DNA BARCODING OF KWAZULU-NATAL AFROMONTANE FOREST *PARMELIA* (PARMELIACEAE) SPECIES: A MOLECULAR APPROACH TO ACCURATE SPECIMEN IDENTIFICATION AND SENSITIVITY TO CLIMATE CHANGE

By

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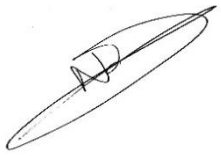
PREFACE

The research contained in this thesis was completed while based in the School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal Pietermaritzburg, South Africa under the supervision of Prof. R.P Beckett and Dr S. Willows-Munro from February 2017 to November 2018

These studies represent original work by the candidate and have not otherwise been submitted in any form of any degree or diploma to another University. Where use has been made of the work by other authors it has been duly acknowledged in the text

Student: 

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DECLERATION OF PLAIGARISM

I, Nqobile Ndhlovu, declare that:

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2. This dissertation has not been submitted in full or in part for any degree or examination to any other university.
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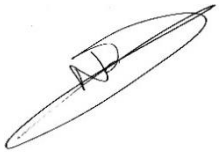
Student: 

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ABSTRACT

Accurate species identification is challenging, especially in groups with subtle taxonomically diagnostic characters such as lichens. Molecular-based techniques have shown to be a valuable tool for accurate specimen identification in fungi, in particular the use of DNA barcoding has become popular. Specifically, the internal transcriber spacer (ITS) region has been shown to successfully discriminate a broad range of fungal species. In this study, the utility of the ITS DNA barcode for use as a species diagnostic tool in the cosmopolitan lichen-forming fungus, *Parmelia* (Parmeliaceae) was investigated. Sixty-eight ITS sequences were generated from specimens collected from five sites around the province of KwaZulu-Natal and analysed. Phylogenetic analysis indicated that unlike European *Parmelia* species that form strong monophyletic clades, what appeared to be morphologically very similar *Parmelia* species in KwaZulu-Natal are paraphyletic or polyphyletic. No barcode gap was detected between the intra and interspecific distances. This suggests that the taxonomy of *Parmelia* lichens in South Africa needs to be thoroughly revised. The molecular data presented in this study provides evidence of previously hidden species-level diversity in *Parmelia* and as such contributes to the knowledge and understanding of the biodiversity of lichenized fungi in South Africa. The thermotolerance of *Parmelia* collected from different sites along an altitudinal gradient around Kwa-Zulu Natal was invesigated Chlorophyll fluorescence was used to assess the performance of lichen photobionts following stress, while ion leakage that of the mycobiont. For heat tolerance, results suggested that tolerance was correlated with the climatic conditions in which the lichens grow. Material from the coastal site of Hawaan were more heat tolerant than that from the three Midlands sites. Counter to our expectations, the coastal collections were more cold tolerant than those from the other sites.

However, the genus clearly contains genetic variation with respect to stress tolerance, suggesting that it may have the potential to adapt to climate change.

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LIST OF ABBREVIATIONS

AFP	antifreeze proteins
Chl	chlorophyll fluorescence
Cf	final conductivity
Cv	initial conductivity
DNA	deoxyribonucleic acid
ETR	electron transport rate
F_M	maximum fluorescence
$F_{M'}$	maximum fluorescence when saturating pulse given in the light
F_O	minimum fluorescence
F_t	stable fluorescence signal in the light
F_V/F_M	maximal quantum yield of PSII photochemistry
HSPs	heat-shock proteins
IPCC	intergovernmental Panel on Climate Change
ITS	internal transcriber spacer
matK	megakaryocyte-associated tyrosine kinase
PSII	photosystem II
PFD	photon influx density
rbcl	ribulose bisphosphate carboxylase large
ROS	reactive oxygen species
-196°C	liquid nitrogen
KZN	KwaZulu-Natal
unp.	unpublished

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CHAPTER 1: LITERATURE REVIEW

1.1 Lichens

Lichenized fungi are a mutualistic association between a fungal partner (mycobiont) and a photosynthetic partner (photobiont). The photosynthetic taxon is either a cyanobacterium or a green alga, or in some cases both (Mitrović et al., 2011; Biosca et al., 2016). Lichens exist in four different morphological forms, namely: fruticose, foliose, crustose and gelatinous (Watson, 1929). These morphological forms allow lichens to successfully grow in different habitats around the world (Kosanić et al., 2015). The fungal component of the association provides water, shelter and minerals for the photobiont which in turn performs photosynthesis (Nguyen et al., 2013). This symbiotic partnership has been successful within the Ascomycota, as there are more than 18 000 lichen species currently described out of a total of 28 000 species of Ascomycetes worldwide (Leavitt et al., 2012b). Lichens play important ecological roles, such as stabilizing bare soils, contributing to nitrogen fixation (Mark et al., 2016; Leavitt et al., 2012a), and serving as food for reindeers (Kanz et al., 2015) and small invertebrates such as spiders (Mukherjee et al., 2010). Additionally, because lichens are sensitive to environmental changes, they are often used as biomonitors of air pollution (Nascimbene et al., 2010; Boch et al., 2013). Even though they are successful and widely distributed, the genetic structure of a typical lichen population, and the distribution patterns of lichen species remain poorly explored (Amo de Paz et al., 2011). Trying to understand population structures using phenotypic-based approaches has proven hard because of morphological convergence or parallelism (Del-Prado et al., 2010; Leavitt et al., 2012a). The result has been that real species biodiversity is often underestimated or inaccurate (Del-Prado et al., 2010; Leavitt et al., 2013a). Modern revisions that include genetic data have revealed previously

overlooked lineages (Kroken and Taylor, 2001), increasing our understanding of factors that drive diversification, biogeographical distribution and evolutionary patterns (Kress et al., 2015).

1.2 South African lichen biota

"*Many groups of lichens were poorly studied, and it was difficult to find a competent lichenologist to work on them*"- Almborn (1987). Although written about southern African species over thirty years ago, unfortunately, this statement is still true today as many southern African species-rich regions remain unexplored (Maphangwa, 2010). In South Africa a comprehensive checklist on lichen biota was last produced by Ethel Doige (Doige, 1950). It is estimated that the South African biota consists of about 2500-3000 taxa, but only 1750 have been reported (Fryday, 2015). An online checklist by Feuerer (2013) is available but the problem with this list is that some taxa and microlichens are not included, and it only reviewed literature published up to 2002 (Fryday, 2015). However, there are other checklists published by Jürgens and Lohman (1995), and Schultz et al. (2009). It is clear that much work still needs to be done on South African lichen biodiversity, especially amongst microlichens (Crous et al., 2006).

1.3 Traditional vs molecular based approaches in Parmeliaceae

Parmeliaceae (Ascomycota, Lecanorales) is the largest family of lichenized fungi (Molina et al., 2004; Divakar et al., 2005). It comprises large well-known genera such as *Parmelia* and *Usnea*. To date, this family is known to consist of 2726 species placed in 79 genera (Thell et al., 2012). Members of the Parmeliaceae occur widely in the southern hemisphere, with centers of distributions in southern Africa, Australia and South America (Thell et al., 2012). When

Eschweiler (1824) first described the Parmeliaceae family, he made use of the structure of the apothecia (Del-Prado et al., 2010) and ever since, this morphological character has become the most important for delimiting this family (Thell et al., 2012). However, there have been challenges with circumscriptions made on phenotypically-based methods. First, the characters used for separation are not always consistent (Eriksson and Hawksworth, 1998). Furthermore, specimens need to be in a state where the characters used for identification are clearly visible, which may exclude juvenile or fragmentary samples (Leavitt et al., 2011). Second, some species are phenotypically cryptic – in other words, the presence of distinct species that are morphologically similar is masked (Crespo and Pérez-Ortega, 2009; Bickford et al., 2007). In addition, lichens can be phenotypically plastic, resulting in their morphology being influenced by the environment (Pérez-Ortega et al., 2012). Third, there are semi-cryptic species that lack similar morphological characters but share the same ecology and geographical patterns (Crespo et al., 2010). Lastly, some groups are taxonomically challenging i.e. some lichen structures are difficult to discern. For these reasons, morphological-based analyses may be inaccurate, and often underestimate diversity (Pino-Bodas et al., 2012), as cryptic species may be overlooked, with certain lineages "hiding" within well described, recognized lineages (Kelly et al., 2011). In a survey of the Parmeliaceae, Elix (1993) concluded that while generative characters are constant, a combination of vegetative and chemical characters should be used to delineate genera in this family. Since then, no attempt has been made to try and circumscribe the genera in Parmeliaceae (Thell et., 2012). Unfortunately, there are many species in which identification remains questionable due to the ambiguity of key features. Therefore, the use of morphological characters alone to identify lichenized species, sometimes even to the level of genus, can prove difficult (Crespo and Lumbsch, 2010).

Molecular data provides an alternative to phenotypic-based approaches (Crespo et al., 2002). As in other groups, in lichens, molecular techniques have helped understand evolution and species delimitation, improving species circumscription (Lumbsch and Leavitt, 2011). Furthermore, in lichenized fungi molecular research has provided a tool for accurate species identification (Leavitt et al., 2013b). Accurate and improved species recognition and estimation of diversity are important as they help us understand biogeographical patterns, diversification (Leavitt e al., 2013a; Leavitt et al., 2012b) and address more fundamental biological questions (Del-Prado et al., 2010). Linking genetic data with information such as GPS coordinates and environmental variables could also help us to better understand the distribution and diversity in lichenized fungi (Leavitt et al., 2013b). However, the success of DNA-based approaches is dependent on the availability of a strong and well-curated reference sequence database from expertly identified specimens (Seifert, 2009). Molecular-based studies carried out so far have shown that most clades in this family are monophyletic and has allowed the better delimitation of genera (Divakar et al., 2016). However, although the taxonomy of Parmeliaceae is steadily being amended as result of increased publications with sequence data, there are still many genera that require attention.

1.4 The effects of climate change on lichens

Human activities such as urbanization, habitat destruction and burning fossil fuels have resulted in a large increase in the emission of greenhouse gases such as carbon dioxide (CO_2), a major factor that is contributing to global climate change (Karl and Tranberth, 2003). The consequence of the emission of greenhouse gases is that heat is trapped in the atmosphere, causing the "greenhouse effect" (Hungate et al., 2003; www.nws.noaa.gov/os/brochures/climate/Climatechange.pdf). Climate change can be defined as

the changes in global climatic patterns that are taking place as a result of human activities (Hungate et al., 2003). A report compiled by the Intergovernmental Panel on Climate Change (IPCC) predicted that temperatures over the next 30-50 years will rise between 3-5°C. The heat waves we will experience in future will be even more intense than those that we are currently experiencing (Hungate et al., 2003). Precipitation will increase because of increased evapotranspiration, sea levels will rise, changes in vegetation will occur, and droughts, and wildfires will become more common (Karl and Tranberth, 2003). Therefore, it is important to monitor changes in climate to be able to forecast the response of species, and for general conservation purposes (Ellis et al., 2007; Allen and Lendemer, 2016).

Lichens lack a cuticle and are perennial organisms. For reasons that remain unclear, they are extremely sensitive to certain pollutants such as sulphur dioxide (Conti and Cecchetti, 2001). As a result, they respond rapidly to climate change, and the resulting habitat changes that it causes (Aptroot and van Heck, 2007; Stapper and John, 2015). Apart from air pollutants, the distribution of lichens is also influenced by humidity, light and temperature (Stapper and John, 2015). According to Kershaw (1985), the exact reasons why increased temperatures are harmful to lichens are unknown but are likely to include severe physiological damage in the cell membrane (Pisani et al., 2007). As discussed above, the IPCC suggested that temperatures are likely to increase 2.0-4.5°C between the years 1990-2100 because of climate change (Hungate et al., 2003). Because of the difficulty in definitively ascribing damage to specific climatic shifts, it is important to determine the sensitivity of organisms' components to climate change to enable early detection of injury caused by climate change (Parmesan and Yohe, 2003). A 1°C increase in annual temperature can drastically change lichen distribution (Pisani et al., 2007), and therefore some species have been used as indicators of temperature shifts. For example, *Flavoparmelia caperata* has been used

as an indicator of increased temperatures in Denmark (Søchting, 2004). A study in the Netherlands showed that warm temperature species are now increasing, and cold-tolerant species are either decreasing or disappearing. It seemed clear that climate change was the driving force behind the trend observed (Aptroot and van Heck, 2007; Stapper and John, 2015). This work was the first long-term study on the biological monitoring of terrestrial systems, and the conclusions of this study were that climate change may be affecting lichen populations globally (Aptroot and van Heck, 2007). It seems clear that climate change can cause shifts in the boundaries between biomes, resulting in changes in species assemblages, habitat loss and potentially extinction (Leavitt et al., 2014; Allen and Lendemer, 2016). Therefore, detailed monitoring of sensitive environments is needed to understand the significance of these climatic shifts (Leavitt et al., 2016).

1.5 Temperature stress

Perhaps the greatest effect of climate change on lichens will be an increase in temperature. Increased temperatures have, and will continue to, caused profound effects on all terrestrial ecosystems, resulting in changes in the length of the seasons available for growing and reproducing (Steinhäuser et al., 2016). With climate change, extreme temperature events are likely to become even more intense (Hatfield and Prueger, 2015; Steinhäuser et al., 2016), and harsh temperatures may affect the most important physiological processes of plants such as respiration, photosynthesis, and primary and secondary metabolite production (Awasthi et al., 2015). Plants can survive environmental changes either by migrating to favorable habitats, or acclimatization through increased heat tolerance (Kai and Iba, 2014; Steinhäuser et al., 2016). The ability of organisms to adapt to changes in their environments is essential for them to be able to persist in

changing environments. Without these survival strategies, loss of species may result, which in turn, will degrade the whole ecosystem (Steinhäuser et al., 2016).

Lichens are known for their ability to tolerate a wide range of harsh environments (Nash, 1996), such as conditions that occur in Antarctica, deserts, and high alpine regions. Lichens form the dominant ground cover on about 8% of the Earth's land surface (Ahmadjian, 1995). One of the reasons for the success of lichens is that they are poikilohydric (Kranner et al., 2008). Poikilohydric organisms can tolerate desiccation, but as soon as water becomes available resume metabolic activity (Smith et al., 1997; Li and Wei, 2016). As they lack a cuticle, heat stress will cause rapid drying, and when dry they show high resistance to extreme temperatures (Kappen and Lange, 1972; Gauslaa and Solhaug, 1999; Solhaug et at. 2018).

a. Response to low temperatures

Lichens are tolerant to low temperatures (Solhaug et al., 2018). Becquerel (1950) showed that *Xanthoria parietina* could survive freeze drying to absolute zero. However, hydrated lichens are not all resistant to cold temperatures. Tolerance is species dependent; and also depends on the micro-environment in which a lichen grows. For example, *Usnea dasypoga* collected from tropical mountains of Argentina showed great resistance and normal respiration following freezing of up to -78°C, but surprisingly collections from temperate regions that received the same treatment as the latter were less tolerant (Kappen and Lange, 1970, 1972). In general, these authors found that tolerance to low temperatures was poorly correlated with habitat temperature. For example, high tolerance to cold was found in a great number of lichen species found in areas where this tolerance

would not be expected such as the Negev desert in Israel and central Europe (Kappen and Lange, 1972).

Solhaug et al., (2018) attempted to explain the distribution of two *Lobaria* species (*L. virens* and *L. pulmonaria*) based on freezing tolerance by comparing the short- and long-term effects of freezing of these species. The short-term viability (F_V/F_M and membrane leakage, assessed as a "conductivity index") of the coastal *L. virens* were greatly affected by chilling, consistent with the distributional pattern of this species. Interestingly, the long-term indicators of stress (relative growth rate) of the two species was unaffected, meaning that it is impossible to definitively conclude that the tolerance to freezing temperatures determines distribution.

b. Response to high temperatures

Warm temperatures increase evaporation, and it can be difficult to distinguish the effects of desiccation and heat stress in lichens. According to Thomas (1939), the photobiont is more sensitive than the mycobiont but in some cases, both symbionts are equally sensitive. When moist, many lichen species seemed to be sensitive to temperatures ranging between 20°-30°C. For example, spores of *Xanthoria parietina* did not germinate at temperatures higher than 24°C (Thomas, 1939). By contrast, the temperature of dry lichen thalli in open habitats ranges between 50°C and 60°C for crustose and foliose lichens (Ahmadjian and Hale, 1973). Thermal tolerance also differs from species to species, for example, *Cladonia pyxidata* species occurring in the southwest of Germany can be heated up to 66°C, but this temperature is lethal to other species (Kappen, 1973). In the case of black hydrophilous lichens, thalli of these lichen species can be become overheated by direct sunlight because of their colour. Therefore dark-coloured species

such as *Ephebe canata* occurring on irrigated rocks or soil gutters in strongly insolated areas are threatened by heat damage (Wirth, 1972).

c. Mechanisms of tolerance to temperature stress

Tolerance to cold Stress

Various physiological mechanisms to cold resistance by animals, plants and fungi have been proposed, and it is likely that some of these have been selected for in lichens (Robinson, 2001). Lichens are normally rich in trehalose and sugar alcohols (Roser et al., 1992), and these mixes have been proposed to act as general cryoprotectants in fungi (Hoshino et al., 2003). Freezing temperatures in lichens result in the release of RNase enzymes from the cell wall of both the mycobiont and photobiont (Fontaniella et al., 2000). The presence of the two polyols, ribitol produced by the photobiont and mannitol produced by the mycobiont, delays solubilization of the RNases (Fontaniella et al., 2000). The recently discovered antifreeze proteins (AFPs) (Griffith and Yaish, 2004) are uncommon proteins: they have numerous, hydrophilic ice-restricting areas that seem to work as inhibitors of ice recrystallization and ice nucleation (Duman et al., 1993). These proteins are known from microbes, fungi, plants and invertebrates. Although there are no published reports from lichens, interestingly, a United States patent was recently enlisted asserting that an AFP from a lichen, with a clear sub-atomic weight of from 20 to 28 kDa, might be utilized as a part of keeping food from solidifying (Beckett et al., 2008). In the meantime, the lichens that have AFPs additionally contain extracellular proteinaceous ice nucleators that trigger solidifying at high below zero temperatures (Kieft and Ruscetti, 1990). These have significantly higher atomic masses than AFPs, and either give cold protection assurance from the discharged warmth of combination

or on the other hand set up a defensive sheath of extracellular ice in freeze tolerant species. Clearly, lichens can manage ice formation on and in their thalli very well (Beckett et al., 2008).

Tolerance to heat stress

In free-living fungi, both heat and cold shock induce the synthesis of set of proteins called heat-shock proteins (HSPs) (Tiwari et al., 2015). These proteins occur almost everywhere in the cell e.g. mitochondria, nucleus, cell membrane (Kregel, 2002) and are involved in many processes, such as in protein folding, replication, transcriptional and signal pathways (Verghese et al., 2012). These proteins can be induced by different stresses such as temperature, osmotic, oxidative or pH (Tereshina, 2005). The overexpression of HSPs12 induces thermotolerance in *S. cerevisiae* by causing the accumulation of trehalose (Pacheco, 2009), and trehalose accumulation protects cell membranes and acts as a reserve carbohydrate that may be utilized during stress (Hounsa et al., 1998).

No data are available on the presence of heat shock proteins in the mycobionts of lichenized fungi to act against various stresses, but as these proteins exist in free-living fungi, they are likely to be important in lichens. In photobionts however, a transcriptomic approach was recently used to study the effect of desiccation on *Treboxia* (Carniel et al., 2016). This study showed that HSPs are constitutively expressed in lichen photobionts, and also indicated that other tolerance mechanisms are upregulated during desiccation stress e.g. aquaporins, antioxidants i.e. ROS related systems (Kranner et al., 2008) and genes related to the photosynthetic apparatus. A similar study on mycobionts would undoubtedly provide useful information on mechanisms of heat tolerance in lichens.

