An investigation of the symbiotic association between the sub-aquatic fungus *Dermatocarpon luridum* var. *luridum* and its green algal photobiont

by

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SPECIAL DEDICATION

This thesis is dedicated to my Mother, Karen D. Fontaine (July 1955 – February 2013), who sadly passed away after a lengthy battle with cancer. Mom's death was untimely, transpiring three weeks before my thesis defense, and three months before graduation. I know Mom would be, and is very proud of my accomplishments. I owe my success to the devotion Mom showed as a parent; she raised me with great patience and love, and was always supportive of my goals. Mom was, and always will be, a positive influence in my life.

“Consider the Lichen. Lichens are just about the hardiest visible organisms on Earth, but the least ambitious.”

— Bill Bryson

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Abstract

The biology of the sub-aquatic lichen, *Dermatocarpon luridum*, was investigated. This lichen is sparsely distributed within the temperate climatic zones around the world, colonizing rock along watercourses that regularly experience water level fluctuations. Specimens collected from Canada and Austria were cultured using standard growth media. Brightfield microscopy was used for algal species identification, while fungal ITS, algal ITS and algal actin gene sequences were used for phylogenetic and population genetic evaluation. Results were: 1) axenic cultures of the photobiont were successful, while those of the mycobiont were not successful; 2) *Diplosphaera chodatii* is the photobiont associated with *D. luridum* var. *luridum* and allies, suggesting algal sharing between mycobiont species; 3) genetic diversity is high, and gene flow was high within local populations, but low between continental populations. *Diplosphaera chodatii* may be a keystone species contributing to the survival of *D. luridum* var. *luridum* along with other sub-aquatic, aquatic and terrestrial lichens.

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Chapter 1

Introduction

1.1 Introduction

Communities of organisms on the exposed bedrock that borders freshwater lakes and rivers is dominated by algae, lichens, and bryophytes (Hale 1984; Gilbert and Giavarini 1997; 2000). Lichens and bryophytes mask another community of microorganisms living among their stems and thalli. These communities are adapted to continually changing conditions of the lake margin such as the fluctuation in water levels throughout a single growing season, but little is known about their adaptive mechanisms or dispersal ability. In boreal regions of Manitoba, members of the community found at the water level growing directly on the bedrock include a number of lichens in the genera *Verrucaria, Staurothele, Leptogium*, and *Dermatocarpon*, and sometimes aquatic mosses. Lichens, the interactions between fungi and algae resulting in the development of complex morphological and physiological symbiotic associations, are mainly composed of members of the Ascomycota (filamentous fungi) and microscopic Chlorophyta (green algae), respectively. A small fraction of lichens are considered to be aquatic because the surfaces they grow on (rock, trees and soil) are associated with water bodies (streams, rivers, lakes and soil water interfaces) that inundate the lichens continuously or for a portion of the year. Truly aquatic lichens should, however, have features similar to those of aquatic fungi that allow them to survive in aquatic conditions. Many fungal groups are aquatic or include some aquatic species, either marine or freshwater. Zoosporic fungi (eg. Chytrids and Oomycetes), Hyphomycetes, Ascomycetes, Basidiomycetes and Zygomycetes are comprised of aquatic species (Goh and

Hyde 1996). A recent phylogenetic study suggested that terrestrial fungi evolved from aquatic fungi that are most closely related to the Chytridiaceae (James et al. 2006). There is considerably less known about the biology and dispersal ability of aquatic lichenized-fungi than aquatic non-lichenized fungi or terrestrial lichens. The majority of aquatic lichens are Ascomycetes belonging to the Verrucariales.

Dermatocarpon luridum (Verrucariaceae)

Approximately 45 genera and 750 species are estimated to comprise the Verrucariaceae (Eriksson, 2006). Representatives are found inhabiting either marine, freshwater or terrestrial ecosystems primarily in the tropics and temperate regions (Honegger 2009). Studies investigating the Verrucariaceae were minimal in the past, focusing primarily on the extensive morphological variation exhibited within the family. Without the use of molecular techniques the assortment of genera and species within the family was artificial, leading to varied phylogenetic conclusions. Within the past decade, research by lichen biologists has greatly increased the knowledge of the morphological, molecular and systematic biology of the Verrucariaceae (Gueidan et al. 2007; 2009). The most recent hypothesis for the phylogenetic history of the Verrucariaceae reveals that the species *D. luridum* is a distant relative showing a wide range of morphological variation between genera of the Verrucariaceae (Gueidan et al. 2009).

Dermatocarpon luridum is a member of the Verrucariaceae and is commonly found growing on acidic bedrock or other acidic rock types (eg.

gneiss, granite, schist, etc.) at the edge of persistent water bodies. *Dermatocarpon luridium* displays a foliose (umbilicate) growth habit; thick-walled lower cortical cells and pycnidia (Gueidan et al. 2009 and references therein); immersed thalloid perithecia, containing eight simple spores per ascus and the absence of hymenial algae, which are commonly found in other members of the Verrucariaceae. Other members of this community include *Staurothele fissa*, a crustose lichen with a pseudocortex and perithecia which are flattened or immersed inside wart-like areoles, containing two muriform spores per ascus and possessing hymenial algae (Thomson 1991; Gueidan et al. 2009). The recent morphological and phylogenetic studies have addressed many fundamental questions about the group as a whole such as character state evolution, diversity, and relatedness in an evolutionary classification. Information pertaining to population genetics of taxa within the Verrucariaceae is, however, needed to understand how these communities survive under rapidly changing conditions.

Fungal taxa within the Verrucariaceae associate with Chlorophytes in the class Trebouxiophyceae. Honegger (2009) reported that the highest photobiont diversity is found within the Verrucariaceae. *Dermatocarpon luridum* is believed to associate with species of *Myrmecia* (Order Trebouxiales) or *Stichococcus* (Order Prasiolales), although other genera such as *Desmococcus* and *Trebouxia* are also hypothesized to be photobionts of *D. luridum* (Brodo et al. 2001; Friedl and Büdel 2008). *Myrmecia* species are small (< 10 µm), non-flagellated spherical to ellipsoidal ("*Trebouxia*-like") cells that possess a single parietal chloroplast lacking a pyrenoid (Friedl and Büdel 2008). *Stichococcus* species are

highly variable morphologically ranging from cylindrical to spherical cells that may be observed as unicellular or forming readily dissociating pseudo-filaments with cells ranging from < 10 µm to 15 µm and a parietal chloroplast that may or may not contain a pyrenoid (John 2003; Neustupa et al. 2007). The phylogenetic history has been constructed for three orders and a few species within the Trebouxiophyceae (Friedl and Büdel 2008). *Myrmecia* is nested within *Trebouxia* and *Asterochloris*, while *Stichococcus* falls in a sister clade among more diverse algae such as *Prasiola*, *Desmococcus* and *Chlorella.* Little is known about the species richness and biology of *Myrmecia* and *Stichococcus*. Both genera are reasonably well documented as free-living algae occupying various substrata and lichen-forming fungal associations belonging to the Verrucariaceae (Tschermak-Woess 1978; Rambold et al. 1998).

The mycobiont of most lichens is thought to grow and reproduce in an obligate association because a free-living counterpart has yet to be discovered. While the mycobiont is capable of reproducing sexually in the lichen association; algal sexual reproductive structures have not been reported for any Trebouxioid alga. Aquatic lichens are thought to primarily utilize water for the dispersal of their spores, but wind and animals may also contribute to local and long distance dispersal. Species in the trimline community on rocks at the margins of water bodies may have different strategies for dispersal and survival. *Staurothele fissa* produces fewer and larger spores per ascus in comparison to *D. luridum.* However, *S. fissa* has the advantage of co-dispersing its spores with a suitable photobiont favoring the likelihood of successful colonization and establishment of

another lichen. Some lichen-fungi sporulate and disperse their spores more readily during the winter and early spring months (Bailey and Garrett 1968), while others will sporulate and disperse year round (Pyatt 1969). Pyatt's (1969) spore germination success studies of six apothecial lichens revealed low spore germination percentages from monthly collections. Collections from September to January were the only ones that displayed germination values equal to or greater than 10% for one or more of the six species. All six species possessed asci with mature spores following sectioning of apothecia even in months when discharge was not observed. Asci and spore development are favored during long periods of hydration (Honegger 1978), followed by a dry period suitable for spore dispersal. Ascus release from perithecia is usually by a forceful ejection of spores through fissitunicate asci that release spores one ascus at a time through the perithecial ostiole. Therefore, *D. luridum* and *S*. *fissa* may not conform to the same sporulation and developmental trends reported for many of the terrestrial apothecial lichens studied, because of their aquatic nature.

In Manitoba and the surrounding region, *D. luridum* and its associated community are relatively common constituents of lake and stream ecosystems (Thomson 1991; Brodo et al. 2001). The role of these lichens within their community is speculative, and may provide bacteria (Grube et al. 2009) and invertebrates (Sowter 1971) with food and shelter, they may actively be breaking down the rock on which they live (de los Ríos et al. 2002), potentially supplying the ecosystem with whole and/or modified mineral nutrients. Environmentally, *D.*

luridum and *S. fissa* have served as bioindicator species to assess water levels, water quality and pollution (Monnet et al. 2005; USDA).

The condition of the water in the lakes where these lichens are found is fundamental to their survival and success. Lichens (terrestrial and aquatic) are very sensitive to pollution (Nash 2008). Atmospheric pollutants as well as aquatic pollutants impact these species. “Greenhouse gases” are altering the climate and the atmosphere. As a result, water levels, temperature, and nutrient composition may be altered. Human impacts such as colonization around the water bodies directly impact the quality and function of the water system and have been shown to affect the survival of another aquatic lichen, *Peltigera hydrotheria* (Davis et al. 2000; 2003). Furthermore, Manitoban waterways in the vicinity of these lichens and their communities provide many prospects for the development of hydroelectric generating systems. The impacts of hydroelectric dams are well known and the potential alteration to immediate and surrounding ecosystems could be devastating to the survival and/or distribution of aquatic lichens, in addition to other flora and fauna. Payuk Lake is currently the only known ecosystem in Manitoba that harbors the rare and threatened aquatic lichen *Leptogium rivulare* (Lee 2004). Alteration to the watershed and its nutrient composition by flooding could not only put the other lichens and organisms at risk, but may eradicate *Leptogium rivulare*. Conservation biologists and the hydroelectric industry may benefit from a better understanding of the population structure of these lichens.

The general goal of this study was to investigate the biology of the sub-aquatic lichen *Dermatocarpon luridum* var. *luridum.* In chapter two the objective was to explore optimal culture conditions for each of the symbionts of the lichen *Dermatocarpon luridum* var. *luridum*. To understand the biology of *D. luridum* var. *luridum* the objectives of the third chapter were: a) to determine the species of photobiont(s) that associate with *D. luridum* var. *luridum*, b) to determine the extent of algal sharing between different species of *Dermatocarpon*, c) to estimate the phylogenetic placement of Canadian and Austrian *Dermatocarpon luridum* var. *luridum* along with three additional aquatic species: *D. luridum* var. *decipiens, D. arnoldianum,* and *D. rivulorum*. Lastly, once the identity of the photobiont had been determined, the objective of chapter four was to investigate and compare the population structure of the photobiont associated with *Dermatocarpon luridum* between Manitoba, Canada, and Austria. It is hypothesized that there will be low gene flow between North American and Austrian populations but high gene flow within each of the geographic regions. Long-distance dispersal between Manitoban and Austrian populations was not expected to be present.

1.2 Literature Review

Free-living aquatic fungi and algae

Nearly 71% of the Earth's surface is comprised of water (Babkin 2003). Of the entire hydrosphere – all water on and above the Earths surface – 96.5% is oceanic, while the remaining 3.5% is distributed amongst the following glaciers, permafrost, seas, lakes, river/stream systems, atmosphere, ground water, biota, etc. (Babkin 2003). Because water constitutes such a large proportion of the Earth, it is not surprising that a diversity of biological organisms have evolved to live permanently or temporarily within water. About 2.5% of all water on Earth is freshwater and dynamic free-living fungal and algal assemblages inhabit fresh water lakes, swamps, ponds, rivers, and streams (the major freshwater habitats ~0.008% (Babkin 2003)).

The vast majority of free-living algae are aquatic, in freshwater and marine habitats, while many species are terrestrial within or on the surface of soil, on rock, on plants, etc. In contrast, free-living fungal diversity is best known from terrestrial habitats, but with aquatic species known from both marine and freshwater habitats. Both groups of organisms contribute to biogeochemical cycles and dynamic food webs of aquatic environments.

Freshwater fungi are best described from temperate regions (Goh and Hyde 1996; Wong 1998) where they are known to inhabit numerous substrata: algae, plants, leaf litter, other fungi and invertebrates that are primarily aquatic or semi-aquatic. Fungi are considered aquatic if the life cycle relies fully or partially on the presence of free freshwater (Thomas 1996). The hyphomycetes (Ingold

1942; 1943) or “Ingoldian fungi” are the best-known group of aquatic fungi (Goh and Hyde 1996; Wong et al. 1998). The group belongs to the Deuteromycotina, which is an artificial grouping of species, most of which are anamorphs of Ascomycete or Basidiomycete origin (Belliveau and Bärlocher 2005). Other fungal groups: Ascomycetes, Basidiomycetes, Zygomycetes, Coelomycetes, and Trichomycetes, along with groups that were once considered true fungi (eg. Chytridomycetes, Oomycetes, Slime molds) are found inhabiting aquatic environments (Goh and Hyde 1996; Wong et al. 1998; Wurzbacher et al. 2010). Many propagules of terrestrial fungal species are introduced into aquatic habitats via wind, water, and other vectors where some of these propagules may retain some degree of activity and are thus considered adapted to aquatic habitats (Smirnov 1964).

Truly aquatic fungi and fungal-like protists, have evolved different strategies for ensuring successful dispersal, such that they can thrive in aquatic environments. The zoosporic fungi possess the ability to move throughout the water medium until they find a suitable colonization location. Many of the ascomycetes and other higher aquatic fungi have evolved their spores in such a way that colonization of wet environments is attainable. Such spores often have mucilaginous sheaths or sticky pads at the poles that swell over time in water becoming extra capable of maintaining position on suitable substrata. Other spores have branches or appendages that facilitate entrapment on suitable substrata (Jones 1994; Goh and Hyde 1996). In many cases the conidia produced have special adaptations, such as branched or sigmoidal shape,

mucilaginous pads or special flotation apparatus. These adaptations all help with dispersal and promote rapid germination and production of apressoria when conditions are adequate.

The Lichen Symbiosis

In biology, the term symbiosis defines the interaction of two or more different organisms (symbionts) living in close physical association, such that each partner benefits from the other(s) (Frank 1877; de Bary 1879). Nature presents many different symbiotic associations, such as the soil bacteria and mycorrhizal fungi that associate with plant roots and the dinoflagellate algae (zooxanthellae) that associate with Cnidarians forming corals. However many more symbioses are known, perhaps arguably the most well known symbiotic interactions involve fungi and algae (and/or cyanobacteria). The interactions between fungi, algae and/or cyanobacteria that result in the development of complex morphological and physiological symbiotic associations are referred to as lichens.

Lichens have received the attention of scientists for centuries. Linnaeus, a botanist, and so-called “father of taxonomy” paid little attention to the lichens. Of the 80 or so lichens to which Linnaeus paid any attention, he noted they were different from most other fungi and thus collectively classified all within the genus *Lichen.* It was Erik Acharius, who in the latter half of the 18th century, correctly began classifying lichen fungi, earning himself the honorary title of today, “father of lichenology”. During these times (early to late 18th century) lichens were

thought of and studied as fungi. Schwendener (1867; 1869) put a dual hypothesis forth in 1867, suggesting what we now know as fact; lichens consist of two organisms, a fungus and an alga (Honegger 2000). Initially, the dual hypothesis suggested that the fungus is no more than a parasite of algal cells. The hypothesis was not well supported among lichenologists due to the lack of experimental evidence. For the remaining years of Schwendener's life, he insisted that culturing would prove his theory true. A mere 14 years following Schwendener's death in 1939, his dual hypothesis received proof when results of the first successful re-synthesis experiment using the lichen *Cladonia pyxidata* was published (Thomas 1939; Honegger 2000 (review article)).

Lichen associations are ubiquitous and contribute to most ecosystems of the world (excluding open waters) including places uninhabitable to most other organisms (Hale 1983). One of the main reasons for the successful colonization of virtually all ecosystems comes from their ability to survive successive hydration and dehydration events (Seymore et al. 2005). This attribute means that lichens are poikilohydric organisms. Poikilohydric organisms rely directly on the environment for water and as the environment experiences moisture fluctuations lichen thalli also experience moisture fluctuations. In times of drought lichens will become dehydrated to a state of dormancy and will quickly resume metabolic activity with re-hydration.

Another reason for the success of lichens comes from their dual nature. Each symbiont provides a benefit to the other partner; something that cannot be derived without the other. Thus, in an environment where most other fungi or

algae cannot survive due to moisture or nutritive needs, a lichen may survive. Since lichens are mutually beneficial associations between fungal and photosynthesizing partners, bilateral exchange of nutrients is of utmost importance for survival (Quispel 1943). Algae are autotrophic organisms and as such can provide the fungus, which is heterotrophic, with necessary carbon for metabolism. Depending on the photobiont, green alga or cyanobacterium, the two most common photobionts, the form of carbon received by the fungus will vary. Green algae provide polyhydric sugar alcohols, such as: ribitol, sorbitol and erythritol; Cyanobacteria provide glucose (Honegger 1991; Feige and Jensen 1992; Fehselt 1994; Palmqvist 2008). The fungus performs the conversion of sugar alcohols to mannitol, thus converting the carbon source to an unavailable form for metabolism by the alga (Galun 1988; Honegger 1991; Fehselt 1994). Likewise, the alga receives nutrients from the fungus. Additionally, the algae are surrounded by the fungal hyphae, which provide needed protection from the elements of the environment that would otherwise render the environment uninhabitable for the algal partner (Ahmadjian 1964; Lewis 1973; Nash 2008).

The mycobiont and photobiont form a thallus which may be stratified (heteromerous) or non-stratified (homiomerous) (Honegger 1991). The lichen thallus is morphologically dissimilar to the morphology of either symbiont when found alone or grown in culture. This morphological dissimilarity is especially true in the case of the fungus, since most lichen fungi are not free-living and in nature the fungi comprise the majority of the thallus (Honegger 1991).

The heteromerous lichen thallus is the more typical or common thallus arrangement. Figure 1.1 illustrates the typical lichen thallus when observed in cross section, where the algae are arranged among loosely woven hyphae forming the medulla, which is located under a tightly woven layer of fungal hyphae, the upper cortex. The majority of lichen fungi (mycobionts) and green algae (photobionts) are members of the Ascomycota and Chlorophyta (green algae), respectively.

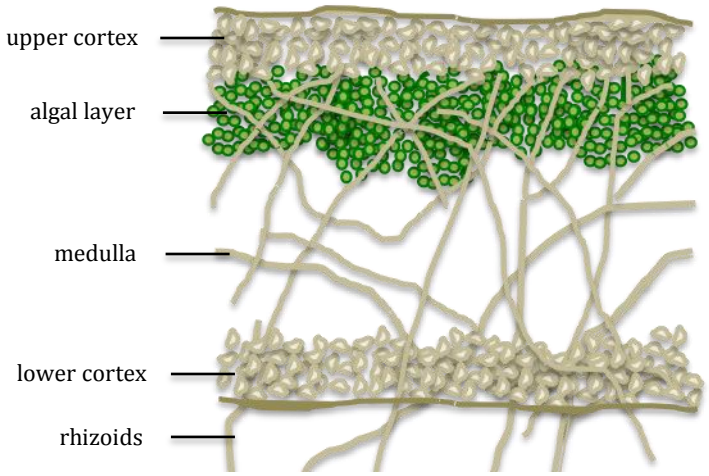


Figure 1.1. A typical representation of a lichen thallus illustrating the upper thallus, algal layer, medulla, lower cortex and rhizoids.

Symbiont Diversity

Mycobionts

There is a vast diversity of fungi on Earth. It has been proposed that the number of fungi outnumber land plants 11:1 (O'Brien et al. 2005). This makes the once accepted estimate of fungal diversity by Hawksworth (1991) of 1.5 million species highly conservative (Blackwell 2011). Current estimates suggest that fungal species diversity is likely between 3.5 and 5.1 million (O'Brien et al. 2005). Currently, according to Kirk et al. (2008) the total number of all globally described fungal species is around 99,000 species.

Following former numbers of the described fungal diversity, 65,000 species, almost 20% of the fungi are lichen–forming (DePriest 2004; Lutzoni and Miadlikowska 2009). It is approximated that 13,750 fungal species are lichenized, and that most of these species (98%) belong to the Ascomycota (Honegger 2001; DePriest 2004; Lutzoni and Miadlikowska 2009). Approximately 50 and 200 species of known lichenized fungi are basidiomycetes or anamorphs (DePriest 2004; Honegger 2008; Lutzoni and Miadlikowska 2009). According to Feuerer and Hawksworth (2007), the estimated total number of lichen fungi and lichenicolous fungi is now around 20,000 species.

Photobionts

It is unknown how many algae there are on Earth, but as with other organisms there are educated estimates. AlgaeBase, the online catalogue of identified algae, provides a means for making such estimates. The current global

estimate of algal diversity, including cyanobacteria, is 72,536 species (Guiry 2012). Currently, there are 43,918 described algal species, of which 73% or 32,360 species are catalogued in AlgaeBase.

Lichen fungi are primarily associated with green algae (Chlorophyta) or cyanobacteria (Cyanophyta), which account for about 85% (12,000) and 10% (1,250), respectively (Ahmadjian 1993). The remaining lichens are believed to be associated with both types of photobionts simultaneously (Friedl and Büdel 2008; Högnabba et al. 2009). There are approximately 100 algal/cyanobacterial species from 40 genera that associate with lichen–fungi (Honegger 1997; Friedl and Büdel 2008). The number of algal and cyanobacterial species available for lichenized-fungi is small, considering that 8,000 green algal and 3,300 cyanobacterial species have been described of an estimated 13,000 and 8,000 total species, respectively (Guiry 2012). Still, most lichen fungi associate with a green algal species.

Green algae are present in all sorts of habitats, primarily those that are marine and freshwater; however, it is typical that green algae also inhabit an array of terrestrial habitats (Holzinger 2009; Holzinger et al. 2011). The Trebouxiophyceae is the class of green algae to which most photobionts belong. The photobiont of this class are typically associated with drier habitats or are aerophytic. Many free-living and obligate photobiont species comprise the Trebouxiophyceae. By far the most common order of photobionts are those from the Trebouxiales, of which there are currently three genera: *Trebouxia,* *Asterochloris*, and *Myrmecia* (Friedl and Büdel 2008). The most well common

and well-studied lichen photobiont is *Trebouxia*. Overall species diversity within this genus remains a mystery. New species are continuously being reported and systematic investigations refine the classification and diversity assessment of previously identified species (Friedl and Büdel 2008; Skaloud and Peksa 2010).

