	157	EL1042	Falkland 2	157
158	EL1258	Elephant Island	5	158	EL1479	Livingston Island	151	158	EL1044	Falkland 2	158
159	EL1259	Elephant Island	5	159	EL1480	Livingston Island	152	159	EL1045	Falkland 2	159
160	EL1260	Elephant Island	5	160	EL1481	Livingston Island	153	160	EL1046	Falkland 2	160
161	EL1261	Elephant Island	5	161	EL1482	Livingston Island	154	161	EL1047	Falkland 2	161
162	EL1262	Elephant Island	5	162	EL1483	Livingston Island	155	162	EL1049	Falkland 2	162
163	EL1263	Elephant Island	5	163	EL1484	Livingston Island	156	163	EL1050	Falkland 2	163
164	EL1264	Elephant Island	5	164	EL1485	Livingston Island	157	164	EL1052	Falkland 2	164
165	EL1265	Elephant Island	5	165	EL1486	Livingston Island	158	165	EL1053	Falkland 2	165
166	EL1266	Elephant Island	5	166	EL1487	Livingston Island	159	166	EL1054	Falkland 2	166
167	EL1267	Elephant Island	5	167	EL1488	Livingston Island	160	167	EL1055	Falkland 2	167
168	EL1268	Elephant Island	5	168	EL1489	Livingston Island	161	168	EL1057	Falkland 2	168
169	EL1269	Elephant Island	5	169	EL1490	Livingston Island	162	169	EL1058	Falkland 2	169
170	EL1270	Elephant Island	5	170	EL1491	Livingston Island	163	170	EL1059	Falkland 2	170
171	EL1271	Elephant Island	5	171	EL1492	Livingston Island	164	171	EL1060	Falkland 2	171
172	EL1272	Elephant Island	5	172	EL1493	Livingston Island	163	172	EL1061	Falkland 2	172
173	EL1198	King George Island	114	173	EL1494	Livingston Island	164	173	EL1063	Falkland 2	173
174	EL1199	King George Island	115	174	EL1495	Livingston Island	165	174	EL1064	Falkland 3	174
175	EL1200	King George Island	116	175	EL1496	Livingston Island	166	175	EL1065	Falkland 3	175
176	EL1201	King George Island	117	176	EL1497	Livingston Island	167	176	EL1066	Falkland 3	176
177	EL1203	King George Island	118	177	EL1498	Livingston Island	168	177	EL1067	Falkland 3	177
178	EL1204	King George Island	119	178	EL1499	Livingston Island	169	178	EL1069	Falkland 3	178
179	EL1205	King George Island	120	179	EL1500	Livingston Island	170	179	EL1070	Falkland 3	179
180	EL1206	King George Island	117	180	EL1501	Livingston Island	171	180	EL1072	Falkland 3	180
181	EL1207	King George Island	121	181	EL1502	Livingston Island	172	181	EL1074	Falkland 3	181
182	EL1208	King George Island	117	182	EL1503	Livingston Island	173	182	EL1076	Falkland 3	182
183	EL1209	King George Island	120	183	EL1547	Livingston Island	174	183	EL1077	Falkland 3	183
184	EL1210	King George Island	120	184	EL1548	Livingston Island	175	184	EL1078	Falkland 3	184
185	EL1211	King George Island	120	185	EL1549	Livingston Island	176	185	EL1079	Falkland 3	185
186	1086	King George Island	122	186	EL1550	Livingston Island	177	186	EL1083	Falkland 3	186
187	1087	King George Island	122	187	EL1551	Livingston Island	178	187	EL1085	Falkland 3	187
188	1088	King George Island	123	188	EL1552	Livingston Island	179	188	EL1086	Falkland 3	188
189	1089	King George Island	124	189	EL1553	Livingston Island	180	189	EL1087	Falkland 3	189
190	1090	King George Island	125	190	EL1554	Livingston Island	181	190	EL1089	Falkland 3	190
191	1101	King George Island	125	191	EL1555	Livingston Island	182	191	EL416	Elephant Island	191
192	1103	King George Island	122	192	EL1556	Livingston Island	182	192	EL417	Elephant Island	192
193	1104	King George Island	124	193	EL1557	Livingston Island	183	193	EL418	Elephant Island	193
194	1106	King George Island	122	194	EL1558	Livingston Island	184	194	EL419	Elephant Island	194
195	1107	King George Island	125	195	EL1559	Livingston Island	185	195	EL420	Elephant Island	195
196	1108	King George Island	123	196	EL1560	Livingston Island	186	196	EL421	Elephant Island	196
197	1110	King George Island	126	197	EL1561	Livingston Island	187	197	EL422	Elephant Island	197
198	1111	King George Island	127	198	EL1562	Livingston Island	188	198	EL423	Elephant Island	198
199	1112	King George Island	117	199	EL1563	Livingston Island	189	199	EL424	Elephant Island	199
200	1113	King George Island	122	200	EL1564	Livingston Island	190	200	EL425	Elephant Island	200
201	1114	King George Island	122	201	EL1565	Livingston Island	191	201	EL427	Elephant Island	201