1.6 DNA barcoding

DNA barcoding is a molecular technique making use of a standard DNA region as a rapid tag for taxon identification (Hollingsworth, 2007; Valentini et al., 2008). The mitochondrial gene cytochrome *c* oxidase (COI) is often used for animals as the standard DNA region, *matK* and *rbcL* for plants, and internal transcriber spacer (ITS) was recently proposed as a marker for fungus (Kaur, 2015; Schoch et al., 2012). DNA barcoding was first used by the science community in 1993, but initially was not extensively used. However, in 2003 a group of researchers from the University of Guelph, led by Paul Hebert, published a paper titled "*Biological identifications through DNA barcodes*" (Hebert et al., 2003). This publication resulted in a more widespread adoption of the technique. DNA barcoding uses molecular data to cluster together organisms with the same genetic makeup into groups that represent species (Kanz et al., 2015). For DNA barcoding to be successful there must be a gap between the inter- and intraspecific genetic distances. This gap is referred to as the barcoding gap (Meyer and Paulay, 2005), and is important for testing the accuracy of the technique and separating distinct species (Čandek and Kuntner, 2015). DNA barcoding aims to improve the accuracy of species identification (Leavitt et al., 2013b), address issues where the taxonomy is unstable (Kanz et al., 2015, Divakar et al., 2016) and establish a good reference database against which unknown samples can be compared (Leavitt et al., 2013b). DNA barcoding is applied in such diverse fields such as the identification of medicinal plants, the control of agricultural pests, the protection of endangered species, and monitoring water quality (Kaur, 2015). As in other groups of fungi, molecular data has helped understand evolution and species delimitation in lichenized fungi (Leavitt et al., 2013a).

1.7 Challenges associated with DNA-based techniques

As much as DNA-based approaches have the potential to aid species identification and discovery, there are still concerns about these approaches as there is only a limited number of well sampled datasets available to test its performance (Meyer and Paulay, 2005). In theory, all species show variability at the molecular level, and therefore it is important to define and establish a threshold that will highlight genetic differentiation for distinct groups of species (Kanz et al., 2015). DNA barcoding is most effective when there is only a small overlap between closely related sister species (Meyer and Paulay, 2005). There are problems with sequencing DNA using the Sanger technique especially in lichenized fungi. The lack of characters used for delimitation in lichens results in challenges when designing efficient primers sufficient enough to reduce fungal contamination (Hodkinson and Lendemer, 2013). Therefore, amplicon pools derived from Sanger techniques usually fail to produce reliable results or readable sequences especially for lichens (Lendemer, 2012). Also, the use of high-throughput sequencing HTS has raised questions as to whether Sanger-based analyses underestimate within lichen photobiont diversity (Eva et al., 2013). The questionable performance of Sanger techniques which normally yields unambiguous photobiont sequence per individual, however, has never been formally tested (Paul et al., 2018). DNA-based methods work effectively with thoroughly, well sampled and documented clades with previously recognized traditional characters (Meyer and Paulay, 2005). Therefore, for DNA barcoding to work and be used as a tool, there is a certain criterion that has to be met by the samples used and the correct technique must be applied to obtain valid results.

1.8 Use of ITS gene as a barcode marker

The Internal Transcriber Spacer (ITS) was proposed as the primary marker for fungi by the Consortium of DNA Barcoding (Schoch et al., 2012), and ever since it has been used more than any other DNA region (Kelly et al., 2011; Leavitt et al., 2014; Mark et al., 2016). ITS has been extensively used to test for species boundaries and the correlation between genetic and morphological diversity in many fungal taxa (Del-Prado et al., 2010; Kelly et al., 2011). In particular, Del-Prado et al., (2010) and Divakar et al., (2010) used the ITS for species delimitation in the Parmeliaceae. While modern revisions may uncover hidden lineages and facilitate accurate species identification and delimitation, the taxonomy of lichens is nowhere near complete, and is being continually updated as new research is carried out (Leavitt et al., 2013a).

1.9 DNA barcode gap

As mentioned above, the "DNA barcoding gap" is the gap between intra and interspecific divergences or variation (Meyer and Paulay, 2005). The interspecific variation needs to exceed the intraspecific genetic distance in such a way that there is a clear gap that will allow for the identification of unknown individuals to their species with, of course, negligible errors (Hebert et al., 2003; Barret and Hebert, 2005). Errors may occur when a group possesses semi-species pairs with incomplete lineage sorting (Hebert et al., 2004). As a result, to separate two species a threshold has been proposed, which is suggested should be 10 times greater than that which occurs within species (Hebert et al., 2004). Differences above this threshold would indicate the existence of a new taxon (Meyer and Paulay, 2005), while differences below this threshold would indicate that the specimens belong to the same species (Wiemers and Fiedler, 2007). So, the presence of a

DNA barcode gap would enable the identification of species previously undescribed (Hebert et al., 2004; Smith et al., 2006).

There are few possible errors that could occur when using a predefined DNA barcode gap; a false positive occurs if populations within one species are genetically distinct, for example when there are high levels of genetic diversity below species level or interrupted gene flow in allopatric populations (Wiemers and Fiedler, 2007). In such cases, depending on the morphological variation and species concept to be applied, such populations may be called "cryptic species". In contrast, a false negative occurs when there is little or no sequence variation in the barcoding fragment between biospecies (Wiemers and Fiedler, 2007). Therefore, checking for the existence of false negatives is crucial for the barcoding approach, because such an error would reveal cases where DNA barcoding would prove less powerful than other holistic approaches used for delimitation (Wiemers and Fiedler, 2007). When a barcode gap is present (Figure 1A) then there is a difference between the inter and intraspecific genetic variation. When there is an overlap i.e. no barcode gap, then the inter and intraspecific genetic distances are continuous (Figure 1B).

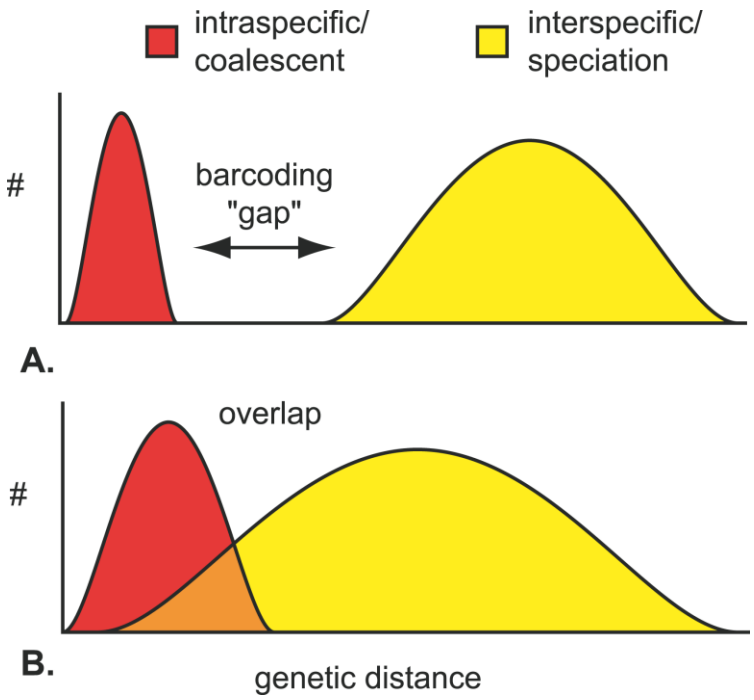


Figure 1: The DNA barcode gap formed by the (A) intraspecific variation shown in yellow, the distribution of interspecific divergence shown in red and (B) the overlap of the intra-intraspecific variation. Figure adopted from Meyer and Paulay (2005).

1.10 Barcode of Life data system (BOLD)

BOLD is a platform that provides an online database that is used for collection and management of specimens (e.g. voucher specimens), molecular data (sequences, primers) and analytical tools. This platform is freely available to researchers interested in DNA barcoding all over the world (Barcode of Life Data Systems Handbook, 2013). BOLD provides a variety of tools such as: "Database" (which includes BIN database, public data portal, primer database etc.), "Taxonomy" (which contains distribution maps, images and any other information necessary for taxon), "Identification" (this tool gives access to plant, animal and fungi search engines based on gene markers), "Workbench" and "Resources" (Barcode of Life Data Systems Handbook, 2013).

For access to public sequences (as in this study) or specimen data, the public data portal can be used for that, it also provides access to geographical distributions, sample ID, institution keywords etc. The public data portal tool allows you to search using any kind of keywords combination e.g. *Lepidoptera*, Canada, this search will show you all *Lepidoptera* records collected in Canada. Results will also display BINs, record lists and will also show public records (Barcode of Life Data Systems Handbook, 2013). A search for specimen record will show information on identifier, collection data such as location, taxonomy, specimen image, specimen details and sequence information. Sequence data stored on the public data portal stores information about sequence data for specimens such as forward and reverse sequences. This information allows for the search of sequence data information for different markers, sequence trace files on this page can be viewed and downloaded (Barcode of Life Data Systems Handbook, 2013).

BOLD allows for different types of submissions to the platform, and each submission has its own process and requirements. One type of submission is "Specimen data submission" where you are required to create an excel file that you will use for submission. "Specimen submissions" must be accompanied by images, these must be formatted according to requirements listed on the BOLD systems and one must familiarize themselves with image licensing and use. "Sequence submission" includes assembling the package such as making sure the file contains sequences that are aligned and in a FASTA file format then you can upload that file into the system. A new workbench for sequence data submission now includes an option to do sequence contigs using an online Sequence Editor (Barcode of Life Data Systems Handbook, 2013)

1.11 Problem statement

Most taxonomic classifications of lichens are based on traditional approaches where a taxonomist will classify plant species according to characters such as morphology or chemical properties. As

data about taxa has accumulated, the taxonomic statuses for many species have changed due to additional information and therefore a revision is required. Molecular methods are the latest technology used to identify and reclassify species. Lichen species tend to be morphologically similar, and "cryptic diversity" (when species cannot be clearly separated using morphological characters) is common in this group. As a result, misidentification and inaccurate taxonomic status is common amongst lichens (Crespo and Lumbsch, 2010). The genus *Parmelia* is a model study system, because, as discussed above (section 1.3), identification through morphology-based methods is challenging because of the unavailability of diagnostic characters (Divakar et al., 2016). The first aim of this study is to test for the ability of ITS sequences to accurately identify species in the genus *Parmelia*, by estimating overlaps within and between species. Second, the stress tolerance of a widespread *Parmelia* species (or group of species) collected from sites with different environmental conditions around Kwazulu-Natal will be estimated. These experiments will help us understand how adaptable this species is, and therefore how the species will be affected by changes in weather patterns caused by climate change, as most lichen species are known to be sensitive to climatic shifts (Insarov and Insarova, 1996; Insarov and Schroeter, 2000).

This study therefore combines two disciplines. The first is conservation genetics, where the ability of a barcode to clarify species boundaries, facilitate specimen identification and estimate the level of biodiversity will be tested in the genus *Parmelia* from South Africa. To achieve this, sequencing the ITS region will be used to test for the presence of a DNA barcode gap.

The second approach was ecophysiological. It was hypothesized that lichens collected from the coast i.e. warmer sites would be more tolerant to heat and more susceptible to cold than lichen species collected from other sites i.e. inland sites. Ecophysiological techniques were used to test the thermotolerance and cold tolerance of lichen species collected from different sites along an

altitudinal transect in KZN. It was hoped to use the results of these experiments to predict the likely effects of climate change on lichen communities.

References

Amo de Paz, G., Cubas, P., Divakar, P.K., Lumbsch, H.T. Crespo, A., 2011. Origin and diversification of major clades in parmelioid lichens (Parmeliaceae, Ascomycota) during the Paleogene inferred by Bayesian analysis. PLOS One 6, e28161.

Ahmadjian, V., Hale, M.E., 1973. *Lichens*. Academic Press, New York and London.

Ahmadjian, V., 1995. Lichens are more important than you think. BioScience, 45, 124.

Allen, J.L., Lendemer, J.C., 2016. Climate change impacts on endemic, high-elevation lichens in a biodiversity hotspot. Biodiversity and Conservation 25, 555–568.

Almborn, O., 1987. Lichens at high altitudes in southern Africa. Bibliotheca Lichenologica 25, 401–417

Aptroot, A., Van Herk, C.M., 2007. Further evidence of the effects of global warming on lichens, particularly those with *Trentepohlia* phycobionts. Environmental Pollution 146, 293–298.

Awasthi, R., Bhandari, K., Nayyar, H., 2015. Temperature stress and redox homeostasis in agricultural crops. Frontiers in Environmental Science 3, 11.

Beckett, R., Kranner, I., Minibayeva, F., 2008. Stress physiology and the symbiosis. In T. Nash, III (Ed.), Lichen Biology, Cambridge: Cambridge University Press, pp. 134–151.

Becquerel, P., 1950. La suspension de la vie au-dessous de 1/20° K absolu par demagnetization adiabatique de l'alun de fer dans le vide le plus eleve. C. R. Proceedings of the National Academy of Sciences of the United States of America 231, 261–263.

Barrett, R.D.H., Hebert, P.D., 2005. Identifying spiders through DNA barcodes. Canadian Journal of Zoology 83,481–491.

Bickford, D., Lohman D.J., Sodhi, N.S, Ng, P.K.L., Meier, R., Winker, K., Ingram, K.K., Das, I., 2007. Cryptic species as a window on diversity and conservation. Trends in Ecology and Evolution 22, 148–155.

Biosca, E.G., Flores, R., Santander, R.D., Díez-Gil, J.L., Barreno, E., 2016. Innovative approaches using lichen enriched media to improve isolation and culturability of lichen associated bacteria. PLOS One 11, e0160328.

Boch, S., Prati, D., Hessenmöller, D., Schulze, E.D., Fischer, M., 2013. Richness of lichen species, especially of threatened ones, is promoted by management methods furthering stand continuity. PLOS One 8, e55461.

Čandek, K., Kuntner, M., 2015. DNA barcoding gap: reliable species identification over morphological and geographical scales. Molecular Ecology Resources 15, 268–277.

Carniel, F.C., Gerdol, M., Montagner, A., Banchi, E., De Moro, G., Manfrin, C., Tretiach, M., 2016. New features of desiccation tolerance in the lichen photobiont *Trebouxia gelatinosa* are revealed by a transcriptomic approach. Plant Molecular Biology 91, 319–339.

Conti, M.E., Cecchetti, G., 2001. Biological monitoring: lichens as bioindicators of air pollution assessment — a review. Environmental Pollution 114, 471–492.

Crespo, A,, Molina, M.C., Blanco, O., Schroeter, B., Sancho, L.G., Hawksworth, D.L., 2002. rDNA ITS and β-tubulin gene sequence analyses reveal two monophyletic groups within the cosmopolitan lichen *Parmelia saxatilis*. Mycological Research 106, 788–795.

Crespo, A., Pérez-Ortega, S., 2009. Cryptic species and species pairs in lichens: a discussion on the relationship between molecular phylogenies and morphological characters. In Anales del Jardín Botánico de Madrid 6. Consejo Superior de Investigaciones Científicas.

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

Crespo, A., Ferencova, Z., Pérez-Ortega, S., Elix, J.A., Divakar, P.K., 2010. *Austroparmelina*, a new Australasian lineage in parmelioid lichens (Parmeliaceae, Ascomycota). Systematics and Biodiversity 8, 209–221

Crous, P.W., Rong, I.H., Wood, A., Lee, S., Glen, H., Botha, W., Slippers, B., De Beer, W.Z., Wingfield, J., Hawksworth, D.L., 2006. How many species of fungi are there at the tip of Africa. Studies in Mycology 55, 13–33.

Del-Prado, R., Cubas, P., Lumbsch, H.T., Divakar, P.K., Blanco, O., de Paz, G.A., Crespo, A., 2010. Genetic distances within and among species in monophyletic lineages of Parmeliaceae (Ascomycota) as a tool for taxon delimitation. Molecular Phylogenetics and Evolution 56, 125–133.

Divakar, P.K., Upreti, D.K., 2005. A new species in *Melanohalea* (Parmeliaceae, Ascomycotina) and new lichen records from India. The Lichenologist 37, 511–517.

Divakar, P.K., Figueras, G., Hladun, N.L., Crespo, A., 2010. Molecular phylogenetic studies reveal an undescribed species within the North American concept of *Melanelixia glabra* (Parmeliaceae). Fungal Diversity 42, 47–55.

Divakar, P.K., Leavitt, S.D., Molina, M.C., Del-Prado, R., Lumbsch, H.T., Crespo, A., 2016. A DNA barcoding approach for identification of hidden diversity in Parmeliaceae (Ascomycota): *Parmelia sensu stricto* as a case study. Botanical Journal of the Linnean Society 180, 21–29.

Doidge, E.M., 1950. The South African fungi and lichens to the end of 1945. Bothalia 5, 1–1094.

Duman, J.G., Olsen, T.M., 1993. Thermal hysteresis protein activity in bacteria, fungi, and phylogenetically diverse plants. Cryobiology 30, 322–328.

Ellis, C.J., Coppins, B.J., Dawson, T.P., Seaward, M.R., 2007. Response of British lichens to climate change scenarios: trends and uncertainties in the projected impact for contrasting biogeographic groups. Biological Conservation 140, 217–235.

Elix, J.A., 1993. Progress in the generic delimitation of *Parmelia* (*sensu lato*) lichen (Ascomycotina: Parmeliaceae). Bryologist 96, 359–383.

Eriksson, O.E., Hawksworth, D.L., 1998. Outline of the ascomycetes. Systema Ascomycetum 16, 83–301

Eschweiler, F.G., 1824. Systema lichenum, genera exhibens rite distincta, pluribus novis adaucta: Commentatio. Schrag.

Eva, M.D.C., Santiago, C., Jacinta, G., Alicia, D.H., Fernando, M.A., Leonardo M.C., Eva, B., 2013. The genetic structure of the cosmopolitan three-partner lichen *Ramalina farinacea* evidences the concerted diversification of symbionts. FEMS Microbiology Ecology 83, 310–323.

Feuerer, T., 2013, Checklists of lichens and lichenicolous fungi, viewed February–April 2014, from http://www.checklists.de Index Fungorum Partnership, 2014, Index Fugorum, CABI, CBS and Landcare Research (custodians), viewed February–April 2014, from http://www.indexfungorum.org

Fontaniella, B., Vicente, C., Legaz, M.E., 2000. The cryoprotective role of polyols in lichens: Effects on the redistribution of RNase in *Evernia prunastri* thallus during freezing. Plant Physiology and Biochemistry 38, 621–627.

Fryday, A.M., 2015. A new checklist of lichenised, lichenicolous and allied fungi reported from South Africa. Bothalia 45, 4 pages.

Gauslaa, Y., Solhaug, K.A., 1999. High-light damage in air-dry thalli of the old forest lichen *Lobaria pulmonaria*—interactions of irradiance, exposure duration and high temperature. Journal of Experimental Botany 50, 697–705.

Griffith, M., Yaish, M.W.F., 2004. Antifreeze proteins in overwintering plants: a tale of two activities. Trends in Plant Science 9, 399–405.

Hatfield, J.L., Prueger, J.H., 2015. Temperature extremes: effect on plant growth and development. Weather and Climate Extremes 10, 4–10.