Since photobionts from the Trebouxiophyceae prefer drier habitats most of the associations are with terrestrial saxicolous, ground dwelling and epiphytic species. Lichens that prefer increased water association, many from the Verrucariaceae and several other families, tend to associate with a variety of green algal orders and genera, or algae from other orders (eg. Trentepohliales, Ulvales, Xanthophyceae), or domains (Cyanobacteria); not the Trebouxiophyceae alone (Honegger 2009; Thüs et al. 2011).

Aquatic Lichens and Ecology

There are numbers of lichens that are adapted to growing along the banks of freshwater bodies, such as lakes, rivers, streams and seepages where water levels are subject to highly variable change. Many other lichens have evolved to tolerate the saltwater conditions of tidal zones of the oceans and seas (marine). These freshwater and marine lichens have received considerably less attention than their terrestrial counterparts. The total number of freshwater and marine lichens is seldom reported and the best estimate came from Santesson (1939), where it was predicted that about 700 lichens colonize marine habitats, while 200 colonize freshwater habitats.

It is well known that lichens are poikilohydric in nature and that they

require an alternation of dry and wet conditions in order to metabolize and carry out their life cycles (Nash 2008). The degree of water exposure is important, where excessive hydration or desiccation become harmful to the lichen species (Ried 1960a; Ried 1960b; Dietz and Hartung 1999).

Aquatic lichen communities have been assigned two group designations; those that are ekreophilic (aquatic) and those that are hydrophilic (amphibious or semi-aquatic) (James et al. 1977; Roux et al. 2006; Coste 2010). The first group represents lichens growing on rocks that are subjected to long-term water contact, while the second group represents lichens growing on rock that is subject to variable water levels with periodic immersion (James et al. 1977; Roux et al. 2006). Abiotic and biotic environmental factors determine the species diversity along the banks of watercourses. Mineral content and pH of the water, as well as substratum, current strength and water temperature (James et al. 1977; Thüs 2002; Aptroot and Seaward 2003; Coste 2005) are all likely to influence community composition (Reid 1960a; 1960b; James et al. 1977). Additionally, the duration of contact with the water (Coste 2010), or as past research reported the “duration of immersion” (Santesson 1939), together with the rate at which the substrate (rock) dries determines the structure of aquatic and sub-aquatic lichen communities (Santesson 1939; Pentecost 1977; Coste 2005; 2010). In many regions canopy cover provides ample shade, which compensates for potential lack of continual water contact by delaying the dehydration process (Coste 2005; Nascimbene et al. 2007).

Fully submerged lichens, such as the aquatic lichens, *Peltigera gowardii*

and *P. hydrothyria* require full submergence in combination with periodic relief above water so ascospores can be ejected into the air. These aquatic lichens are cyanobacterial lichens that do not tolerate long periods of dehydration (Davis et al. 2003). In general cyanobacerial lichens tolerate desiccation less than green algal lichens (Kopecky et al. 2005; Heber et al. 2006a; 2006b; Beckett et al. 2008). Periodically submerged lichens, such as *Dermatocarpon luridum* var. *luridum*, *Staurothele fissa* and *Leptogium rivulare*, display tolerance to long-periods of submersion (primarily during early spring thaw) and desiccation.

The lichen communities inhabiting rock substrata surrounding freshwater and marine waters can be differentiated into eco-zones perpendicular to the water surface. These zones (commonly the supra-littoral, littoral and sub-littoral) reflect the relative length of time that species within each zone are in constant contact with the water. Several studies have been conducted to test the confidence of assessing community structure of rock substrates in and around water bodies. Santesson (1939) investigated lake margins in Sweden, and coined the phrase “lichen-line” – a zone where foliose lichens, particularly those associated with *Trebouxa* sp. photobionts are incapable of survival. The “lichen line” has also been shown for lichens growing as epiphytes on trees in regions of annual or regular flooding (Hale 1984).

An analogous method can be used for assessing epiphytic lichen communities in forested habitats. However, vertical zonation of lichen epiphytes on trees is not readily evident upon inspection (Kershaw 1964). As done with aquatic zonation differentiation, Hale (1952) tried a presence/absence approach

at standardized height intervals on trees. Although much overlap is associated with data obtained using this approach, distinct zonation patterns of the lichen community can be shown (Hale 1952; Edwards et al. 1960; Kershaw 1964; Campbell and Coxson 2001).

The designation and study of vertical zonation of marine lichen communities received attention prior to that of freshwater lichen communities. For both marine and freshwater communities a four-zone classification system is accepted, with the exception of additional subzones for marine habitats (Scott 1967; Fletcher 1973a; 1973b; Gilbert 1996; Gilbert and Giavarini 1997; 2000; Aptroot and Seaward 2003). Freshwater zonation is arranged in four zones (Gilbert 1996; Gilbert and Giavarini 1997; 2000; Aptroot and Seaward 2003): 1) The submerged zone contains organisms that can tolerate long inundation periods. On average species colonizing this zone are within 10 cm of the summer water level, and only specialized species are reported up to 50 cm below the summer water level. 2) The fluvial mesic zone, this zone typically contains most of the lichen diversity inhabiting watercourse habitats. The lichens in this zone are located below the water line during spring flood levels and at/or slightly above the water line during summer dry periods. Rain events easily cause this zone to periodically become submerged and thus only aquatic, semi-aquatic and few terrestrial species that prefer rock surfaces can colonize this zone. 3) The fluvial xeric zone is a transitional zone where few semi-aquatic species and terrestrial species that prefer moist rock surfaces dominate. 4) The fluvial terrestrial zone contains lichens that are truly terrestrial occupy this zone; however, competition

is reduced in this zone relative to other sites located away from the water body because of annual or common flood events, preventing vascular plant dominance.

Marine zonation is very similar to the freshwater sematic. The four zones include (Fletcher 1973a; 1973b): 1) Sublittoral, 2) Littoral comprised of eulittoral and littoral fringe subzone, 3) Supralittoral with mesic and xeric subzones, and 4) terrestrial consisting of halophilic and halophobic subzones. Ultimately the role that lichens play within their respective habitats, be it marine or freshwater, remains speculative.

Reproduction and dispersal mechanisms

One of the most fundamental and underlying themes in biology is that organisms live out their lives with the purpose of reproducing viable offspring. Little is known about sexual reproduction of the algal partner. To date no sexual reproduction has been documented within the lichenized thallus for photobionts. It is believed that the mycobiont may control the growth and reproduction of the photobiont. In general lichen thalli are primarily composed of the mycobiont, while the photobiont forma a small portion of the lichen thallus. The photobiont is believed to only undergo asexual reproduction by mitotic division within the thallus. Dispersal of photobionts may occur in a variety of ways within many lichen associations.

Meanwhile, much information is known about the reproductive modes and dispersal mechanisms of lichen mycobionts. The majority of mycobionts are

ascomycetes and some mycobionts are predominantly reproducing by asexual (eg. *Cetraria* sp., *Thamnolia* sp., etc.) or sexual (eg. *Rhizocarpon* sp, *Dermatocarpon* sp., etc.) means, while others have evolved to accommodate both modes of reproduction (eg. *Lobaria* sp., *Cladonia* sp., etc.). Asexual reproduction can be carried out by conidia, thallus fragmentation, or the production of "symbiotic" propagules such as soredia and isidia.

Sexual reproduction facilitates dispersal of the mycobiont if the spores are forcefully ejected into the air. The only downfall of this method is that once spores land and germinate they have a short window to come into contact with a suitable algal partner to reestablish the lichen condition. Several lichens, such as *Staurothele fissa*, or *Endocarpon pussilum* will co-disperse their ascospores with a photobiont from the hymenial layer of the perithecium. This is an adaptive strategy that may greatly increase the chance that spores will germinate within the vicinity of a compatible algal partner, and develop a new lichen thallus with a new haplotype combination. Wind, water, and animals may disperse asexual propagules to new locations. Asexual propagules, with the exception of conidia, are less likely to disperse over great distances relative to ascospores because of their larger size (Walser 2004). However, this vegetative method of dispersal by "symbiotic" propagules results in the co-dispersal of already compatible symbionts simultaneously. The negative aspect of asexual reproduction is lower levels of genetic variation within the gene pool of the mycobiont and photobiont.

Population genetics of lichen fungi and photobionts

Population biology examines the structure and dynamics of populations and how they change over time (Thompson 1998). As environments change, populations become altered as communities adapt to the changes. Population genetics examines the genetic variation within and between populations to assess the potential for adaptation as influenced by natural selection, genetic drift, gene flow and mutation (McDonald 1983; Milton and Grant 1984; Cooper 2007). This information can provide insight into the role of a species within its ecosystem and the ability of the species to adapt to changing environmental conditions (natural or induced through human interference). Genetic studies have been conducted to assess population structure, and infer gene flow within and between populations of numerous lichens (Wright 1943). In the past such studies were limited and complicated by the symbiosis between two genetically distinct organisms. Hypotheses have been derived from field experiments studying dispersal of vegetative propagules and variation of chemical (secondary metabolites) and physiological (eg. photosynthetic gas exchange, growth rate and cyanogenesis) properties (Fahselt 2008). Once lichen biologists began using molecular techniques, isozyme markers were used to assess populations (Hageman and Fahselt 1992). Rapid advancements to molecular and culturing procedures are providing further means for gathering more specific information.

Currently, population structure and gene flow studies of lichen symbionts are conducted using a variety of molecular techniques such as gene sequencing, restriction fragment length polymorphisms (RFLP), random amplification of

polymorphic DNA (RAPD), and simple sequence repeats (SSR; microsatellites). RAPDS are dominant markers and they have been used for population and paternity studies showing multiple fragments, and hence multiple loci, for each individual, but the heterozygote and homozygote recessive cannot be distinguished from the homozygote dominant. RFLPs and SSRs are co-dominant markers and they can detect both heterozygote and homozygotes showing fewer fragments because that usually represent a single locus. Co-dominant markers are more advantageous than dominant markers, when multiple loci can be examined, because they provide distinction between heterozygous and homozygous genotypes and thus gene frequencies can be derived for the population revealing the allelic variation present. Co-dominant molecular markers that span the entire genome and are highly polymorphic, such as SSRs, are valuable because they provide considerably more information than RFLPs, which usually require prior knowledge of the gene before application. Axenic cultures of the symbionts are required for the development of SSR primers within the genome of each of the mycobiont or photobiont. Species-specific priming regions can then be identified and primers designed to allow amplification of either the algal or fungal SSR from the whole lichen thallus. Walser et al. (2003) was the first lichen biologist to isolate and utilize microsatellite SSRs from the lichen *Lobaria pulmonaria*. Before this, although microsatellites had proven to be very effective and reliable markers for genetic research of many pro- and eukaryotic organisms, they were not available for lichen symbionts. Only a few studies since Walser et al. (2003) have used highly informative co-dominant microsatellite

SSRs to investigate the genetics of other lichen symbionts. This is likely due to the difficulty of obtaining axenic mycobiont and photobiont cultures.

Buschbom (2007) examined the potential of long distance gene flow for the mycobiont *Porpidia flavicunda* between geographic sites located in Canada, Greenland and Scandinavian countries of Europe. Nucleotide sequences of three polymorphic gene regions of the fungus provided support for long distance gene flow in *Porpidia flavicunda*. It was suggested that explosively dispersed ascospores could be distributed globally via high-altitude wind currents in the jet streams. Two research studies, Heibel et al. (1999) and Robertson and Piercey-Normore (2007) investigated the genetic variation present within two separate species of lichen fungi (the latter also included the photobiont). The former used a dominant RAPD marker while the latter used a co-dominant RFLP marker and group I intron DNA sequences. Robertson and Piercey-Normore (2007) concluded that high levels of genetic variation are present within a small geographic region and that gene flow is occurring between populations for both symbionts.

Genetic variation as a result of recombination or mutation is important for the sustainability of species because it provides a means of adapting to new and changing habitats (McDonald 1983; Cooper 2007). Increasing levels of adversity as a result of climate change, pollution and hydroelectric generation systems could potentially alter or destroy the environment and ecosystems where aquatic lichens are living. The knowledge gained from this study will accompany the recent works of lichen biologists studying the Verrucariaceae and other lichens.

This research will provide implications on the conservation of rare species in lichen communities with *D. luridum* such as *Leptogium rivulare*. Also, hypotheses regarding the impacts of global warming, pollution and hydroelectric generating systems, on aquatic lichens will be possible. Investigating the population genetics and reproductive fitness of the symbionts of *D. luridum,* will provide a greater understanding of aquatic lichen biology and ecology.

Chapter 2

Exploration of culture conditions for the cultivation of the mycobiont and photobiont(s) of the sub-aquatic lichen *Dermatocarpon luridum* var. *luridum*

Introduction

The many disciplines of science (eg. chemistry, biology, mathematics, medicine, etc.) have benefited from the ability to axenically culture organisms (eg. fungi, algae, bacteria, invertebrates, etc.) and/or organismal tissues (eg. skin cells, cancer cells, etc.) using both artificial and natural growth media. One of the most famous examples, the discovery of penicillin and its anti-bacterial properties, illustrates how culture experiments may benefit human health (Fleming 1929). The culture experiments of Fleming (1929) led to investigative studies of natural products, which won Fleming a Nobel Prize in 1945 for his discovery. Culturing techniques have been established and optimized for a large number of fungal-like organisms (Oomycetes, Myxomycetes), primitive and imperfect fungi (Chytridiomycetes and Deuteromycetes, respectively), and true fungi (Zygomycetes, ascomycetes, basidiomycetes). Likewise, culture techniques for many different algal groups have been well established and optimized. Both mycological and phycological experimentation is progressing. On the contrary, lichenology has been trailing behind other biological disciplines in regard to many aspects of experimental study because of the challenges of culturing the symbionts (Nash 2008). Since lichens are ubiquitous and the symbionts are seldom known free-living (Nash 2008), nutrient and environmental conditions required to axenically culture the symbionts apart from one another becomes challenging. There is much to learn about the more or less mutualistic relationship between lichen symbionts. Therefore, one of the ongoing challenges

in lichenology is to determine the conditions required to axenically culture lichen symbionts.

One of the problems with culturing lichen symbionts is the slow growing nature of the organisms (Yoshimura et al. 2002). Aseptic conditions are of utmost importance to prevent unwanted contaminants. If nutrient rich media are used, contaminating organisms, principally bacteria, non-lichen fungi and foreign algae, grow faster and overgrow the desired symbiont (Stocker-Wörgötter 2001; Yoshimura et al. 2002). There have been two well-conceived methods for isolating axenic symbiont cultures; the “spore rain” method and the ““Yamamoto method”” (Yamamoto 1990; Stocker-Wörgötter and Hager 2008). For the mycobiont, Ahmadjian (1973) indicated the importance of spores over thallus fragments for obtaining axenic culture. Spores signify the true lichen fungus and there is a better probability that contaminant organisms, which are believed to be more abundant within the thallus, will be less represented than those in thallus fragments (Ahmadjian 1973). For lichens that are rarely observed producing apothecia or pycnidia, thallus fragments are the only way of culturing the mycobiont. Since the majority of lichens do not discharge algae like they do fungal spores, thallus fragments and growth media suited for algal growth must be used to isolate the photobiont. For the last half-century, interest and dedication by lichenologists to determine culture conditions for isolating axenic symbiont cultures and even study the in vitro resynthesis of lichens has made great strides forward.

The impact of improved culture conditions and abilities to obtain axenic

cultures has resulted in many exploratory studies of secondary metabolites, developmental and resynthesis capabilities, and the use of a wide variety of molecular techniques for phylogenetic and population genetic inquiries. Research has improved our understanding of lichen fungi and our ability to culture photobionts. Honegger (1991) stated that only 2% of all lichen photobionts can be identified at the species level. Photobionts grown in culture display different shape and structure than is presented in the lichenized state ("i.e. they are not licheniforms"). Although most currently used culture conditions and growth media are optimized for mycobiont growth of terrestrial lichen fungi, the aquatic and semi-aquatic lichens have received little attention. The objective of this study was to investigate the culture conditions most favourable for growth of the sub-aquatic mycobiont *Dermatocarpon luridum* var. *luridum* and the associated photobiont using standard media and culture conditions.

Materials and Methods

Lichen collection within Manitoba, Canada

In west central Manitoba, Canada, four large boreal forest lakes (Payuk Lake, Neso Lake, Nisto Lake and Naosap Lake) were sampled for the sub-aquatic lichen *Dermatocarpon luridum* var. *luridum*. These lakes constitute a chain of 15 lakes where water exits the northern most lake (Naosap Lake) and passes through all other lakes in a more or less flow toward the south, where ultimately water passes through the southern most lake (Payuk Lake). Payuk Lake is one location in Canada where the threatened sub-aquatic lichen

Leptogium rivulare is known to exist (listed as threatened by COSEWIC in 2004; Lee 2004). Additionally, this particular water way has historic and modern ties to Aboriginal hunting, fishing and trapping practices (Pers. comm. Tom Booth, July, 2010). Seasonal water level fluctuations, high and low granitic rock shorelines and clean water conditions support the colonization of aquatic and sub-aquatic lichens (habitat photos Figure 2.1).

Dermatocarpon luridum var. *luridum* was collected in Manitoba from: 28 sites from Payuk Lake (54°38'31"N; 101°31'40"W; Figure 2.1); 32 sites from Neso Lake (54°39'51"N; 101°32'44"W; Figure 2.2); 31 sites from Nisto Lake (54°42'02"N; 101°30'17"W; Figure 2.3); and 25 sites from Naosap Lake (54°50'38"N; 101°26'12"W). Vouchers representing collections are labeled with the following sight abbreviations: P = Payuk Lake, Ne = Neso Lake, Ni = Nisto Lake and Na = Naosap Lake. Site and collection number follow the site abbreviations, for example P1-1 represents collection Payuk Lake site one collection one. These sites consisted of open forest margins with large granite boulders around the margin of open lakes. The forest was dominated by black spruce and fir with some larch, jack pine and other minor species such as poplar, alder etc. (Fig. 2.1).

Figure 2.1. Habitat of *D. luridum* showing typical locations along the periphery of the lake water bodies in west central Manitoba, Canada. **A.** Drainage outflow from Payuk Lake where three collection sites were located. **B.** Typical location of *D. luridum* var. *luridum* colonization among the crevices of rock in shaded locations. **C.** One of the sites along Nisto Lake where *D. luridum* var. *luridum* thalli inhabited the rock right at the water line during mid summer when water levels were lowest. **D.** A thallus of *D. luridum* var. *luridum* growing in the crevice of granite rock with a portion of the thallus in the water and the rest above the water line during mid summer when water levels are lowest. Photographs taken by Dr. T. Booth, Chris Deduke and Kyle Fontaine.

Figure 2.2. Google Earth™ image of Payuk Lake, Manitoba, Canada with all collection sites indicated by red dots. Scale bar = 500 meters. © 2012 Cnes/Spot Image; Image © 2012 Digital Globe; © 2012 Google.

Figure 2.3. Google Earth™ image of Neso Lake, Manitoba, Canada with all collection sites indicated by yellow dots. Scale bar = 500 meters. © 2012 Cnes/Spot Image; Image © 2012 Digital Globe; © 2012 Google.

Image has been removed due to potential copyright issues

Figure 2.4. Google Earth™ image of Nisto Lake, Manitoba, Canada with all collection sites indicated by blue dots. Scale bar = 500 meters. © 2012 Cnes/Spot Image; Image © 2012 Digital Globe; © 2012 Google.

Figure 2.5. Google Earth™ image of Naosap Lake, Manitoba, Canada with all collection sites indicated by green dots. Scale Bar = 500 meters. © 2012 Cnes/Spot Image; Image © 2012 Digital Globe; © 2012 Google.

Lichen collection within Upper Austria, Austria

Two watercourses in Upper Austria, Austria (Figure 2.8): a hill side stream at Schlogener Schlinge in the Danube Valley (site abbreviation DV; 48°25'59''N; 13°52'28''E; elevation average 305 meters above sea level - ASL) and the Waldaist River (site abbreviation M; site one 48°19'44''N; 13°52'28''E; elevation 337 meters ASL; and site two 48°23'41''N; 13°36'06''E; elevation 429 meters ASL) were sampled for *Dermatocarpon luridum* var. *luridum* (Figure 2.6). At the Schlogener Schlinge site, 12 samples were collected along the watercourse that is only a few meters across and ~130 meters long. The site consisted of many overlapping rocks and boulders with a light consistent flow of water. This site is heavily shaded by lime and maple tree dominated forest, and the rocks were mostly bare except for the cover of *D. luridum* var. *luridum* thalli and some moss and liverwort species (Figure 2.6A and B). At the Waldaist sites, which flow through the center of deep valleys that are littered with large granitic boulders and shaded by an overhanging canopy consisting of fir, alder, pine, ash, poplar, and spruce (Figure 2.6C). Boulders were dominated by *D. luridum* var. *luridum* thalli just above, below, and at the waterline (Figure 2.6D). Dry rock was covered by moss species, liverwort species and terrestrial lichens primarily *Peltigera sp*. From Waldaist sites one and two, 13 and 15 samples were collected along approximately 220 meters of the river, respectively.

Figure 2.6. Typical habitats of the sub-aquatic lichen, *D. luridum* var*. luridum* in Upper Austria, Austria. **A.** Schlogener Schlinge site in Upper Austria, a stream running down the slope of a large hill in the Danube Valley that drains in to the Danube River. **B.** Thalli of *D. luridum* var. *luridum* growing in the crevices of overlapping granitic rocks where water flow is constant. **C.** Waldaist site in Upper Austria, a long-range north to south flowing river with lots of granitic rock boulders that flows into the Danube River and is prone to regular flooding events. **D.** Thalli of *D. luridum* var. *luridum* growing on one of the many boulders just above the water line at time of collection. Photographs taken by Dr. T. Booth and Kyle Fontaine.

Lichen collection within the mountains of the Austrian Alps

Three sites (mountains) in the Austrian Alps (Figure 2.8): Preberkessel, Salzburg state (site abbreviations Pr1 and Pr3 and A2 for three separate but very close watercourses; Pr1 = 47°12'37"N; 13°51'05"E, elevation ~2070 meters ASL; Pr3 = 47°13'16"N; 13°51'13"E, elevation 2270 meters ASL; A2 = 47°12'47"N; 13°51'10"E, elevation ~2070 meters ASL); Schladminger Tauern, Styria state (site abbreviation S; 47°16'17"N; 13°43'47"E; elevation 1960 meters ASL); and Hohe Tauern, Carinthia state (site abbreviation G; 46°56'13"N; 13°00'25"E; elevation ~2350 meters ASL) were sampled for sub-aquatic *Dermatocarpon* species. The habitats of the watercourses, Pr1, A2, S and G, were very similar to one another, in that they were very cold glacier and seepage streams flowing down from mountain slopes (Figure 2.7A and C). These watercourses were not very wide (several meters) or deep (maximum 50 cm) at the time of collection, and consisted of small to large overlapping granitic rock types. Growing on the rocks, the sub-aquatic lichens are found above and below the water (Figure 2.7B and D), and growing alone as there were almost no mosses. No *D*. *luridum* var. *luridum* thalli were collected in these sites, only *D. luridum* var. *decipiens*, *D. arnoldianum* and *D. rivulorum* thalli were collected. The last site Pr3 was unique to all others such that it was a vertical rock face covered in *D. luridum* var. *decipiens* with consistent water flow from above moving over and among the thalli.