202	1115	King George Island	125	202	EL1566	Livingston Island	192	202	EL428	Elephant Island	202
203	EL1212	King George Island	127	203	144-01a	Deception Island	193	203	EL429	Elephant Island	203
204	EL1213	King George Island	122	204	144-01b	Deception Island	194	204	EL430	Elephant Island	204
205	EL1214	King George Island	127	205	144-02	Deception Island	195	205	EL431	Elephant Island	205
206	EL1215	King George Island	122	206	144-03	Deception Island	196	206	EL432	Elephant Island	206
207	EL1216	King George Island	122	207	144-06	Deception Island	197	207	EL433	Elephant Island	207
208	EL1217	King George Island	127	208	144-07	Deception Island	198	208	EL434	Elephant Island	208
209	EL1218	King George Island	127	209	144-09	Deception Island	199	209	EL63	King George Island	209
210	EL1219	King George Island	127	210	144-10	Deception Island	198	210	EL64	King George Island	210
211	EL1220	King George Island	127	211	144-11	Deception Island	196	211	EL65	King George Island	211
212	EL1221	King George Island	127	212	EL0602	Primavera Base	200	212	EL66	King George Island	212
213	EL1222	King George Island	127	213	EL0603	Primavera Base	201	213	EL67	King George Island	213
214	EL1223	King George Island	127	214	EL0604	Primavera Base	202	214	EL68	King George Island	214
215	EL1224	King George Island	127	215	EL0605	Primavera Base	203	215	EL69	King George Island	215
216	EL1225	King George Island	127	216	EL0606	Primavera Base	204	216	EL70	King George Island	216
217	EL1226	King George Island	127	217	EL0607	Primavera Base	205	217	EL72	King George Island	217
218	EL1227	King George Island	127	218	EL0608	Primavera Base	206	218	EL73	King George Island	218
219	EL1228	King George Island	127	219	EL0609	Primavera Base	207	219	EL74	King George Island	219
220	EL1229	King George Island	127	220	EL0610	Primavera Base	208	220	EL75	King George Island	220
221	EL1230	King George Island	127	221	EL0611	Primavera Base	209	221	EL76	King George Island	221
222	EL1231	King George Island	127	222	EL0612	Primavera Base	210	222	EL77	King George Island	222
223	EL1232	King George Island	127	223	EL0614	Primavera Base	211	223	EL78	King George Island	223
224	EL1273	Primavera Base	128	224	EL0615	Primavera Base	212	224	EL79	King George Island	224
225	EL1275	Primavera Base	129	225	EL0616	Primavera Base	213	225	EL80	King George Island	225
226	EL1276	Primavera Base	128	226	EL0617	Primavera Base	214	226	EL81	King George Island	226
227	EL1277	Primavera Base	128	227	EL0618	Primavera Base	209	227	EL82	King George Island	227
228	EL1278	Primavera Base	130	228	EL0619	Primavera Base	215	228	EL36	King George Island	228
229	EL1279	Primavera Base	128	229	EL0620	Primavera Base	78	229	EL85	King George Island	229
230	EL1281	Primavera Base	128	230	EL0621	Primavera Base	216	230	EL86b	King George Island	230
231	EL1282	Primavera Base	128	231	EL0622	Primavera Base	217	231	EL87b	King George Island	231
232	EL1283	Primavera Base	130	232	EL0623	Primavera Base	218	232	EL88	King George Island	232
233	EL1284	Primavera Base	128	233	EL0624	Primavera Base	219	233	EL90	King George Island	233
234	EL1285	Primavera Base	128	234	EL0625	Primavera Base	216	234	EL91	King George Island	234
235	EL1286	Primavera Base	128	235	EL0630	Primavera Base	220	235	EL92b	King George Island	235
236	EL1287	Primavera Base	128	236	EL0631	Primavera Base	221	236	EL93	King George Island	236
237	EL1288	Primavera Base	128	237	EL0632	Primavera Base	222	237	EL94	King George Island	237
238	EL1290	Primavera Base	128	238	EL0634	Primavera Base	223	238	EL95	King George Island	238
239	EL1291	Primavera Base	128	239	EL0635	Primavera Base	224	239	EL96	King George Island	239
240	EL1292	Primavera Base	128	240	EL0636	Primavera Base	225	240	EL97	King George Island	240
241	EL1293	Primavera Base	128	241	EL0637	Primavera Base	226	241	EL98	King George Island	241
242	EL1294	Primavera Base	128	242	EL0642	Primavera Base	227	242	EL99	King George Island	242
243	EL1295	Primavera Base	128	243	EL0643	Primavera Base	219	243	EL100	King George Island	243
244	EL1296	Primavera Base	128	244	EL0644	Primavera Base	228	244	EL101b	King George Island	244
245	EL1297	Primavera Base	128	245	EL0645	Primavera Base	229	245	EL102	King George Island	245