Hebert, P.D., Cywinska, A., Ball, S.L., 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London B: Biological Sciences 270, 313–321.

Hebert, P.D., Stoeckle, M.Y., Zemlak, T.S., Francis, C.M., 2004. Identification of Birds through DNA Barcodes. PLOS Biology 2, e312.

Hodkinson, B.P., Lendemer, J.C., 2013. Next-generation sequencing reveals sterile crustose lichen phylogeny. Mycosphere 4, 1028–1039.

Hollingsworth, P.M., 2007. DNA barcoding: potential users. Genomics, Society and Policy 3, 44–47.

Hoshino, T., Kiriaki, M., Ohgiya, S., Fujiwara, M., Kondo, H., Nishimiya, Y., Tsuda, S., 2003. Antifreeze proteins from snow mold fungi. Canadian Journal of Botany 81, 1175–1181.

Hounsa, C.G., Brandt, E.V., Thevelein, J., Hohmann, S., Prior, B.A., 1998. Role of trehalose in survival of *Saccharomyces cerevisiae* under osmotic stress. Microbiology, 144, 671–680.

http://biodiversityadvisor.sanbi.org/wp-content/uploads/2016/05/BOLD_Handbook_Oct2013.pdf

Hungate, B.A., Dukes, J.S., Shaw, M.R., Luo, Y., Field, C.B., 2003. Nitrogen and climate change. Science 302, 1512–1513.

Insarov, G., Insarova, I., 1996. Assessment of lichen sensitivity to climate change. Israel Journal of Plant Sciences, 44, 309–334.

Insarov, G., Schroeter, B., 2002. Lichen monitoring and climate change. In Monitoring with Lichens—Monitoring Lichens (pp. 183-201). Springer, Dordrecht.

Jürgens, N., Niebel-Lohmann, A., 1995. Geobotanical observations on lichen fields of the southern Namib Desert. Mitteilungen aus dem Institut für allgemeine Botanik in Hamburg 25, 135–156.

Kai, H., Iba, K., 2014. Temperature stress in plants. Essential for Life Science.

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

Kappen, L., Lange, O.L., 1970. Kalteresistenz von Flechten aus verschiedenen Klimagebieten. Deutsche Botanische Gesellschaft 4, 61–65.

Kappen, L., Lange, O.L., 1972. Die Kalteresistenz einiger Makrolichenen. Flora Jena 161, 1–29**.**

Kappen, 1973. Response to extreme environments, in; Ahmadjian, V., Hale, M.E., 1973. *Lichens*. Academic Press, New York and London, pp 311–368.

Karl, T.R., Trenberth, K.E,. 2003. Modern global climate change. Science 302, 1719–1723.

Kaur, S., 2015., DNA Barcoding and Its Applications. International Journal of Engineering Research and General Science 3, 602–604.

Kelly, L.J., Hollingsworth, P.M., Coppins, B.J., Ellis, C.J., Harrold, P., Tosh, J., Yahr, R., 2011. DNA barcoding of lichenized fungi demonstrates high identification success in a floristic context. New Phytologist 191, 288–300.

Kershaw, K.A., 1985. Physiological Ecology of Lichens. Cambridge: Cambridge University Press.

Kieft, T.L., Ruscetti, T., 1990. Characterization of biological ice nuclei from a lichen. Journal Bacteriology 172, 3519–3523.

Kosanić, M., Ranković, B., Stanojković, T., Stošić, I., Grujičić, D., Milošević-Djordjević, O., 2015. *Lasallia pustulata* lichen as possible natural antigenotoxic, antioxidant, antimicrobial and anticancer agent. Cytotechnology 1–10.

Kranner, I., Beckett, R., Hochman, A., Nash III, T. H., 2008. Desiccation-tolerance in lichens: a review. The Bryologist 111, 576–593.

Kregel, K.C., 2002. Invited review: heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. Journal of Applied Physiology 92, 2177–2186.

Kress, W.J., Garcıa-Robledo, C., Uriarte, M., Erickson, D.L., 2015. DNA barcodes for ecology, evolution, and conservation. Trends in Ecology and Evolution 30, 25–35.

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

Leavitt, S.D., Johnson, L., St. Clair, L.L., 2011 Species delimitation and evolution in morphologically and chemically diverse communities of the lichen-forming genus *Xanthoparmelia* (Parmeliaceae, Ascomycota) in western North America. American Journal of Botany 98, 175–188.

Leavitt, S.D., Esslinger, T.L., Lumbsch, H.T., 2012a. Neogene-dominated diversification in Neotropical montane lichens: Dating divergence events in the lichen-forming fungal genus *Oropogon* (Parmeliaceae). American Journal of Botany 99, 1764–1777.

Leavitt, S.D., Esslinger, T.L., Divakar, P.K., Lumbsch, H.T., 2012b. Miocene and Pliocene dominated diversification of the lichen-forming fungal genus *Melanohalea* (Parmeliaceae, Ascomycota) and Pleistocene population expansions. BMC Evolutionary Biology 12, 176.

Leavitt, S.D., Fernández-Mendoza, F., Pérez-Ortega, S., Sohrabi, M., Divakar, P.K., Vondrák, J., Clair, L.L.S., 2013a. Local representation of global diversity in a cosmopolitan lichen-forming fungal species complex (*Rhizoplaca*, Ascomycota). Journal of Biogeography 40, 1792–1806.

Leavitt, S., Fernández-Mendoza, F., Pérez-Ortega, S., Divakar, P., Lumbsch, T., Clair, L.S. 2013b. DNA barcode identification of lichen-forming fungal species in the *Rhizoplaca melanophthalma* species-complex (Lecanorales, Lecanoraceae), including five new species. MycoKeys 7, 1.

Leavitt, S.D., Esslinger, T.L., Hansen, E.S., Divakar, P.K., Crespo, A., Loomis, B.F., Lumbsch, H.T., 2014. DNA barcoding of brown *Parmeliae* (Parmeliaceae) species: a molecular approach for accurate specimen identification, emphasizing species in Greenland. Organisms Diversity and Evolution 14, 11–20.

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

Lendemer, J. C., 2012. Lichen Taxonomy for the 21st Century: A Revision of the Genus Lepraria SL in North America North of Mexico (Doctoral dissertation, City University of New York).

Li, H., Wei, J.C., 2016. Functional analysis of thioredoxin from the desert lichen-forming fungus, *Endocarpon pusillum* Hedwig, reveals its role in stress tolerance. Scientific Reports 6, 27184.

Lumbsch, H.T., Leavitt, S.D., 2011. Goodbye morphology? A paradigm shift in the delimitation of species in lichenized fungi. Fungal Diversity 50, 59–72.

Maphangwa, K.W., 2010. Lichen thermal sensitivities, moisture interception and elemental accumulation in an arid South African ecosystem (Doctoral dissertation, University of the Western Cape).

Mark, K., Cornejo, C., Keller, C., Flück, D., Scheidegger, C., 2016. Barcoding lichen-forming fungi using 454 pyrosequencing is challenged by artifactual and biological sequence variation 1. Genome 59, 685–704.

Meyer, C.P., Paulay, G., 2005. DNA barcoding: error rates based on comprehensive sampling. PLOS Biology 3, e422.

Mitrović, T., Stamenković, S., Cvetković, V., Tošić, S., Stanković, M., Radojević, I., Marković, S., 2011. Antioxidant, antimicrobial and antiproliferative activities of five lichen species. International Journal of Molecular Sciences 12, 5428–5448.

Molina, M.,C, Crespo, A., Blanco, O., Lumbsch, H.T., Hawksworth, D.L., 2004. Phylogenetic relationships and species concepts in *Parmelia s. str.* (Parmeliaceae) inferred from nuclear ITS rDNA and β-tubulin sequences. The Lichenologist 36, 37–54.

Mukherjee, A., Wilske, B., Navarro, R.A., Dippenaar-Schoeman, A., Underhill, L.G., 2010. Association of spiders and lichen on Robben Island, South Africa: a case report. Journal of Threatened Taxa 2, 815–819.

Nascimbene, J., Brunialti, G., Ravera, S., Frati, L., Caniglia, G., 2010. Testing *Lobaria pulmonaria* (L.) Hoffm. as an indicator of lichen conservation importance of Italian forests. Ecological Indicators 10, 353–360.

Nash, T. H., 1996. *Lichen biology*. Cambridge University Press.

Nguyen, K.H., Chollet-Krugler, M., Gouault, N., Tomasi, S., 2013. UV-protectant metabolites from lichens and their symbiotic partners. Natural Product Reports 30, 1490–1508.

NOOA National Weather Service., 2007. www.nws.noaa.gov/os/brochures/climate/Climatechange.pdf

Pacheco, A., Pereira, C., Almeida, M.J., Sousa, M.J., 2009. Small heat-shock protein Hsp12 contributes to yeast tolerance to freezing stress. Microbiology 155, 2021–2028.

Parmesan, C., Yohe G., 2003. A globally coherent fingerprint of climate change impacts across natural systems. Nature 421, 37–42.

Paul, F., Otte, J., Schmitt, I., Dal Grande, F., 2018. Comparing Sanger sequencing and high-throughput metabarcoding for inferring photobiont diversity in lichens. Scientific Reports 8, 8624.

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

Pino-Bodas, R., Burgaz, A.R., Martin, M.P., Lumbsch, H.T., 2012. Species delimitations in the *Cladonia cariosa* group (Cladoniaceae, Ascomycota). The Lichenologist 44, 121–135.

Pisani, T., Paoli, L., Gaggi, C., Pirintsos, S.A., Loppi, S., 2007. Effects of high temperature on epiphytic lichens: issues for consideration in a changing climate scenario. Plant Biosystems 141, 164–169.

Robinson, C.H., 2001. Cold adaptation in Arctic and Antarctic fungi. New Phytologist 151, 341–353.

Roser, D.J., Melick, D.R., Ling, H.U., Seppelt, R.D., 1992. Polyol and sugar content of terrestrial plants from continental Antarctica. Antarctic Science 4, 413–420.

Seifert, K.A., 2009. Progress towards DNA barcoding of fungi. Molecular Ecology Resources 9, 83–89.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Miller, A.N., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences 109, 6241–6246.

Schultz, M., Zedda, L., Rambold, G., 2009. New records of lichen taxa from Namibia and South Africa. Bibliotheca Lichenologica 99, 315–334.

Smith S.D., Monson, R.K., Anderson, J.E., 1997. Poikilohydric Plants. In: Physiological Ecology of North American Desert Plants. Adaptations of Desert Organisms. Springer, Berlin, Heidelberg.

Smith, M.A., Woodley, N.E., Janzen, D.H., Hallwachs, W., Hebert, P.D., 2006. DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). Proceedings of the National Academy of Sciences of the United States of America 103, 3657–3662.

Søchting, U., 2004. *Flavoparmelia caperata* – a probable indicator of increased temperatures in Denmark. Graphis Scripta 15, 53–56.

Solhaug, K.A., Chowdhury, D.P., Gauslaa, Y., 2018. Short-and long-term freezing effects in a coastal (*Lobaria virens*) versus a widespread lichen (*L. pulmonaria*). Cryobiology 82, 124–129.

Stapper, N.J., John, V., 2015. Monitoring climate change with lichens as bioindicators. 2268–3798.

Steinhäuser, S.S., Andrésson, Ó.S., Pálsson, A., Werth, S., 2016. Fungal and cyanobacterial gene expression in a lichen symbiosis: Effect of temperature and location. Fungal biology 120, 1194–1208.

Tereshina, V.M., 2005. Thermotolerance in fungi: the role of heat shock proteins and trehalose. Microbiology 74, 247–257.

Tiwari, S., Thakur, R., Shankar, J., 2015. Role of heat-shock proteins in cellular function and in the biology of fungi. Biotechnology Research International.

Thell, A., Crespo, A., Divakar, P.K., Kärnefelt, I., Leavitt, S.D., Lumbsch, H.T., Seaward, M.R., 2012. A review of the lichen family Parmeliaceae–history, phylogeny and current taxonomy. Nordic Journal of Botany 30, 641–664.

Thomas, E.A., 1939. Uber die Biologie von Flechtenbildnern. Beitr. Kryptogamenflora Schweiz 9, 1–208

Valentini, A., Pompanon, F., Taberlet, P., 2008. DNA barcoding for ecologists. Trends in Ecology and Evolution 24, 110–117.

Verghese, J., Abrams, J., Wang, Y., Morano, K.A., 2012. Biology of the heat shock response and protein chaperones: budding yeast (*Saccharomyces cerevisiae*) as a model system. Microbiology and Molecular Biology Reviews, 76, 115–158.

Watson, W., 1929. The classification of lichens. New Phytologist, 28, 1–36.

Wiemers, M., Fiedler, K., 2007. Does the DNA barcoding gap exist?–a case study in blue butterflies (Lepidoptera: Lycaenidae). Frontiers in Zoology 4, 8.

Wirth, V., 1972. Die Silikatflechten-Gemeinschaften im aufier-alpinen Zentraleuropa. "Dissertationes Botanicae 17. Cramer, Lehre.

CHAPTER 2: MATERIALS AND METHODS

2.1 Sample collection

Healthy thalli of lichens from the genus *Parmelia* were collected from February 2017- April 2018 from five different sites along a longitudinal transect in KwaZulu-Natal, South Africa (see map, Figure 3). For the thermotolerance (physiological) experiments, all material was collected in summer or early autumn. Collections were made along a series of plateaus over a distance of 345.5 km from the north coast to the mountainous regions of Drakensburg (Figure 2). From the five sites, 68 *Parmelia* specimens were collected (see Table 7). Specimens were identified as belonging to *Parmelia* based on morphological characters described by Hale Jr. (1987). Loosely, material keyed out to *Parmelia perlata* (*sensu lato*) (Figure 2). Therefore, the characteristics used to collect the lichen material were specimens that had a well-developed foliose thallus, a dark (presumably melanised) lower surface with rhizines, an upper side that varied from pale grey when dry to a greenish colour when damp, well developed soralia, and a tan-coloured marginal zone (Purvis et al., 1992; van Herk et al., 2004; Allen, 2008; Frahm et al., 2010). This method of collection was used for lichen material to be used for both physiology and molecular studies (DNA barcoding) experiments. Sequences for a range of *Parmelia* species were downloaded from BOLD systems and GenBank. Accession numbers for these are given in Table 8.

Figure 2: *Parmelia perlata* (*sensu lato*) species used in this study.

Table 1: Table showing the five sites, GPS coordinates and altitude where the lichen material was collected.

Collection sites	**GPS coordinates**	**Altitude**	**Location**	**Dates of sample collections**
Hawaan forest (uMhlanga, Durban), RSA	-29.7128816°S, 29.9133356°E	0 m	Coastal	Jun 2017
uMlalazi nature reserve (Mtunzini), RSA	-28.9541203°S, 31.766569°E	30 m	Coastal	Nov 2017 Jan/Feb 2018
University of KwaZulu-Natal (Scottsville, Pietermaritzburg), RSA	-29.6258172°S, 30.4019928°E	688 m	Inland	Mar 2017 Jul 2017 Oct 2017
Fort Nottingham nature reserve (Fort Nottingham), RSA	-29.4145055°S, 29.9133356°E	1491 m	Inland/Afromontane	Feb 2017 Jun 2017 Jan/Feb 2018 Apr 2018
Monks Cowl (Drakensberg), RSA	-29.0485156°S, 29.4064394°E	1600 m	Montane	April 2017 June 2017

				Mar 2018

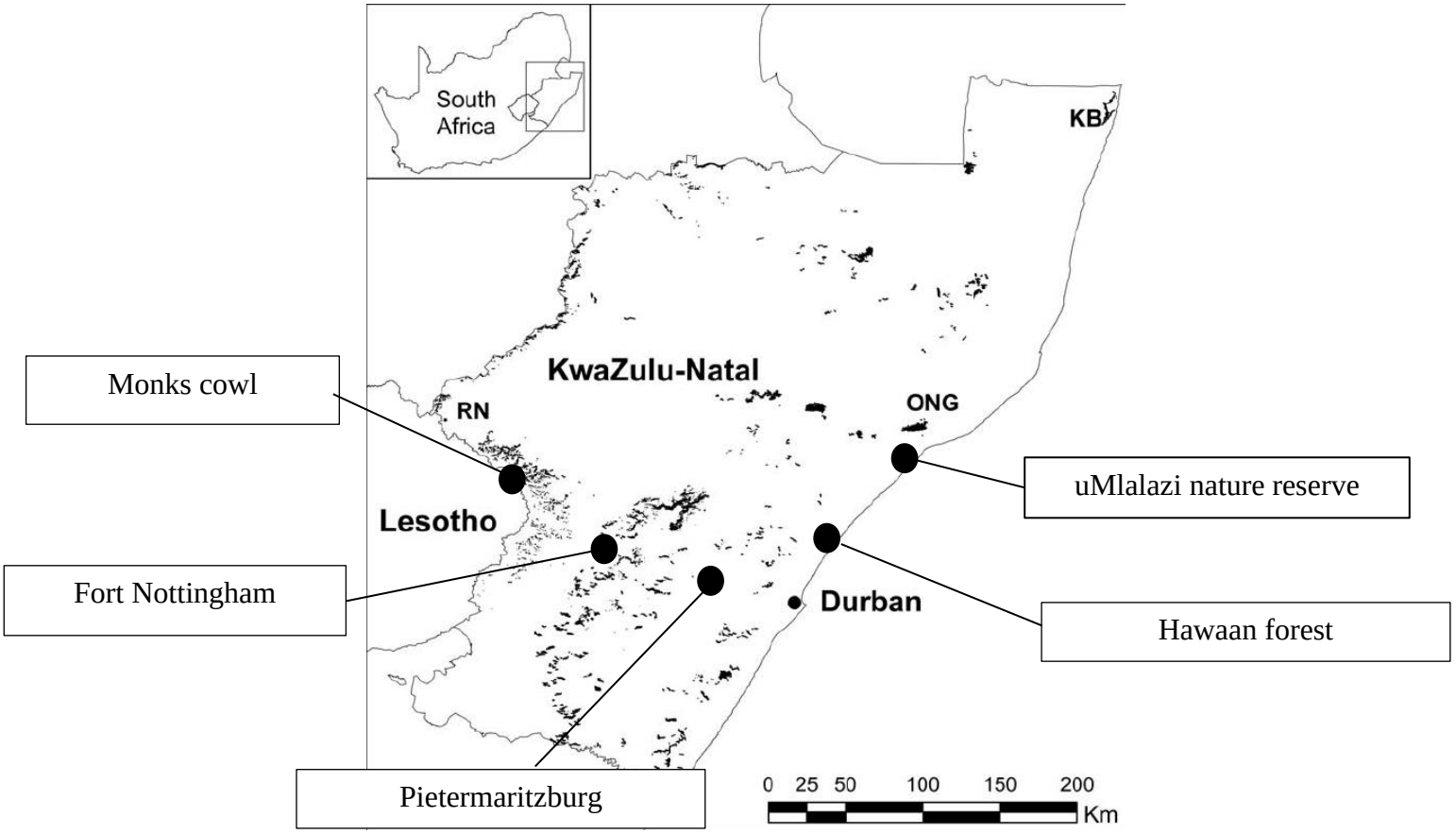


Figure 3: Map showing the five sites in KwaZulu-Natal where the *Parmelia* species were collected.
Map by: Adie and Lawes (2011).