Identifications of all lichen specimens collected in Austria were confirmed by Dr. R. Türk, lichen taxonomist, University of Salzburg, Salzburg, Austria.

Samples from both continents were collected by scraping the thallus from the rock with a knife and allowing it to air dry in a paper bag. Vouchers are deposited in the University of Manitoba Herbarium (WIN).

Figure 2.7. Typical mountain habitats of sub-aquatic lichens, *D. luridum* var. *luridum*, *D. luridum* var. *decipiens*, *D. arnoldianum*, and *D. rivulorum* in the Austrian Alps. **A.** Preberkessel Mountain site in Salzburg state, a glacier and seepage fed stream running down the slope of the mountain. **B.** Thallus of *D. arnoldianum* growing on the edge of a granitic rock at the waters edge of constantly flowing watercourse. **C.** Hohe Tauern Mountain site in Carinthia state, a glacier and seepage fed stream running down the slope of the mountain. **D.** Thalli of *D. rivulorum* growing over the surface of granitic rock above and below the water of a constantly flowing watercourse. Photographs taken by Kyle Fontaine.

Figure 2.8. Google Earth™ Image showing Austria and surrounding European countries, where within Austria, five collection sites were visited: Schlogener Schlinge, Upper Austria; Waldaist, Upper Austria; Schladminger Tauern, Styria; Preberkessel, Salzburg; and Hohe Tauern, Carinthia. Collection sites are indicated on the map using red dots labeled with respective site names, with the collection abbreviations in brackets. Scale bar = 50 kilometers. © 2009 GeoBasis-DE/BKG; © 2012 Cnes/Spot Image; © 2012 MapLink/Tele Atlas; © 2012 Google.

"Spore rain" method of culturing mycobionts

In order to obtain axenic mycobiont cultures, spores offer the best opportunity to derive "true" representatives of the mycobiont (Ahmadjian 1973). The study species, *Dermatocarpon luridum* var. *luridum*, produces perithecia immersed within the thallus (Figure 2.9) where 8 simple hyaline ascospores are produced within each bitunicate ascus (13.5–18 µm long) (Gueidan et al. 2009; Heiðmarsson 2000). If perithecia are developed, tiny brown to black dots, which are perithecial openings (ostules), will be apparent on the surface of the thallus (Purvis et al. 1992; Heiðmarsson 2000). However, *Dermatocarpon luridum* var. *luridum* also produces pycnidia that are immersed within the thallus showing brown to black ostules on the thallus surface. The only way to distinguish between perithecia and pycnidia is through the observation of cross-sections under with a microscope, although pycnidia are somewhat smaller and often do not protrude above the thallus surface (Heiðmarsson 2000).

The "spore rain" procedure (Yoshimura et al. 2002; Stocker-Wörgötter and Hager 2008) was performed using small (approximately 6mm^2) pieces of thallus where perithecia were numerous. Unfortunately, selection of thallus fragments with single perithecia was not possible because of the abundance and close proximity of perithecia to one another within the thallus.

To clean the thallus and perithecia of potential contaminating organisms trapped among the hyphae of the thallus and the perithecia, the following wash procedure was followed. The thallus fragments were washed in a 50 mL Erlenmeyer flask containing 25 mL of sterile double distilled water for 15 min. on

a stir table. One drop of Tween 80 was added to the water and the fragments were washed for an additional 15 min. on a magnetic stirrer. The lichen thallus fragments were transferred using sterile forceps to a clean 50 mL Erlenmeyer flask and washed in 25 mL of fresh sterile double distilled water for 20 min. to remove the Tween 80. Under sterile conditions of a laminar flow bench, the freshly washed thallus fragments were dried on sterile wipes and mounted to the lid portion of a petri dish (3 per lid) using a small dab of petroleum jelly. Lids were placed on the petri dish bottoms (ostioles facing down), containing pre-poured growth medium (see Appendix A for recipes), either Malt-Yeast agar medium (MY; Yamamoto 1990) or Lilly and Barnett Medium (LBM; Lilly and Barnett 1951). Parafilm was used to seal the petri dishes (2 or 3 replicates) and they were placed in a growth chamber maintained at 22 °C. As the perithecia dried out in the incubator, the spores were discharged to the surface of the growth medium.

If contamination was present on the plates after spore release from perithecia, a thin piece of medium with spores on the surface was transferred to a new petri dish with fresh growth medium. During the growth phase, the fungus may be further sub-cultured to new growth media.

The “spore rain” procedure was carried out using eight *Dermatocarpon* samples: one *D. miniatum* (terrestrial*)* from Manitoba, Canada; five *D. luridum* var. *luridum* (sub-aquatic) from Manitoba; one *D. arnoldianum* (sub-aquatic*)* from Austria*;* and one *D. luridum* var. *decipiens* (sub-aquatic) from Austria. Three plate replicates (each with 3 thallus fragments) were used for the Manitoban samples,

whereas two plate replicates were used for the Austrian samples. Unfortunately, *D. luridum* var. *luridum* thalli collected in Austria had no perithecia present (Figure 2.9), and thus the "spore rain" procedure could not be attempted using these thalli.

"Yamamoto method" of culturing mycobionts and photobionts

In the event that the "spore rain" method did not work, or was not possible because perithecia were not produced, the "Yamamoto method" (1990) was used since it separated the fungal hyphae from the algae in thallus frgaments. Additionally, because so few lichens disperse algae from their apothecia or perithecia along with ascospores, the "Yamamoto method" is the primary method for culturing lichen photobionts.

Isolation of the sub-aquatic mycobiont, *Dermatocarpon luridum* var. *luridum* and the associated photobiont was performed according to the "Yamamoto method" (Yamamoto 1990) with some modifications as specified in Stocker-Wörgötter (2002). Eight different samples were used for mycobiont isolation: four *D. luridum* var. *luridum* from Manitoba; three *D. luridum* var. *luridum* from Upper Austria; and one *D. luridum* var. *decipiens* from Salzburg, Austria. Seven samples were used for photobiont isolation, which were the same as those for the mycobiont isolation except for one *D. luridum* var. *luridum* sample from Manitoba.

A lobe of each lichen thallus, approximately 20 mm^2 was cut into smaller fragments and washed using the same wash protocol as above for the "spore

rain" procedure. Under the sterile conditions of a laminar flow bench, the washed lichen fragments were then ground in 3–4 mL of sterile water using mortar and pestle, filtered through a sieve with mesh pore size of 500 µm, and the fragments that passed through the sieve filtered for a second time through a mesh with pore size 150 µm. The filtrate consisting of fragments of lichen tissues (about 150 µm) were selected (fragments with minimal algal content for mycobiont isolations and fragments with abundant algal content for photobiont isolations) under a stereomicroscope using sterile bamboo sticks and transferred to slanted agar media.

Eight standard mycobiont growth media were used [MY, MY 1% mannitol, MY 0.5% mannitol, LBM, Georg modified-LBM = G-LBM (Pers. Comm. Georg Brunauer May 2011), Potato Dextrose Agar (Stocker-Wörgötter and Hager 2008), Murashinge and Skoog Medium = MS (Stocker-Wörgötter 2001), 2% Sabouraud (Stocker-Wörgötter and Hager 2008); (Appendix A for recipes), with 5 to 30 replicates per sample. A fraction (40%) of all mycobiont replicates were incubated in dark cool (~4-5 °C) conditions of a refrigerator, while the remainder were incubated at 22 °C in a growth chamber (the majority in the dark while some were left with 14:10 hour light:dark regime). The presence of algae with the fungus was tried using several replicates of four samples of *D. luridum* var. *luridum* to see if algal presence could stimulate mycobiont growth (suggested by Dr. Martin Grube, University of Graz, Graz, Austria) under both light (14:10 regime) and consistent dark conditions of the growth chamber at 22 °C. Slant

replicates were left to grow for up to nine months if no contaminant organisms were present within the tubes.

To culture the photobiont, three photobiont growth media were used (see Appendix B for recipes): 1) Modified Woods Hole MBL (WHM; Nichols 1973); 2) Modified Bolds Basal Medium plus soil extract (BBM + Soil Extract; Deason and Bold 1960); and 3) Optimal Haematococcus Medium (OHM; Fábregas et al. 2000). There were typically 25 replicates of slants per thallus sample, but 10 and 15 replicates were used for some samples. Cultures were grown for 2–3 months at 22 °C under a changing light:dark regime of 14:10 hours with a light intensity of 100 μE $m^{-2}s^{-1}$. Sub-culturing of the photobiont to liquid medium was performed, once the algal colony size reached approximately 2–3 mm diameter. An inoculation needle was flamed and the colony was fractioned into tiny drops within the liquid growth medium. A small number of algae were transferred to a sterile 50 mL Erlenmeyer flask containing 25–30 mL of liquid medium.

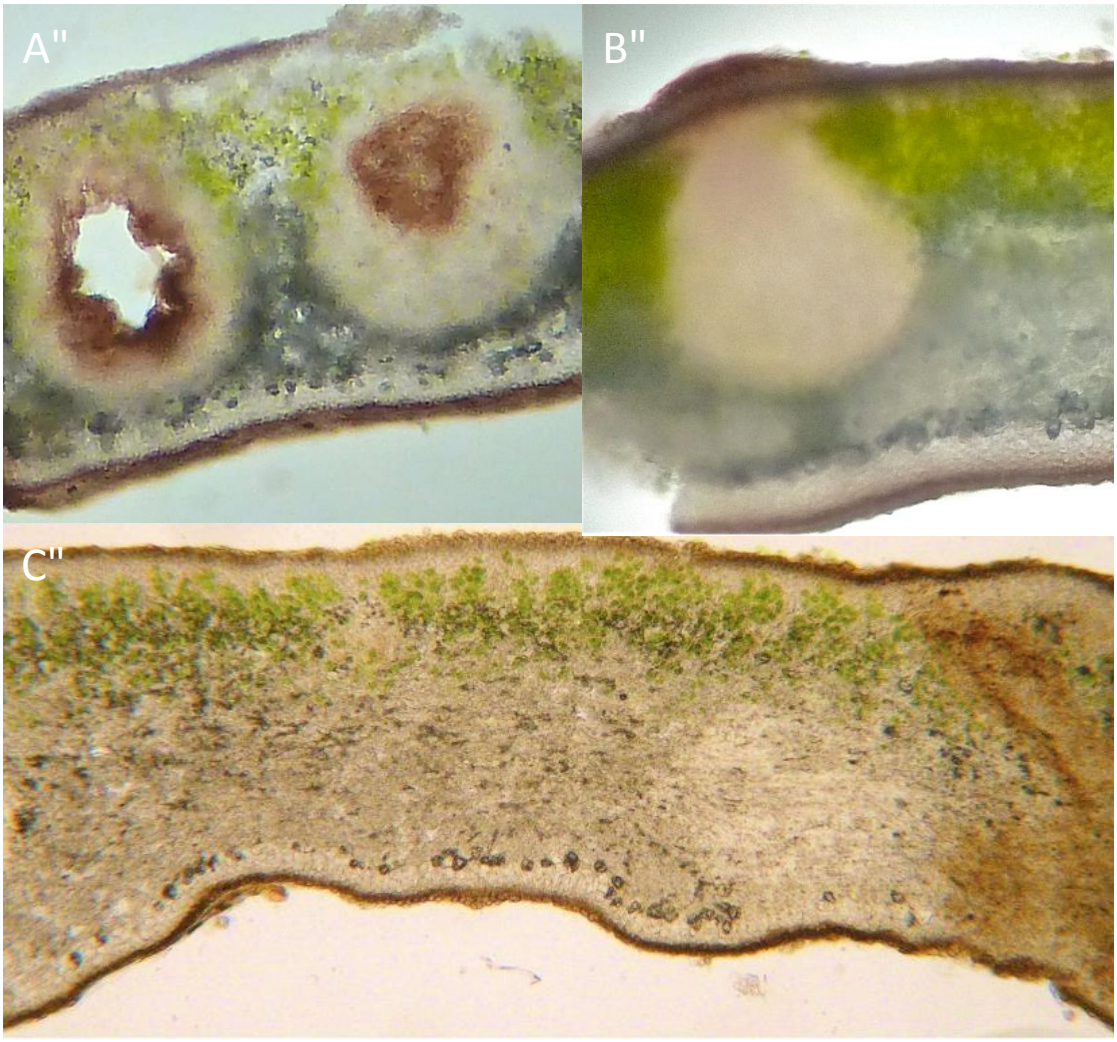


Figure 2.9. Thallus sections of the sub-aquatic *Dermatocarpon luridum* var. *luridum.* **A–B.** Manitoban sample showing the immersed perithecia (mature and immature, left and right, respectively). **C.** Austrian sample showing the lack of perithecia in the thallus; a typical thallus fragment that would be used to culture the algae using the "Yamamoto method" (Yamamoto 1990) of culturing.

Results

Mycobiont culturing using the "spore rain" method

Use of the "spore rain" method resulted in spore discharge from the perithecia of all samples investigated (Table 2.1). Contaminating growth (in most cases bacterial) also covered the area where spores were present in some cases. After 4-7 days the spores that were discharged and were free of contamination showed evidence of germination in the form of germ hyphae extending from individual spores. Almost all plates had contamination; therefore, spores required sub-culturing to fresh media. Germination was observed in three of five samples of *D. luridum* but they had moderate to high levels of contamination. The other species, *D. miniatum, D. luridum* var. *decipiens*, and *D. arnoldianum*, showed moderate, low, or no contamination and all cultures showed germination. However, none of the samples showed any growth after germination. *D. arnodianum* and *D. luridum* var. *decipiens,* displayed similar results in spore discharge and germination between Austrian and Manitoban samples, but there was much less contamination in the cultures prepared at Salzburg University than there was in the cultures prepared at the University of Manitoba. After nine months of incubation, the mycobiont of *Dermatocarpon luridum* var. *luridum* could not be cultured under the conditions tested. Additionally, none of the other lichen-fungal cultures progressed past the germination stage (Table 2.1).

Table 2.1. Results from culturing of sub-aquatic lichen fungi, using the "spore rain" method and standard media (MY is malt yeast medium; LBM is Lilly and Barnett medium) showing number of replicates for each sample, presence (+) or absence (-) of spores released, germination, and growth, and degree of contamination (none. Low, moderate, high).

Lichen identification (voucher number)	Culture medium	Number of replicates	Spores released	Germination	Growth	Bacterial/fugal contamination
*D. miniatum**[1] (P22-2)	MY	3	+	+	-	Moderate
D. luridum var. *luridum*[1] (P28-1)	MY	3	+	+	-	Moderate to High
D. luridum var. *luridum* (Ne4-1)	MY	3	+	-	-	High
D. luridum var. *luridum*[1] (Ni15-3)	MY	3	+	+	-	Moderate to High
D. luridum var. *luridum*[1] (Ni23-2)	MY	3	+	+	-	Moderate to High
D. luridum var. *luridum* (Na23-2)	MY	3	+	-	-	High
D. arnoldianum (A2-1)	MY	2	+	+	-	None
	LBM	2	+	+	-	None
D. luridum var. *decipiens* (Pr1-1)	MY	2	+	+	-	Low
	LBM	2	+	+	-	Low

* Terrestrial lichen; [1] uncontaminated region of plates containing spores were sub-cultured to fresh MY agar plates.

Mycobiont culturing using the "Yamamoto method"

Four Manitoban and three Austrian samples of *Dermatocarpon luridum* var. *luridum*, along with one Austrian sample of *Dermatocarpon luridum* var. *decipiens* were subjected to various culturing conditions (Table 2.2). The largest number of replicated treatments were conducted using LBM, G-LBM and MY (with mannitol added). None of the fungal fragments showed any signs of growth in the light or dark conditions. Additional growth trials were performed using PDA, MS and Sabouraud with lower number of replicates to determine if growth would be stimulated in different media. Similarly, no growth occurred under the same temperature and light conditions with these media (Table 2.2)

Table 2.2. Results of culturing the sub-aquatic fungus *Dermatocarpon luridum* var. *luridum* following the "Yamamoto method" with standard growth media, showing number of replicates for each media and specimen treatment, and presence (+) or absence (-) of growth under three incubation conditions (dark, light, and with algae). n/a = not applicable, therefore was not attempted.

Lichen identification	Growth medium	Number of replicates	Growth in dark[1]	Growth in light[2]	Growth with many algae present[3]
D. luridum var. *luridum* (P28-1)	LBM	25	-	-	n/a
	G-LBM	20	-	-	n/a
	PDA	10	-	-	n/a
	MY	10	-	-	n/a
	MY1%	10	-	-	n/a
	MY0.5%	10	-	-	n/a
D. luridum var. *luridum* (Ne18-1)	LBM	10	-	-	n/a
	G-LBM	10	-	-	n/a
	MY	10	-	-	n/a
	MS	10	-	-	n/a
D. luridum var. *luridum* (Ne25-2)	LBM	20	-	-	n/a
	G-LBM	20	-	-	n/a
	PDA	10	-	-	n/a
	MY	10	-	-	n/a
	MY0.5%	10	-	-	n/a
D. luridum var. *luridum* (Ni15-3)	LBM	30	-	-	-
	G-LBM	30	-	-	n/a
	MY	25	-	-	-
	MY1%	25	-	-	n/a
	MY0.5%	25	-	-	n/a
	2% Sabouraud	10	-	-	n/a
D. luridum var. *luridum* (DV4-1)	LBM	35	-	-	-
	MY	35	-	-	-
	MY0.5%	10	-	-	n/a
	PDA	10	-	-	n/a
	MS	10	-	-	n/a
	2% Sabouraud	10	-	-	n/a
D. luridum var. *luridum* (M3-1)	LBM	30	-	-	-
	MY	30	-	-	-
	MS	10	-	-	n/a
	2% Sabouraud	5	-	-	n/a
D. luridum var. *luridum* (M18-1)	LBM	30	-	-	-
	MY	30	-	-	-
	MS	10	-	-	n/a
	2% Sabouraud	5	-	-	n/a
D. luridum var. *decipiens* (Pr1-1)	LBM	10	-	-	n/a
	G-LBM	10	-	-	n/a
	MY	10	-	-	n/a
	PDA	10	-	-	n/a
	MS	10	-	-	n/a

[1] Incubator and refrigerator temperature conditions; [2] incubator temperature condition; [3] incubator (light and dark) and refrigerator (dark) temperature conditions; LBM is Lilly and Barnett medium, G-LBM is Georg modified-LBM, MY is malt-yeast medium, MY 1% mannitol, MY 0.5% mannitol, MS is Murashinge and Skoog medium.

Photobiont culturing using the "Yamamoto method"

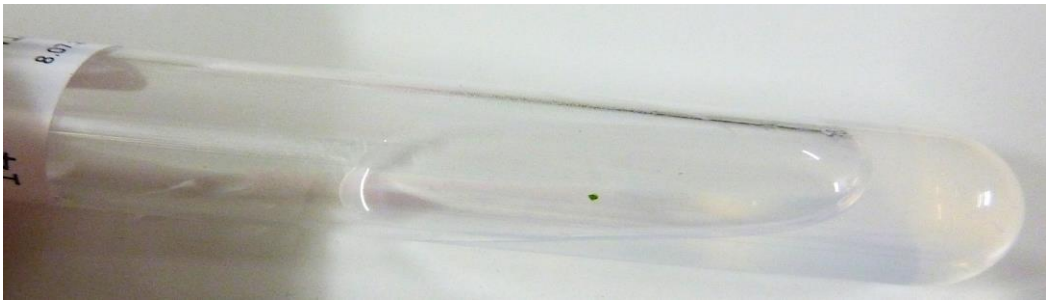
Growth was present from the photobionts associated with six samples of *Dermatocarpon luridum* var. *luridum* and one sample of *D. luridum* var. *decipiens* using different growth media (Table 2.3). Three kinds of media were used: WHM, BBM+S and OHM. Even though cultures were obtained for OHM, it was not as effective as the other two growth media. Growth seemed to be slow for both WHM and BBM+S slants, producing equal sized colonies, 1-1.5 mm in diameter, after one month of culturing (Figure 2.10). Contamination of slant colonies was most more prevalent with the WHM medium, although both media types produced considerable levels of contamination, which warranted the need for many replicates per sample (Figure 2.11). The larger number of replicates allowed a better chance of obtaining uncontaminated cultures. Bacterial colonies were the most common contamination observed for both media types, while fungal contamination was more common with WHM than BBM+S. If algal colonies are overgrown by a fungal contaminant, the alga cannot be subcultured (Fig. 2.11b). However, if the contamination has not reached the alga, the algal colonies could be sub-cultured to either a new slant or liquid medium (Figure 2.11a). Liquid WHM sub-cultures from slant isolates greatly accelerated algal replication, where after one and a half months of growth the medium was very dark green (Figure 2.12). In contrast, after sub-culturing to BBM+S liquid medium from slant colonies growth remained slow. After one and a half months of growth, the BBM+S medium remains predominantly transparent with algal cells much less apparent than in WHM cultures (Figure 2.12).

Table 2.3. Results from culturing of the photobiont associated with the sub-aquatic lichen fungus *Dermatocarpon luridum* var. *luridum* using the "Yamamoto method" with three standard growth media (WHM, BBM+S, OHM), showing number of replicates for each media type and each specimen, and the presence (+) or absence (-) of colony growth.

Voucher number	Growth medium	Number of replicates	Growth of axenic algal culture
P28-1	WHM	25	+
	WHM (liquid)	1	+
	BBM+S	25	+
	BBM+S (liquid)	1	+
Ne18-1	WHM	25	+
	OHM	10	+
Ni15-3	WHM	25	+
	WHM (liquid)	2	+
	BBM+S	25	+
	BBM+S (liquid)	2	+
	OHM	15	+
DV4-1	WHM	25	+
	WHM (liquid)	1	+
	BBM+S	25	+
	BBM+S (liquid)	1	+
M3-1	WHM	25	+
	BBM+S	10	+
M18-1	WHM	25	+
	WHM (liquid)	1	+
	BBM+S	25	+
	BBM+S (liquid)	1	+
Pr1-1	WHM	10	+
	BBM+S	10	+

WHM is Wood's Hole medium; BBM+S is Bold's Basal medium with Soil extract; and OHM is Optimal Haematococcus Medium.

A.



B.