246	EL1299	Primavera Base	128	246	EL0646	Primavera Base	230	246	EL103	King George Island	246
247	EL1300	Primavera Base	128	247	EL0647	Primavera Base	226	247	EL104	King George Island	247
248	EL1301	Primavera Base	128	248	EL0648	Primavera Base	231	248	EL105	King George Island	248
249	EL1302	Primavera Base	128	249	EL0649	Primavera Base	232	249	EL106	King George Island	249
250	EL1303	Primavera Base	128	250	EL0650	Primavera Base	233	250	EL107	King George Island	250
251	EL1304	Primavera Base	128	251	EL0651	Primavera Base	234	251	EL108	King George Island	251
252	EL1305	Primavera Base	128	252	EL0652	Primavera Base	235	252	EL109	King George Island	252
253	EL1306	Primavera Base	128	253	EL0653	Primavera Base	234	253	EL110	King George Island	253
254	EL1307	Primavera Base	128	254	EL0674	Primavera Base	236	254	EL111	King George Island	254
255	EL1308	Primavera Base	128	255	EL0675	Primavera Base	237	255	EL113	King George Island	255
256	EL1309	Primavera Base	128	256	EL0676	Primavera Base	238	256	EL114	King George Island	256
257	EL1310	Primavera Base	128	257	EL0677	Primavera Base	238	257	EL115	King George Island	257
258	EL1311	Primavera Base	128	258	EL0654	Primavera Base	239	258	EL116	King George Island	258
259	EL1312	Primavera Base	128	259	EL0655	Primavera Base	240	259	EL117	King George Island	259
260	EL1313	Primavera Base	128	260	EL0656	Primavera Base	241	260	EL118	King George Island	260
261	EL1314	Primavera Base	128	261	EL0657	Primavera Base	242	261	EL119	King George Island	261
262	EL1315	Primavera Base	128	262	EL0658	Primavera Base	243	262	EL120	King George Island	262
263	EL1316	Primavera Base	128	263	EL0659	Primavera Base	244	263	EL121	King George Island	263
264	EL1317	Primavera Base	128	264	EL0660	Primavera Base	245	264	EL122	King George Island	264
265	EL1318	Primavera Base	128	265	EL0661	Primavera Base	246	265	EL123	King George Island	265
266	EL1319	Primavera Base	128	266	EL0662	Primavera Base	247	266	EL124	King George Island	266
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				268	EL0664	Primavera Base	249	268	EL436	King George Island	268
				269	EL0665	Primavera Base	250	269	EL437	King George Island	269
				270	EL0666	Primavera Base	251	270	EL439	King George Island	270
				271	EL0667	Primavera Base	252	271	EL440	King George Island	271
				272	EL0668	Primavera Base	253	272	EL441	King George Island	272
				273	EL0669	Primavera Base	254	273	EL442	King George Island	273
				274	EL0670	Primavera Base	243	274	EL443	King George Island	274
				275	EL0671	Primavera Base	255	275	EL444	King George Island	275
				276	EL0672	Primavera Base	66	276	EL445	King George Island	276
				277	EL0673	Primavera Base	256	277	EL446	King George Island	277
				278	EL0678	Primavera Base	257	278	EL447	King George Island	278
				279	EL0679	Primavera Base	258	279	EL448	King George Island	279
				280	EL0696	Esperanza Base	259	280	EL449	King George Island	280
				281	EL0697	Esperanza Base	260	281	EL450	King George Island	281
				282	EL0698	Esperanza Base	261	282	EL451	King George Island	282
				283	EL0699	Esperanza Base	262	283	EL452	King George Island	283
				284	EL0700	Esperanza Base	263	284	EL454	King George Island	284
				285	EL0701	Esperanza Base	264	285	EL455	King George Island	285
				286	EL0702	Esperanza Base	265	286	EL456	King George Island	286
				287	EL0703	Esperanza Base	266	287	EL457	King George Island	287
				288	EL0704	Esperanza Base	267	288	EL458	King George Island	288
				289	EL0705	Esperanza Base	268	289	EL459	King George Island	289
				290	EL0706	Esperanza Base	269	290	EL460	King George Island	290
				291	EL0707	Esperanza Base	270	291	EL461	King George Island	291
				292	EL0708	Esperanza Base	271	292	EL462	King George Island	292
				293	EL0709	Esperanza Base	272	293	EL463	King George Island	293
				294	EL0710	Esperanza Base	273	294	EL464	King George Island	294
				295	EL0711	Esperanza Base	274	295	EL465	King George Island	295
				296	EL0712	Esperanza Base	275	296	EL466	King George Island	296
				297	EL0713	Esperanza Base	276	297	EL467	King George Island	297
				298	EL0714	Esperanza Base	277	298	EL468	King George Island	298