2.2 Monthly max and min temperatures of the 5 sites where the lichen material used for this study was collected

Table 2: Table showing weather by month // weather averages of uMhlanga rocks (site near Hawaan forest)

	January	February	March	April	May	June	July	August	September	October	November	December
Avg. Temperature (°C)	24	24.3	23.7	21.7	19.4	17.3	16.8	17.9	19.3	20.6	21.8	23.2
Min. Temperature (°C)	20.3	20.6	19.7	17.3	14.3	11.7	11.3	12.7	14.8	16.7	18	19.5
Max. Temperature (°C)	27.7	28.1	27.7	26.2	24.6	23	22.4	23.2	23.8	24.5	25.6	27
Avg. Temperature (°F)	75.2	75.7	74.7	71.1	66.9	63.1	62.2	64.2	66.7	69.1	71.2	73.8
Min. Temperature (°F)	68.5	69.1	67.5	63.1	57.7	53.1	52.3	54.9	58.6	62.1	64.4	67.1
Max. Temperature (°F)	81.9	82.6	81.9	79.2	76.3	73.4	72.3	73.8	74.8	76.1	78.1	80.6
Precipitation / Rainfall (mm)	118	116	120	72	59	34	32	42	60	92	111	111

The difference in precipitation between the driest month and the wettest month is 88 mm. During the year, the average temperatures vary by 7.5 °C.

Table 3: Table showing weather by month // weather averages of Mtunzini (site near uMlalazi nature reserve)

	January	February	March	April	May	June	July	August	September	October	November	December
Avg. Temperature (°C)	24.7	24.7	24.1	21.9	19.6	17.2	17.1	18.4	19.8	21	22.4	23.9
Min. Temperature (°C)	20.3	20.4	19.6	17.2	14.3	11.4	11.2	12.9	14.8	16.4	17.9	19.4
Max. Temperature (°C)	29.2	29.1	28.7	26.7	24.9	23.1	23	24	24.9	25.7	26.9	28.5
Avg. Temperature (°F)	76.5	76.5	75.4	71.4	67.3	63.0	62.8	65.1	67.6	69.8	72.3	75.0
Min. Temperature (°F)	68.5	68.7	67.3	63.0	57.7	52.5	52.2	55.2	58.6	61.5	64.2	66.9
Max. Temperature (°F)	84.6	84.4	83.7	80.1	76.8	73.6	73.4	75.2	76.8	78.3	80.4	83.3
Precipitation / Rainfall (mm)	125	133	146	78	74	45	42	51	69	98	121	122

Between the driest and wettest months, the difference in precipitation is 104 mm. Throughout the year, temperatures vary by 7.6 °C.

Table 4: Table showing weather by month // weather averages of Pietermaritzburg (site where University of KwaZulu-Natal is situated)

	January	February	March	April	May	June	July	August	September	October	November	December
Avg. Temperature (°C)	22	22.2	21.4	19	15.7	12.9	12.9	15	17.1	18.8	19.9	21.4
Min. Temperature (°C)	16.6	16.9	15.7	12.6	8.2	4.2	4.2	6.9	10	12.7	14.4	15.8
Max. Temperature (°C)	27.5	27.6	27.1	25.5	23.3	21.6	21.7	23.1	24.3	25	25.5	27.1
Avg. Temperature (°F)	71.6	72.0	70.5	66.2	60.3	55.2	55.2	59.0	62.8	65.8	67.8	70.5
Min. Temperature (°F)	61.9	62.4	60.3	54.7	46.8	39.6	39.6	44.4	50.0	54.9	57.9	60.4
Max. Temperature (°F)	81.5	81.7	80.8	77.9	73.9	70.9	71.1	73.6	75.7	77.0	77.9	80.8
Precipitation / Rainfall (mm)	140	123	113	64	31	12	12	28	48	87	115	124

The variation in the precipitation between the driest and wettest months is 128 mm. The variation in annual temperature is around 9.3 °C.

Table 5: Table showing weather by month // weather averages of Howick (site near Fort Nottingham nature reserve)

	January	February	March	April	May	June	July	August	September	October	November	December
Avg. Temperature (°C)	20.5	20.5	19.4	16.9	13.7	10.7	10.6	13	15.5	17.5	18.2	19.8
Min. Temperature (°C)	14.7	14.7	13.5	10.2	6.1	2.4	2.3	4.9	8.1	10.9	12	13.9
Max. Temperature (°C)	26.4	26.4	25.3	23.7	21.3	19	18.9	21.2	22.9	24.1	24.5	25.7
Avg. Temperature (°F)	68.9	68.9	66.9	62.4	56.7	51.3	51.1	55.4	59.9	63.5	64.8	67.6
Min. Temperature (°F)	58.5	58.5	56.3	50.4	43.0	36.3	36.1	40.8	46.6	51.6	53.6	57.0
Max. Temperature (°F)	79.5	79.5	77.5	74.7	70.3	66.2	66.0	70.2	73.2	75.4	76.1	78.3
Precipitation / Rainfall (mm)	133	128	114	51	24	15	16	21	37	83	109	130

The difference in precipitation between the driest month and the wettest month is 118 mm. The variation in temperatures throughout the year is 9.9 °C.

Table 6**:** Table showing weather by month // weather averages of Monks Cowl

	January	February	March	April	May	June	July	August	September	October	November	December
Avg. Temperature (°C)	19.9	19.5	18.4	15.9	12.8	9.3	9.5	12.5	15.3	17.1	18	19.2
Min. Temperature (°C)	14.2	13.9	12.4	9.1	4.9	1.1	0.9	4.2	7.7	10.3	11.8	13.3
Max. Temperature (°C)	25.6	25.2	24.5	22.7	20.8	17.6	18.1	20.9	23	23.9	24.3	25.2
Avg. Temperature (°F)	67.8	67.1	65.1	60.6	55.0	48.7	49.1	54.5	59.5	62.8	64.4	66.6
Min. Temperature (°F)	57.6	57.0	54.3	48.4	40.8	34.0	33.6	39.6	45.9	50.5	53.2	55.9
Max. Temperature (°F)	78.1	77.4	76.1	72.9	69.4	63.7	64.6	69.6	73.4	75.0	75.7	77.4
Precipitation / Rainfall (mm)	180	167	149	64	26	11	10	24	40	76	123	162

There is a difference of 170 mm of precipitation between the driest and wettest months. Throughout the year, temperatures vary by 10.6 °C.

2.3 Storage and selection of lichen material

Lichen material was partially cleaned in the laboratory and placed in a growth cabinet under low light conditions (20 µmol m^2 s^{-1}) at 17°C for up to 4 d until the beginning of experiments. After cleaning, material was sometimes dried, in this state, the lichens' water content is below 10% and in this desiccated state, lichens are able to survive unharmed for longer periods of time and metabolic activities can be restored within seconds upon rehydration (Kappen, 1988 and Hogenner, 2003) and were then stored at -24^{o}C until required. Generally, freezing is recommended as a storage method for lichens that will be used for physiological experiments (Larson, 1978; Jensen and Feige, 1987). Freezing ensures that thalli remains healthy for physiological experiments and measurements such as integrity of cell membrane (ion leakage), assimilation pigments (chlorophyll *a*), and chlorophyll *a* fluorescence emission (Fv/Fm) (Paoli et al., 2013). Freezing preserves the viability of lichens (Yamamoto et al., 1998) and frozen samples can be moved back and forth between room temperature and freezing without losing viability (Honegger,

2003). Before any physiology experiments, frozen material was thawed, hydrated and stored in the growth cabinet for 24 h.

Lichen thalli had varying morphology, colour and size of lobes, therefore necessitating random assignment of the lobes to the different treatments. For experiments involving chlorophyll fluorescence measurements, 20 x 1 cm discs per site were used. For experiments that involved measuring ion leakage, 0.3 g x 25 replicates per site were used.

MATERIALS AND METHODS FOR PHYSIOLOGY EXPERIMENTS

2.4 Thermotolerance

The response of the lichens to heat stress was tested for the mycobiont (ion leakage assay: conductivity index) and the photobiont (chlorophyll fluorescence; F_V/F_M and ETR).

2.4.1. Heat tolerance of the photobiont (F_V/F_M and ETR)

After overnight acclimation as described above, heat stress was given at a temperature of 35°C for 4 h in the darkness, with F_V/F_M measured before heat stress ("time zero"), and during the experiment at 1 h intervals.

Chlorophyll fluorescence was measured using two chlorophyll fluorometers: Hanstech FMS 2 (Hanstech instruments, King's Lynn, England) and an OS-30p chlorophyll fluorometer (Opti-Sciences Inc., Hudson, USA). The Hansatech is a modulated device that allows the calculation of ETR, while the Opti-Sciences device simply measures F_V/F_M. F_V/F_M is the maximum photochemical efficiency of photosystem II, and ETR is the electron transport rate, an approximation of the rate of photosynthesis (Kalaji et al., 2014). A saturating flash was initially

given to determine F_V/F_M. In experiments where ETR was measured, thalli were then exposed to PAR at 30 µmoles m^{-2} s^{-1} for 10 min, after which fluorescence had reached a stable value. Another saturating flash was then applied, and F_V/F_M and ΦPSII were calculated as:

$F_V/F_M = (F_M – F_O)/F_M$. Where F_M is the maximum fluorescence (reaction centres closed) and F_O is the minimum fluorescence (reaction centres open), and:

$ΦPSII = (F_{M'} – F_t)/F_{M'}$. Where F_t is the stable fluorescence signal in the light, and $F_{M'}$ is the maximum fluorescence when a saturating pulse is given in the light and ETR (electron transfer rate) = ΦPSII x 0.5 x PFD.

2.4.2. Heat tolerance of the mycobiont (Conductivity - µS/cm)

To assess the effect of heat on the mycobionts, material was acclimated as described above, and then 25 replicates of 0.3 g of hydrated lichen material placed under a thermal stress of 40°C for 8 h, and ion leakage assessed in material stressed for 0, 2, 4, 6 and 8 h. Controls were immersed in distilled water for 5 minutes, and then immediately boiled. Conductivity was measured as initial (before boiling) and final (after boiling) with a water conductivity meter (Mettler-Toledo AG, Analytical Schwerzenbach, Switzerland). After heat stress, the lichen material was then immersed in 10 ml of distilled water for then was gently shaken for 30 min. After the removal of the lichens, the electric conductivity of the solution was measured (initial conductivity, or conductivity lost due to stress: Cv) in µS cm^{-1}. Lichens were then boiled in 5 ml of H_2O for 30 min, 5 ml of H_2O added, and conductivity (Cf) again measured. The average of the two blanks was always subtracted from solution measurements. Damage to membranes was assessed as the initial conductivity expressed as the percentage of the total conductivity lost, calculated as follows: 100*Cv/(Cv+Cf) (n=25).

2.5 Freezing tolerance

Experimental design

The aim of this experiment was to investigate the freezing tolerance of lichen species growing in contrasting localities with different environmental conditions to estimate how the material from the communities will behave under extreme cold conditions. Out of the five sites we chose uMlalazi nature reserve which is warm, humid and with a high average temperature throughout the year (see Table 2). The other site was Fort Nottingham nature reserve which is montane with summer rainfalls and cold winters with snow sometimes. So, out of the five sites used here, these two would enable us to compare relatively warm with relatively cool.

Before the start of experiments, 100 x 1 cm discs of thalli were acclimated as above then two experiments were run simultaneously, using -24°C and -75°C with lichen material from two sites: Fort Nottingham nature reserve (inland) and uMlalazi nature reserve (along the coast). As a control, unstressed material was kept in the growth cabinet under conditions described above.

The maximal quantum yield of PSII photochemistry (F_V/F_M) and the electron transport rate (ETR) were measured before and after freezing using 20 randomly selected discs from each site. Thalli were placed in plastic petri dishes with moist filter paper, and then sprayed with H_2O in the beginning and end of the experiment as recommended by Solhaug et al., (2018). The short-term effects of freezing were measured after 24 and 48 h and the long-term effects were measured after 7 d; each treatment had a separate set of discs. After the freezing stress, the discs were thawed, then dark adapted for 10 min, then a FMS 2 fluorimeter (Hanstech Instruments Ltd, Kings's Lynn, England) was used to measure fluorescence parameters.

2.6 Statistical analysis

The significance of differences ($P < 0.05$) between the different sites and treatments (for cold tolerance) were checked by a one and two-way analysis of variance (ANOVA) using the Bonferroni and Turkey's HSD tests for post-hoc comparisons for both the chlorophyll fluorescence parameters and cell membrane integrity. Data not meeting the assumptions of normality for a parametric test (K-S or Shapiro Wilk W-test) at the 95% confidence interval were log-transformed to correct skewed distribution. All statistics analysis were performed using IBM SPSS v25.

MATERIALS AND METHODS FOR MOLECULAR STUDIES (DNA BARCODING)

2.7 DNA extraction, Polymerase Chain Reaction (PCR) and sequencing

Following freezing, the lichen material was thawed, hydrated and left in room temperature to air-dry for 24 h. then DNA was extracted from a small portion of a healthy lichen thallus using the Zymo Research Quick-DNA^{TM} Plant/Seed Miniprep Kit (ZYMO research, USA). To reduce the possibility of contamination and inhibition due to other lichen substances, a method adapted from Divakar et al. (2016) with slight modification was used. Instead of using liquid nitrogen to dry the lichen material as per Divakar et al. (2016), in this study we simply air-dried our material at room temperature for 24 h. before use (this method/technique was only applied on lichen material used for molecular studies (DNA barcoding) and not for the physiology experiments. Dry thalli were soaked in acetone for 2-3 h then air dried overnight. The thallus was then chopped into small pieces and ground in lysis buffer for 5 min at room temperature prior to extraction. Amplification of nuclear ribosomal ITS was performed using fungal-specific primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes and Bruns, 1993), ITS4A (3'-

CGCCGTTACTGGGGCAATCCCTG-5') (Larena et al., 1999). Each 12.5 µl PCR reaction contained: 7.7 µl dH_2O; 1 µl 10x Dream Taq green buffer; 1.25 mL, 1 µl (mg ml^{-1}) Bovin Serum Albumin (BSA); 1 µl $MgCl_2$ (25 Mm); 0.25 µl of each (10 µM) primer; 0.05 µl DreamTaq DNA polymerase (Fermentas, South Africa); 0.25 µl deoxynucleoside triphosphate (dNTP) (10 µM) and (0.5-1 ng) DNA template. Negative controls (no template reactions) were included to check for contamination of reagents. PCR cycling conditions were the following: 95°C initial heating step of 3 min, followed by 35x cycles of 95°C for 30 s, 30 s at 65°C and 1 min at 72°C then a final extension step of 10 min at 72°C.

The PCR products were viewed on a 2% (w/v) TBE agarose gel stained with Ethidium Bromide (0.2mg, EtBr). PCR products were sized using a 100bp molecular weight ladder (Soils, BioDyne), which was also loaded onto the gel. The expected product size of ITS was 500-700bp. Gels were viewed under an ultra violet light using the FOTODYNE Incorporation gel imager. Successfully amplified products were excised from the gel and submitted for sequencing at the Central Analytical Facility (CAF), Stellenbosch University, South Africa. BLASTn (https://blast.ncbi.nlm.nih.gov) searches were conducted against GenBank to verify all sequences obtained.

2.8 Sequence alignments, phylogenetic analysis and genetic distances estimates

The electropherogram of the each ITS sequences generated was checked using BioEdit v7.2.6.1 (Hall, 1999). Sequences were aligned using ClustalX 2.1 (Larkin et al., 2007). Alignments were checked manually to ensure homology. The sequences generated from this study (n=68) (Table 7) were aligned with 60 previously published ITS sequences (Table 8) downloaded from GenBank

(https://www.ncbi.nlm.nih.gov/genbank/) and BOLD Systems (http://boldsystems.org/). We downloaded sequences by searching for genus name on the database and every sequence for that genus name was downloaded. The final aligned data matrix included 128 taxa and 521 aligned characters.

2.9 Phylogenetic analysis

Phylogenies were constructed using two model-based approaches, maximum likelihood (ML) and Bayesian inference (Bayes). The best-fit substitution model (GTR+G) for the ITS data set was estimated using the Akaike Information Criterion (AIC) in jModel Test v. 0.1.1 (Darriba et al., 2012). The program Garli v2.0 (Zwickl, 2008) was used to perform ML analysis. Branch support values for each node were estimated using 1000 bootstrap (BS) replicates. These trees were viewed and edited on the program FigTree v.1.4.3 (Drummond and Rambant, 2007).

The program MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003) was used to perform the Bayes analysis. For this analysis, two separate runs were conducted, each run consisting of four parallel MCMC chains. Chains were run for 20 million generations with a sampling frequency of 300. Once the runs were completed, convergence of the two runs was checked using Tracer v1.6 (Rambaut and Drummon, 2012). Convergence was assumed when all the effective sampling size (ESS) values exceeded 200. The first 4 million (20%) trees were removed from the tree file as burn-in. A 50% majority rule consensus tree was generated using the Consense module available in Phylip v3.6.9.5 (Felsenstein, 2005). Branch support was assessed using posterior probability values.

2.10 DNA barcode gap analysis

The best-fit model (GTR+G) was used to generate pairwise distances in RaxmlGUI v. 1.5b1 (Silvestro and Michalak, 2012). Intra- and interspecific distances were then plotted to visualize the DNA barcode gap. To test for the statistical separability of the intraspecific and interspecific distances, the Jeffries-Mutusita Distance (J-M) function was calculated in R Studio (https://www.r-project.org/). Jeffries-Mutusita Distance (J-M) function is a widely used statistical tool to assess the potential seperability two classes (Trigg and Flasse, 2001). The J-M test has a threshold of 1.414, so anything equal to or greater than 1.414 suggests statistical separability (Trigg and Flasse, 2001).