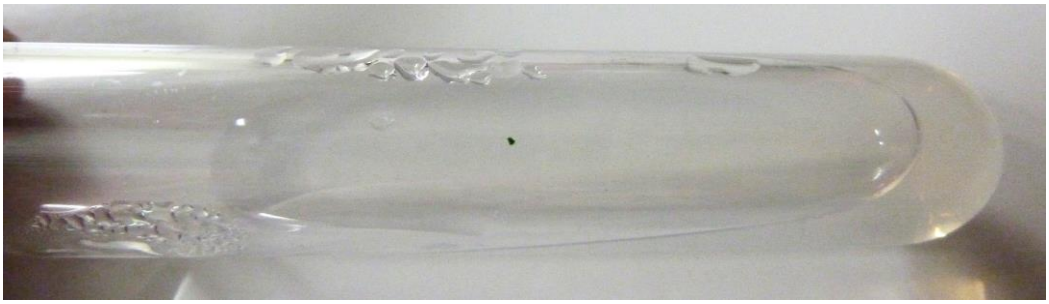
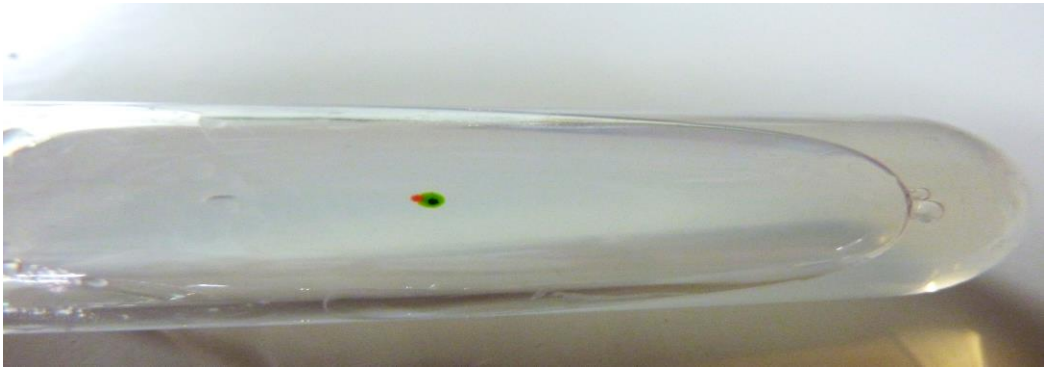


Figure 2.10. The photobiont of *Dermatocarpon luridum* var. *luridum*: **A.** collection Ni15-3 from Nisto Lake, Canada, one month after inoculation, grown on a slant of WHM growth medium; **B.** collection M18-1 from Waldaist, Austria, one month after inoculation, grown on a slant of BBM+S growth medium. Photographs taken by Kyle Fontaine.

A.



B.

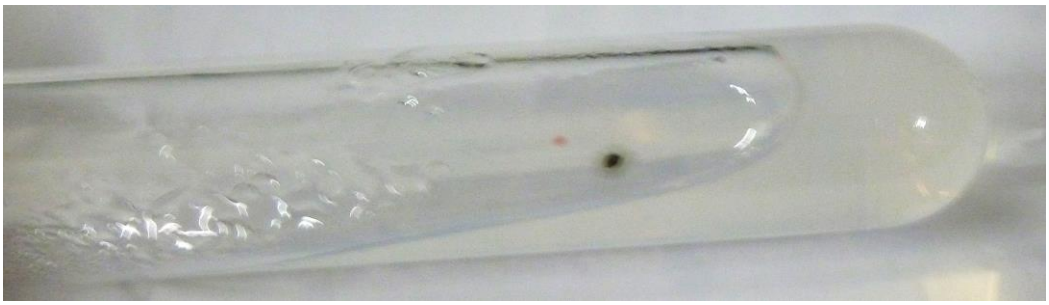


Figure 2.11. The photobiont of *Dermatocarpon luridum* var. *luridum*: **A.** collection, M18-1 from Waldaist Austria, shown with a pink bacterial colony growing on the periphery of the algal colony one month after inoculation, grown on a slant of BBM+S growth medium; **B.** collection Ni15-3 from Nisto Lake Canada showing a fungal contaminant overgrowing the algal colony and a bacterial colony growing apart from the fungal contaminated algal colony one month after inoculation, grown on a slant of WHM growth medium. Photographs taken by Kyle Fontaine

A. B.

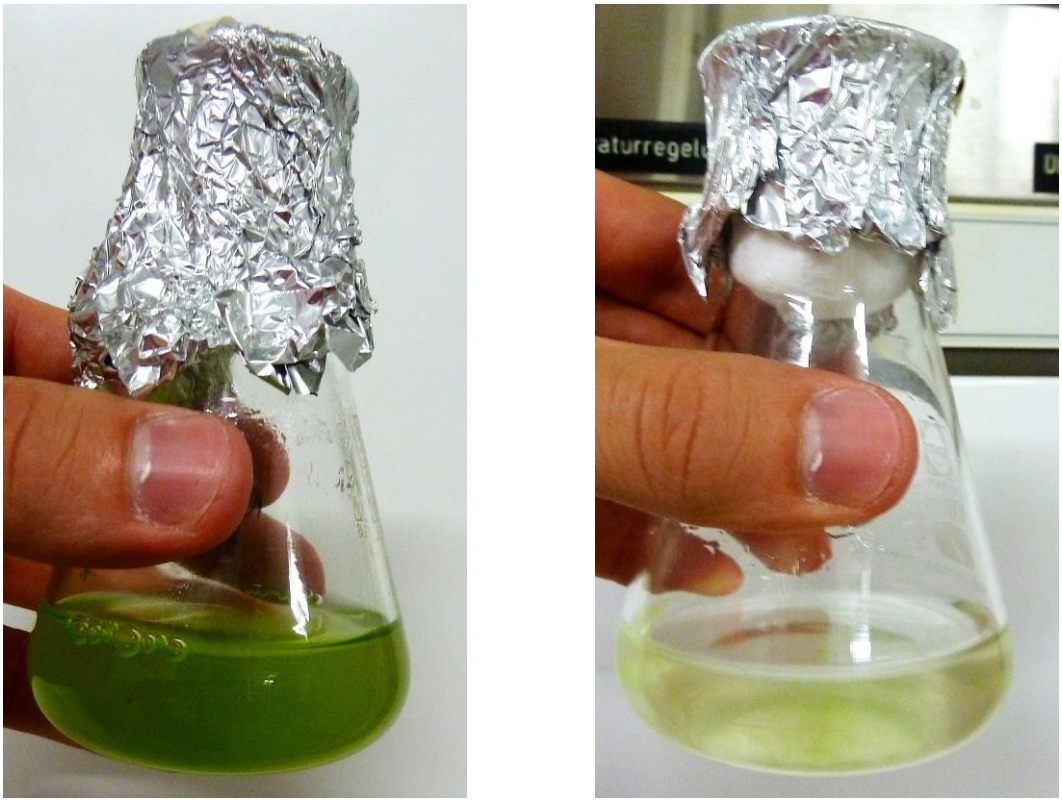


Figure 2.12. The photobiont of *Dermatocarpon luridum* var. *luridum*: **A.** collection # Ni15-3 from Nisto Lake, Manitoba, Canada growing in liquid WHM medium, one and a half months after sub-culturing from a slant with WHM growth medium; **B.** collection M18-1 from Waldaist, Upper Austria, Austria growing in liquid BBM+S medium, one and a half months after sub-culturing from a slant with BBM+S growth medium. Photographs taken by Kyle Fontaine.

Discussion

This study has successfully isolated and grown in culture the photobiont of *Dermatocarpon luridum*. It also was successful at isolating the spores and initiating germination of the spores of the lichen-fungus. However, it was not successful at the continued growth in culture of the fungus. In other studies, the algae of sub-aquatic and aquatic lichens have been successfully cultured. For example, Kele (1964) cultured the cyanobacterial symbiont of the aquatic lichen *Peltigera hydrothyria* (synonym = *Hydrothyria venosa*) and was able to identify the species as *Nostoc sphaericum.* However, the mycobiont of *P. hydrotheria* could not be cultured. Failure to culture the aquatic lichen fungus has repeatedly been the outcome over the years even though culturing methodologies have greatly improved with time. The lack of knowledge with respect to several factors may influence culture success for aquatic lichen fungi. The lichen fungi grow in close association with water. It is not known how critical the adaptation to the aquatic environment is when determining culture conditions. It is also not known how important the rock substratum or the presence of the photobiont is to the growth of the lichen fungus. Some mycorrhizal fungi require the presence of the plant host in order to grow in culture.

While growth of the *D. luridum* var. *luridum* was not successful, the release of the spores during dehydration of the petri dish environment suggests that the spores are released according to environmental changes that would be similar to those for terrestrial lichen fungi. Dehydration cycles are needed for spore release in many ascomycetes (Kendrick 2001). Germination of the spores

suggests that the ascospore must contain sufficient nutrients for initial germination but culture conditions used do not favour continued growth of the germ tube. Lichenized fungi are ecologically obligate biotrophic fungi (Honegger 2009), where the fungus invades living tissue of the algae, and can obtain nutrients directly from the living cells. Non-lichenized biotrophic fungi such as *Entomosporium* spp., causing leaf spot disease in members of the Rose family (Horie and Kobayashi 1980; Baudoin 1986), have been difficult to culture and have slow growth rates in culture. Most lichen-forming fungi have presented challenges for successful growth in culture, but necessary conditions have been established for a growing number of species. Members of the genus *Peltigera* have been successfully cultured (Stocker-Wörgötter and Türk 1994; Stocker-Wörgötter 1995). However, Stocker-Wörgötter et al. (1994) reported that most lichens amenable to culture were those growing on soil or sand natural substrata. *Dermatocarpon luridum* var. *luridum* grows on rock substratum and partially submerged in water, making the prediction of environmental factors more complex and less well known than those of many terrestrial lichens.

Mycobiont culture experiments of the sub-aquatic lichen, *Dermatocarpon luridum* var. *luridum* using standard media in this study have shown that the lichen fungus produces viable spores suggesting that the optimal culture conditions have yet to be discovered for this lichen. Nutrient composition of the growth medium, reflecting the natural environmental conditions of the lichen, is of utmost importance for successful cultivation of any organism in vitro; especially lichen symbionts (Stocker-Wörgötter and Hager 2008). For example, the mineral

composition of the growth media used may create an unfavorable environmental pH for the growth of *D. luridum* var. *luridum* or the concentration of one or more minerals may prevent growth. Alternatively, the absence of a critical mineral or environmental condition may prevent fungal growth. The aquatic lichens collected for this thesis were all found growing on acidic (granitic) rock. There seems to be a preferred condition for environmental pH to be slightly acidic (5-6 pH). This aquatic lichen has been shown to be highly sensitive to copper (Monnet et al. 2005), suggesting that it may be sensitive to other essential and trace minerals. It is this intolerance to mineral concentrations that have made *D. luridum* var. *luridum* an ideal indicator of environmental health.

Dermatocarpon luridum var. *luridum* may acquire nutrients from the associated photobiont, and also freely from the environment especially where the water may flow among the thalli lobes carrying nutrients with it (eg. waterbody, atmosphere, substrate, etc.) and other organisms living in association with the thallus, such as bacteria (Brown and Wilson 1969; Jacobs and Ahmadjian 1969; Cardinale et al. 2006; Liba et al. 2006; Cardinale et al. 2008; Grube et al. 2009; Hodkinson & Lutzoni 2009). It is not known whether bacteria associated with the thallus are necessary for thallus development. Jacob and Ahmadjian (1973) claimed that the aquatic lichen, *Peltigera hydrothyria* is without bacterial flora and suggested that the lichen is therefore capable of sufficiently deriving the necessary nutrients from the water in which it lives submerged.

The results of this study show that the photobiont associated with the sub-aquatic lichen *Dermatocarpon luridum* var. *luridum* is readily culturable using two

standard media to isolate axenic photobiont cultures, and one medium (OHM) designed to specifically culture species of the green algal genus, *Haematococcus* Flotow. The fact that levels and occurrences of bacterial and fungal contaminants appeared to be much reduced with the growth medium, BBM+S, suggests that this medium is more favorable for growing axenic cultures of green algal photobionts. However, rate of growth of algal colonies is accelerated by the growth medium, WHM, which is a more universal medium for culturing green algae. BBM+S or BBM in general, is more commonly used for the culturing of a wide range of photobionts. Rate of growth differences would then be attributed to the fact that photobiont growth media are more nutrient deprived than universal media types, preventing the growth of unwanted faster growing bacterial and/or fungal contaminant organisms.

In conclusion, the study was successful with culturing the photobiont that associates with *D. luridum*, which is consistent with that of other studies. Although the fungal partner could be isolated and genermination occurred through the spore method only, the growth of the fungal partner could not be sustained. Clearly more research is needed to determine the conditions for fungal growth. A quotation from a leading researcher on the culturing of lichen fungi is that "Recent advances in culturing techniques have destroyed the myth that lichen fungi or lichens are difficult to culture." Stocker-Wörgötter and Hager (2008). However, this is a very bold statement, and it would be more accurate if the authors were to acknowledge that the culture experiments that provided them the opportunity to make the statement were conducted using *terrestrial* lichen

fungi. A search of the literature fails to provide evidence that any sub-aquatic and/or aquatic lichen fungi have been successfully cultured.

Chapter 3

Photobiont Relationships and Phylogenetic History of *Dermatocarpon luridum* var. *luridum* and Related *Dermatocarpon* Species

Introduction

The vast majority of lichens involve the association of a heterotrophic fungus of the Ascomycota and a photoautotrophic alga of the Chlorophyta (Nash 2008). Terrestrial ecosystems harbor nearly all known lichens, while some occupy fully or partially submerged rocks of marine or freshwater lake, river/stream and spring ecosystems (Thüs 2002; Nascimbene et al. 2007). Considerably less knowledge exists about aquatic and sub-aquatic lichens than aquatic fungi and terrestrial lichens. The majority of lichen species that live in or are associated with aquatic ecosystems are systematically positioned within the Verrucariaceae. Many genera within the Verrucariaceae do not form monophyletic clades, but the genus *Dermatocarpon* Eschw. is considered to be monophyletic (Gueidan et al. 2007; 2009) with an umbilicate growth habit, thick-walled lower cortical cells of the thallus, pycnidia, immersed thalloid perithecia containing eight simple spores per ascus, and an absence of hymenial algae in the perithecia, as found in species of *Endocarpon* and *Staurothele* (Gueidan et al. 2009). While species of *Dermatocarpon* are globally distributed, North America has 19 to 24 species (Esslinger 2011), and Austria has six species (Türk et al. 2010). Some *Dermatocarpon* species colonize rock substrata that are associated with watercourses, which periodically inundate the lichen thalli; such lichens are considered “sub-aquatic” lichens (Santesson 1939; Aptroot and Seaward 2003). Other members of *Dermatocarpon* are not associated with watercourses and live on rocks and occasionally on soil. In general, members of this genus exhibit rather diverse morphologies, while other commonly known sub-

aquatic species include: *Dermatocarpon arnoldianum*, *D. luridum* var. *luridum* and var. *decipiens*, *D. meiophyllizum* and *D. rivulorum*, that share more or less similar morphological characters. Even sub-aquatic *Dermatocarpon* species such as *D. luridum* are not fully submerged like the aquatic lichens, *Peltigera gowardii* and *P. hydrothyria*. A comprehensive taxonomic treatment of the genus *Dermatocarpon* was published by Amtoft et al. (2008) and Heiðmarsson (2000; 2002).

Symbiotic interactions between lichenized fungi and their algal partners are described by their degree of selectivity and specificity (Beck et al. 2002; Yahr et al. 2006). Selectivity is the preferred interaction between two organisms (Galun and Bubrick 1984; Piercey-Normore 2006) or the frequency of compatible symbiotic partners in lichen associations (Casano et al. 2011). Selectivity is assessed from the perspective of one partner in lichen associations and the level of selectivity can be graded as high to low (Beck et al 2002). Specificity is defined as, the coevolutionary pattern between symbionts and it is the result of the selectivity of the partaking bionts (Beck et al. 2002; Piercey-Normore 2006). Specificity refers to the taxonomic range of acceptable partners, which could be influenced by the environment (Casano et al. 2011). Like selectivity, specificity is rank ordered from high to low from the aspect of the fungus and alga, respectively. Some genera of the Verrucariaceae express low selectivity (e.g., *Bagliettoa* sp. and *Verrucaria* sp.), such that they are known to associate with multiple genera of green algae, of different phylogenetically assigned families of photobionts (Thüs et al. 2011). However, the genus *Verrucaria* is not a good

example for selectivity because it is polyphyletic. The model generated by Yahr et al. (2004) summarized the mycobiont-photobiont relationship (between *Cladonia perforata* and *Asterochloris*) and the intricate systems that structure these interactions.

The diversity of photobiont partners associated with lichen-forming fungi in the Verrucariaceae is thought to be among the greatest of all lichen fungi (Beck and Peršoh 2009; Honegger 2009). Recent phylogenetic investigations of photobiont algae using nuclear small subunit and ribulose-bisphosphate carboxylase gene sequences by Thüs et al. (2011) revealed photobionts in the genera *Stichococcus* and *Diplosphaera* associated with the fungi of the Verrucariaceae. The photobiont of *Dermatocarpon luridum* and several other lichens were phylogenetically identified as belonging to the genus *Diplosphaera* (Thüs et al. 2011). However, culture experiments of *Dermatocarpon* photobionts were not performed in this study and their identification was based only on nucleotide sequences despite morphological based identifications having been performed by Thüs and Schultz (2008). Early culture experiments in the Verrucariaceae investigated the squamulose rock/concrete inhabiting lichen *Endocarpon pussilum* and crustose rock inhabiting *Staurothele clopima* (Ahmadjian and Heikkilä 1970). Further experiments, performing an “artificial” re-synthesis between the isolated lichen fungus and algae were conducted with *Dermatocarpon miniatum* (Stocker-Wörgötter and Türk 1989), as well as with *Verrucaria macrostoma* (Stocker-Wörgötter and Türk 1989; Stocker-Wörgötter 2010). The algal symbionts of *Dermatocarpon miniatum* were identified as

Hyalococcus dermatocarponis. The photobionts of *Endocarpon pusillum* were already known as *Stichococcus diplosphaera*, whereas the algal partner associated with *Verrucaria macrostoma* was unidentified at this time. In these early investigations, it was found that some algal species, although associated with members of the fungal family Verrucariaceae were incapable of re-synthesizing with other lichen mycobionts. Thus, the natural pairing and interaction of algal symbionts with the "appropriate" mycobionts was thought to be species specific (Ahmadjian and Heikkilä 1970). Certainly, such hypotheses have to be tested de-novo with the use of nucleotide sequence markers. But until recently, there is little known about the photobionts associated with fungi of the Verrucariales.

The major objective of this study was to determine the relationship between Canadian and Austrian *Dermatocarpon luridum* var. *luridum* along with three additional sub-aquatic *Dermatocarpon* species. Phylogenetic analysis was expected to shed light on the uncertain taxonomic status of sub-aquatic *Dermatocarpon* species, *D. arnoldianum* and *D. luridum* var*. decipiens*, which until now have eluded molecular investigation. This is the first study to examine the selected sub-aquatic *Dermatocarpon* species and their photobionts simultaneously using a DNA sequence marker as the framework. Further goals were to determine the species of photobiont(s) that associate with *D. luridum* var. *luridum* and finally to investigate the algal sharing potential of selected species of the genus *Dermatocarpon.*

Materials and Methods

Lichen Material

Four lakes in west central Manitoba, Canada, and five locations in Austria were sampled for *D. luridum* var*. luridum* and other *Dermatocarpon* lichens. In Canada, ten specimens were collected (Table 3.1). Thirteen samples were collected from five locations in Austria (Table 3.1). All lichen thalli were moistened to aid the scraping of the thalli off rock surfaces and reduce damage to the thalli before they were air dried and processed. All vouchers (Table 3.1) are maintained in the cryptogamic division of the University of Manitoba herbarium (WIN), Winnipeg, Manitoba, Canada.

Table 3.1. List of samples and sequence sources for symbionts used in this study, including collection information and accession (Acc.) numbers for the DNA sequences deposited to NCBI GenBank. NS is not sequenced. * Specimens used for algal culture experiments.

Taxon identification	Sample collection number or source	Collection site	Fungal ITS Acc.	Algal ITS Acc.
Dermatocarpon arenosaxi	Amtoft et al. (2008)	USA, Missouri	EF014161	NS
Dermatocarpon arenosaxi	Amtoft et al. (2008)	USA, Missouri	EF014164	NS
Dermatocarpon arenosaxi	Amtoft et al. (2008)	USA, Arkansas	EF014168	NS
Dermatocarpon arnoldianum	Fontaine A2-1	Austria, Salzburg, Preberkessel, 2011; 47°12'47"N; 13°51'10"E	JX645038	NS
Dermatocarpon arnoldianum	Fontaine Pr1-8	Austria, Salzburg, Preberkessel, 2011; 47°12'37"N; 13°51'05"E	JX645037	JX645019
Dermatocarpon leptophyllum	Heiðmarsson (2003)	Sweden	AF333155	NS
Dermatocarpon leptophyllum	Heiðmarsson (2003)	Sweden	AF333156	NS
Dermatocarpon luridum	Heiðmarsson (2003)	USA, Minnesota	AF333133	NS
Dermatocarpon luridum	Heiðmarsson (2003)	Sweden	AF333132	NS
Dermatocarpon luridum var. *decipiens* *	Fontaine Pr1-1	Austria, Salzburg, Preberkessel, 2011; 47°12'37"N; 13°51'05"E	JX645039	NS
Dermatocarpon luridum var. *decipiens*	Fontaine Pr3-9	Austria, Salzburg, Preberkessel, 2011; 47°13'16"N; 13°51'13"E	JX645040	NS
Dermatocarpon luridum var. *decipiens*	Fontaine S1-1	Austria, Styria, Schladminger Tauern, 2011; 47°16'17"N; 13°43'47"E	JX645041	NS
Dermatocarpon luridum var. *luriudm*	Amtoft et al. (2008)	USA, North Carolina	EF014194	NS
Dermatocarpon luridum var. *luridum*	Amtoft et al. (2008)	USA, Arkansas	EF014195	NS
Dermatocarpon luridum var. *luridum*	Amtoft et al. (2008)	USA, Missouri	EF014196	NS
Dermatocarpon luridum var. *luridum*	Amtoft et al. (2008)	USA, Alabama	EF014197	NS
Dermatocarpon luridum var. *luridum*	Amtoft et al. (2008)	USA, Missouri	EF014198	NS
Dermatocarpon luridum var. *luridum*	Fontaine P19-1	Canada, Manitoba, Payuk Lake, 2010; 54°38'31"N; 101°31'40"W	JX645023	JX645008
Dermatocarpon luridum var. *luridum*	Fontaine P23-3	Canada, Manitoba, Payuk Lake, 2010; 54°38'31"N; 101° 31'40"W	JX645024	JX645018
Dermatocarpon luridum var. *luridum* *	Fontaine P28-1	Canada, Manitoba, Payuk Lake, 2010; 54°38'31"N; 101°31'40"W	JX645025	JX645015
Dermatocarpon luridum var. *luridum*	Fontaine Ne14-2	Canada, Manitoba, Neso Lake, 2010; 54°39'51"N; 101°32'44"W	JX645026	JX645016
Dermatocarpon luridum var. *luridum* *	Fontaine Ne18-1	Canada, Manitoba, Neso Lake, 2010; 54°39'51"N; 101°32'44"W	JX645027	JX645009
Dermatocarpon luridum var. *luridum*	Fontaine Ne29-2	Canada, Manitoba, Neso Lake, 2010; 54°39'51"N; 101°32'44"W	JX645028	JX645013
Dermatocarpon luridum var. *luridum* *	Fontaine Ni15-3	Canada, Manitoba, Nisto Lake, 2010; 54°42'02"N; 101°30'17"W	JX645029	JX645011
Dermatocarpon luridum var. *luridum*	Fontaine Ni10-2	Canada, Manitoba, Nisto Lake, 2010; 54°42'02"N; 101°30'17"W	JX645030	JX645012
Dermatocarpon luridum var. *luridum*	Fontaine Na15-1	Canada, Manitoba, Naosap Lake, 2010; 54 50'38"N; 101°26'12"W	JX645031	JX645010
Dermatocarpon luridum var. *luridum* *	Fontaine M3-1	Austria, Waldaist, 2011; 48°19'44"N; 13°52'28"E	JX645033	JX645020
Dermatocarpon luridum var. *luridum* *	Fontaine M18-1	Austria, Waldaist, 2011; 48°23'41"N; 13°36'06"E	JX645032	JX645045
Dermatocarpon luridum var. *luridum*	Fontaine DV1-1	Austria, Schlogener Schlinge, 2011; 48°25'59"N; 13°52'28"E	JX645034	JX645021
Dermatocarpon luridum var. *luridum* *	Fontaine DV4-1	Austria, Schlogener Schlinge, 2011; 48°25'59"N; 13°52'28"E	JX645035	JX645014
Dermatocarpon luridum var. *xerophilum*	Amtoft et al. (2008)	USA, Missouri	EF014199	NS