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					300	EL0680	Esperanza Base	279		300	EL470	King George Island	300
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					302	EL0682	Esperanza Base	281		302	EL126	King George Island	302
					303	EL0692	Esperanza Base	260		303	EL127	King George Island	303
					304	EL0693	Esperanza Base	282		304	EL130	King George Island	304
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					306	EL0695	Esperanza Base	284		306	EL132	King George Island	306
					307	EL0716	Esperanza Base	285		307	EL134	King George Island	307
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					313	EL0727	Esperanza Base	291		313	EL141	King George Island	313
					314	EL0728	Esperanza Base	292		314	EL142	King George Island	314
					315	EL0729	Esperanza Base	291		315	EL146	King George Island	315
					316	EL0730	Esperanza Base	291		316	S01	King George Island	316
					317	EL0731	Esperanza Base	293		317	S02	King George Island	317
					318	EL0733	Esperanza Base	294		318	S03	King George Island	318
					319	EL0734	Esperanza Base	295		319	S04	King George Island	319
					320	EL0736	Esperanza Base	296		320	S05	King George Island	320
					321	EL0737	Esperanza Base	291		321	S06	King George Island	321
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					323	EL0741	Esperanza Base	298		323	S14	King George Island	323
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					326	EL0744	Esperanza Base	301		326	EL1743	King George Island	326
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					329	EL0690	Esperanza Base	276		329	EL1746	King George Island	329
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					361	EL0791	Esperanza Base	333		361	EL1366	Livingston Island	361
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										386	EL1400	Livingston Island	386
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										414	EL1422	Livingston Island	414
										415	EL1423	Livingston Island	415
										416	147-02	Deception Island	416
										417	147-04	Deception Island	417
										418	147-06	Deception Island	418
										419	147-07	Deception Island	419
										420	147-09	Deception Island	420
										421	147-10	Deception Island	421
										422	147-11	Deception Island	422
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										436	147-26	Deception Island	436
										437	147-27	Deception Island	437
										438	147-28	Deception Island	438
										439	147-29	Deception Island	439
										440	147-30	Deception Island	440
										441	147-32	Deception Island	441