Table 7: Specimens of *Parmelia* from which new sequences were obtained from this study loosely, keyed out to *Parmelia perlata* (*sensu lato*)

Specimen ID	Locality	GPS coordinates
uMlalazi_001	Mthunzini	-28.9541203°S, 31.766569°E
uMlalazi_003	Mthunzini	-28.9541203°S, 31.766569°E
uMlalazi_004	Mthunzini	-28.9541203°S, 31.766569°E
uMlalazi_005	Mthunzini	-28.9541203°S, 31.766569°E
uMlalazi_006	Mthunzini	-28.9541203°S, 31.766569°E
uMlalazi_007	Mthunzini	-28.9541203°S, 31.766569°E
uMlalazi_008	Mthunzini	-28.9541203°S, 31.766569°E
uMlalazi_009	Mthunzini	-28.9541203°S, 31.766569°E
uMlalazi_010	Mthunzini	-28.9541203°S, 31.766569°E
*NT_HF_001_ITS_*	uMhlanga, Durban	-29.7128816°S, 31.0927580°E
HFor_002	uMhlanga, Durban	-29.7128816°S, 31.0927580°E
HFor_003	uMhlanga, Durban	-29.7128816°S, 31.0927580°E
HFor_004	uMhlanga, Durban	-29.7128816°S, 31.0927580°E
HFor_005	uMhlanga, Durban	-29.7128816°S, 31.0927580°E
HFor_006	uMhlanga, Durban	-29.7128816°S, 31.0927580°E
HFor_007	uMhlanga, Durban	-29.7128816°S, 31.0927580°E
*NT_HF_010_ITS_*	uMhlanga, Durban	-29.7128816°S, 31.0927580°E
UKZN_001	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_002	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_003	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_004	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_007	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_009	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_010	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_013	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_014	Pietermaritzburg	-29.6258172°S, 30.4019928°E

Table 7: cont…

Specimen ID	Locality	GPS coordinates
UKZN_015	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_001	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_003	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_004	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_005	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_007	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_008	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_009	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_010	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_011	Pietermaritzburg	-29.6258172°S, 30.4019928°E
M. Cowl_001	Drakensburg	-29.0485156°S, 29.4064394°E
M. Cowl_002	Drakensburg	-29.0485156°S, 29.4064394°E
M. Cowl_003	Drakensburg	-29.0485156°S, 29.4064394°E
M. Cowl_004	Drakensburg	-29.0485156°S, 29.4064394°E
M. Cowl_005	Drakensburg	-29.0485156°S, 29.4064394°E
M. Cowl_006	Drakensburg	-29.0485156°S, 29.4064394°E
FN_001	Fort Nottingham	-29.4145055°S, 29.9133356°E
FN_002	Fort Nottingham	-29.4145055°S, 29.9133356°E
FN_003	Fort Nottingham	-29.4145055°S, 29.9133356°E
FN_004	Fort Nottingham	-29.4145055°S, 29.9133356°E
FN_005	Fort Nottingham	-29.4145055°S, 29.9133356°E
FN_006	Fort Nottingham	-29.4145055°S, 29.9133356°E
UKZN_007	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_008	Pietermaritzburg	-29.6258172°S, 30.4019928°E
UKZN_009	Pietermaritzburg	-29.6258172°S, 30.4019928°E
FN_007	Fort Nottingham	-29.4145055°S, 29.9133356°E

Table 7: cont…

Specimen ID	Locality	GPS coordinates
FN_008	Fort Nottingham	-29.4145055°S, 29.9133356°E
FN_009	Fort Nottingham	-29.4145055°S, 29.9133356°E
FN_010	Fort Nottingham	-29.4145055°S, 29.9133356°E
FN_011	Fort Nottingham	-29.4145055°S, 29.9133356°E
FN_012	Fort Nottingham	-29.4145055°S, 29.9133356°E
HFor_008	uMhlanga, Durban	-29.7128816°S, 29.9133356°E
HFor_009	uMhlanga, Durban	-29.7128816°S, 29.9133356°E
HFor_010	uMhlanga, Durban	-29.7128816°S, 29.9133356°E
HFor_011	uMhlanga, Durban	-29.7128816°S, 29.9133356°E
HFor_013	uMhlanga, Durban	-29.7128816°S, 29.9133356°E
HFor_014	uMhlanga, Durban	-29.7128816°S, 29.9133356°E
HFor_015	uMhlanga, Durban	-29.7128816°S, 29.9133356°E
uMlalazi_011	Mthunzini	-28.9541203°S, 31.766569°E

Table 8: Specimens of *Parmelia* downloaded from GenBank and BOLD systems with localities, accession number and publication sources

Specimen ID	Locality	GenBank accession numbers	References
P. reticulatum	Unknown	EU266116	Han et al., 2007 (unp.)
P. reticulatum	Argentina	EU853257	Adler et al., 2008 (unp.)
P. tinctorum	Unknown	EU643593	Lei et al., 2008
P. tinctorum	Unknown	EU643592	Lei et al., 2008
P. subtinctorium	Unknown	GU593037	Hur, 2010 (unp.)
P. tinctorum	Unknown	JF831050	Hur, 2010 (unp.)
P. tinctorum	Unknown	HQ650684	Schmull et al., 2011
P. austrosinense	Unknown	HQ650683	Schmull et al., 2011
P. reticulatum	Uruguay	AY251450	Thell et al., 2004
P. cetratum	Uruguay	AY251449	Thell et al., 2004
P. sp. Feuerer s.n.	Chile	AY251448	Thell et al., 2004
P. tinctorum	Yemen	AY251443	Thell et al., 2004
P. crinitum	Yemen	AY251442	Thell et al., 2005
P. fistulatum	Argentina	AY251415	Thell et al., 2004
P. fistulatum	Uruguay	AY581057	Blanco et al., 2004
P. pilosum	Uruguay	AY581056	Blanco et al., 2004
P. haitiense	Australia	AY581055	Blanco et al., 2004
P. hypoleucinum	Morroco	HM017035	Del-Prado et al., 2010

Table 8: cont…

Specimen ID	Locality	GenBank accession numbers	References
P. norsticticatum	South Africa	GU994576	Crespo et al., 2010
P. pseudoreticulatum	Spain	HM017053	Del-Prado et al., 2010
P. pseudoreticulatum	Spain, Balearic Islands	JN166399	Del-Prado et al., 2011 (unp.)
P. pseudotinctorum	Chamoli, India	KF129421	Roca-Valiente et al., 2013
P. reticulatum	Morocco	HM016953	Del-Prado et al., 2010
P. reticulatum	Spain	HM016954	Del-Prado et al., 2010
P. reticulatum	Morocco	HM017057	Del-Prado et al., 2010
P. reticulatum	Spain	HM017060	Del-Prado et al., 2010
P. reticulatum	Spain, Canary Islands	JN166381	Del-Prado et al., 2010 (unp.)
P. species	Unknown	HM016957	Del-Prado et al., 2010
P. species	Unknown	HQ335207	Zhang and Shi, 2010 (unp.)
P. subtinctorium	China	KC978853	Dong, 2013 (unp.)
P. tinctorum	Unknown	KF129455	Roca-Valiente et al., 2013
P. aff. cetratum Lucking 15116A	Costa Rica, Puntarens	AY642850	Divakar et al., 2005
P. aff. cetratum Lucking 15096	Costa Rica, Puntarens	AY642849	Divakar et al., 2005
P. aff. cetratum Lucking 15593A	Costa Rica, Puntarens	AY642848	Divakar et al., 2005
P. cetratum	Uruguay, Maldonado	AY642847	Divakar et al., 2005
P. clavuliferum	Spain, Pontevedra	AY642846	Divakar et al., 2005
P. reticulatum	Kenya	AY642845	Divakar et al., 2005
P. reticulatum	Spain, Canary Islands	AY642844	Divakar et al., 2005
P. pseudoreticulatum	Portugal, Estremadura	AY642842	Divakar et al., 2005
P. pseudoreticulatum	Portugal: Estremadura	AY642841	Divakar et al., 2005
P. pseudoreticulatum	Portugal, Estremadura	AY642839	Divakar et al., 2005

Table 8: cont…

Specimen ID	Locality	GenBank accession numbers	References
P. reticulatum	Portugal, Portalegre	AY642838	Divakar et al., 2005
P. reticulatum	Portugal, Evora	AY642837	Divakar et al., 2005
P. reticulatum	Portugal, Evora	AY642836	Divakar et al., 2005
P. reticulatum	Portugal, Evora	AY642835	Divakar et al., 2005
P. reticulatum	Spain, Tenerife, Canary Islands	AY642834	Divakar et al., 2005
P. clavuliferum	Spain, Pontevedra	AY642833	Divakar et al., 2005
P. clavuliferum	Spain, Cies islands	AY642832	Divakar et al., 2005
P. reticulatum	Spain, Cies islands	AY642831	Divakar et al., 2005
P. pseudoreticulatum	South Africa, Eastern Cape	AY642830	Divakar et al., 2005
P. pseudoreticulatum	South Africa, Eastern Cape	AY642829	Divakar et al., 2005
P. pseudoreticulatum	South Africa, Eastern Cape	AY642828	Divakar et al., 2005
P. reticulatum	Portugal, Santarem	AY642827	Divakar et al., 2005
P. reticulatum	Portugal, Santarem	AY642826	Divakar et al., 2005
P. reticulatum	Spain, Tenerife, Canary Islands	AY642825	Divakar et al., 2005
P. clavuliferum	China: Chu Xiong County, Yunnan Province	AY642824	Divakar et al., 2005
P. clavuliferum	China: JianChuan County, Yunnan Province	AY642823	Divakar et al., 2005
P. clavuliferum	China: Chen Xirg County, Yunnan Province	AY642822	Divakar et al., 2005
P. reticulatum	Spain, Parque Natural de los Alcornocales, Malaga	AY642820	Divakar et al., 2005
P. reticulatum	China: Lu Nan County, Yunnan Province	AY642819	Divakar et al., 2005

References

Adie, H., Lawes, M.J., 2011. Podocarps in Africa: temperate zone relicts or rainforest survivors?

Allen, A., 2008. Lichen specialties of Lundy: an overview. Journal of the Lundy Field Society, 1, 33–40.

Blanco, O., Crespo, A., Elix, J.A., Hawksworth, D.L., Thorsten Lumbsch, H., 2004. A molecular phylogeny and a new classification of parmelioid lichens containing *Xanthoparmelia*-type lichenan (Ascomycota: Lecanorales). Taxon 53, 959-975.

Crespo, A., Kauff, F., Divakar, P.K., del Prado, R., Pérez-Ortega, S., de Paz, G.A., Cubas, P. ,2010. Phylogenetic generic classification of parmelioid lichens (Parmeliaceae, Ascomycota) based on molecular, morphological and chemical evidence. Taxon 59, 1735-1753.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9, 772.

Del-Prado, R., Cubas, P., Lumbsch, H.T., Divakar, P K., Blanco, O., de Paz, G. A., Crespo, A., 2010. Genetic distances within and among species in monophyletic lineages of Parmeliaceae (Ascomycota) as a tool for taxon delimitation. Molecular Phylogenetics and Evolution 56, 125-133.

Divakar, P.K., Blanco, O., Hawksworth, D.L., Crespo, A., 2005. Molecular phylogenetic studies on the *Parmotrema reticulatum* (*syn. Rimelia reticulata*) complex, including the confirmation of *P. pseudoreticulatum* as a distinct species. The Lichenologist 37, 55-65.

Divakar, P.K., Leavitt, S.D., Molina, M.C., Del-Prado, R., Lumbsch, H.T., Crespo, A., 2016. A DNA barcoding approach for identification of hidden diversity in Parmeliaceae

(Ascomycota): *Parmelia sensu stricto* as a case study. Botanical Journal of the Linnean Society 180, 21–29.

Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology 7, 214.

Felsenstein, J., 2005. PHYLIP (phylogeny inference package) Distributed by the author. Department of Genome Sciences, University of Washington, Seattle), Version, 3.

Frahm, J.P., Schumm, F., Stapper, N.J., 2010. Epiphytische Flechten als Umweltgütezeiger: eine Bestimmungshilfe. BoD–Books on Demand.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Molecular Ecology 2, 113–118.

Hale Jr, M.E., 1987. A monograph of the lichen genus *Parmelia* Acharius *sensu stricto* (Ascomycotina: Parmeliaceae).

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

Honegger, R., 2003. The impact of different long-term storage conditions on the viability of lichen-forming Ascomycetes and their green algal photobiont, *Trebouxia* spp. Plant Biology 5, 324-330.

Jensen, M., Feige, G.B., 1987. The effect of desiccation and light on the 77 K chlorophyll fluorescence properties of the lichen *Peltigera aphthosa*. Bibliotheca Lichenologica 25, 325–330.

Kalaji, H.M., Schansker, G., Ladle, R.J., Goltsev, V., Bosa, K., Allakhverdiev, S.I., Elsheery, N.I., 2014. Frequently asked questions about in vivo chlorophyll fluorescence: practical issues. Photosynthesis research, 122, 121–158.

Kappen, L., 1988 Ecophysiological relationships. In CRC Handbookof Lichenology, Vol. 2 (Galun, M., ed.), Boca Raton: CRC Press, 37-100.

Larena, I., O., Salazar, V., González, M.C., Julián V., Rubio., 1999. Design of a primer for ribosomal DNA internal transcribed spacer with enhanced specificity for ascomycetes. Journal of Biotechnology 75, 187–194.

Larkin, M.A., Blackshields, G., Brown N.P., Chenna, R., McGettigan, P.A., McWilliam H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J. Higgins, D.G. 2007 Clustal W and Clustal X version 2.0. Bioinformatics (Oxford, England) 23, 2947–2948.

Larson, D.W., 1978. Patterns of lichen photosynthesis and respiration following prolonged frozen storage. Canadian Journal of Botany 56, 2119–2123.

Paoli, L., Munzi, S., Pisani, T., Guttová, A., Loppi, S., 2013. Freezing of air-dried samples of the lichen *Evernia prunastri* (L.) Ach. ensures that thalli remain healthy for later physiological measurements. Plant Biosystems 147, 141-144.

Purvis, O.W., 1992. Lichen Flora of Great Britain and Ireland. Natural History Museum Publications in association with the British Lichen Society.

Rambaut, A., Drummond, A.J., 2012. Tracer v1. 4. 2007. Computer program and documentation. Retrieved from a http://beast.bio.ed.ac.uk/Tracer. Date assessed:26 May 2016.

Roca-Valiente, B.E.A.T.R.I.Z., Divakar, P.K., Ohmura, Y., Hawksworth, D.L., Crespo, A., 2013. Molecular phylogeny supports the recognition of the two morphospecies *Parmotrema pseudotinctorum* and *P. tinctorum* (Parmeliaceae, Ascomycota). Vieraea, 41, 333-348.

Ronquist, F., Huelsenbeck, J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Schmull, M., Miadlikowska, J., Pelzer, M., Stocker-Wörgötter, E., Hofstetter, V., Fraker, E., Kauff, F., 2011. Phylogenetic affiliations of members of the heterogeneous lichen-forming fungi of the genus *Lecidea* sensu Zahlbruckner (Lecanoromycetes, Ascomycota). Mycologia 103, 983-1003.

Silvestro, D., Michalak, I., 2012. raxmlGUI: a graphical front end of RAxML. Organisms, Diversity and Evolution 12, 335-337.

Solhaug, K.A., Chowdhury, D.P., Gauslaa, Y., 2018. Short-and long-term freezing effects in a coastal (*Lobaria virens*) versus a widespread lichen (*L. pulmonaria*). Cryobiology 82, 124–129.

Thell, A., Feuerer, T., Kärnefelt, I., Myllys, L., Stenroos, S., 2004. Monophyletic groups within the Parmeliaceae identified by ITS rDNA, β-tubulin and GAPDH sequences. Mycological Progress 3, 297-314.

Thell, A., Herber, B., Aptroot, A., Adler, M.T., Feuerer, T., Kärnefelt, I., 2005. A preliminary phylogeographic study of *Flavopunctelia* and *Punctelia* inferred from rDNA ITS-sequences.

Trigg, S., Flasse, S., 2001. An evaluation of different bi-spectral spaces for discriminating burned shrub-savannah. International Journal of Remote Sensing 22, 2641–2647.

van Herk, C. M., Aptroot, A., Sparrius, L. B., Spier, J. L., 2004. Paleispark Het Loo bij Apeldoorn, een ware hotspot voor epifytische korstmossen. Buxbaumiella 69, 9–16.

Yamamoto, Y., Kinoshita, Y., Takahagi, T., 1998. Factors affecting discharge and germination of lichen ascospores. Journal of the Hattori Botanical Laboratory 85, 267-278.

Zwickl, D. J., 2008. GARLI (Genetic algorithm for rapid likelihood inference), version 0.96. URL http://www. bio. utexas. edu/faculty/antisense/garli/garli. html [accessed on May 2008].

CHAPTER 3: THERMOTOLERANCE OF *PARMELIA* SPECIES FROM CONTRASTING LOCALITIES IN KWAZULU-NATAL

1. Introduction

Considering the predicted changes caused by climate change, such as the increase in average temperatures, severe droughts and intense rainfall (Karl and Tranberth, 2003) different regions with varying environmental conditions are predicted to react differently to these sudden changes in climate. As discussed in Chapter 1, Aptroot and van Heck (2007) showed that species found in cold regions are in danger of declining in numbers and potentially extinction because of failure to adapt to new climatic conditions. In light of this, the current study hopes to shed some light on how extreme temperatures will affect lichen species occurring in different regions: coast (warm and humid), inland (summer rainfalls and extreme cold winters which may sometimes include snow) and montane regions within KZN. The experiments carried out in this study will allow us to investigate how lichens from these different regions will react to the possible "extreme" temperature we might experience as a result of climate change, with the prediction that lichen species from the "warm" sites normally in the coast will have higher tolerance to high temperatures than lichens from places like the Drakensburg and vice versa with cold tolerance. Results from these experiments will allow us to predict how lichen communities occurring in different regions will be affected by climate change.

The aim of the work presented here was to test if variations exist in the heat and cold tolerance of lichens collected from different sites along an altitudinal transect in the province of KwaZulu-Natal, with the aim of predicting the likely effects of climate change. Here we used a variety of techniques to assess the tolerance of symbionts to different stresses. Techniques such as chlorophyll fluorescence (Chl) have been widely used to assess viability, recovery and

performance of lichen photobionts following stress (Green et al., 1998). Furthermore, measuring the loss of membrane integrity through conductivity index has also been suggested as reliable tools to measure the effects of stress on lichen mycobionts (Solhaug et al., 2018). Ion leakage has been previously used as a tool to investigate the effects of desiccation in mosses (Beckett, 2001) and lichens (Buck and Brown, 1979). In some cases, the use of ion leakage as a viability of measure has proven to be a faster and a more sensitive indicator of damage of the mycobiont compared to other methods e.g. measuring respiration (Fields and St. Clair, 1984; Shirazi et al., 1996). Therefore, the stress tolerance of photobionts was assessed by measuring chlorophyll fluorescence parameters, and ion leakage (measured as electrical conductivity/ conductivity index) to assess the performance of the lichens mycobiont and photobiont respectively under high and freezing temperatures.

2. Results

2.1 Thermotolerance (chlorophyll fluorescence)

Treating samples of *Parmelia* at 35°C for 4 h greatly reduced F_V/F_M in lichen material collected from all sites except the coastal location (Figure 4). Clearly, the photobionts in the lichens from the coastal site (Hawaan forest) were more tolerant to heat stress compared to the photobionts from all the other sites. Using the Bonferroni test for post-hoc comparisons, the *Parmelia* samples from the four different sites at 35°C for 4 h resulted in Hawaan forest showing a significant difference from the other three sites ($p<0.05$) for the investigated parameter (F_V/F_M).

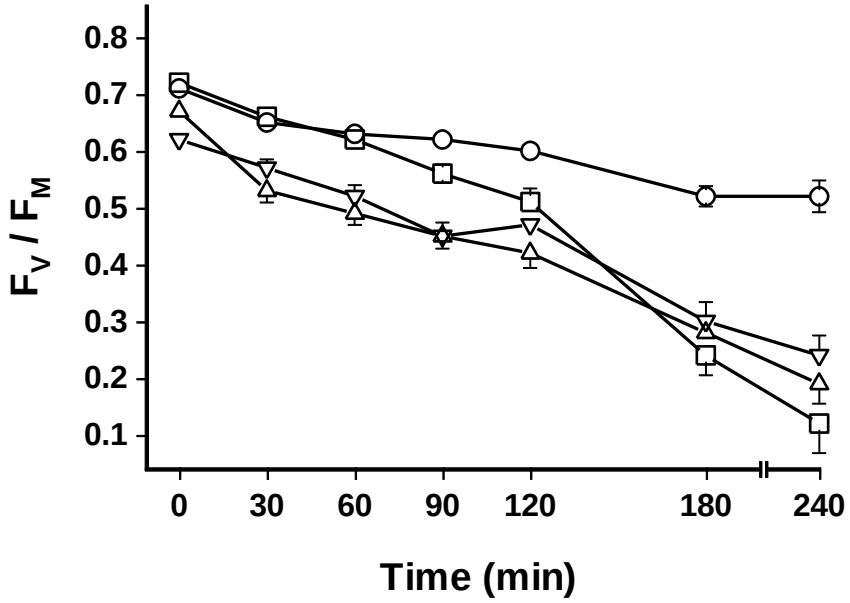


Figure 4: The effect on maximal quantum yield of PSII photochemistry of treating hydrated *Parmelia sp.* at 35°C for 4 h. Samples were collected from Hawaan forest (open circles), Monks cowl (triangles down), Fort Nottingham (triangles up) and Pietermaritzburg (squares). Values are given ±S.E, n=20.