Table 1. *Continued*

Taxon identification	Sample collection number or source	Collection site	Fungal ITS Acc.	Algal ITS Acc.
Dermatocarpon luridum var. *xerophilum*	Amtoft et al. (2008)	USA, Oklahoma	EF014200	NS
Dermatocarpon luridum var. *xerophilum*	Amtoft et al. (2008)	USA, Arkansas	EF014201	NS
Dermatocarpon luridum var. *xerophilum*	Amtoft et al. (2008)	USA, Arkansas	EF014202	NS
Dermatocarpon luridum var. *xerophilum*	Amtoft et al. (2008)	USA, Arkansas	EF014203	NS
Dermatocarpon luridum var. *xerophilum*	Amtoft et al. (2008)	USA, Arkansas	EF014204	NS
Dermatocarpon miniatum	Fontaine P22-2	Canada, Manitoba, Payuk Lake, 2010; 54°38'31''N; 101°31'40''W	JX645036	JX645017
Dermatocarpon miniatum var. *complicatum*	Heiðmarsson (2003)	USA, Minnesota	AF333146	NS
Dermatocarpon miniatum var. *complicatum*	Heiðmarsson (2003)	Sweden	AF333150	NS
Dermatocarpon miniatum var. *complicatum*	Heiðmarsson (2003)	Iceland	AF333151	NS
Dermatocarpon miniatum var. *complicatum*	Heiðmarsson (2003)	Austria	AF333154	NS
Dermatocarpon miniatum var. *complicatum*	Heiðmarsson (2003)	Norway	AF333163	NS
Dermatocarpon miniatum var. *miniatum*	Heiðmarsson (2003)	Sweden	AF333144	NS
Dermatocarpon miniatum var. *miniatum*	Heiðmarsson (2003)	USA, Minnesota	AF333148	NS
Dermatocarpon miniatum var. *miniatum*	Heiðmarsson (2003)	Iceland	AF333153	NS
Dermatocarpon miniatum var. *miniatum*	Heiðmarsson (2003)	India	AF333162	NS
Dermatocarpon miniatum var. *miniatum*	Amtoft et al. (2008)	Wales	EF014192	NS
Dermatocarpon multifolium	Amtoft et al. (2008)	USA, Missouri	EF014170	NS
Dermatocarpon multifolium	Amtoft et al. (2008)	USA, Missouri	EF014171	NS
Dermatocarpon multifolium	Amtoft et al. (2008)	USA, Missouri	EF014172	NS
Dermatocarpon multifolium	Amtoft et al. (2008)	USA, Virginia	EF014177	NS
Dermatocarpon multifolium	Amtoft et al. (2008)	USA, North Carolina	EF014184	NS
Dermatocarpon rivulorum	Heiðmarsson (2003)	Sweden	AF333166	NS
Dermatocarpon rivulorum	Türk 49780	Austria, Carinthia, Hohe Tauern, 2011; 46°59'18''N; 13°15'28''E	JX645042	NS
Dermatocarpon rivulorum	Fontaine G1	Austria, Carinthia, Hohe Tauern, 2011; 46°56'13''N; 13°00'25''E	JX645044	JX645022
Dermatocarpon rivulorum	Fontaine G10	Austria, Carinthia, Hohe Tauern, 2011; 46°56'13''N; 13°00'25''E	JX645043	NS
Diplosphaera chodatii	Zhang and Wei (2011)	China	NS	HM237335
Diplosphaera chodatii	Zhang and Wei (2011)	China	NS	HM237336
Diplosphaera chodatii	Zhang and Wei (2011)	UTEX culture collection #1177	NS	HQ129931
Stichococcus bacillaris	Marin (2012)	Unknown	NS	HE610125
Stichococcus bacillaris	Unpublished	Unknown	NS	AJ431678
Stichococcus mirabilis	Unpublished	Unknown	NS	AJ431679
Uncultured eukaryote	Khan et al. (2011)	Antarctica	NS	HM490287
Uncultured eukaryote	Khan et al. (2011)	Antarctica	NS	HM490288

Photobiont Isolation for Culturing

Photobiont isolation from *Dermatocarpon luridum* var. *luridum* was performed using seven thalli (Table 3.1) according to the "Yamamoto method" (Yamamoto 1990), and isolations were performed using the procedure as described in Stocker-Wörgötter (2002) and Stocker-Wörgötter and Hager (2008). A lobe of lichen thallus, approximately 20 mm^2 was cut into smaller fragments and washed in a 50 mL Erlenmeyer flask containing 25 mL of sterile double distilled water for 15 min on a stir table. One drop of Tween 80 (a surfactant) was added to the water and the fragments were washed for an additional 15 minutes on a magnetic stirrer. The lichen thallus was transferred using sterile forceps to a clean 50 mL Erlenmeyer flask and washed in 25 mL of fresh sterile double distilled water for 20 min to remove the Tween 80.

Under sterile conditions, the washed lichen fragments were then ground in 3–4 mL of sterile water using mortar and pestle which was filtered through a sieve with mesh pore size of 500 µm and the fragments that passed through were then filtered for the second time through a mesh with pore size 150 µm. The filtrate consisting of fragments of lichen "tissue" (about 150 µm maximum) were selected under a stereomicroscope using sterile bamboo sticks and transferred to slanted agar media.

Woods Hole MBL (Nichols 1973) was used to culture and isolate axenic colonies of the photobiont. Cultures were grown for 2–3 months at 22 °C under a changing light:dark regime of 14:10 hours with a light intensity of 100 µE $m^{-2}s^{-1}$. Sub-culturing of the photobiont to liquid medium was performed, once the algal colony size reached 2–3 mm diameter. An inoculation needle was flamed and the colony was fractioned into tiny drops. A small number of algae were transferred to a

sterile 50 mL Erlenmeyer flask containing 25–30 mL of liquid medium. Single cell isolates were not feasible, because of the small size of the *Diplopshaera* cells.

Microscopic Examination

To investigate the morphological features of the algal cells in culture, cells were streaked into water on a microscope slide and observed using a Univar Light Microscope (Reichert INC., Vienna, Austria) and photographs were taken with a Canon PowerShot G5 camera (Canon INC., Tokyo, Japan). Algal species cultures were sent to A. Beck and temporarily maintained at the Culture Collection of the Botanische Staatssammlung Munich, Germany.

DNA Extraction, Amplification, and Purification

Extraction of DNA was accomplished using a protocol modified from Grube et al. (1995). The ITS rDNA of both the mycobiont and photobiont of 22 *Dermatocarpon* thalli was amplified by Polymerase Chain Reaction (PCR). The DNA was initially amplified using 20 µL reactions to determine the optimal DNA concentration for PCR and then amplified in larger quantities (four to eight 20 µL PCR reactions) for sequencing. One 20 µL reaction consisted of 5–25 ng of genomic DNA, 1× PCR buffer (50 mM KCl; 100 mM Tris-HCl [pH 8.3]), 2.0 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer, and 2 units of Taq DNA Polymerase (Invitrogen, Burlington, ON, Canada).

The fungal and algal ITS genes were amplified using the fungal specific forward primer ITS1F-5' (5'-CTTGGTCATTTAGAGGAAGTAA-3'; Gardes and Bruns

1993) and the newly designed algal specific primer STICHO-ITS-F-5' (5'-GGATCATTGAATCTATCAACAAC-3'), respectively, both together with the universal reverse primer ITS4-3' (5'-TCCTCCGCTTATTGATATGC-3'; White et al. 1990). All amplifications of the ITS genes were performed using a Biometra T-Gradient thermal cycler (Fisher Sci, Nepean, ON, Canada). The PCR cycle used to amplify the fungal ITS consisted of an initial denaturation of the DNA at 94 °C for 3 min.; then 30 cycles of denaturation at 94 °C for 1 min., annealing at 61.5 °C for 30 sec. and extension at 72 °C for 45 sec.; followed by 6 °C soak. Amplification of the algal ITS was accomplished with an initial denaturation of the DNA at 94 °C for 3 min.; then 30 cycles of denaturation at 94 °C for 1 min., annealing at 58.5 °C for 35 sec., and extension at 72 °C for 45 sec.; followed by 6 °C soak.

Precipitation of the combined PCR products was carried out by adding 0.2 volumes of 5 M NaCl and 2.5 volumes of 100% ethanol. The tubes were gently inverted several times, placed in the fridge (4 °C) for 30 min., and centrifuged for 10 min. at 13,000 rpm. The supernatant was poured off and the pellet was washed with 300 µL of cold 80% ethanol and the pellets were left to air dry for 30 min. This pellet was dissolved in 20 µL of sterile distilled water and the entire 20 µL PCR volume was electrophoresed on a 1% agarose gel, the bands were excised from the gel, and purified using Promega's Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA). The purified DNA was resuspended in 35 µL of sterile distilled water, and quantified by gel electrophoresis. Band intensities were compared with the 1650 base pair band (80 ng/µL) of a 1 kb plus DNA ladder

(Invitrogen, Burlington, ON, Canada) and visualized using an AlphaImager 2200 transilluminator (Alpha Innotech, Fisher Scientific, Nepean, ON, Canada).

DNA Sequencing

PCR products were cycle sequenced with BigDye v.3.1 (Applied Biosystems, Foster City, CA, USA) following the manufacturer's directions. Precipitation and cleanup of the cycle sequenced product to remove excess fluorescent dyes was carried out by the EDTA method following manufacturer's directions, resuspended in Hi-Di formamide **(**Applied Biosystems, Foster City, CA, USA) and loaded to a 96-well plate for sequencing with an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Data Analysis

Nucleotide sequences obtained from samples collected for this study were edited using Sequencher v. 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences retrieved from NCBI GenBank were selected by first performing a nucleotide BLAST [62] search on the entire nucleotide collection using each algal sequence of the photobionts from *D. luridum* var. *luridum* as the query sequence. Only those with significant e-values of $10e^{-50}$ (O'Rourke et al. 2009) and high query coverage (>75%) were selected for phylogenetic analysis. Sequences were aligned manually using Se-Al v. 1.0 (Rambaut 2001) and two ambiguous regions were delimited based on visual inspection where substitutions were too numerous for a homologous alignment. The alignment was subjected to parsimony analysis in PAUP* 4.0b10 (Swofford 2003) and Bayesian analysis in MrBayes v3.1.2

Huelsenbeck et al 2001; Ronquist and Huelsenbeck 2003). Four MP analyses were performed for the algal alignment to assess the effect of the ambiguous regions on the phylogeny: (1) one ambiguous region was removed from the analysis; (2) the other ambiguous region was removed from the analysis; (3) both ambiguous regions were removed from the analysis; and (4) both ambiguous regions were included in the analysis. Since the topology was the same and the bootstrap values fluctuated between 3 and 5%, the final analysis presented includes both ambiguous regions. The fungal data set that was analyzed consisted of 58 fungal ITS sequences, 22 derived from the current study and 36 from NCBI GenBank (Table 3.1). Likewise, the algal data set that was analyzed consisted of 22 algal ITS sequences, 16 derived from the current study and 6 from NCBI GenBank (Table 3.1). The fungal outgroup species were *Endocarpon pallidulum* and *Verrucaria viridula*, while the algal outgroup was designated as uncultured Eukaryotic algae. The sequences obtained from this study have been deposited in NCBI GenBank and accession numbers are indicated in Table 3.1.

Maximum Parsimony settings were set to run a full heuristic search with a tree bisection and reconnection (TBR) branch swapping algorithm, with a stepwise addition and 5,000 replicates (Fungal data set) or 10,000 replicates (Algal data set) with random addition of taxa. Swapping was performed on only the best trees, and only the best trees were kept with the saving of Multrees option off. The most parsimonious trees were analyzed for bootstrap support within PAUP with the following MP settings: 1,000 bootstrap replicates (Felsenstein 1985) were performed and only scores greater than 70% were presented in the phylogenetic trees. All other

settings were the same as the initial heuristic search; all but the random addition of taxa setting, which was set to 10 for the fungal analysis and 100 for the algal analysis. Sequence alignment contained ambiguous regions in the algal data set from base pair positions 76–107 and 415–465 of the unaligned UTEX 1177 (HQ129931) sequence. Each of these regions was individually excluded from subsequent analyses and then all together excluded to see if tree topology was altered significantly. All ambiguous regions had minimal effect on the topology and clade scores, therefore all character data were used for the phylogenetic analysis presented.

Bayesian analysis was performed following the best evolutionary model estimated with JModeltest 0.1.1 (Posada and Crandall 1998; Guindon and Gascuel 2003; Posada 2008). Both ITS data sets were analyzed according to a TrN+G (Tamura-Nei plus Gamma model), which was presented as the best model according to hLRTs (Hierarchial Likelihood Ratio Tests) output within JModeltest. This model follows a six parameter with a gamma shaped distribution. The TrN+G analysis parameters were set as follows for the fungal dataset: Lset Base = (0.2398, 0.2504, 0.3044); Nst = 6; Rmat = (1.0000, 4.7694, 1.0000, 1.0000, 1.7829); Rates = Gamma; Shape = 0.4751; and Pinvar = 0. Likewise, the TrN+G analysis parameters were set as follows for the algal dataset: Lset Base = (0.2323, 0.3015, 0.2792); Nst = 6; Rmat = (1.0000, 1.8102, 1.0000, 1.0000, 3.8085); Rates = Gamma; Shape = 0.3942; and Pinvar = 0. The model information for both analyses was incorporated into PAUP for ML Analysis (outcomes not presented). The settings of this model correspond to the GTR+G evolutionary model. Bayesian analyses were performed using 2,000,000

generations and were terminated well after the standard deviation of split frequencies fell below 0.01. The number of trees discarded following the analysis included 500 burn-in trees. Posterior probability scores greater than 90 are reported on the phylogenies.

A phenetic analysis of both data sets using NJ was also performed in PAUP using the default settings and a Kimura 2-parameter model (outcomes not shown). Outcomes of the ML and Neighbour Joining analyses are not presented because the results were largely congruent with the MP and Bayesian analyses.

Results

Microscopy

Algal cells were isolated and cultured from *D. luridum* var. *luridum* showing spherical (4–5 µm) to oval (4–8 µm × 3–5 µm) cell shape with the presence of a parietal plate shaped chloroplast (Figure 3.1A–C). Algal cultures were obtained from four Austrian collected samples and two Canadian samples (Table 3.1) that were morphologically identified as *Diplosphaera chodatii* (e.g., Figure 3.1A–C). An additional algal culture, which was isolated from *D. luridum* var. *luridum* thallus collected in Canada, revealed two different algal cell types (Figure 3.1D). The first cell type is comprised of spherical to oblate spheroid (7.5–11.5 µm length) shaped cells with the presence of a parietal chloroplast and many globular bodies throughout the cell. The second type is oval to oblong (6–8 µm length) with a width equal to or slightly greater than the width observed in cells of the *D. chodatii* cultures. The second cell type presented characteristics similar to the characteristics described for the first cell type.

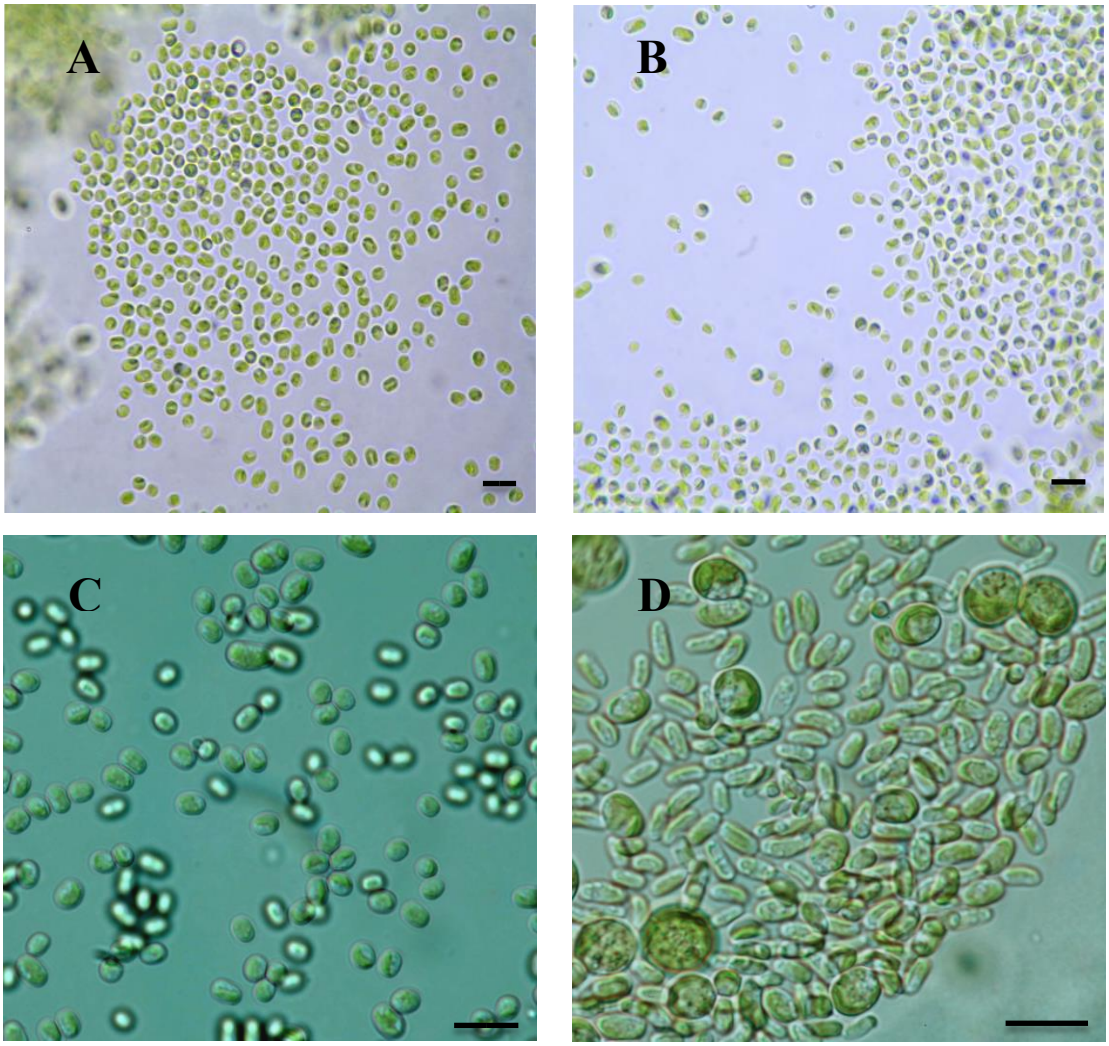


Figure 3.1. Photobionts isolated from *Dermatocarpon luridum* var. *luridum* and *D. luridum* var. *decipiens* grown in liquid medium. (**A**–**C**) *Diplosphaera chodatii* isolated from *D. luridum* var. *luridum* (DV4-1, P28-1 and M3-1, respectively); (**D**) Algal cells isolated from *D. luridum* var. *luridum* (Ne18-1). Scale bars = 12 μm.

Mycobiont Internal Transcribed Spacer (ITS) Phylogeny

One of 32 most parsimonious trees (Figure 3.2) summarizes the phylogenetic placement of 22 Internal Transcribed Spacer (ITS) nuclear ribosomal DNA (rDNA) sequences obtained from this study and 36 NCBI GenBank sequences; two of which were used to root the tree. From a dataset of 451 total characters, 140 were parsimony-informative and the final trees shared a tree length of 376 changes with CI and RI scores of 0.6968 and 0.9132, respectively.

The mycobiont tree may be interpreted by identifying four primary clades (Clades I, II, III, and IV), all of which are well supported by bootstrap and posterior probability support. Clade I is supported by 99% bootstrap and 100% posterior probability, and is comprised of all *D. luridum* samples including two varieties. *D. luridum* var. *decipiens* samples are outside this clade and fall into Clade II, which also includes: *Dermatocarpon miniatum* var. *miniatum*, *D. miniatum* var. *complicatum*, *D. arnoldianum*, and *D. leptophyllum*. With the exception of this intraspecific taxon, *D. luridum* might be considered a monophyletic group.

Clade I may be divided into four sub-clades: a, b, c and d. There are 12 samples that constitute sub-clade Ia with 85% bootstrap and 100% posterior probability support. Four samples constitute sub-clade Ib with 85% bootstrap and 99% posterior probability support. Both sub-clades Ia and Ib represent the species *D. luridum* var. *luridum* and are supported with 99% bootstrap and 85% posterior probability support. One sample in sub-clade Ia (M18-1) is from Austria and all others from North America (Manitoba, Minnesota and North Carolina). Sub-clade Ib represents additional *Dermatocapon luridum* var. *luridum* sequences from Europe

(Austria and Sweden) and is well supported with 85% bootstrap and 99% posterior probability supports. NCBI GenBank fungal ITS sequences of *Dermatocarpon luridum* var. *luridum* representing individuals of the Southern U.S. comprise sub-clade Id. These samples are segregated from the *D. luridum* var. *luridum* collected from Northern Canada and U.S. (sub-clade Ia) and Austria and Sweden (sub-clade Ib). Strong bootstrap and posterior probability, both at 100%, support the placement and segregation of the Southern U.S. from the rest of the *D. luridum* var. *luridum* samples. There are six taxa from NCBI GenBank representing *D. luridum* var. *xerophilum* that comprise sub-clade Ic which is supported by 100% bootstrap and posterior probability support.

Clade II is supported by 95% posterior probability but the taxa: *Dermatocarpon miniatum* var. *miniatum*, *D. miniatum* var. *complicatum*, *D. luridum* var. *decipiens* and *D. leptophyllum* comprising Clade II, with the exception of *D. arnoldianum*, are not monophyletic. Clade III is represented by two monophyletic species *D. multifolium* and *D. arenosaxi* each with 100% support. Clade IV is also monophyletic being comprised of *D. rivulorum* with 100% support.