ANNEX 2: DEUTSCHE ZUSAMMENFASSUNG

Diese manuskriptbasierte Dissertation ist in vier Kapitel unterteilt. Das erste Kapitel ist eine Einführung in die Flechten und die Antarktis. Es führt in das Ziel der Arbeit und die mit der Flechtensystematik und dem mangelnden Wissen über antarktische Flechten verbundenen Probleme ein. Die Antarktis ist eine der letzten vom Menschen weitgehend unbeeinflussten Regionen der Erde und ist von den anderen Kontinenten durch den antarktischen Zirkumpolarstrom, die Subantarktische Front, die Antarktische Polarfront und die Drake-Passage isoliert. Das terrestrische Leben in der Antarktis beschränkt sich auf weit voneinander entfernte und kleine eisfreie Gebiete, die nur 0,3% des Kontinents bedecken. Die Kolonisierung der Antarktis ist für viele Taxa eine Herausforderung und hängt mit ihrer Fähigkeit zur Fernverbreitung und ihrer Anpassung an die raue Klimabedingungen zusammen. Die terrestrischen Ökosysteme der Antarktis sind durch den Klimawandel, das Eindringen invasiver Arten und Wechselwirkungen zwischen diesen Faktoren erheblich bedroht. Der durch überdurchschnittlich hohe Temperaturen verursachte Gletscherrückgang legt neue Lebensräume frei, die leicht von lokalen Biota besiedelt werden können. Aber auch nicht-einheimische Arten können durch die veränderten klimatischen Bedingungen begünstigt werden. Die anthropogene Ausbreitung von Diasporen kann zur Ansiedlung neuer Arten in der Antarktis beitragen oder die Populationsstruktur vieler Taxa verändern. Die terrestrische Biota besteht fast

ausschließlich aus "niederen Organismen" (wirbellose Tiere, Moose, Algen, lichenisierte Pilze und Mikroorganismen). Flechten sind die dominierende Komponente und die wichtigsten Primärproduzenten. Sie sind symbiotische Systeme, die aus einem Pilz (Mykobionten) und einem oder mehreren photosynthetischen Partnern (Photobionten) bestehen und können sich sexuell oder vegetativ verbreiten. Die symbiotische Natur der Flechten schafft verschiedene Probleme, die eine einfache Artbestimmung bei Flechten erschweren. Selbst mit molekularen Daten ist die Artabgrenzung bei Flechten noch immer nicht einfach. Die wahre Anzahl der Arten wird aufgrund der Anwesenheit kryptischer Arten und Artenpaaren häufig unterschätzt. Die empfohlenen universellen Marker für das DNA-Barcoding (z. B. ITS) können manchmal keine Artenpaare abgrenzen. Bevor mit der Analyse von Flechtenpopulationen begonnen werden kann, ist es daher notwendig, schnell evolvierende Marker zu finden, die die Abgrenzung eng verwandter Arten ermöglichen. Das Ziel dieser Arbeit ist es, die bisher unbekannte genetische Struktur antarktischer Flechtenpopulationen wegen ihrer unmittelbaren Konsequenzen für den Artenschutz aufzuklären. Die Arbeit konzentriert sich dabei nicht nur auf Differenzierungsmuster und Genfluss, sondern untersucht auch die Frage des vom Menschen vermittelten Diasporentransfers in die Antarktis und zwischen antarktischen Standorten. Sie liefert Daten über die genetische Struktur antarktischer Flechten, die dringend benötigt werden, um angesichts der globalen Erwärmung und der zunehmenden menschlichen Aktivitäten in der Region Artenschutzstrategien zu entwickeln. Da es wegen ihrer symbiontischen Natur nicht möglich ist, unspezifische Fingerprinting-Methoden auf Flechten anzuwenden, sind Mikrosatelliten oder „Simple Sequence Repeats“ (SSRs) eines der besten Werkzeuge zur Untersuchung der genetischen Struktur von Flechtenpopulationen. SSRs ermöglichen es, zwischen den Flechtenpartnern zu unterscheiden, aber artspezifische Mikrosatelliten wurden nur für einige wenige Arten entwickelt. In der Antarktis ist bisher nur eine Art mit SSRs untersucht worden.

Das zweite Kapitel beschreibt neue Methoden und Werkzeuge zur Abgrenzung eng verwandter Flechtenarten und präsentiert schnell evolvierende Marker zur Charakterisierung ihrer genetischen Struktur. Das Kapitel stellt die in dieser Arbeit analysierten Flechtenarten und die Probleme im Zusammenhang mit ihrer korrekten morphologischen und molekularen Identifizierung vor. Im zweiten Kapitel wird die Besammlungsmethode erläutert: Probenahme in kleinen Arealen, in