2.2 Conductivity index (cell membrane integrity and ion leakage)

Loss of cell membrane expressed as ion leakage (µS cm^{-1}) was log transformed to meet the normality requirement for a one-way ANOVA. Ion leakage increased significantly with time for all *Parmelia* species collected from the four different sites (Figure 5) but was lowest from the coastal site: Hawaan. As with F_V/F_M, the post-hoc comparison for conductivity was significant ($p<0.05$) for the *Parmelia* lichens collected from the four sites.

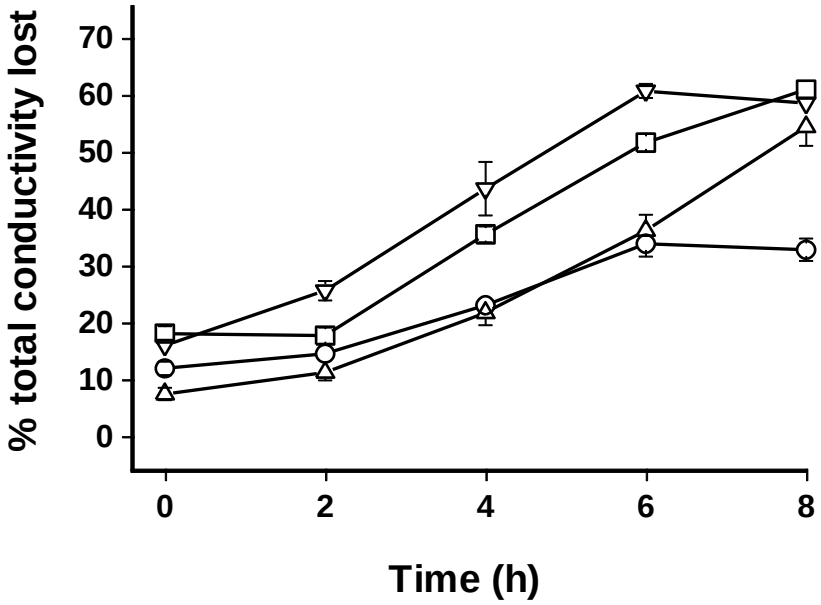


Figure 5: The effect on conductivity index (µS/cm) of treating *Parmelia* sp. at 40°C for 8h. Samples were collected from Monks cowl (triangles down), Pietermaritzburg (squares), Fort Nottingham (triangles up) and Mtunzini (circles). Values are given ±S.E, n=25.

2.3 Freezing tolerance (chlorophyll fluorescence)

Both the short (24 and 48 h) and the long-term (7 d) effect of freezing were assessed by exposing the lichen material collected from Fort Nottingham (inland) and uMlalazi (coastal) to -24 and -75^{o}C freezing temperatures. Freezing had little effect at either temperature on ETR (Figure 4A, B), and freezing at -24^{o}C had little effect on F_V/F_M (Figure 3A). However, freezing at -75^{o}C significantly reduced F_V/F_M in the material from both sites, with the material from the colder Fort Nottingham being slightly more sensitive (Figure 3B). A two-way ANOVA results showed that at -75°C there was a significant difference between the two sites ($p<0.05$), suggesting that the species are behaving differently in response to temperature but no significance difference between treatments at -24°C.

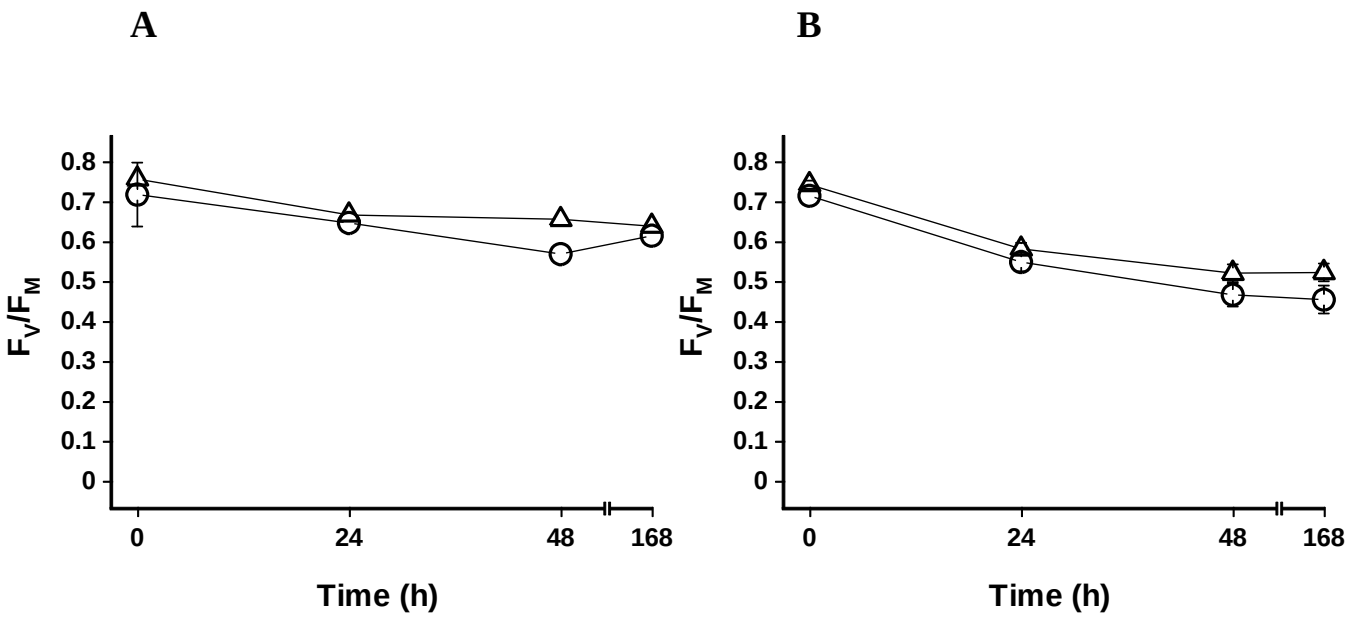


Figure 6: The effect on F_V/F_M of treating hydrated *Parmelia sp.* at -24°C (Fig 6A) and -75°C (Fig 6B) for up to 168 h. Samples were collected from Mtunzini (open circles) and Fort Nottingham (triangles up). Values given ±S.E, n=20.

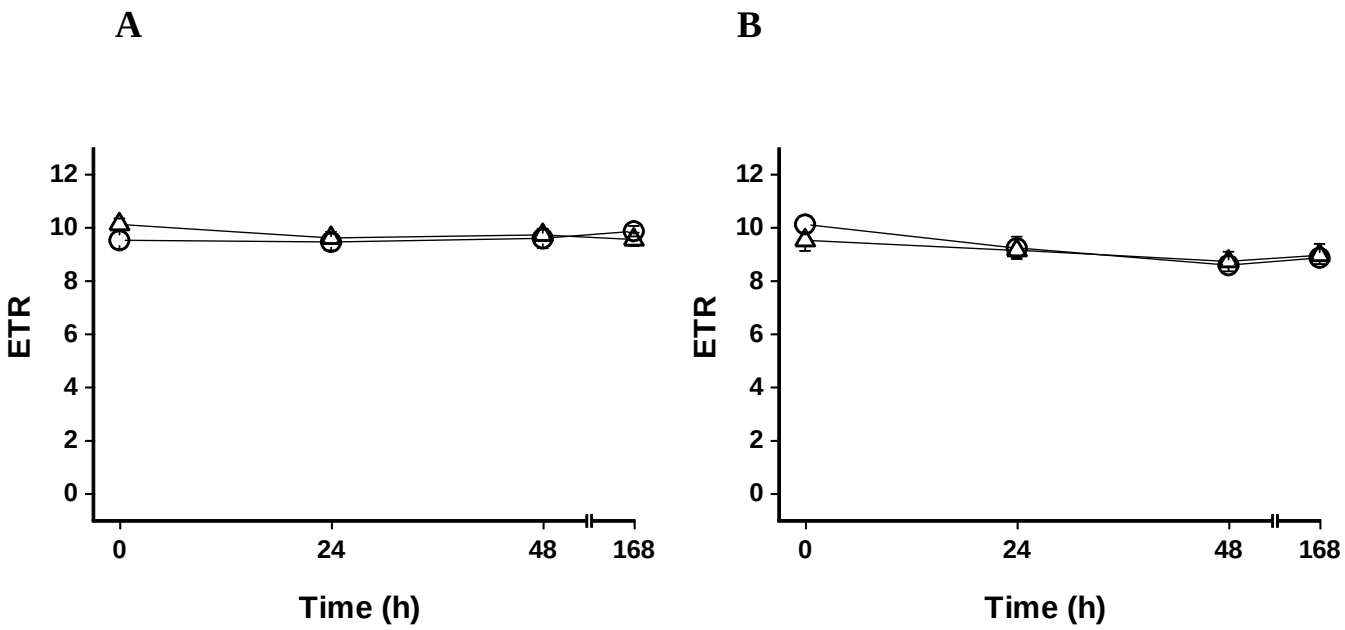


Figure 7: The effect on ETR of treating *Parmelia* sp. at -24°C (Fig 7A) and -75°C (Fig 7B) for 168 h. Samples collected from Mtunzini (open circles) and Fort Nottingham (triangles up). Values given ±S.E, n=20.

3. Discussion

By exposing *Parmelia* species to high and freezing temperatures, short-term viability parameters such as F_V/F_M, ETR and membrane integrity were impaired. The reductions in the chlorophyll fluorescence parameters indicated that the photobiont was affected by both freezing and heat treatments, while the increased ion leakage indicated heat damage to the mycobiont (Yemets et al., 2015). Results presented here show that both the photobionts and mycobionts of the closely related members of *Parmelia* from warm coastal localities are more heat tolerant than those from inland or montane sites. However, the photobionts display only very small differences in freezing tolerance. It would appear that *Parmelia* possesses an ability to acclimate to changes in temperature, which should mean that members of this genus will be able to adapt to future increases in temperature.

The work presented in this chapter indicated that both the photobiont (assessed by measuring photosynthesis) and mycobiont (assessed by measuring ion leakage) of the *Parmelia* species collected here differ in their tolerance to thermal stress (Figures 4 and 5). Thermal stress caused a decline in the lichen's health (F_V/F_M and ion leakage) over a 4 h and 8 h experimental periods at 35°C and 40°C respectively. While material from the three Midlands sites behaved similarly, material from the warmer coastal site of Hawaan was clearly more heat tolerant. The differences in heat tolerance could be because of phenotypic acclimation or genetic (ecotypic) variation; to test out these possibilities, one would have to carry out transplant experiments or possibly carry out these experiments at different times of the year. There have been relatively few studies on difference in tolerance to heat within a single species of lichen. In a study by Tegler and Kershaw (1981), the heat tolerance of *Cladonia rangiferina* was assessed at different times of the year. While net photosynthesis in a late winter collection was reduced after 7 d at 35°C, in a mid-summer collection the same treatment had no deleterious effects. These results suggest that phenotypic acclimation to warm temperatures can occur in lichens. Phenotypic adaptation in mosses were reported by Lange (1955) in the genera *Ctenidium*, *Fissidens* and *Syntrichia*. For example, the temperature required to cause stress in *Ctenidium* increased by 15°C between winter and summer. Both these studies indicate that there can be a 10°C or more difference in heat tolerance between the winter and the summer. Similar data exist for higher plants. Larcher (2000) reports that seasonal changes in heat resistance in Angiosperms can occur in response to time of year. Season variation of heat tolerance is often of the "summer type" where increases in resistance are associated with increases in ambient temperature. Thus, it is clearly possible for phenotypic acclimation can take place in higher and lower plants. While the material used in the present study was all collected in summer or early autumn, it would be interesting to test whether season variations in heat tolerance occurs in *Parmelia*. Apart from seasonal variations within a single population, a few studies have

compared heat tolerance in different populations of the same species collected at the same time of year. MacFarlane and Kershaw (1980) showed that two populations of *Peltigera canina* significantly differed in their response to heat stress (when air-dried). While one population was affected at 35°C, the other was only affected at 45°C. These varying levels of thermal sensitivity in the different populations of *P. canina* were correlated exactly with their ecology (MacFarlane and Kershaw, 1980). Interestingly, not all studies have shown differences in heat tolerance of a lichen species from different habitats. Shirazi et al., (1996) found no differences in heat tolerance in *Lobaria* species sampled from a mountain and a valley.

By comparison to heat tolerance, only very small differences in freezing tolerance were observed in this study (Figures 3 and 4). Lichens with *Trentepohlia* and *Dictyochloropsis* photobionts are considered to be more susceptible to freezing stress compared to lichens associated with other green algal photobionts (Kallio and Heinonen, 1971; Kappen, 1973; Lange, 1953; Nash et al. 1987). For example, in the study of Nash et al., (1987) lichens with *Trentopohlia* exhibited a significant reduction in net photosynthesis following 6 h at -12, -20 and -46°C treatments, accompanied by a decrease in total chlorophyll content. By contrast in lichens with *Treboxia* as their photobiont, damage only occurred when temperatures of -46°C were used. As lichen genus used in the present study, *Parmelia*, has *Treboxia* as a photobiont, it was probably not surprising that all collections had an inherently high tolerance to freezing. However, differences in the freezing tolerance of the mycobionts of lichens, assessed by ion leakage, have been reported between lichen species within the same genus (e.g. *Lobaria* (Solhaug et al. 2018)). The effects of freezing stress on the mycobiont the *Parmelia* species were not tested in the present study. Anecdotal observations suggest that while the material from the inland regions e.g. Drakensburg and Fort Nottingham experiences frost during the winter, the material from the coast almost certainly never does. Therefore, future work should determine if there are differences in the freezing tolerance of the mycobiont. However, results

presented here suggest that the sensitivity of the photobiont to freezing does not differ between the different sites.

4. Conclusion

The results of our study showed that both the photobiont and the mycobiont of *Parmelia* lichen species collected from the coast can better tolerate a temperature stress of 35°C for 4 h compared to those collected from inland sites. These temperatures are common in KwaZulu-Natal, especially in summer, although unless rainfall or other forms of precipitation was occurring at these temperatures' thalli would quickly dry. Possibly in future, F_V/F_M could be used as a parameter to assess the tolerance of the photobiont to high temperatures in both dry and moist state. However, there is little difference in the sensitivity of the photobionts of different populations to freezing, consistent with earlier literature indicating that the photobiont of this lichen, *Trebouxia*, has an inherently high tolerance to freezing. Taken together, results suggest that should global warming occur, these types of species are unlikely to go extinct as they seem to adapt well to changes. While more work is needed to test the freezing tolerance of the mycobiont, the photobiont at least shows an extraordinarily high resistance to freezing, consistent with earlier reports in the literature.

References

Aptroot, A., Van Herk, C.M., 2007. Further evidence of the effects of global warming on lichens, particularly those with *Trentepohlia* phycobionts. Environmental Pollution 146, 293–298.

Beckett, R.P., 2001. ABA-induced tolerance to ion leakage during rehydration following desiccation in the moss *Atrichum androgynum*. Plant Growth Regulation 35, 131–135.

Buck, G.W., Brown, D.H., 1979. The effect of desiccation on cation location in lichens. Annals of Botany 44, 265–277.

Fields, R.D., St. Clair, L.L., 1984. A comparison of methods for evaluating SO_2 impact on selected lichen species: *Parmelia chlorochoa, Collema polycarpon* and *Lecanora muralis*. The Bryologist 87, 297-301.

Green, T. G. A., Schroeter, B., Kappen, L., Seppelt, R. D., Maseyk, K., 1998. An assessment of the relationship between chlorophyll a fluorescence and CO_2 gas exchange from field measurements on a moss and lichen. Planta 206, 611-618.

Kallio, P., Heinonen, S., 1971. Influence of short-term low temperature on net photosynthesis in some subarctic lichens. Department of Botany, University of Turku.

Kappen, L., Lange, O. L., Schulze, E. D., Evenari, M., Buschbom, U., 1973. Primary production of lower plants in the desert and its physiological bases. In "Photosynthesis and Productivity in Different Environments" (J.P. Cooper, ed). Cambridge Univ. Press. (in press).

Karl, T.R., Trenberth, K.E., 2003. Modern global climate change. Science 302, 1719–1723.

Lange, O. L., 1953. Hitze- und Trockenresistenz der Flechten in Beziehung zu ihrer Verbreitung. Flora Jena 140, 39-97.

Lange, O. L., 1955. Untersuchungen iiber die Hitzeresistenz der Moose in Beziehung zu ihrer Verbreitung. Die Resistenz stark ausgetrockneter Moose. Flora Jena 142, 381-399

Larcher, W., 2000. Temperature stress and survival ability of Mediterranean sclerophyllous plants. Plant biosystems 134, 279-295.

MacFarlane, J. D., Kershaw, K. A., 1980. Physiological-environmental interactions in lichens. New Phytologist 84, 669-685.

Nash III, T. H., Kappen, L., Lösch, R., Larson, D. W., Matthes-Sears, U., 1987. Cold resistance of lichens with *Trentepohlia*-or *Trebouxia*-photobionts from the North American west coast. Flora, 179, 241-251.

Orsenigo, S., Abeli, T., Rossi, G., Bonasoni, P., Pasquaretta, C., Gandini, M., Mondoni, A., 2015. Effects of autumn and spring heat waves on seed germination of high mountain plants. PloS one 10, e0133626.

Schröder, W., Pesch, R., 2005. Time Series of Metals in Mosses and their Correlation with Selected Sampling Site-Specific and Ecoregional Characteristics in Germany*(9 pp). Environmental Science and Pollution Research 12, 159-167.

Shirazi, A. M., Muir, P. S., McCune, B., 1996. Environmental factors influencing the distribution of the lichens *Lobaria oregana* and *L. pulmonaria*. Bryologist, 12-18.

Solhaug, K. A., Chowdhury, D. P., Gauslaa, Y., 2018. Short-and long-term freezing effects in a coastal (*Lobaria virens*) versus a widespread lichen (*L. pulmonaria*). Cryobiology 82, 124-129.

Tegler, B., Kershaw, K. A., 1981. Physiological-environmental interactions in lichens: xii. The seasonal variation of the heat stress response of *Cladonia rangiferina*. New Phytologist, 87, 395-401.

Vieira, B. J., Freitas, M. C., Wolterbeek, H. T., 2017. Vitality assessment of exposed lichens along different altitudes. Influence of weather conditions. Environmental Science and Pollution Research 24, 11991-11997.

Yemets, O.A., Gauslaa, Y., Solhaug, K.A., 2015. Monitoring with lichens – conductivity methods assess salt and heavy metal damage more efficiently than chlorophyll fluorescence. Ecological Indicators 55, 59–64.

Zechmeister, H.G., 1995. Correlation between altitude and heavy metal deposition in the Alps. Environmental Pollution 89, 73–80.