Photobiont ITS Phylogeny

The ITS rDNA phylogeny represents sequences from 22 algal samples (Figure 3.3), 16 sequences from this study, and six NCBI GenBank accessions. One of nine most parsimonious trees (Figure 3.3) is presented from a data set of 699 characters, of which 178 were parsimony-informative characters. The tree length is 707 changes and CI and RI scores were 0.8925 and 0.8281, respectively. Since the

Neighbor Joining (NJ) and Maximum Likelihood (ML) trees were consistent with those of the Maximum Parsimony (MP) and Bayesian analyses, the NJ and ML trees are not presented.

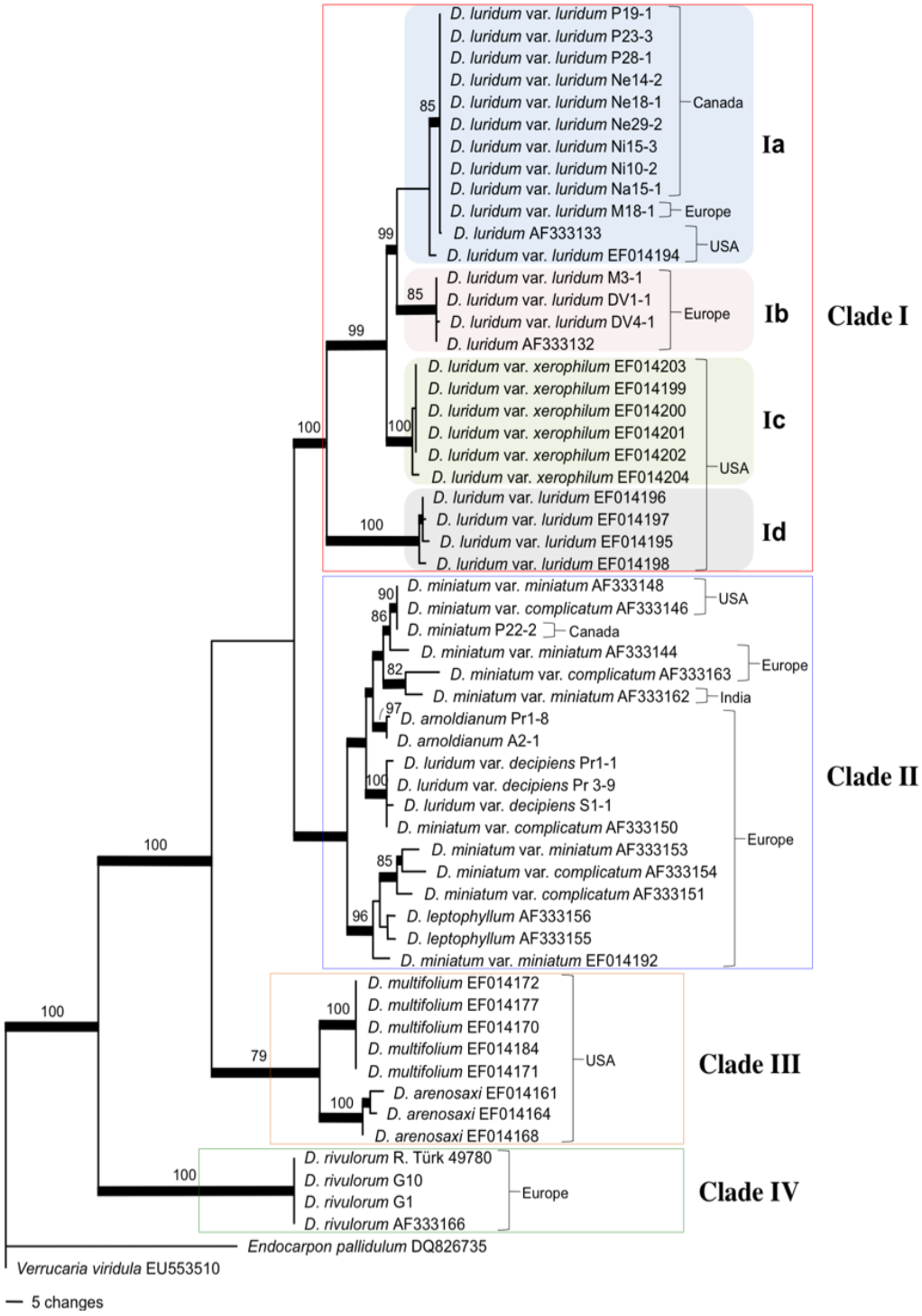


Figure 3.2. One of 32 most parsimonious phylograms showing the phylogenetic placement of *Dermatocarpon* species from this study relative to species of *Dermatocarpon* from the NCBI GenBank database. The tree is constructed based on the ITS rDNA nucleotide sequence of the mycobiont of sub-aquatic and terrestrial *Dermatocapon* specimens. Numbers above the branch internodes are bootstrap values greater than 70% as presented in PAUP, and thickened branch internodes are supported by ≥95% posterior probability as presented from Bayesian analysis in Mr. Bayes. Taxon identifications are presented followed by collection number or NCBI GenBank Accession numbers. The outgroup was designated as *Endocarpon pallidulum* and *Verrucaria viridula*.

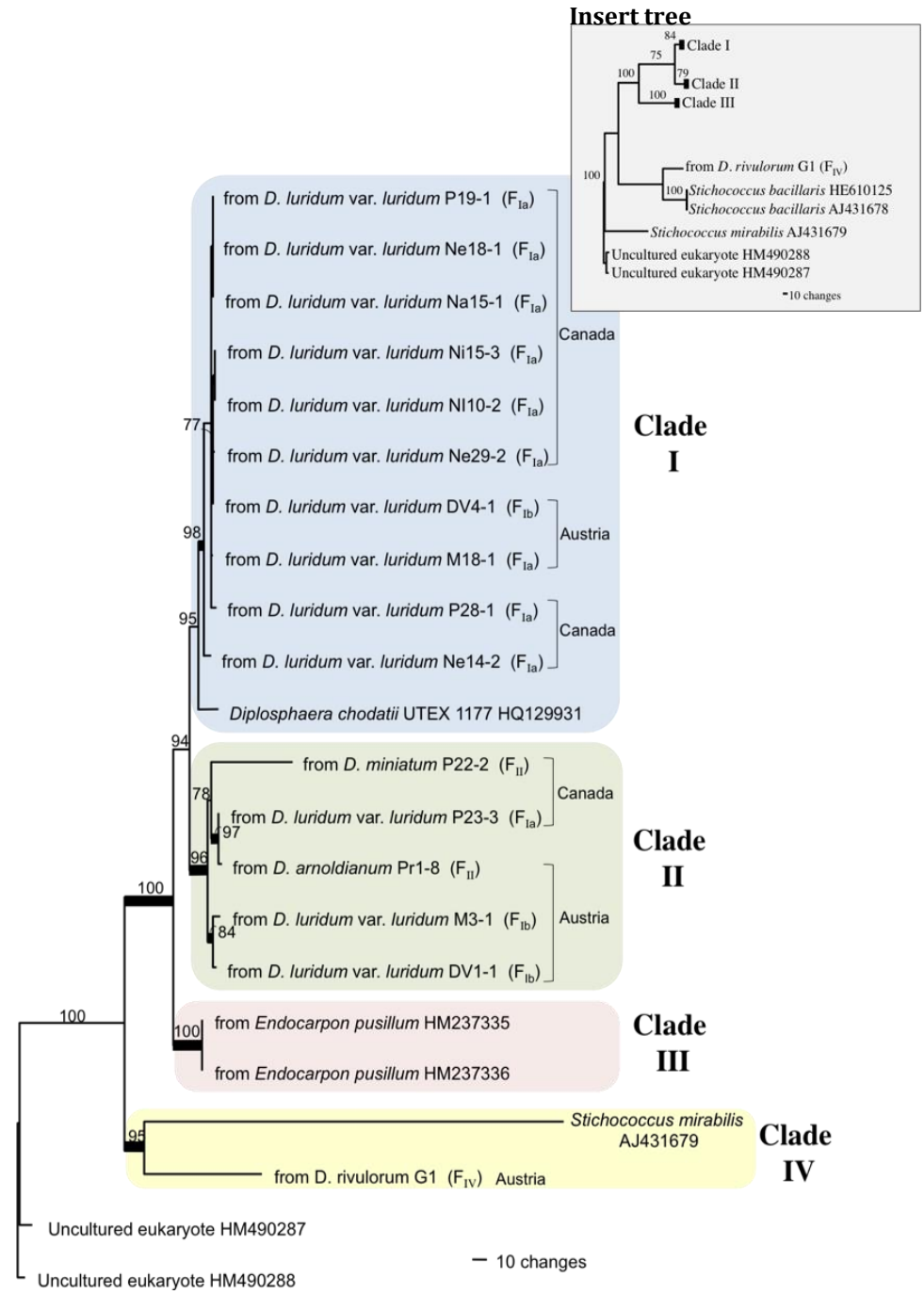


Figure 3.3. One of nine most parsimonious phylograms showing the phylogenetic placement of the photobiont of the *Dermatocarpon luridum* var. *luridum* and related lichens obtained from this study with sequences of *Diplosphaera chodatii* and similar nucleotide sequences obtained from NCBI GenBank database. The tree is constructed based on the ITS rDNA nucleotide sequences. Numbers above the branch internodes are bootstrap values ≥70%, and thickened branch internodes are supported by ≥95% posterior probability. Samples are presented followed by collection number for those collected in this study or NCBI GenBank Accession numbers for those retrieved from GenBank database. Information presented within parentheses represents the clade from the fungal tree containing the mycobiont from which the photobiont was associated. Two uncultured eukaryotes from NCBI GenBank were chosen as the outgroup taxa.

Four highly supported clades are presented in the algal phylogenetic tree (Figure 3.3). Clade I is supported by 95% bootstrap support and it contains algal sequences representing both Austrian and Canadian (Manitoban) photobionts. This clade is composed of the photobionts associated with *D. luridum* var. *luridum* and one NCBI GenBank reference. Clade II, supported by 96% bootstrap support and 95% posterior probability support, contains the algae of three different taxa, *D. miniatum*, *D. luridum* var. *luridum* and *D. arnoldianum*. Algae from three species of *Dermatocarpon*, in Clades I and II form a highly supported group with 94% bootstrap support and cluster with the UTEX sample of *Diplosphaera chodatii*. Clade III contains two NCBI GenBank accessions of *Stichococcus* with 100% of both bootstrap and posterior probability. Clade IV contains a *Stichococcus* species and the alga from *D. rivulorum*, and is supported by 95% bootstrap support and 100% posterior probability support.

Algal clade I are all photobionts of *D. luridum* var. *luridum* from Fungal Clade Ia (Figure 3.3; F_{Ia}), with the exception of one DV4-1 of Fungal Clade Ib (F_{Ib}). Algal Clade II contains algae that associate with fungal species that are in three different fungal clades (Figure 3.3; F_{Ia}, F_{Ib}, and F_{II}).

The insert photo (Figure 3.3) reveals how the addition of two NCBI GenBank sequences of *Stichococcus bacillaris* disrupts the clade consisting of *Stichococcus mirabilis* and the photobiont sequence from *D. rivulorum.* The photobiont from *D. rivulorum* is grouped in a Clade with the two *S. bacillaris* sequences*.* There is 100% bootstrap support for the pairing of the two *S. bacillaris* sequences. The generation of Clades I, II, and III was identical with 100% bootstrap support. Clade I and II are

supported by 84% and 79%, respectively, which are lower support values than the primary tree. Clade III is supported once again by 100% bootstrap support.

Discussion

Intercontinental Gene Flow

The finding that fungal sequences from Austria clustered with sequences from North American specimens (Figure 3.2) suggested that gene flow of *D. luridum* var. *luridum* occurred between continents and is likely ongoing (Figures 3.2 and 3.3). Other studies of lichen phylogenetic differentiation at the global scale have provided mixed results. The crustose lichen *Porpidia flavicunda* was shown to share identical fungal haplotypes between Northern North America, Greenland and Northern Europe (Buschbom 2007) and was hypothesized to have undergone long distance dispersal. Geographic segregation between continents was shown to occur for *Lobaria pulmonaria* (Walser 2005) and for *Letharia vulpina* (Högberg et al. 2002). Both local variation within populations and variation between populations especially as distance increased suggested isolation–by–distance for *Lobaria pulmonaria*. This study suggests low levels of variation in global and local populations of *Dermatocarpon luridum* var. *luridum* with some sharing between continents, but the small sample size does not support conclusive comments to be made on the extent of gene flow. Since long distance dispersal of lichen diaspores was hypothesized for *Cetraria aculeata* and *Cavernularia hultenii* (Buschbom 2007; Printzen et al. 2002; Fernández-Mendoza et al. 2011), this may also be true for the dispersal of the mycobiont, *Dermatocarpon luridum* var. *luridum*. The sharing of genetic information

between photobiont populations associated with *D. luridum* var. *luridum* from both continents is implied, but further study is underway to determine the extent of photobiont exchange/movement between continents.

Fungal Evolution: D. luridum and *D. miniatum Are Paraphyletic*

The fungal phylogeny obtained from the ITS sequence data reveals the paraphyletic nature of both *Dermatocarpon luridum* and *Dermatocarpon miniatum* (Figure 3.2). The placement of *D. luridum* var. *decipiens* into Clade II makes the species paraphyletic. *D. luridum* var. *decipiens* may belong as varietal status within *D. miniatum*. The taxonomic status of *D. arnoldianum* was questioned by Heiðmarsson (2000), suggesting it be incorporated into the species as a variety of *Dermarocarpon miniatum* (Heiðmarsson 2000). In his review, Heiðmarsson (2000) mentions the prior troubles of taxonomically classifying *D. arnoldianum* by Degelius (1934) based on morphological character states and interpretations of iodine reactions relative to *D. linkolae*. The placement of *D. arnoldianum* and *D. luridum* var. *decipiens*, nested within Clade II suggests that the species maybe more appropriately considered a subspecies of *D. miniatum* rather than an independent species. The use of the ITS rDNA gene to address species delimitation is useful because it contains regions that are faster evolving than the surrounding subunit sequences. The ITS gene is routinely used as a phylogenetic marker for fungal species and genera (Begerow et al. 2010) and it has been proposed for use as a bar coding gene (Schoch et al. 2012). Limitations of the locus include the use of the ITS rDNA as a single marker where additional markers will provide more data for

phylogenetic reconstruction. Also, the potential failure of concerted evolution in the multiple tandem repeating units and paralogous ITS regions may obscure phylogenetic relationships (Linder and Banik 2011). Further study is also needed to resolve *D. miniatum* var. *complicatum* and var. *miniatum* since all samples in this tree were obtained from NCBI GenBank database.

Although *D. rivulorum* and *D. luridum* are similar in morphology (Goward et al. 1994), *D. rivulorum* is the most basal of the seven species in the tree (Figure 3.2). *Dermatocarpon rivulorum* and *D. luridum/D. miniatum* may have evolved from an ancestor with similar sub-aquatic habitat requirements and adapted to a terrestrial environment while maintaining the same photobiont. The aquatic nature of the lichen may have been lost multiple times through evolution of these morphologically similar species. However, the limited sample size in this study prevents an assessment of the ancestral state reconstruction of aquatic and terrestrial species.

Algal Identity

The microscopic analysis of the six cultured photobiont isolates from *Dermatocarpon luridum* var. *luridum* and nucleotide sequencing analyses suggest these algae are *Diplosphaera chodatii* Bialosuknia. The morphology of the cells cultured in this experiment (Figure 3.1) are consistent with those shown by Zhang and Wei (2011), photobiont culture isolates from *Endocarpon pusillum* and the UTEX algal collection 1177 of *D. chodatii*. Algal identity is further supported by the cell morphology and dimension information reported for *Diplosphaera chodatii* (syn.

Stichococcus diplosphaera) by Ahmadjian and Heikkila (1970) in the hymenium of *Endocarpon pusillum.*

Blast search in NCBI GenBank of ITS gene sequences of the photobiont of *D. luridum* var. *luridum* obtained from this study provided six significantly similar matches with query coverage greater than 80%, which have been incorporated into the algal phylogeny (Figure 3.3). The sequences of *Diplosphaera chodatii* UTEX 1177 and those generated by Zhang and Wei (2011) were most similar to my sequences. Three *Stichococcus* sequences with lower similarity were also identified through the BLAST search. The phylogenetic analysis included my algal ITS nrDNA sequences combined with the six NCBI GenBank accessions that supported the morphological identification. The strongly supported clades support that *D. chodatii* is the preferred photobiont of *Dermatocarpon luridum* var. *luridum, Dermatocarpon arnoldianum* and *Dermatocarpon miniatum* (Thüs et al. 2011; Reháková 1968).

One of the cultures obtained from *D. luridum* var. *luridum* (Ne18-1; Figure 3.1) contained two types of algal cells (autospores and mature vegetative cells). The morphology does not disagree with that of the green alga *Elliptochloris bilobata* Tschermak-Woess (Figure 3.1) even though the ITS sequence was amplified from *D. chodatii*. *Elliptochloris bilobata* was earlier shown to be a photobiont of *Verrucaria sublobulata* (Thüs et al. 2011) and is comprised of the morphological features shown in Figure 3.1. However, the algal cell types observed in the culture (Figure 3.1) were not observed within the lichen thallus. The finding of an additional algal species using culture methods may be explained if *D. luridum* var. *luridum* can associate with more than one photobiont simultaneously; if *Elliptochloris bilobata*, was present on the

thallus as a free-living alga. Even though the thallus was examined and washed before culturing, small amounts of an additional species could be selected under certain culture conditions. Other studies have shown multiple photobionts in one culture (Grube and Muggia 2010) and multiple photobionts or photobiont genotypes present in the same thallus (Piercey-Normore 2006; Ohmura et al. 2006; Guzow-Krzeminska 2006; Casano et al. 2011; Bates et al. 2012). The phylogeny in Thüs et al. (2011) shows that *Diplosphaera* sp. is shared with four lichen forming fungi including *D. miniatum*, supporting the findings of this study. The re-synthesis experiments of Stocker-Wörgötter and Türk (1989) identified the photobiont associated with *Dermatocarpon miniatum* to be *Hyalococcus dermatocarponis* H. Warén, which does not disagree with the findings in this study. The low selectivity of *D. luridum* and *D. miniatum* provides an explanation for the fungal partner associating with different algal partners in different geographic locations. *Hyalococcus dermatocarponis* was also isolated from *Dermatocarpon luridum* (syn. *Dermatocarpon fluviatilis*) and deposited in the UTEX algal collection (UTEX number 908) by V. Ahmadjian. The finding that six of the seven cultures were morphologically identified as *D. chodatii* provides support for a one-fungus and one-photobiont hypothesis, but it does not eliminate the possibility that more than one photobiont may associate with *D. luridum*.

Photobiont Evolution and Algal Selection

The broad geographic origin of the algal sequences from *Dermatocarpon* species that fall within Clades I and II (Figure 3.3) suggests that there may be

multiple strains of the alga distributed throughout North America and Europe. This broad distribution may be explained by the low selectivity of *D. chodatii* by three mycobiont species, and that in sexual reproduction the fungal ascospores must come into contact with a different compatible algal strain at each dispersal event. Lichen asexual propagules such as isidia and soredia have been hypothesized to serve as vectors for photobiont dispersal (Beck et al. 1998; Piercey-Normore 2006; Ohmura et al. 2006) to promote algal dispersal and algal switching. Members of *Verrucaria*, *Staurothele* and *Endocarpon* illustrate another means of algal dispersal by releasing the photobiont into the environment together with ascospores (Thüs et al. 2011). As with all sexually reproducing lichens, ascospores must come in contact with a suitable alga in order to establish a symbiosis. The ability to tolerate lengths of desiccation stress up to and exceeding two months (Zhang and Wei 2011) and their free-living presence in soils, on bark, on rock and as epiphytes on lichens and other living and dead plants (Lukešová and Hoffmann 1996; Flechtner 1998; Handa et al. 2001; Flechtner et al. 2008; Hill 2009; Voytsekhovich 2011) increases the likelihood that spores will come in contact with *D. chodatii* for a symbiosis to establish.

Fungal and algal taxa from both Canada and Austria are placed throughout the trees, suggesting that fungi from both continents share some of the same photobiont species. Additionally, the clustering of algal sequences obtained from different *Dermatocarpon* species suggests sharing of algal genotypes among *Dermatocarpon* taxa and low specificity. *Dermatocarpon miniatum*, a terrestrial species, shares the same photobiont, *Diplosphaera chodatii*, as *Dermatocarpon luridum* var. *luridum* and *Dermatocarpon arnoldianum*, two sub-aquatic species

(Reháková 1968; Thüs et al. 2011). The sub-aquatic *Dermatocarpon luridum* var. *luridum* and terrestrial *Dermatocarpon miniatum* are typically separated by physical distance from one another, where *D. luridum* var. *luridum* is located in the submerged zone and *D. miniatum* is in the upper splash zone (Gilbert 2000) addressing the original question as to whether they share the same alga in these different habitats.

Both algal and fungal phylogenies place *Dermatocarpon rivulorum* and its algal partner in a basal position to other taxa (Figure 3.2). Since the DNA sequences of the algal and fungal partners were obtained from the same lichen thallus extract, it is possible that the sequence in the algal tree may represent an epiphyte or contaminant by a *Stichococcoid*-like alga. Assuming that the photobiont sequence is from *D. rivulorum*, the placement of the photobiont ITS sequence from *Dermatocarpon rivulorum* in the basal position in the tree with *Stichococcus* spp. suggests that the alga that associates with *D. rivulorum* may be a *Stichococcoid*-like alga. The degree of morphological similarity between *Stichococcus* species and *Diploshpaera* species greatly exceeds the dissimilarity. One difference is the formation of two-celled or short chain clusters Ettl and Gärtner 1995). However, the absence of morphological evidence and the long-branch connecting *Stichococcus* (Figure 3.3) might suggest that the photobiont associated with *D. rivulorum* could be a *Diplosphaera*-like alga. Long-branch attraction (Bergsten 2005) might also account for the placement of *Stichococcus* and photobiont of *D. rivulorum* together in the tree. Culture evidence and additional molecular analysis of cultured photobionts and thallus extracted DNA may help to resolve this issue. The photobiont genus

associated with *Dermatocarpon rivulorum* was not reported by Thüs et al. (2011); however, Nascimbene et al. (2007) stated the photobiont is *Diplosphaera chodatii* [38]. Thüs and Schultz (2008) also indicated that the photobiont of *D. rivulorum* is *D. chodatii*. The divergence of the photobiont associated with *D. rivulorum* in the algal tree suggests that the species of photobiont is more specific to *D. rivulorum* than to *D. luridum* or *D. miniatum* and may reflect the limit of algal sharing between fungal taxa. Since both *D. rivulorum* and *D. luridum* var. *luridum* grow in similar habitats and in close proximity to one another, it is reasonable to assume that the two fungi associate with the same photobiont species. Even though the single sequence in this study suggests a distant relationship between photobionts of *D. luridum* and *D. rivulorum*, the identity of the *D. rivulorum* photobiont(s) needs more investigation.