denen die Artenpaare gemeinsam vorkommen. Dann werden die Methoden zur Entwicklung und Validierung pilzspezifischer, aber artübergreifend amplifizierender Mikrosatelliten beschrieben. Bei dem Artenpaar *Usnea antarctica* und *U. aurantiacoatra* handelt es sich um die häufigsten Flechten in der maritimen Antarktis. Der empfohlene DNA-Barcode für Pilze, der „internal transcribed spacer“ der ribosomalen RNA (ITS) unterscheidet nicht klar zwischen diesen Arten, und einige Autoren haben deshalb vorgeschlagen, die beiden Namen als Synonyme zu betrachten. Um die generelle Eignung von SSRs zur Unterscheidung eng verwandter Flechtenarten zu bestätigen, werden hier auch unveröffentlichte Ergebnisse eines anderen antarktischen Artenpaares, *Placopsis antarctica* und *P. contortuplicata*, dargestellt. Diese Arbeit ist die erste Studie, die Draft-Genome zweier Flechtenarten verwendet, um SSR-Marker mit gleicher Länge in flankierenden Regionen zu identifizieren, die über Artgrenzen hinweg amplifizieren. Mit Hilfe der neu entwickelten SSRs ist es möglich, die beiden eng verwandten Arten klar zu unterscheiden und gleichzeitig genetische Variabilität auf Populationsebene zu erfassen. Am Ende des Kapitels werden ITS-Sequenzen, Mikrosatelliten und SNPs zur Abgrenzung der Arten *Usnea antarctica* und *U. aurantiacoatra* verwendet. Das Kapitel zeigt die Bedeutung einer korrekten Artabgrenzung und die Vorteile von SSRs und SNPs im Vergleich zu der empfohlenen universellen Pilz-Barcode-Sequenz ITS bei der Abgrenzung antarktischer Usnea-Arten auf.

Im dritten Kapitel wird die genetische Vielfalt und Differenzierung von Flechtenpopulationen untersucht, um den Einfluss von Ausbreitungsstrategien und Migrationsgeschichte auf die populationsgenetische Struktur antarktischer Flechten zu ermitteln. Proben aus Südamerika und der maritimen Antarktis wurden analysiert, um einen möglichen vom Menschen vermittelten Genfluss zwischen den beiden Kontinenten und zwischen antarktischen Standorten zu identifizieren. Im dritten Kapitel werden populationsgenetische Analysen von drei Flechtenarten mit unterschiedlichen Ausbreitungsstrategien (sexuell, vegetativ) basierend auf einer hohen Anzahl von Proben vorgestellt. Usnea aurantiacoatra pflanzt sich sexuell fort und kommt disjunkt in Südamerika und der Antarktis vor. An ihr wird interkontinentaler Genfluss von Südamerika in die maritime Antarktis untersucht. Die sterile, sorediöse Usnea antarctica konnte überraschenderweise nur in der Antarktis gefunden werden und ist dort vermutlich endemisch. Um Genfluss zwischen den Kontinenten auch an einer asexuellen Flechtenart untersuchen zu können, wurde auf

Cetraria aculeata zurückgegriffen. Die beiden vegetativen Arten (*C. aculeata* und *U. antarctica*) weisen eine geringere genetische Vielfalt auf als *U. aurantiacoatra*. Die geringe genetische Differenzierung innerhalb der antarktischen Populationen und die höhere genetische Differenzierung innerhalb der patagonischen weisen auf ein altes Vorkommen von *U. aurantiacoatra* in der Antarktis und eine Ausbreitung von dort nach Südamerika hin. Die genetische Differenzierung antarktischer Populationen von *U. antarctica* ist vergleichbar mit der von *U. aurantiacoatra*, aber die Art wurde nicht in Südamerika gefunden. Die geringe Diversität und starke genetische Differenzierung antarktischer Populationen von *C. aculeata* bestätigt, dass die Art von Patagonien aus die Antarktis besiedelt hat. Glaziale Refugien wurden auf Navarino Island und auf den Südshetlandinseln identifiziert. Es gibt keine Hinweise auf vom Menschen verursachte Migration oder einen anhaltenden Genfluss in die Antarktis.

Kapitel vier enthält die wichtigsten Schlussfolgerungen. Mikrosatelliten sind geeignete Instrumente zur Unterscheidung von Artenpaaren und zur Untersuchung der genetischen Struktur von Flechtenpopulationen. Die phylogeographische Geschichte erklärt die genetische Struktur der Population jeder Art besser als die Art der Vermehrung (sexuell oder vegetativ). Kontrastierende Muster der genetischen Differenzierung liefern Hinweise darauf, dass *U. antarctica* und *U. aurantiacoatra* die letzte Eiszeit in der Antarktis überdauert haben. Es gibt keine eindeutigen Hinweise auf eine vom Menschen vermittelte Ausbreitung zwischen Südamerika in die Antarktis. Die starke genetische Differenzierung antarktischer Populationen von *C. aculeata* erfordert Schutzmaßnahmen, um Genfluss zwischen isolierten Populationen und die Ausrottung lokaler Populationen zu verhindern.