CHAPTER 4: DNA BARCODING OF *PARMELIA* (PARMELIACEAE) SPECIES: MOLECULAR APPROACH TO ACCURATE SPECIMEN IDENTIFICATION OF SPECIES FROM KWAZULU-NATAL

1. Introduction

Species delimitation is important for evolutionary and ecological biologists (Kress et al., 2015). However, it is challenging to establish diagnostic characters and suitable analytical methods to delimit species (Parnmen et al., 2012), as morphological-based approaches are proving inadequate for some groups (Crespo and Perez-Ortega, 2009). This is a major limitation especially for groups with simple morphologies such as fungi where characters used for delimitation may not always be available or visible during certain life stages (Crespo and Lumbsch, 2010). This is especially true for lichens as obtaining an accurate estimate of the number of lichen species is complicated by several factors such as unclear taxonomic status of certain groups (Sipman and Aptroot, 2001; Feuerer and Hawksworth, 2007), and under collection and description of lichens in some regions. This is particularly true for tropical areas where it has been estimated that 50% of lichens are understudied (Aptroot and Sipman, 1997). Furthermore, in some groups only subtle morphological characters separate species (Divakar et al., 2016). Morphological and chemical characters in lichens are commonly used as taxonomic characters without a clear concept of their evolutionary origins. The evolution of some taxonomic characters in lichens have been examined in some studies (Blanco et al., 2006) but high plasticity has been demonstrated within life cycle of some fungi making them difficult to study (Wedin et al., 2004). This is unfortunate, given the importance of these characters in lichen symbiosis and their frequent use in taxonomy (Blanco et al., 2006).

The genus *Parmelia* belongs to the largest family of lichenized fungi, Parmeliaceae which was established by Acharius in 1803. This family contains a large number of foliose

species with lecanorine apothecia including diverse genera such as *Xanthoria*, *Lobaria*, *Cetraria*, *Parmelia* and *Physcia* (Hale Jr.,1987). Traditionally, two large genera (*Parmelia* and *Cetraria*) are distinguished as core groups in the Parmeliaceae; these more or less correspond to the Parmeloid and Cetraroid groups (DePriest, 1999). Morphological and chemical characters used to delimit *Parmelia* include foliose thallus, shape of the lobes e.g. adnate or sub-irregular, presence of pseudocyphellae, a black lower surface with simple or branched rhizines (Crespo et al., 2007 & 2010b), and the presence of atranorin or chloroatranorin in the cortex (Hale Jr., 1987). Currently, *Parmelia* is a widely distributed genus with three centers of distribution, one in the boreal temperate regions of Europe and North America, a second in eastern Asia and a third in Australasia (Ferencova et al., 2014). The Parmeloid group consists of ~1500 taxa which are placed in *Parmelia sensu lato* (Hale and DePriest, 1999).

Crespo et al., (2001) demonstrated monophylly of parmeloid lichen groups using a mitochondrial rDNA gene, a result parallel to that found by Mattson et al., (2004) who used four different mitochondrial loci. However, the relationship amongst major clades of parmeloids remain unexplored and poorly studied (Blanco et al., 2006). Despite the use of molecular techniques in resolving taxonomic confusion or accurate specimen identification in parmeloid lichens - a group widely known for morphologically complex lichenized fungi (Henssen and Jahns, 1974) with disjunct cosmopolitan distribution and high chemical diversity (Thell et al., 2012), species delimitation remains challenging especially when phenotypic-based methods are used. Therefore, this genus provides a good study system for testing the utility of DNA barcoding as a method of clarifying species boundaries, accurate identification and estimating the level of biodiversity (Divakar et al., 2016).

In this study, using South African members of *Parmelia*, tested for the utility of DNA barcoding as a rapid tool for accurate specimen identification. Gene markers such as *β-tubulin* and translation elongation factor 1-α (*tef-1α*) together with ITS have been used for fungal

phylogenetics (see Walker et al., 2012) but in this study only ITS was used as a gene marker. To achieve our aim the standard DNA barcode marker, internal transcriber spacer (ITS) region of the nuclear ribosomal DNA (Schoch et al., 2012) will be used. Combining data collected from *Parmelia* specimens collected along an altitudinal transect in KZN with data available from previous studies on GenBank, this study had two main objectives. The first aim was to examine the diversity of the South African *Parmelia* along an altitudinal transect. The second aim was to test for the accuracy of species delimitation of DNA barcoding on the South African *Parmelia* genus, by testing for the presence of a DNA barcode gap, to see if these specimens are separable and can be identified to species-level. Although this study was a small-scale study focused in one province, conclusions and outcomes drawn from this study will be used in the future research of lichens in South Africa such as improving our knowledge on the diversity and taxonomy of lichenized fungi.

2. Results

2.1 Data description

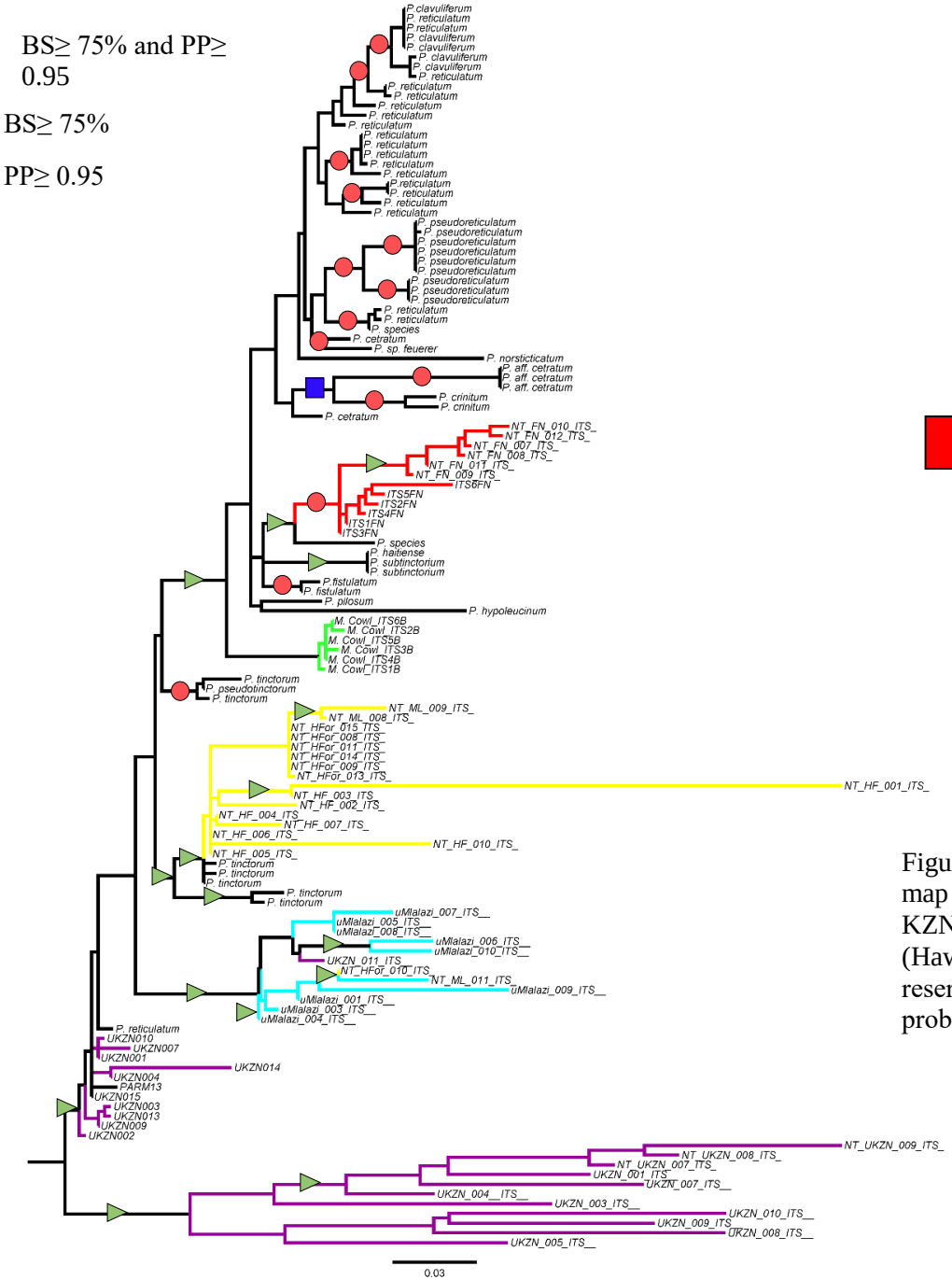
DNA was extracted from 105 *Parmelia perlata* (*sensu lato*) specimens. The ITS gene region was successfully amplified and sequenced from 68 specimens. The 68 barcodes were >500 bp in length and contained no contamination, misidentification or stop codons. These sequences are considered barcode compliant by the Consortium for DNA barcoding. These new sequences were aligned with 60 sequences downloaded from GenBank and BOLD (Table 2). The sequences were easily aligned with no insertions and or deletions. After trimming, the final alignment dataset was 519bp in length (Table 1), contained 364 variable characters of which 267 were parsimony informative (PI). Consistency (CI) of and retention index (RI) values were 0.550259 and 0.838722 respectively, indicating the presence of high numbers of homoplasious characters (Farris, 1989). High homoplasy results in “true” phylogenetic signal being obscured

(Brandley et al., 2009). In such cases, the use of model-based phylogenetic methods are warranted.

2.2 Phylogenetic analysis

The same topologies were recovered from the ML and Bayes analysis, for this reason only the most likely tree is shown (Figure 8) with both bootstrap and posterior probability values annotated onto the branches. Branches with bootstrap values ≥ 65% and posterior probability values ≥ 0.95 were considered well supported. Generally, branches supporting lineages were better supported than branches along the backbone of the phylogeny e.g. the branch leading to the taxon of samples that were collected from Fort Nottingham. The barcode marker ITS, is known to resolve species-level associations well, but does less well when examining higher-level relationships such as associations among different genera. The phylogenetic analysis indicates that despite the lichens collected in KZN being morphologically similar, they do not form a single monophyletic lineage. Instead at least five separately evolving lineages are seen in the phylogeny (Figure 8). What is also clear from the phylogeny is that the phylogenetic structure seen in the South African samples is strongly influenced by geography, with individuals from the same locality grouping together.

- BS≥ 75% and PP≥ 0.95
- BS≥ 75%
- PP≥ 0.95



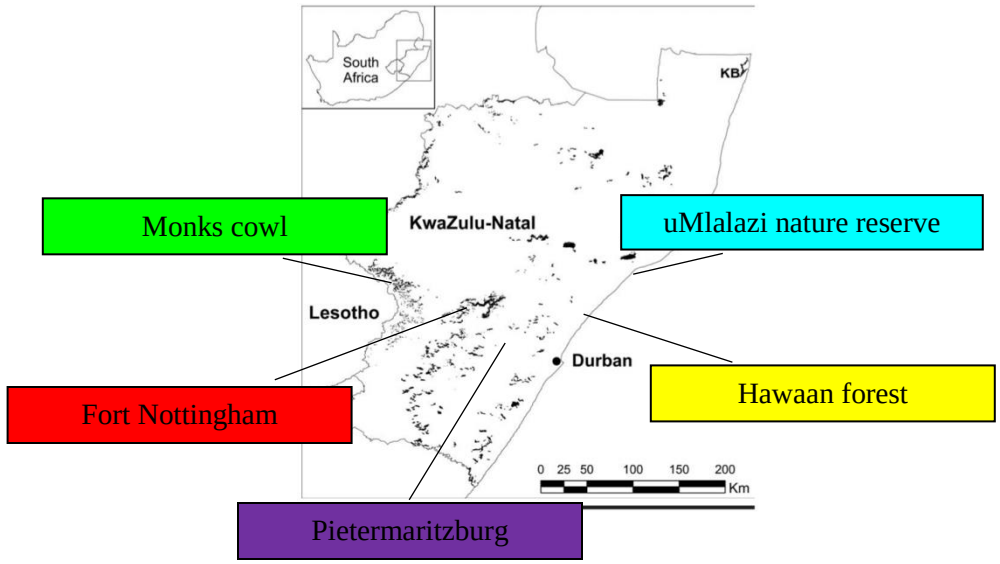


Figure 8: Maximum likelihood topology of *Parmelia* analysed in this study (left) and map of sampling localities (right). The 68 specimens collected from five sites around KZN are colored by locality: turquoise blue (uMlalazi nature reserve), yellow (Hawaan forest), purple (Pietermaritzburg, UKZN), red (Fort Nottingham nature reserve), green (Monks cowl). The only support values BS≥65% and posterior probability≥0.95 are annotated onto the tree

2.3 DNA barcode analysis

Interspecific (between) species genetic distances (0.00-0.451) overlapped completely with intraspecific (within) distances (0.00-0.07) and therefore no gap was not detected between the two taxonomic classes (Figure 9). This was also confirmed by the J-M value for this dataset (1.05), which was far below the threshold (1.414) for separability, therefore suggesting that the two classes from this study are not separable based on the ITS data alone.

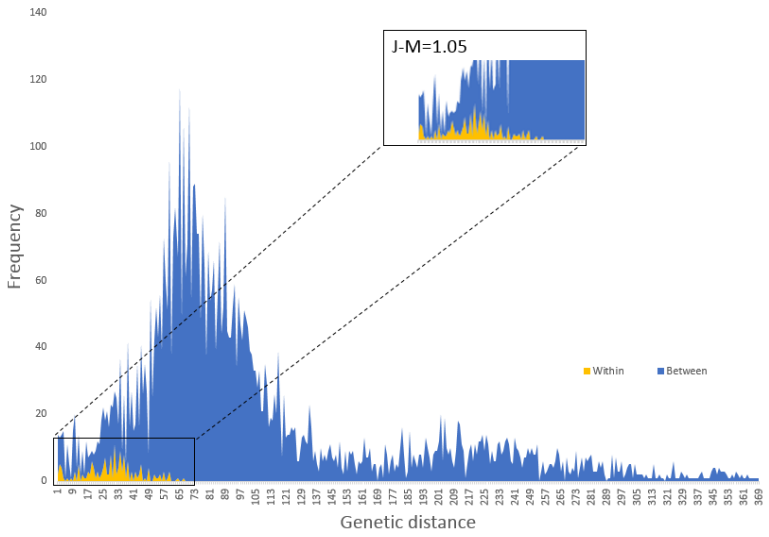


Figure 9: Histogram showing the number of observations/frequencies of the pairwise intra- and inter-specific genetic distances based on a GTR+G model. The area graph shows and overlap between the intraspecific and the interspecific genetic divergence.

2. Discussion

Despite the recent increase in lichenized fungi descibed, a large number of lichen-forming groups still remain unexplored (Amo de Paz et al., 2011; Sérusiaux et al., 2011). Because phenotypically-based methods have proven difficult to work with in groups such as lichens (Kroken and Taylor, 2001; Divakar et al., 2010), DNA barcoding has been suggested to provide a quick accurate species identification for lichens, which are amongst the most taxonomically challenging groups (Del-Prado et al., 2016). As discussed in Chapter 1, for DNA barcoding to be successful, a well-curated reference library is needed, and the standard marker selected for the group should also be able to separate species and as such a DNA barcode gap has to be met.

In the current study, the ability of ITS DNA barcoding for the identification of species belonging to the lichen-forming genus *Parmelia* collected across a wide geographical range in the province of KZN was tested for. Results from the phylogenetic analysis of the nuclear ITS sequence data shows that the traditionally circumscribed *Parmelia* species are in some cases polyphyletic (Figure 8). The phenomenon of species and even genus level polyphyly is not unusual in Parmeliaceae and also in other groups of fungi (Divakar et al., 2010). Such patterns have been previously observed in many groups of lichen-forming fungi (see Kroken and Taylor, 2001; Högnabba and Wedin, 2003; Myllys et al., 2003; Molina et al., 2004; Divakar et al., 2007). This unstable taxonomy also led to a lack of DNA barcode gap for the ITS data used in this study, as without clear species boundaries there is a complete overlap in inter- and intraspecific genetic distances. This limits the utility of barcoding in this group (Kvist, 2016). This highlights the need for a careful taxonomic re-examination of the genus. In this case, the DNA data may provide a valuable tool for highlighting unique lineages that can be examined further by taxonomic experts.

Most specimens in this study show a much higher intraspecific divergence compared to the previously proposed threshold of 0.015-0.017 substitutions per site suggested by Del-Prado et al., (2010). Fourteen specimens exceeded this threshold (Figure 9), and the presence of such high intraspecific genetic distances, suggests the possibility of a hidden lineage or unrecognized species. Based on this result, additional research is required to accurately identify or characterize species-level diversity (Leavitt et al., 2014) for *Parmelia* species from KwaZulu-Natal. Instead of relying on standard general thresholds like that of Del-Prado's et al., (2010), thresholds can be estimated directly from the data. Deep divergences within the South African *Parmelia* species should be examined by using additional genetic markers. Meyer and Paulay (2005) stated that if the intra-interspecific divergence overlaps and give rise to polyphyletic or paraphyletic clades, when such overlap is "real" (i.e. not as a result of poor taxonomy), then the gene marker in question is not reliable to distinguish between those species. It seems most likely that in our study the overlap is "not real" because it is a result of poor taxonomy. This is because the ITS gene marker has previously proven to successfully discriminate and delimit a wide range of *Parmelia* species (see Del-Prado et al., 2010; Divakar et al., 2005; Leavitt et al., 2013, 2014; Molina et al., 2004, 2011; Kelly et al., 2011). Although our results are in accordance with previous studies (see Divakar et al., 2016) in using ITS to develop a DNA barcode, however, it should be noted that examples exist where attempting to use the ITS as a marker were not successful; for example, in *Cladonia* and *Physcia* (Myllys et al., 2001 The absence of a barcode gap in *Cladonia* was explained by hybridization, incomplete lineage sorting and radiations (Pino-Bodas et al., 2012).). In a study by Kelly et al., 2011, the lack of a barcode gap in *Cladonia* was explained by the presence of species complexes in which morphological characters between taxa overlap (Fontaine et al., 2010). In this study, delimitation attempt also failed in *Cladonia gracilis* (an easy-to-identify species) suggesting that ITS variation may not always be able to track species delimitation in the genus *Cladonia*

(Kelly et al., 2011). Studies by Kolteko and Piercey-Normore, (2010) revealed that some species of *Cladonia* can't be resolved by the use of ITS or alternative molecular analysis and this due to the likelihood that morphological characters used for delimitation could reflect variation in environmental conditions (phenotypic plasticity) or homoplasious as a result of convergence evolution/morphology (Fontaine et al., 2010). ITS as a gene marker has also been found to have drawbacks such as a lack of sequence variation amongst closely related sister species (Xu et al., 2000). The absence of a DNA barcode gap in the South African *Parmelia* genus can be explained by the lack of research in lichens in the country (Fryday, 2015), incomplete, unreliable checklists or reference libraries (Fryday, 2015), convergence morphology (Parnmen et al., 2012), high cryptic diversity (Thell et al., 2012), lack of modern revisions through the use of DNA-based techniques (Fryday, 2015) to mention but a few. Hybridization, radiation, incomplete lineage sorting, the presence of complexes with a genus, overlapping of morphological characters used for delimitation, phenotypic plasticity, homoplasy and convergence morphology in the genus *Cladonia* might also be used to explain the presence of a DNA barcode gap in the South African *Parmelia* genus. In addition, the drawbacks associated with ITS such as the absence of variation within this gene marker could also account for the absence of the barcode gap

As mentioned above, the gene marker β-tubulin is also used for phylogenetic studies in lichens. In a study by Thell et al. (2002), ITS and β-tubulin were used to clarify the taxonomical position of cetraroid lichens within the family Parmeliaceae and identify monophyletic groups the lichens. Results from the study showed that ITS and β-tubulin sequences showed congruency and a correlation between molecular-based data and morphological analysis (conidial shape). In another study by Park et al. (2018), the gene markers ITS and β-tubulin sequences showed that Korean *Stereocaulon* species were monophyletic and therefore were placed back in their previous phyletic classification. The latter study also revealed two *Stereocaulon* species that

were polyphyletic; these species were morphological and geographically widespread. Högnabba (2006), used ITS and β-tubulin to analyse the phylogenetic status of the genus *Stereocaulon.* Results from this study revealed that morphological analysis of the genus may not specifically agree with molecular data. Despite the success of ITS and β-tubulin in some phylogenetic studies, results are not always as expected. For example, in a study by Articus et al. (2002) the two genes were unable to support the separation of two *Usnea* species. Results from this study showed that the two species form a monophyletic cluster of intermixed species and not two species with consistent morphology. In the current study using β-tubulin was also attempted for use for phylogenetic analysis but was unsuccessful as it kept identifying microlichens or photobiont partners.