In conclusion, the sub-aquatic nature of the lichen is not indicative of the phylogenetic placement using ITS sequence data. *Dermatocarpon luridum* var. *luridum* specimens collected in Canada and Austria are phylogenetically similar and form two strongly supported clades. Additional specimens from Europe are closely associated with the Austrian representatives, while the additional specimens from North America fall out in three sub-clades. *Dermatocarpon luridum* var. *decipiens* and *Dermatocarpon arnoldianum* are phylogenetically positioned with *Dermatocarpon miniatum*. Taxonomic revision of *D. miniatum* is needed and it is recommended that *D. luridum* var. *decipiens* be investigated as a variety of *D. miniatum*. However, phylogenetic study of all species within the genus is needed before taxonomic revisions should be considered. The use of ITS data alone for reconstruction of a fungal phylogeny may pose problems if the ITS region contains

paralogues. The presence of paralogous rDNA regions may overestimate diversity and not truly represent the phylogenetic placement of taxa. The use of further independent markers would be required before proposing taxonomic changes based on an ITS phylogeny. The final sub-aquatic species investigated, *D. rivulorum* is segregated from the rest of the *Dermatocarpon* species as a strongly supported monophyletic clade. Investigation of the algae suggests that *Diplosphaera chodatii* is the photobiont associated with *Dermatocarpon luridum* var. *luridum*, as well as the other species of *Dermatocarpon*. *Dermatocarpon rivulorum*, however is not thought to associate with *Diplospheara chodatii* or at least not one of the strains shown to associate with other *Dermatocarpon* species. The shared ITS sequence similarities of the photobiont between fungal species suggests that algal sharing is occurring within and between fungal species. The presence of common genotypes of both the mycobiont and photobiont in North America and Europe may support long distance dispersal and photobiont replacement.

Chapter 4

The population genetics of *Diplosphaera chodatii* within and between Manitoba, Canada and Upper Austria, Austria

Introduction

Lichens colonize a diversity of substrata within a diverse number of ecosystems all over the world. Many of the ecosystems inhabited by lichens are ecologically too harsh for other macro-organisms. However, in many lichens, neither symbiont can survive alone, or at least for long durations of time without the other. The nature of the lichen mutualism allows for the long-term survival of the symbionts in a diversity of environments. Most lichen fungi cannot survive without an algal partner, rendering lichen fungi obligate symbiotic organisms, such that their life cycle cannot be completed without the symbiosis.

The lichen life cycle must address the life cycles of each of the fungal and algal symbionts (eukaryotic or prokaryotic). Lichen fungi have evolved to reproduce using either sexual, asexual, and in some instances both means of reproduction. The terms horizontal (independent) and vertical (co-dependent) transmission are used to describe the sexual and asexual life cycle of the mycobiont, respectively (Sluiman et al. 1989; Nash 2008; Werth and Sork 2010; Grande et al. 2012). Horizontal transmission occurs during sexual reproduction after the dispersal of sexually derived spores. Sexual reproduction is a primary means of shuffling and diversifying genetic variability of lichen thalli. In this instance, dispersed spores must be viable so they can germinate and encounter a compatible algal species within the immediate vicinity in order to re-lichenize. Vertical transmission occurs in lichens that disperse via vegetative means (soredia, isidia, or thallus fragmentation), where cells of each symbiont are dispersed together and will regenerate a distinctly different individual but the lichen will have the same genetic composition as each parent symbiont

(Doering and Piercey-Normore 2009).

Another method of dispersal is displayed by several lichen fungi and their photobionts within the Verrucariaceae (eg. *Staurothele* and *Endocarpon*). This method is a vertical method of dispersal where sexually derived spores are dispersed with the already suitable photobiont, which is found living amongst the hymenial layer of the perithecia. This method of dispersal is thought to increase the success of lichenization. Honegger (2009), and Beck and Peršoh (2009) suggest that the Verrucariaceae family of lichen fungi associate with the greatest diversity of algal photobionts of all lichen fungal families. In a review of the photobiont diversity associated with more prevalent lichens from the Verrucariaceae, the most commonly presented photobiont was *Diplosphaera,* with 12 out of 17 fungal genera were shown to associate with the photobiont genus.

For successful lichenization to occur following spore dispersal, the germinated fungal spore must come into contact with a compatible algal partner, either a free-living alga or through competition and seizure of a photobiont associated with another lichen-fungus. The majority of lichen photobionts; however, are not known as free-living algae. This is primarily because the majority of lichen fungi associate with green algae of the genera *Trebouxia* and *Asterochloris*. Factors such as the selective nature of the mycobiont for the photobiont(s) and the ecological requirements of the algae, determine the stock of available photobionts within a community (Beck 1999). Some lichen fungi within a community may associate with the same photobiont. Algal switching assists in the distribution of genotypes within a community displaying algal sharing by different mycobiont individuals and species (Ohmura et al. 2006; Piercey-

Normore 2006; Doering and Piercey-Normore 2009).

Many members of the Verrucariaceae, some commonly constituents of the same community (eg. *Staurothele fissa*, *Dermatocarpon luridum*, and other species) associate with green algae from the genus *Diplosphaera* (Thüs et al. 2011; Fontaine et al. 2012). Fontaine et al. (2012) speculates that the lichens, which discharge their spores with their photobiont (eg. *Staurothele* sp. *Endocarpon* sp.), may actively populate the environment with suitable photobionts that have the potential of becoming lichenized by the same species, or germinating spores of other species in the community (eg. *Dermatocaron luridum*). *Diplosphaera chodatii*, the photobiont associated with *Dermatocarpon luridum* (Fontaine et al. 2012) is a hardy species that is documented as free-living on an array of substrata in different climatic zones all over the world. Vectors of dispersal for *Diplosphaera chodatii* and other *Diplosphaera* species have not specifically received attention. However, the dispersal of aerial and aquatic microalgae has been proposed. Common transfer vectors that have been documented include water, wind, insects, mammals, and birds (Nash 2008).

The sub-aquatic lichen fungus *Dermatocarpon luridum* is a globally distributed species, with three currently recognized subspecies (subsp. *luridum,* subsp. *decipiens*, and subsp. *xerophilum*). Fontaine et al. (2012; Chapter 3) suggested that gene flow of *D. luridum* var. *luridum* has occurred or is occurring between the European and North American continental populations based on phylogenetic analysis of ITS gene sequences. Likewise, they found congruent genetic information from both continents for the photobiont *Diplosphaera chodatii*, also implying long-range gene flow.

The dispersal capacity of aquatic lichens may be considered species specific, where ecological factors play a critical role in the ability to disperse. There are a number of sub-aquatic and aquatic lichens around the world occupying substrata of freshwater and marine ecosystems. Attention to these groups of lichens has increased over the last two decades, where it has been found that the survival of many sub-aquatic and aquatic lichens may be affected by water quality. The Committee on the Status of Endangered Wildlife in Canada (COSEWIC), as well as the U.S. Forest Service have prepared several conservation management assessments for subaquatic and aquatic freshwater lichens, including: *Leptogium rivulare, Peltigera hydrothyria, Dermatocarpon luriudum var. luridum,* and *Dermatocarpon meiophyllizum*. Similarly in Europe, many sub-aquatic and aquatic lichens, for example *Dermatocarpon luridum* var. *luridum*, are at risk of becoming endangered (RED list) for many states of the European Union and surrounding regions, such as Slovakia, Czech Republic, Austria, Estonia, and Latvia.

All the aquatic lichens listed above produce sexually derived spores (ascospores) within apothecia or perithecia. Spore dispersal is thought by lichenologists to be the primary means of dispersal for these lichens; however, no studies have been conducted to test this hypothesis. Also, these lichens are foliose and produce lobes that can be torn from the thallus, where the lobes may act as an asexual means for dispersal. If ascospores are released they must recombine with a compatible alga to form another lichen. Therefore the movement of the alga from one population to another would either depend on the fragmented thallus or the dispersal of the free-living alga. If the alga is confined to aquatic habitats and relies on water

for dispersal, the lichenized algal populations should be highly structured with more diversity within the population than between populations. There has been no documentation of sexual reproduction by lichen algae while in the lichenized state, and the dispersal in this state is thought to be very restricted (Sluiman et al. 1989). Nothing is known about the population structure of *Diplosphaera chodatii,* the photobiont of *Dermatocarpon luridum* var. *luridum,* at local, continental, or global scales.

Isolation-by-distance is a facet of population biology that determines whether geographic distance can explain the genetic difference between populations (Wright 1943). Isolation-by-distance can provide evidence to determine the method of dispersal (Slatkin 1994) such as wind blown algal cells or water bourne algal cells. Gene flow is inferred from this indirect method by assuming isolation of populations by geographic distance, because direct observational studies of dispersal and colonization events are not practical (Warren 2003).

The objective of this study was to investigate genetic diversity and infer gene flow of the photobiont (*Diplosphaera chodatii*) associated with the aquatic lichen *D. luridum* var. *luridum* within and between populations on the North American and European continents.

Materials and Methods

Lichen Material

For this experiment, 74 samples of *Dermatocarpon luridum* var*. luridum* were collected from four lakes in west central Manitoba, Canada, and two river locations in

Upper Austria, Austria. Specifically, 36 samples from Canada and 38 from Austria were utilized (Table 4.1). Moistening dry lichen thalli prior to scraping the thalli off rock surfaces facilitated collection. The lichen thalli (vouchers) were air-dried and processed for storage in the cryptogamic division of the University of Manitoba herbarium (WIN), Winnipeg, Manitoba, Canada.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from whole lichen thalli, or in a few cases axenic *Diplosphaera chodatii* cultures, using a modified extraction protocol from Grube et al. (1995). The ITS rDNA of 74 *Diplosphaera chodatii* samples, and the actin gene from 21 samples was amplified by Polymerase Chain Reaction (PCR). Initially, 20 µL amplification reactions were used to determine the optimal DNA concentration for PCR. In order to ensure enough PCR product (15-30 ng/µL) could be obtained for sequencing. Four to eight replicate 20 µL reactions were performed and combined for purification. One 20 µL reaction consisted of 5-25 ng of genomic DNA, 1X PCR buffer (50 mM KCl; 100 mM Tris-HCl [pH 8.3]), 2.0 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer, and 2 units of Taq DNA Polymerase (Invitrogen, Burlington, ON, Canada), or Pfu DNA polymerase (Dr. P. Loewen's research laboratory, Department of Microbiology, University of Manitoba).

The ITS gene was amplified using the newly designed forward specific algal primer STICHO-ITS-F-5' (5'-GGATCATTGAATCTATCAACAAC -3') with the universal reverse primer ITS4-3' (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). The Actin gene was amplified using the primer combination Act1T-5' (5'-

CACACRGTRCCCATCTAYGAGG-3'), and Act4T-3' (5'-GTTGAACAGCACCTCAGGGCA-3') (Kroken and Taylor 2000). All amplifications were performed using a Biometra T-Gradient thermal cycler (Fisher Sci, Nepean, ON, Canada). The PCR cycle used to amplify the ITS was accomplished with an initial denaturation of the DNA at 94 °C for 3 minutes; then 30 cycles of denaturation at 94 °C for 1 minute, annealing at 58.5 °C for 35 seconds, and extension at 72 °C for 45 seconds; followed by a 6 °C soak. For the actin gene, amplification was performed with an initial denaturation of the DNA at 94 °C for 3 minutes; then a touchdown cycle, which consisted of 94 °C for 1 minute, annealing at 63 °C for the first two cycles and dropping by 1 °C to continue with two cycles at each degree, a final annealing temperature of 58 °C for 25 cycles, and extension at 72 °C for 45 seconds; followed by a 6 °C soak.

Precipitation of the combined PCR products (80-160 µL) was carried out by adding 0.2 volumes of 5M NaCl and 2.5 volumes of 100% ethanol. The tubes were gently inverted several times, placed in the fridge (4 ^{o}C) for a minimum of 1 hour, and centrifuged for 10 minutes at 13000 rpm. The supernatant was poured off and the pellet was washed with 300 µL of cold 70% ethanol and the pellets were left to air dry. The amplified DNA was resuspended in 20 µL of sterile distilled water. The entire 20 µL PCR product was mixed with ~10 µL of bromophenol blue loading dye (6x BPB) and loaded into a 1% agarose gel stained with ethidium bromide (0.5mg/mL) in 1x TBE buffer (0.089M Trizma base; 0.089M Boric acid; 2.0 mM EDTA pH 8.0). Gels were electrophoresed at 120 volts until the loading dye was 1 cm from the bottom of the gel. The bands were cut out of the gel under a short wave

(254 nm) UV light box and placed in 1.5 µL Eppindorf tubes. Purification of the DNA from the agarose gel was carried out using Promega Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, Wisconsin, USA). The purified DNA was resuspended in 35 µL of sterile distilled water, and quantified by gel electrophoresis. Band intensities were compared with the 1650 base pair band (80 ng/µL) of a 1 kb plus DNA ladder (Invitrogen, Burlington, ON, Canada). Results were visualized using an AlphaImager 2200 transilluminator (Alpha Innotech, Fisher Scientific, Nepean, ON, Canada).

Precipitation of the cycle sequenced product to remove excess fluorescent dyes was carried out by the addition of 0.25 volumes of 125 mM EDTA and 3 volumes of pure ethanol. Tubes were inverted gently and incubated in the dark at room temperature for 15 minutes. The samples were centrifuged at 4000 rpm for 45 minutes, the supernatant pipetted off and 60 µL of cold 80% ethanol was added. The tubes were once again centrifuged for 15 minutes at 4200 rpm, followed by the removal of the supernatant and drying of the DNA in a ThermoSavant DNA 120 SpeedVac Concentrator (GMI INC., Ramsey, Minnesota, USA). Hi-Di formamide (Applied Biosystems, Foster City, CA, USA) was used to resuspend the DNA (20 µL). The DNA was denatured at 94 °C for 5 minutes, immediately placed on ice, and loaded to a 96-well plate for sequencing with an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Analysis of data

All nucleotide sequences obtained for this experiment were edited using Sequencher v. 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA) and manually aligned using the program Se-Al v. 1.0 (Rambaut 2001). The alignment lengths for the ITS and actin gene sequences were 682, and 528 base pairs, respectively. However, because many of the ITS sequences (n = 74) differed in their lengths, the 5' and 3' ends consisted of varying amounts of missing data (missing nucleotides). This created an issue, because missing data are misread within the analysis program used for population assessment. Therefore, sequences were "cut-down" at the 5' and 3' ends to ensure all sequences were of the same length and missing data were consistently absent. Final ITS, actin, and combined ITS/actin lengths were 618, 528, and 1132 base pairs, respectively.

Each alignment was exported in nexus format for Neighbour-Joining (NJ) analysis and Unweighted pair group with arithmetic mean (UPGMA) cluster analysis in PAUP* 4.0b10 (Swofford 2003) for assessment of genetic structure. Pairwise genetic distance matrices were generated using a maximum likelihood parameter for each NJ and UPGMA analysis within PAUP. The program JModeltest 0.1.1 (Posada and Crandall 1998; Guindon and Gascuel 2003; Posada 2008) was used to determine the adequate evolutionary constraints to use for the maximum likelihood pairwise distance construction. The resulting NJ phylograms were subjected to bootstrap analysis using the NJ method with maximum likelihood parameter and 10000 bootstrap replicates were performed (Felsensten 1985). Bootstrap scores ≥ 50% were presented in the phylograms.

The best model parameters presented for the ITS, actin and combined ITS/actin by JModeltest are respectively: 1) Lset Base = (0.2323, 0.3015, 0.2792); Nst = 6; Rmat = (1.0000, 1.8102, 1.0000, 1.0000, 3.8085); Rates = Gamma; Shape = 0.3942; and Pinvar = 0; 2) Lset Base = equal; Nst = 1; Rates = equal; and Pinvar = 0; 3) Lset Base = (0.2264, 0.3055, 0.2840); Nst = 2; T ratio = 1.7308; rates = Gamma; Shape = 0.0096; and Pinvar = 0.

The ITS, actin and combined ITS/actin nexus files were analyzed within TCS (Clement et al. 2000) for haplotype abundances, using the 5^{th} state criteria and the probability of parsimony was set at 95%.

To supplement the genetic structure outputs of the NJ and UPGMA methods, the multivariate, principal coordinate analysis (PCoA) method, within GenALEx v. 6.5 b3 (Peakall and Smouse 2012; Peakall and Smouse 2006) was used when appropriate. The genetic distances of these haploid sequences were calculated within GenAlEx following Excoffier et al. (1992). Prior to PCoA analysis, pairwise genetic distances were standardized for covariance. According to Hauser and Crovello (1982), the NJ and UPGMA methods are more sensitive to the similarities between individuals, while PCoA is said to be more informative for assessing the distance among groups. To test for isolation-by-distance, a Mantel test was implemented within GenAlEx v. 6.5 b3 (Bohonak 2002). This test is used to assess the correlation strength of pairwise genetic distances and pairwise geographic distance. Global positioning (GPS) coordinates were converted from Degrees - Minutes' seconds" to universal transvers mercator (UTM) and again to kilometers (km) to generate the pairwise geographic distances between sites for the Mantel

Test.

Analysis of genetic diversity was also carried out using GenALEx v. 6.5 b3 in the form of analysis of molecular variance (AMOVA) and PhiPT fixation index (analogous to F_{ST} fixation index). For all AMOVA analyses, 999 permutations were used.

The ITS data set was analyzed in a series of ways: 1) to assess variation and structure between Manitoba, Canada and Upper Austria, Austria; 2) to assess variation and structure within Manitoba, Canada; 3) to asses variation and structure within Upper Austria, Austria. The actin and combined ITS/Actin data sets were analyzed for within Manitoba, Canada alone, however analyses were replicated to incorporate five sequences from Upper Austrian Samples to assess the influence they have on the outcomes. For most analyses, the samples were categorized several ways into different regional and population pairings. To assess variation between Manitoba, Canada and Upper Austria, Austria, regions and populations were defined by the following: 1) Manitoba and Upper Austria represent two regions, where within Manitoba, Payuk Lake, Neso Lake, Nisto Lake and Naosap Lake are four different populations, and within Upper Austria, Schlogener Schlinge, Waldaist (South; collection sites 1-10) and Waldaist (North; collection sites 11-22) represent three different populations (n = 3); 2) Manitoba and Upper Austria represent two regions, where within Manitoba, the four lakes represent one population, and within Upper Austria, Schlogener Schlinge and Waldaist (North and South sites) represent two populations; 3) Manitoba and Upper Austria represent one region, where within Manitoba, Payuk Lake, Neso Lake, Nisto Lake and Naosap Lake are four different

populations, and within Upper Austria, Schlogener Schlinge, Waldaist (South; collection sites 1-10) and Waldaist (North; collection sites 11-22) represent three different populations (n = 3); 4) Manitoba and Upper Austria represent one region, where within Manitoba, the four lakes represent one population, and within Upper Austria, Schlogener Schlinge and Waldaist (North and South sites) represent one population; 5) Lastly, Manitoba and Upper Austria represent one region, where within Manitoba, the four lakes represent one population, and within Upper Austria, Waldaist (North and South sites) represent one population and Schlogener Schlinge was removed from the data set.

To assess the variation within Upper Austria alone, the ITS data were categorized for analysis as follows: 1) Schlogener Schlinge and Waldaist represent two regions, where Schlogener Schlinge is also a population, and Waldaist has two populations (North and South sites); 2) all Upper Austrian sites are part of one region broken into three populations (Schlogener Schlinge, $Waldaist_{1-10}$ and $Waldaist_{11-22}$); 3) all Upper Austrian sites are part of one region broken into two populations i. Schlogener Schlinge and ii. Waldaist; 4) Lastly, a one region two population comparison of Waldaist (north) and Waldaist (south) was assessed.

For all other AMOVA analyses: Manitoba ITS only, Manitoba actin only, and Manitoba combined ITS/actin the data were categorized in one way, one region with four populations (each lake). The actin and combined ITS/actin data set consisted of 16 samples from Manitoba. When the five Upper Austrian samples were added to the analyses (n = 21), the number of regions and populations were set to two.

Fixation index (Phi) scores vary from three (PhiRT, PhiPR, and PhiPT), when

two regions were declared, to two (PhiPR, and PhiPT) when one region was declared. PhiRT describes variation among regions, PhiPR describes variation among populations, and PhiPT describes variation within populations. Euclidean pairwise PhiPT scores were also obtained from the AMOVA analyses. To determine significance (*P* – values) for the scores of the Phi statistics and the pairwise PhiPT scores, an alpha-value of 0.05 was used.

Lastly, Phi scores were used within the GenAlEx v. 6.5 b3 software to estimate the rate of migration per generation among populations, Nm (rate of migration between populations relative to the product of the effective population size). *N* is the effective population size, *m* is the effective proportion of immigrants, and *Phi* (analogous to F_{ST}) is the fixation index, a way of measuring variation in allele frequencies among populations. Values of *Nm* > 1 suggests that there was enough gene flow to negate the effects of genetic drift, and *Nm* values > 4, implies that the population or populations belonged to one panmictic (randomly mating) population (Wright 1931).

RESULTS

*Genetic structure of Diplosphaera chodatii **between** Europe and North America*

A total of 74 ITS rDNA gene sequences were obtained for genetic structure analysis between Canadian and Austrian *Diplosphaera chodatii* photobionts. Thirty-six ITS rDNA sequences were obtained from the Canadian samples, while 38 ITS rDNA sequences were obtained from samples collected in Austria. A subsample of 21 actin gene sequences, 16 from Canadian samples and 5 from Austrian samples,

were obtained.

The number of haplotypes observed in the ITS data set (n = 74) totaled 39, where 20 different haplotypes were found from both Canadian and Austrian data sets. There was one mutual haplotype observed between the two data sets (Table 4.1). The smaller data set comprised of actin sequences revealed nine haplotypes, six in Manitoba, Canada, and three in Upper Austria, Austria. None of the nine haplotypes were mutually shared between these two distant sites (Table 4.1).

After combining the ITS rDNA and actin sequence data sets for the same 21 samples for which this was possible, a total of 16 haplotypes are observed. Once again there is no mutual haplotype shared by samples from Manitoba, Canada and Upper Austria, Austria (Table 4.1).

The ITS rDNA data set was strictly used to assess genetic structure similarities and differences between the Canadian population(s) with those of the Austrian population(s). Neighbour joining (NJ) and unweighted pair group method with arithmetic mean (UPGMA) cluster analysis were used (Figure's 4.1 and 4.2, respectively) to estimate the degree of relationships between the samples analyzed based on differences in genetic distance. The phenogram (Figure 4.1) and dendrograms (Figure 4.2) showed similar topologies. The NJ phenogram is comprised of two large clades showing 62% and 100% bootstrap support for the two clades (A and B). Clade A is comprised of 32 Canadian samples with 13 Austrian samples, while clade B is comprised of 25 Austrian samples and 4 Canadian samples.