ANNEX 3: CURRICULUM VITAE

Elisa Lagostina

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Nationality: Italian
Date of birth: 9th April 1987

EDUCATION & QUALIFICATION

Nov 2015 – Today Goethe-Universität Frankfurt am Main: **PhD student** - DFG found
Dissertation topic: "Dispersal and genetic exchange of lichen populations between the Maritime Antarctic and southern South America (with a focus on human impact)"

Oct 2009 – Sep 2011 University of Pavia: **MS in Experimental and Applied Biology** (Magna cum laude)
Dissertation topic: "The antioxidant response of mesophilic fungi under thermal stress"

Oct 2006 – Feb 2010 University of Pavia: **BA in Biological Science**
Dissertation topic: "Study of energetic biomass in the district of Pavia"

EMPLOYMENT HISTORY

Dic 2019 – Feb 2020 **Researcher** at Agricultural and Environmental Sciences-Production, Landscape, Agroenergy (DISAA) of University of Milan (Italy)

- Extracting RNA from gut of Spodoptora littoralis to microbiota characterization
- Enzymatic assays of insect gut enzymes

Nov 2015 – Mar 2019 **PhD student (found DFG)** at the Department of Botany and Molecular Evolution - Senckenberg Research Institute and Natural Museum Frankfurt

- Population genetics and conservation of Antarctic lichens
- December 2015-January 2016 Antarctic expedition to Carlini Base on King George Island (South Shetland Islands)
- Sequencing metagenomics of lichens and Illumina data management
- Development of fungus-specific microsatellites for 5 species of lichens

Aug 2017 – Sep 2017 **Visiting Researcher** at Field Museum 1400 S. Lake Shore Drive Chicago, (Illinois, USA)

- Library preparations of lichen DNA for RADseq and sequencing with Illumina MiSeq

Jun 2014 – May 2015 **Microbiologist and molecular biologist at Mycology laboratory** - department of Earth Sciences and Environments of University of Pavia (Italy)

- Identification of Fungi species with morphological and molecular techniques

Jun 2013 – May 2014 **Visiting Scholar at Forest Pathology and Mycology laboratory** - department of ESPM of UC Berkeley (California, USA)

- Microsatellite analysis of fungal genomes

Apr 2013 – May 2013 **Microbiologist at Mycology laboratory** - department of Earth Sciences and Environments of University of Pavia (Italy)

- Analysis of antioxidant response and phosphate solubilization of fungi isolated in Siro Negri wood (Pavia)

Jun 2012 - Oct 2012 **Microbiologist at Mycology laboratory** - department of Earth Sciences and Environments of University of Pavia (Italy)

- Analysis of soil micro-organisms isolated in Siro Negri wood (Pavia)

Mar 2010 - Sep 2011 **MS thesis at Mycology laboratory** in the department of Earth Sciences and Environments of University of Pavia (Italy)

Apr 2008 - Feb 2010 **BA thesis at Analysis of Environmental Impact laboratory** in the department of Earth Sciences and Environments of University of Pavia (Italy)

TEACHING

Oct 2014 – Jun 2015 **Second advisor of thesis** Mycology laboratory-department of Earth Sciences and Environments at University of Pavia

- BA science and technology for environment thesis: "Preliminary mycological investigation of wetland Bruschera oasis"
- BA science and technology for environment thesis: "Fungal evaluation of corn tanned before seeding with *Trichoderma asperellum*"
- Pharmacology thesis: "*Penicillium* from extreme cold environment: cultivation, morphological analysis and antioxidant activity evaluation"
- MS experimental and applied biology thesis: "Evaluation of *Thrichoderma* sp. antagonistic activity against several plant pathogens"

Mar 2013 **Tutor at Mycology course** for BA in Biological Science of University of Pavia

- Teaching microscopy techniques and fungal identification.

WORKSHOPS

- **The complexity of lichen symbiosis: novel interdisciplinary approaches from genomic to functional perspectives** at University of Valencia, Spain December 2018
- **Lichen Genomics** at Institute of Plant Sciences, University of Graz, Austria November 2017
- **International Leadership Initiative – ILI** at International House Berkeley CA, USA March - May 2014
- **Female leadership – Grow between carrier and passion** For Soroptimist International Italy at SDA Bocconi School of Management, Milan Italy May 2012.
- **Quality certification – Laboratory** at University of Pavia, Italy December 2010.