The use of conspecific species from different sites did cause a significant increase in the intraspecific divergence when ITS was used. These results contrast with those of Divakar et al. (2016), where they reported that the inclusion of *P. saxatilis* from distant geographical regions such as Antarctica, Asia and Europe, did not result in any significant increase in the intraspecific divergence. This presence of substantial regional variation of ITS sequences may suggest that a compelling identification framework cannot be developed for the mycobiota of *Parmelia* without requiring extensive geographical surveys of *Parmelia* in South Africa. The samples from the *Parmelia* genus included in our study show high cryptic diversity (Divakar et al., 2016), which is a phenomenon that has been previously reported in lichen-forming fungi and the Parmeliaceae family (reviewed by Lumbsch and Leavitt, 2011; Crespo and Lumbsch, 2010). This increasing number of cryptic lineages in fungi is a major issue for current fungal taxonomy (Hawksworth et al., 2001; Crespo and Perez-Ortega, 2009). So, using a molecular barcode technique can help increase our understanding of the diversity, taxonomy and biogeography of the cosmopolitan lichen-forming genus (Kress et al., 2015).

A large number of lichens are used as bioindicators in forest and ecological continuity, biomonitoring of air pollutants and environmental disturbances (Zedda et al., 2011) or monitoring climate change (Aptroot and van Heck, 2007). Therefore, it's important to have a strategy to quickly and accurately identify species (Hawksworth, 2010). Our results showed that Parmeliaceae species cannot be successfully discriminated using the nuclear ITS region alone, this finding is similar to previous studies (See Leavitt et al., 2014; Divakar et al., 2005) that also did not support monophyletic clades. The intraspecific genetic distances of the *Parmelia* genus in this study exceeded the proposed intra-interspecific genetic distances threshold (Fig. 3). But unlike previous studies where *Parmelia* demonstrated monophyly (Crespo et al., 2001; Divakar et al., 2005), the *Parmelia* species found in KZN showed distinct well-supported polyphyletic/paraphyletic clades. However, additional research is required to assess species boundaries and the potential of distinct lineages within these *Parmelia* species occurring in KwaZulu-Natal.

3. Conclusion

In summary, the current study is an example of the complex problems that occur in species delimitation and recognition. It also provides a realistic example of species-polyphyly proving that traditional-based methods can underestimate "real" species diversity true. This study also revealed the presence of previously hidden lineages, meaning further detail taxonomic research or revision is required. Divakar et al. (2010) used morphology, molecular data and geographic data to provide a more robust approach to delimitation of species phylogeny. So, future research can the method mentioned above and use more than one marker previously used for lichens e.g. *rbcl*, beta-tubulin or *matK*, there might be an improvement in our results. So, in

conclusion, the results from this study have set a stage for a detailed future investigation to improve our knowledge the biodiversity and taxonomy of lichens.

References

Amo de Paz, G., Cubas, P., Divakar, P.K., Lumbsch, H.T., Crespo, A., 2011. Origin and diversification of major clades in parmelioid lichens (Parmeliaceae, Ascomycota) during the Paleogene inferred by Bayesian analysis. PLOS One 6, e28161

Aptroot, A., Sipman, H. J., 1997. Diversity of lichenized fungi in the tropics. Biodiversity of Tropical Microfungi, 93–106.

Aptroot, A., Van Herk, C.M., 2007. Further evidence of the effects of global warming on lichens, particularly those with *Trentepohlia* phycobionts. Environmental Pollution 146, 293–298.

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

Blanco, O., Crespo, A., Ree, R.H., Lumbsch, H.T., 2006. Major clades of parmelioid lichens (Parmeliaceae, Ascomycota) and the evolution of their morphological and chemical diversity. Molecular Phylogenetics and Evolution, 39, 52–69.

Brandley, M.C., Warren, D.L., Leaché, A.D., McGuire, J.A., 2009. Homoplasy and clade support. Systematic Biology, 58, 184–198.

Crespo, A., Blanco, O., Hawksworth, D.L., 2001. The potential of mitochondrial DNA for establishing phylogeny and stabilizing generic concepts in the parmelioid lichens. Taxon 50, 807–819.

Crespo, A., Lumbsch, H.T., Mattsson, J.E., Blanco, O., Divakar, P.K., Articus, K., 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.

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, 71–81.

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

Crespo, A., Ferencova, Z., Pérez-Ortega, S., Elix, J.A., Divakar, P.K., 2010b. *Austroparmelina*, a new Australasian lineage in parmelioid lichens (Parmeliaceae, Ascomycota). Systematics and Biodiversity 8, 209–221

Del-Prado, R., Cubas, P., Lumbsch, H.T., Divakar, P.K., Blanco, O., de Paz, G.A., 2010. Genetic distances within and among species in monophyletic lineages of Parmeliaceae (Ascomycota) as a tool for taxon delimitation. Molecular Phylogenetics and Evolution 56, 125–133.

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, e0161031.

DePriest, P.T., 1999. Development of Mason E. Hale's list of epithets in the parmelioid genera (lichen-forming Ascomycotina): a bibliographic review. Bryologist 102, 442–461.

Divakar, P.K, Molina, M.C., Lumbsch, H.T., Crespo, A., 2005. *Parmelia barrenoae*, a new lichen species related to *Parmelia sulcate* (Parmeliaceae) based on molecular and morphological data. Lichenologist 37, 37–46.

Divakar, P.K., Amo, G., Del Prado, R., Esslinger, T.L., Crespo, A., 2007. Upper cortex anatomy corroborates phylogenetic hypothesis in species of *Physconia* (Ascomycota, Lecanoromycetes). Mycological Research 111, 1311–1320.

Divakar, P.K., Figueras, G., Hladun, N.L., Crespo, A., 2010. Molecular phylogenetic studies reveal an undescribed species within the North American concept of *Melanelixia glabra* (Parmeliaceae). Fungal Diversity 42, 47–55.

Divakar, P.K., Leavitt, S.D., Molina, M.C., Del-Prado, R., Lumbsch, H.T., Crespo, A., 2016. A DNA barcoding approach for identification of hidden diversity in Parmeliaceae (Ascomycota): *Parmelia sensu stricto* as a case study. Botanical Journal of the Linnean Society 180, 21–29.

Farris, J.S., 1989. The retention index and the rescaled consistency index. Cladistics, 5, 417–419.

Ferencova, Z., Cubas, P., Divakar, P.K., Molina, M.C., Crespo, A., 2014. *Notoparmelia*, a new genus of Parmeliaceae (Ascomycota) based on overlooked reproductive anatomical features, phylogeny and distribution pattern. The Lichenologist, 46, 51–67.

Feuerer, T., Hawksworth, D.L., 2007. Biodiversity of lichens, including a world-wide analysis of checklist data based on Takhtajan's floristic regions. Biodiversity and Conservation, 16, 85–98.

Fontaine, K.M., Teuvo, A.H.T.I., Piercey-Normore, M.D., 2010. Convergent evolution in *Cladonia gracilis* and allies. The Lichenologist 42, 323-338.

Fryday, A.M., 2015. A new checklist of lichenised, lichenicolous and allied fungi reported from South Africa. Bothalia–African Biodiversity and Conservation 45, 1–4.

Hale Jr, M.E., 1987. A monograph of the lichen genus *Parmelia* Acharius *sensu stricto* (Ascomycotina: Parmeliaceae).

Hale, B.W., DePriest, P.T., 1999. Mason E. Hale's list of epithets in the parmelioid genera. Bryologist 102, 462–544.

Hawksworth, D.L., 2001. The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycological Research 105, 1422–1432.

Hawksworth, D.L. 2010. Cryptic speciation: how common is it and how should it be handled taxonomically? <imc9.info/prog_sig3_detail_hawksworth.htm>

Henssen, A., Jahns, H.M., 1974. Lichenes, Eine Einführung in die Flechtenkunde. Georg Thieme Verlag, Stuttgart.

Högnabba, F., Wedin, M., 2003. Molecular phylogeny of the *Sphaerophorus globosus* species complex. Cladistics 19, 224–232.

Högnabba, F., 2006. Molecular phylogeny of the genus *Stereocaulon* (Stereocaulaceae, lichenized ascomycetes). Mycological Research 110, 1080-1092.

Kelly, L.J., Hollingsworth, P.M., Coppins, B.J., Ellis., C.J., Harrold, P., Tosh, J., Yahr, R., 2011. DNA barcoding of lichenized fungi demonstrates high identification success in a floristic context. New Phytologist 191, 288–300.

Kotelko, R., Piercey-Normore, M.D., 2010. *Cladonia pyxidata* and *C. pocillum*; genetic evidence to regard them as conspecific. Mycologia 102, 534-545.

Kress, W.J., Garcıa-Robledo, C., Uriarte, M., Erickson, D.L., 2015. DNA barcodes for ecology, evolution, and conservation. Trends in Ecology and Evolution 30, 25–35.

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

Kvist, S., 2016. Does a global DNA barcoding gap exist in Annelida? Mitochondrial DNA Part A, 27, 2241–2252.

Leavitt, S.D., Fernández-Mendoza, F., Pérez-Ortega, S., Sohrabi, M., Divakar, P.K., Lumbsch, H.T., Clair, L.L., 2013. DNA barcode identification of lichen-forming fungal species in the *Rhizoplaca melanophthalma* species-complex (Lecanorales, Lecanoraceae), including five new species. MycoKeys 7, 1–22.

Leavitt, S.D., Esslinger, T.L., Hansen, E.S., Divakar, P.K., Crespo, A., Loomis, B.F., Lumbsch, H.T., 2014. DNA barcoding of brown *Parmeliae* (Parmeliaceae) species: a molecular approach for accurate specimen identification, emphasizing species in Greenland. Organisms Diversity and Evolution 14, 11–20.

Lumbsch, H.T., Leavitt, S.D., 2011. Goodbye morphology? A paradigm shift in the delimitation of species in lichenized fungi. Fungal Diversity 50, 59–72.

Mattsson, J.E., Articus, K., Wiklund, E., Wedin, M., 2004. The monophyletic groups within the Parmeliaceae. In: Articus, K. (Ed.), Phylogenetic Studies in *Usnea* (Parmeliaceae) and Allied Genera. Acta Univ. Upsal.931, sine pagin.

Meyer, C. P., Paulay, G., 2005. DNA barcoding: error rates based on comprehensive sampling. PLOS Biology 3, e422.

Molina, M.C., Crespo A., Blanco O., Lumbsch H.T., Hawksworth D.L., 2004. Phylogenetic relationships and species concepts in *Parmelia s. str*. (Parmeliaceae) inferred from nuclear ITS rDNA and β -tubulin sequences. Lichenologist 36, 37–54.

Molina, M., Del-Prado R., Divakar P., Sánchez-Mata D., Crespo A. 2011. Another example of cryptic diversity in lichen-forming fungi: the new species *Parmelia mayi* (Ascomycota: Parmeliaceae). Organisms, Diversity and Evolution 11, 331–342.

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

Myllys, L., Stenroos, S., Thell, A., Ahti, T., 2003. Phylogeny of bipolar *Cladonia arbuscula* and *Cladonia mitis* (Lecanorales, Euascomycetes). Molecular Phylogenetics and Evolution 27, 58–69.

Parnmen, S., Rangsiruji, A., Mongkolsuk, P., Boonpragob, K., Nutakki, A., Lumbsch, H.T., 2012. Using phylogenetic and coalescent methods to understand the species diversity in the *Cladia aggregata* complex (Ascomycota, Lecanorales). PLOS One 7, e52245.

Park, J.S., Park, C.H., Park, S.Y., Oh, S.O., Jayalal, U., Hur, J.S. ,2018. Revision of the Lichen Genus Stereocaulon (Stereocaulaceae, Ascomycota) in South Korea. Mycobiology, 1-13.

Pino-Bodas, R., Burgaz, A.R., Martin, M.P., Lumbsch, H.T., 2012. Species delimitations in the *Cladonia cariosa* group (Cladoniaceae, Ascomycota). The Lichenologist 44, 121–135.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., Fungal Barcoding Consortium. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences 109, 6241–6246.

Sérusiaux, E., Wheeler, T., Goffinet, B., 2011. Recent origin, active speciation and dispersal for the lichen genus *Nephroma* (Peltigerales) in Macaronesia. Journal of Biogeography 38, 1138–1151.

Sipman, H. J., Aptroot, A. 2001. Where are the missing lichens? Mycological Research 105, 1433–1439.

Thell, A., Stenroos, S., Feuerer, T., Kärnefelt, I., Myllys, L., Hyvönen, J., 2002. Phylogeny of cetrarioid lichens (Parmeliaceae) inferred from ITS and b-tubulin sequences, morphology, anatomy and secondary chemistry. Mycological Progress 1, 335-354.

Thell, A., Crespo, A., Divakar, P.K., Kärnefelt, I., Leavitt, S.D., Lumbsch, H.T., Seaward, M.R. 2012. A review of the lichen family Parmeliaceae–history, phylogeny and current taxonomy. Nordic Journal of Botany, 30, 641-664.

Walker, D. M., Castlebury, L.A., Rossman, A.Y., White Jr, J.F., 2012. New molecular markers for fungal phylogenetics: two genes for species-level systematics in the Sordariomycetes (Ascomycota). Molecular phylogenetics and evolution, 64(3), 500-512.

Wedin, M., Döring, H., Gilenstam, G., 2004. Saprotrophy and lichenization as options for the same fungal species on different substrata: environmental plasticity and fungal lifestyles in the *Stictis-Conotrema* complex. New Phytolgist. 164, 459–465.

Xu, J., Vilgalys, R., Mitchell T.G., 2000. Multiple gene genealogies reveal recent dispersion and hybridization in the human pathogenic fungus *Cryptococcus neoformans*. Molecular Ecology 9, 1471-1481.

Zedda, L., Gröngröft, A., Schultz, M., Petersen, A., Mills, A., Rambold, G., 2011. Distribution patterns of soil lichens across the principal biomes of southern Africa. Journal of Arid Environments 75, 215–220.

CHAPTER 5: GENERAL CONCLUSION AND RECOMMENDATIONS

DNA barcoding of KwaZulu-Natal Afromontane forest Parmelia (Parmeliaceae) species: A molecular approach to accurate specimen identification and sensitivity to climate change. The present study was a combination of two disciplines; in Chapter 3 using chlorophyll fluorescence parameters and conductivity we assessed the tolerance of the symbionts of lichens to heat and cold stress, to determine the likely effect of climate change or global warming on these sensitive species. In Chapter 4, we made use of DNA barcoding to test for the effectiveness of the nuclear ITS gene as a standard marker to identify *Parmelia* species from KZN.

High elevation species will continue to face habitat loss and potential threat of extinction as a result of climate change (Allen and Lendemer, 2016). The effects of climate change on lichen distributions have been previously documented by Aproot and van Herk (2007) and Søtching (2004). Results from the present study suggest that with increased temperatures, coastal lichen species have a better chance of survival than those growing in inland sites. This is because species collected along the coast were more tolerant to heat than species collected from inland, more high-lying areas. Presumably, these species could move their distributions inland should temperatures rise, while those in montane areas would have nowhere to go. This research then improves our understanding on the behavior of species collected from different localities in response to high temperatures that would possibly experience as a result of global warming. Our results are consistent with other studies that have shown either phenotypic variation in heat tolerance (e.g. studies comparing the thermotolerance of the same site at different season or more likely genetic differences (e.g. studies comparing different populations). However, although genetic difference may be more likely in the present study, it is not possible to separate genetic variation from phenotypic adaptation. Future research should test for phenotypic

plasticity, to determine if a change in the environment (e.g. transplanting montane material to the coast) could cause acclimation to temperature stress. Because lichens from high elevations have shown sensitivity to increased temperatures, future studies could focus on monitoring changes in distribution and growth of lichens from these sites as early warning indicators of climate change.

Studies that involve modern revisions through the use of molecular data are now being conducted all over the world, particularly in places such as America and Europe. However, such studies are limited in Africa, which is surprising given that southern Africa may be particularly species-rich. As discussed in Chapter 4, South African lichen taxonomy is poorly explored or understood, and traditional methods of identification are proving inadequate or unreliable for this purpose. Therefore, DNA barcoding was explored as a rapid tool for species identification and resolving taxonomic problems (Blanco et al., 2006). Using ITS as a barcode marker, results from our phylogenetic analysis showed that unlike other *Parmelia* species that demonstrated monophyly, species of this lichen-forming genus from KZN are polyphyletic. In testing for the presence of a barcode gap, our nuclear ITS data sequences do not provide evidence for a gap, with a clear overlap between the inter- and intraspecific genetic classes. The significance of this overlap was further supported by the J-M separability test. The absence of the barcode gap limits the effective use of DNA barcoding as a tool for species identification, suggesting the need for a careful taxonomic revision of genus *Parmelia* in South Africa. The paraphyletic clades and the absence of a barcode gap can be explained by the lack of modern revision and research in lichens of South Africa, hence unstable taxonomy and high levels of cryptic diversity. Although our study was conducted on a small regional scale, outcomes from this study will be used in the future research of lichens in South Africa such as improving our knowledge on the diversity and taxonomy of lichen-forming fungi.

Future recommendations

Although testing temperature tolerance at different times of the year appears attractive, there are a number of difficulties associated with this. First, the inland populations were collected in summer, so their heat tolerance would be expected to be optimal. Their heat tolerance is already lower than the coastal populations, but the difference could have been greater if material had been collected in winter. Second, there is only a small amount of annual variation in temperature on the coast, with low temperatures only rarely occurring, so it would be expected that heat tolerance does not greatly vary throughout the year. A better approach may be to use transplant experiments where can take material collected at the coast and transplanted it inland, and test if individuals lose heat tolerance (or *vice versa*). Alternatively, material could be kept cold in a growth cabinet in the lab and any loss in heat tolerance could be monitored. Further work could include testing if temperature adaptation occurs in ways other than simply "tolerance" e.g. how does respiration / photosynthesis vary as a function of temperature in the different populations? This would enable us to test if the coastal populations are generally better adapted to warm conditions. A transcriptomic approach could also be used to investigation how coastal populations achieve greater tolerance to high temperatures. For example, one obvious possibility would be to test whether HSPs are differently induced by temperature shocks in different populations.

The use of DNA barcoding can be improved by sequencing additional markers such as β-tubulin or tef-1α previously used for lichen groups.

References

Allen, J.L., Lendemer, J.C., 2016. Climate change impacts on endemic, high-elevation lichens in a biodiversity hotspot. Biodiversity and Conservation 25, 555–568.

Aptroot, A., Van Herk, C.M., 2007. Further evidence of the effects of global warming on lichens, particularly those with *Trentepohlia* phycobionts. Environmental Pollution 146, 293–298.

Blanco, O., Crespo, A., Ree, R.H., Lumbsch, H.T., 2006. Major clades of parmelioid lichens (Parmeliaceae, Ascomycota) and the evolution of their morphological and chemical diversity. Molecular Phylogenetics and Evolution, 39, 52–69.

Søchting, U., 2004. *Flavoparmelia caperata* – a probable indicator of increased temperatures in Denmark. Graphis Scripta 15, 53–56.