CONFERENCES

- **Koordinations workshop** 2018 in Gißen 13th September 2018

Poster title: "Dispersal and genetic exchange of lichen populations between Patagonia and the Antarctic Peninsula [Pr567/18]" **Lagostina E.** & Printzen C.

Talk title: "A metagenomic approach to unravel symbiotic interactions in Antarctic lichens" **Lagostina E.** & Printzen C.

- **Bhlam symposium** in Frankfurt am Main 20th-22nd April 2018

Poster title: "Insights into species delimitation of lichenized fungi offered by microsatellite data" **Lagostina E.**, Lutsak T., Printzen C.

- **27. International Polartagung** in Rostock 25th-29th March 2018

Talk title: "Fine-scale genetic structure of lichen populations in Patagonia and the Maritime Antarctic dfg pr 567/18-1" **Lagostina E.** & Printzen C.

- **GEOBIODIVERSITY an integrative approach expanding Humboldt's vision** Frankfurt am Main 1st -3rd October 2017

Flash talk and poster title: "Sexuality, clonality and dispersal in two antarctic lichens" **Lagostina E.** & Printzen C.

- **Koordinations workshop** 2016 in Rostock 14th-16th October 2016

Poster title: “Dispersal and genetic exchange of lichen populations between Patagonia and the Antarctic Peninsula (with a focus on human impact) [PR567/18-1]” **Lagostina E.** & Printzen C.

- **LICHENS IN DEEP TIME The 8th IAL Symposium** in Helsinki, Finland 1st-5th August 2016

Poster title: “Local genetic differentiation between *Usnea aurantiaco-atra* and *U. antarctica*” **Lagostina E.** & Printzen C.

- **110th CONGRESS SBI Onlus** in Pavia, Italy 14th-18th September 2015

Poster 1 title: “Agro-residues supplemented with fungi: evidence of its multifunctional properties in agriculture and energy industry” Tosi S., Accossato S., Rodolfi M., Faè M., **Lagostina E.**, Cella R., Picco AM.

Poster 2 title: “The collection of medicinal macrofungi of Pavia University” Girometta C., Savino E., Guglielminetti M., **Lagostina E.**, Rodolfi M., Rossi P., Bernicchia A., Perini C., Salerni E., Picco AM.

PUBLICATIONS

- **Lagostina E.**, Dal Grande F., Scur M., Lorenz A., Andreev M., Ruprecht U., Søchting U., Sancho LG., Wirtz N., Rozzi R., Printzen C. (under review) **Genetic structure and gene flow of three lichen forming fungi in the Maritime Antarctic and southern South America**. Journal of Biogeography.
- Grewe F., **Lagostina E.,** Wu H., Printzen C., Lumbsch HT. (2018) **Population genomic analyses of RAD sequences resolves the phylogenetic relationship of the lichen-forming fungal species *Usnea antarctica* and *Usnea aurantiacoatra*.** MycoKeys 43: 91-113.
- **Lagostina E.,** Dal Grande F., Andreev M., Printzen C. (2018) **The use of microsatellite markers for species delimitation in Antarctic Usnea subgenus Neuropogon**. Mycologia 110(6): 1047-1057.
- **Lagostina E.,** Dal Grande F.. Ott S., Printzen C. (2017) **Fungus-Specific SSR Markers in the Antarctic Lichens Usnea antarctica and U. aurantiacoatra (Parmeliaceae, Ascomycota).** Applications in Plant Sciences 5(9): 1700054.
- Gonthier P., Sillo F., **Lagostina E.,** Roccotelli A., Cacciola OS., Stenlid J., Garbelotto M. (2015) **Selection processes in simple sequence repeats suggest a correlation with their genomic location: Insights from a fungal model system.** BMC Genomics 16(1): 1-12.
- Maggi O., Tosi S., Angelova M., **Lagostina E.,** Fabbri AA., Pecoraro L., Altobelli E., Picco AM., Savino E., Branda E., Turchetti B., Zotti M., Vizzini A., Buzzini P. (2013) **Adaptation of fungi, including yeasts, to cold environments**. Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology: Official Journal of the Societa Botanica Italiana 147(1): 247-258.