Geographic distribution cannot explain the topology in the trees. The Austrian

samples are distributed among both clades A and B. Bootstrap support values vary within each clade showing Waldaist and Schlogener Schlinge site representatives distributed among both clades in the NJ phenogram (Figure 4.1). Representatives from the four Canadian collections sites are positioned throughout clades A and B in both Figures 4.1, and 4.2 and clade supports vary for Canadian samples. Overall, there is a majority of the Canadian samples in clade A (Figures 4.1, and 4.2) and a majority of Austrian samples in clade B. The placement of samples in the two trees (Figure 4.1 and 4.2) is nearly identical. The greatest change is observed in the placement of sample Payuk Lake 11-5. In Figure. 4.1, this sample is positioned within a well-supported clade with two other Payuk Lake samples and one Nisto Lake sample. Within Figure. 4.2 the sample Payuk Lake 11-5 is positioned alone outside the cluster at the bottom of the Clade A (Figure 4.2).

The Clade B of both Figures 4.1 and 4.2 consist of 29 samples; 25 representatives of Upper Austria, and four representatives from Canada. Three distinct clades are evident in both the phenogram (Figure 4.1) and the dendrogram (Figure 4.2). Clade B(i) is comprised of North American and Austrian samples and is supported with 66% bootstrap support in the phenogram (Figure 4.1). This clade within figures 4.1 and 4.2 is consistent with the positioning of all the samples within the Principal Coordinates Analysis (PCoA) ordinations (Figure 4.3 A, and B).

Table 4.1. The number of haplotype revealed for ITS rDNA, Actin, and combined ITS/Actin nucleotide sequence data sets representing samples collected in Manitoba, Canada, and Upper Austria, Austria.

		Number of Haplotypes (h)		
Province/State and Country	Site	ITS Data	Actin Data	Combined ITS and Actin Data
Manitoba, Canada n = 36 *when n = 16*	Payuk Lake	11 *5*	*2*	*5*
	Neso Lake	8 *5*	*1*	*5*
	Nisto Lake	5 *1*	*1*	*1*
	Naosap Lake	4 *3*	*2*	*3*
	Total (shared with Austria)	20 (1) *10*	*6* (0)	*13* (0)
Upper Austria, Austria n = 38 ^when n = 5^	$Waldaist_{1-10}$	9	^1^	^1^
	$Waldaist_{11-22}$	6	^1^	^1^
	Schlogener Schlinge	9	^2^	^2^
	Total (shared with Canada)	20 (1)	^3^ (0)	^3^ (0)
		n = 74 h = 39	n = 21 h = 9	n = 21 h = 16

Two clades (Bii and Biii) are composed of entirely Upper Austrian samples. Clade Bii is entirely comprised of Waldaist samples, which is supported by 89% bootstrap support (Figure 4.1). Clade Biii, the other Upper Austrian clade, is comprised of one Waldaist sample and six Schlogener Schlinge samples. This clade is supported by 76% bootstrap support in Figure 4.1. The Schlogener Schlinge samples represent the smallest proportion of total samples analyzed, and with the exception of one cluster in Clade Biii in Figures 4.1 and 4.2 the samples are scattered throughout the phenogram and dendrogram.

The PCoA ordinations show how the samples representing Canadian sites and Austrian sites fall out together when the pairwise distances are standardized and converted to eigen vectors (Figure 4.3). The primary axis (PCoA axis 1) accounts for nearly 85% of the total variation within the genetic distance data. Two groups are presented along the primary axis (Figures 4.3 A and B). A third cluster of samples is separated along the secondary axis (PCoA axis 2), which accounts for 9.95% of the total variation within the genetic distance data. This small cluster of samples (n = 7) is comprised of the same seven samples that form the clade Bi in the lower portion of Figures 4.1 and 4.2. The eigen vector scores for the PCoA analysis are provided in Table 4.2. The far left cluster of samples along the primary axis is comprised entirely of Upper Austrian samples representing the two clades Bii and Biii in the lower portion of the phenogram (Figure 4.1) and dendrogram (Figure 4.2). The far right cluster represents the samples of the upper portion observed in figures 4.1 and 4.2. Collectively, the phenogram, dendrogram, and PCoA display two distinctly variable groups of samples that are comprised of samples from all locations sampled.

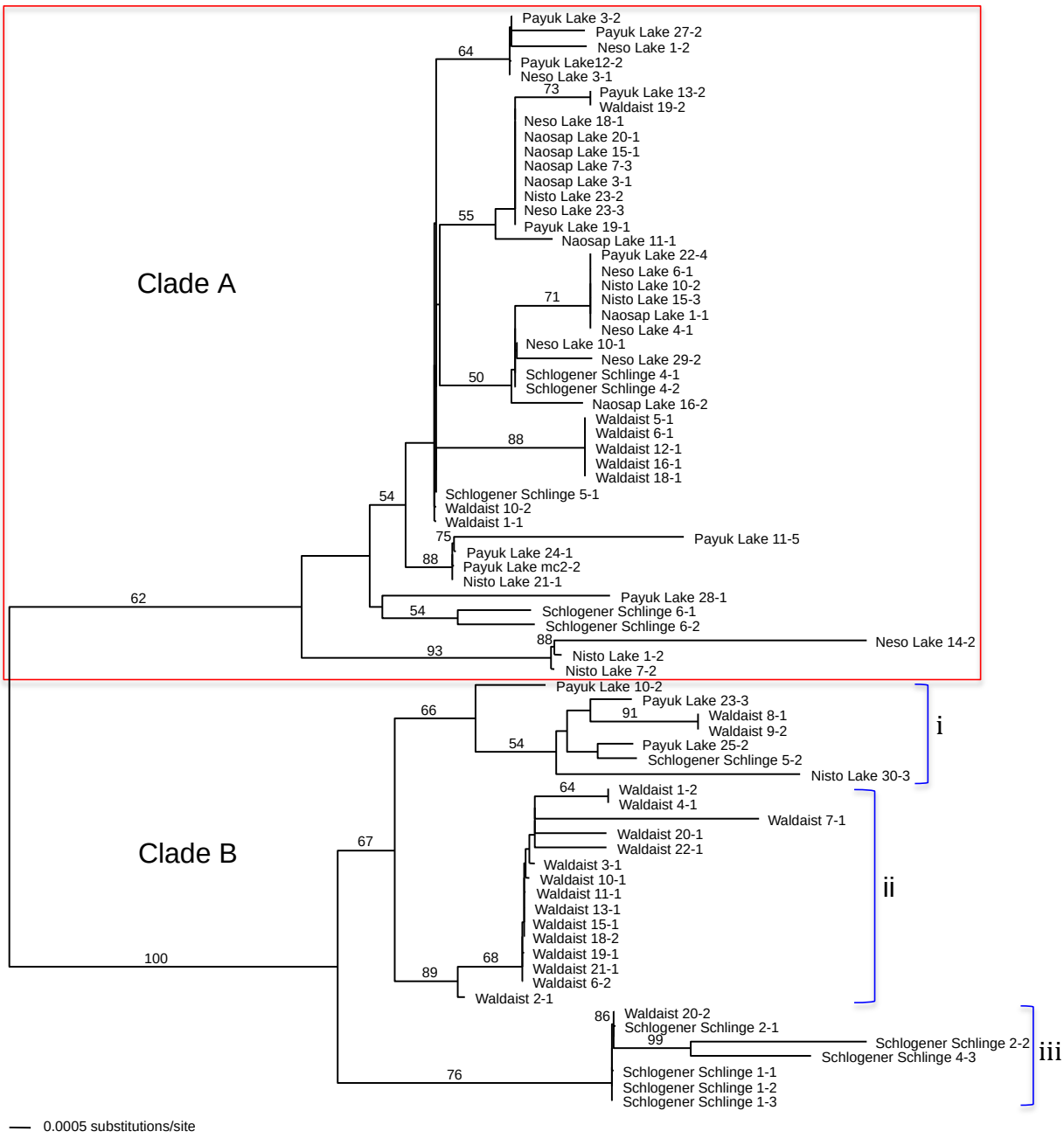


Figure 4.1. Midpoint rooted neighbour joining phenogram constructed using 74 *Diplosphaera chodatii* ITS rDNA gene sequences (36 from Manitoba, Canada, and 38 from Upper Austria, Austria). Pairwise genetic distances were calculated using a maximum likelihood parameter. Bootstrap values ≥ 50% are shown above the branch internodes. Collection site and collection number represents each of the 74 *D. chodatii* samples in the phenogram.

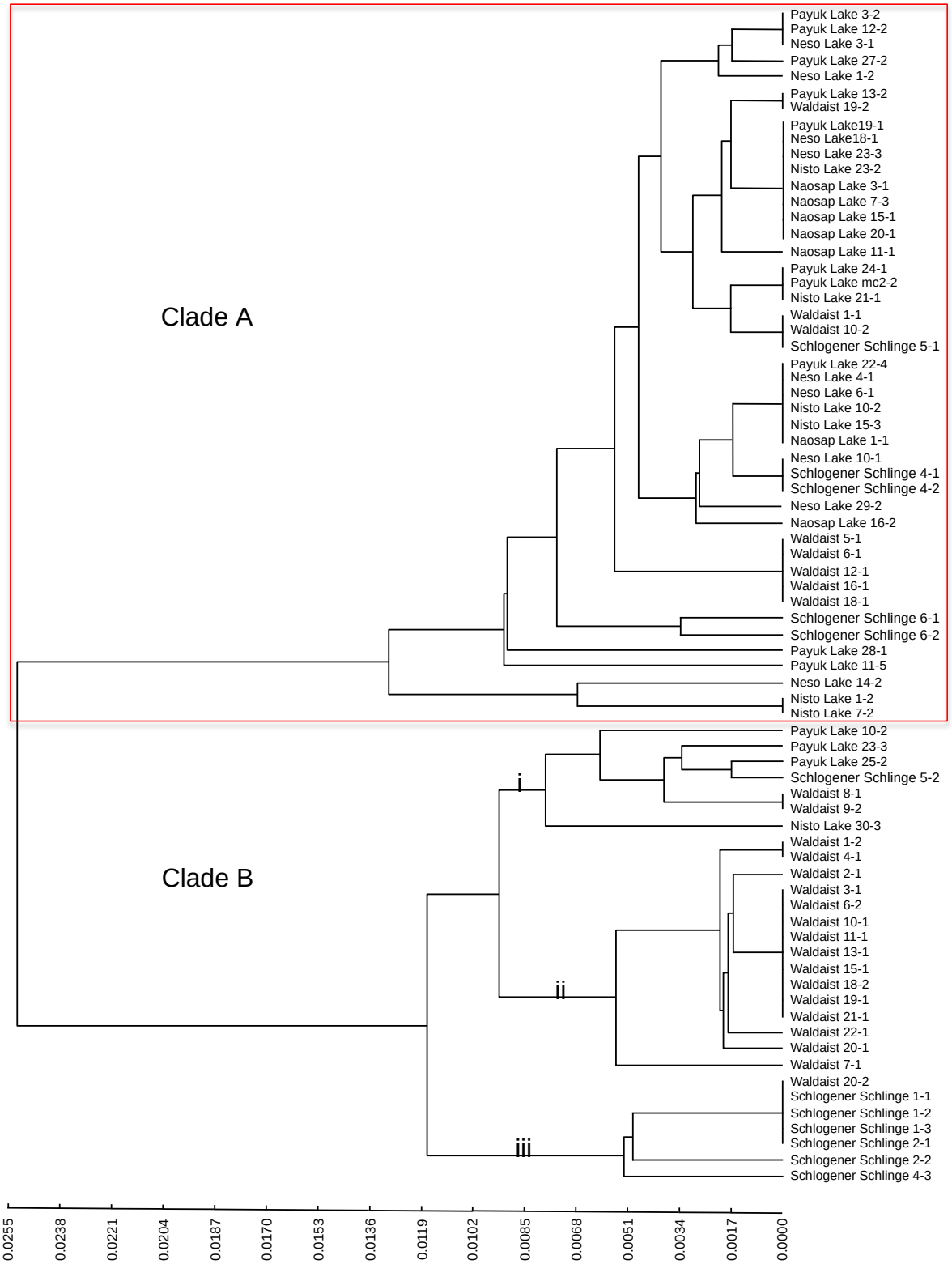
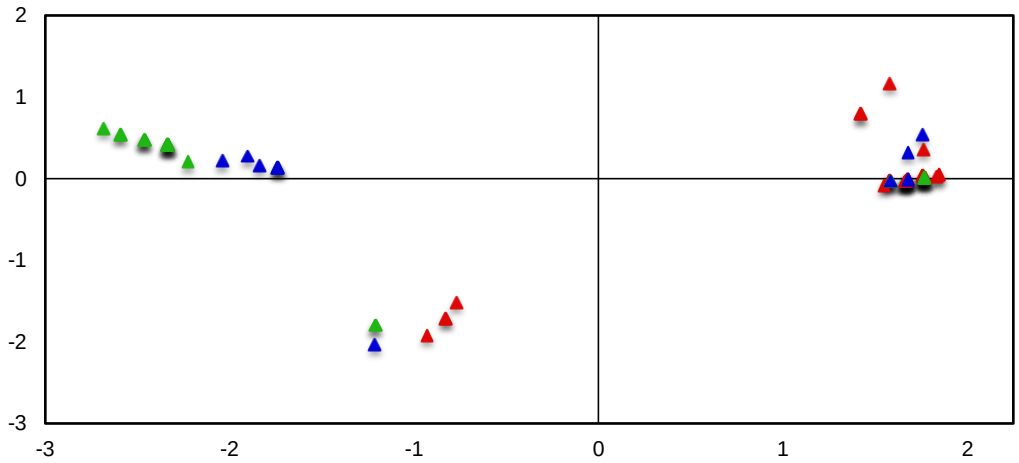


Figure 4.2. UPGMA dendrogram constructed using 74 *Diplosphaera chodatii* ITS rDNA gene sequences (36 from Manitoba, Canada, and 38 from Upper Austria, Austria). Pairwise genetic distances were calculated using a maximum likelihood parameter. Collection site and collection number represents each of the 74 *D. chodatii* samples in the dendrogram.

(A)



(B)

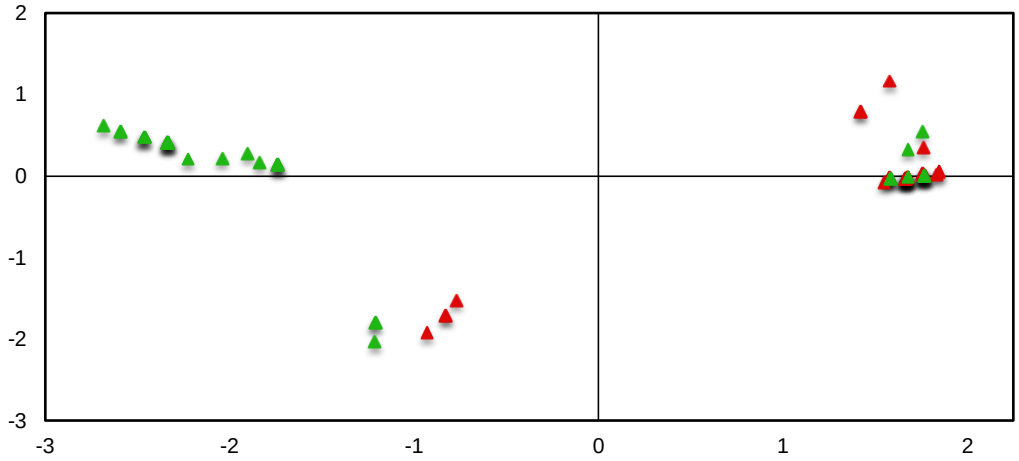


Figure 4.3. Principal Coordinates Analysis (PCoA) constructed using standardized pairwise genetic distances converted to eigen vectors representing the ITS rDNA sequence data of 74 *Diplosphaera chodatii* samples; 36 collected in Manitoba, Canada, and 38 from Upper Austria, Austria. **(A)** Classification of the 74 samples into 3 populations: Red = Manitoba samples, Green = Waldaist, Austria samples, and Blue = Schlogener Schlinge, Austria samples. **(B)** Classification of the 74 samples into 2 populations; Red = Manitoba, Canada, and Green = Upper Austria, Austria. PCoA axis one has an eigen value of 246.935 (84.76%) while PCoA axis two has an eigen value of 28.987 (9.95%). Eigen vector scores for the data are presented in Table 4.2 of this thesis.

Table 4.2. Summary of all eigen vector scores as they correspond to the PCoA Ordination axes one and two in Figure 4.3. Site abbreviations are as follows: P = Payuk Lake, Ne = Neso Lake, Ni = Nisto Lake, Na = Naosap Lake, M = Waldaist, and DV = Schlogener Schlinge.

Axis No.	1	2	Axis No.	1	2
P3-2	1.758	0.035	**M1-1**	1.585	-0.029
P10-2	-0.770	-1.522	**M1-2**	-2.592	0.545
P11-5	1.836	0.021	**M2-1**	-2.226	0.208
P12-2	1.758	0.035	**M3-1**	-2.462	0.480
P13-2	1.768	0.010	**M4-1**	-2.592	0.545
P19-1	1.677	-0.010	**M5-1**	1.767	0.010
P22-4	1.666	-0.027	**M6-1**	1.767	0.010
P23-3	-0.828	-1.714	**M6-2**	-2.461	0.478
P24-1	1.579	-0.021	**M7-1**	-2.683	0.616
P25-2	-0.827	-1.707	**M8-1**	-1.211	-1.796
P27-2	1.847	0.057	**M9-2**	-1.211	-1.796
P28-1	1.762	0.357	**M10-1**	-2.334	0.415
Pmc2-2	1.579	-0.021	**M10-2**	1.585	-0.029
Ne1-2	1.847	0.057	**M11-1**	-2.334	0.415
Ne3-1	1.758	0.035	**M12-1**	1.767	0.010
Ne4-1	1.548	-0.080	**M13-1**	-2.334	0.415
Ne6-1	1.666	-0.027	**M15-1**	-2.334	0.415
Ne10-1	1.665	-0.027	**M16-1**	1.767	0.010
Ne14-2	1.580	1.167	**M18-1**	1.767	0.010
Ne18-1	1.677	-0.010	**M18-2**	-2.334	0.415
Ne23-3	1.677	-0.010	**M19-1**	-2.334	0.415
Ne29-2	1.769	0.009	**M19-2**	1.768	0.010
Ni1-2	1.422	0.795	**M20-1**	-2.461	0.478
Ni7-2	1.422	0.795	**M20-2**	-1.740	0.136
Ni10-2	1.666	-0.027	**M21-1**	-2.334	0.415
Ni15-3	1.666	-0.027	**M22-1**	-2.461	0.478
Ni21-1	1.579	-0.021	**DV1-1**	-1.740	0.136
Ni23-2	1.677	-0.010	**DV1-2**	-1.740	0.136
Ni30-3	-0.929	-1.920	**DV1-3**	-1.740	0.136
Na1-1	1.666	-0.027	**DV2-1**	-1.839	0.162
Na3-1	1.677	-0.010	**DV2-2**	-2.041	0.219
Na7-3	1.677	-0.010	**DV4-1**	1.678	-0.010
Na11-1	1.768	0.010	**DV4-2**	1.678	-0.010
Na15-1	1.677	-0.010	**DV4-3**	-1.903	0.278
Na16-2	1.770	0.009	**DV5-1**	1.585	-0.029
Na20-1	1.677	-0.010	**DV5-2**	-1.214	-2.032
	-	-	**DV6-1**	1.758	0.545
	-	-	**DV6-2**	1.680	0.326

In order to investigate the degree of regional, and population genetic differentiation using the ITS rDNA gene sequence data, AMOVA and Phi (ϕ) statistical methods were used. Regardless of how regions and populations were defined the results provide consistent information. The majority of molecular variation is found within populations, ranging from 54% to 71% depending on how the data were categorized for analysis (Table 4.3). Variation among populations fluctuated more so than seen with variation within populations. Among population variation was 3% among two regions (MB and Austria) and seven populations (four MB lakes and three Austrian lakes). Among population variation was 12% for two regions and three populations were considered. A larger amount of genetic variation, representing genetic distance (28% or 37%) was present between regions than between populations (Table 4.3).

Fixation index (Phi) scores among regions were 0.370 and 0.286 (P = 0.001) for two regions seven populations, and two regions three populations, respectively (Table 4.3). However for the same analyses, phi score for among populations were lower, 0.041 (P = 0.155) and 0.164 (P = 0.015) suggesting low levels of gene flow.

The AMOVA analysis for four compared with three populations in Table 4.3, shows that the Phi scores for were always greater than 0.250. The highest PhiPT score of 0.458 (P = 0.001) was revealed for the one region two populations (Manitoba, and Austria) analysis, where the Schlogener Schlinge data was excluded from the Austrian data set (Manitoba, Canada compared with Waldaist, Austria) (Table 4.3).

Pairwise PhiPT values between collection sites are provided in Table 4.4.

PhiPT scores between the Manitoba lakes show low–level differentiation, the highest being 0.109 (*P* = 0.040), suggesting that some gene flow was occurring between lakes. The PhiPT scores between the Austria lakes also show low–level differentiation where none of the comparisons are significantly different at p=0.05. In this case the highest value is 0.120 (*P* = 0.075) suggesting that gene flow was more common between these sites than it is between Manitoba lakes. Between continents all of the *P* – values are significant (Table 4.4) suggesting that no gene flow or very low levels of gene flow occured between continents. In Table 4.5 the same result is presented at a larger scale reflecting differences between continents. Pairwise PhiPT comparison between Manitoba (as a whole) and Austria, both Waldaist and Schlogener Schlinge, reveal high–level differentiation, 0.458, and 0.358 (both *P* = 0.001) suggesting that no algal haplotypes were shared between continents. The within Austria pairwise PhiPT comparison of Waldaist (as a whole) and Schlogener Schlinge shows less differentiation, 0.115 (*P* = 0.05) suggesting that some gene flow may have occurred.

The PCoA ordination derived using the same sites defined in Table 4.4 shows that the Austrian sites are oriented to the left side of the ordination where the two Waldaist sites are closely associated, and the Schlogener Schlinge site is positioned "lower" along PCoA axis two, and slightly "higher" along PCoA axis one. The Manitoban sites are positioned on the right side of the ordination where Payuk Lake and Nisto Lake are overlapping, both "lower" along PCoA axis one, and both Neso Lake and Naosap Lake positioned at the far right of the ordination "higher" along PCoA axis two (Figure 4.4).

Table 4.3. Analysis of molecular variance (AMOVA) based on ITS gene sequence data representing 74 *Diplosphaera chodatii* samples; 36 collected in Manitoba, Canada, and 38 collected in Upper Austria, Austria. *P* – value estimates are based on 999 permutations. df = degrees of freedom, MS = mean squared deviations, and ϕ = Phi. ϕRT = Among Regions, ϕPR = Among Populations, and ϕPT = Within Population among all populations. Significant *P* – values are highlighted.

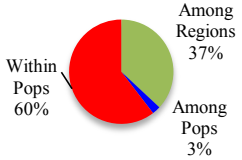
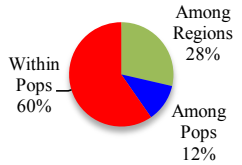
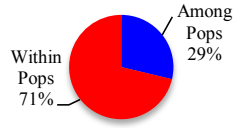
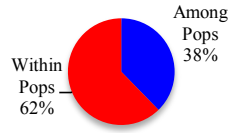
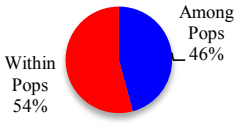
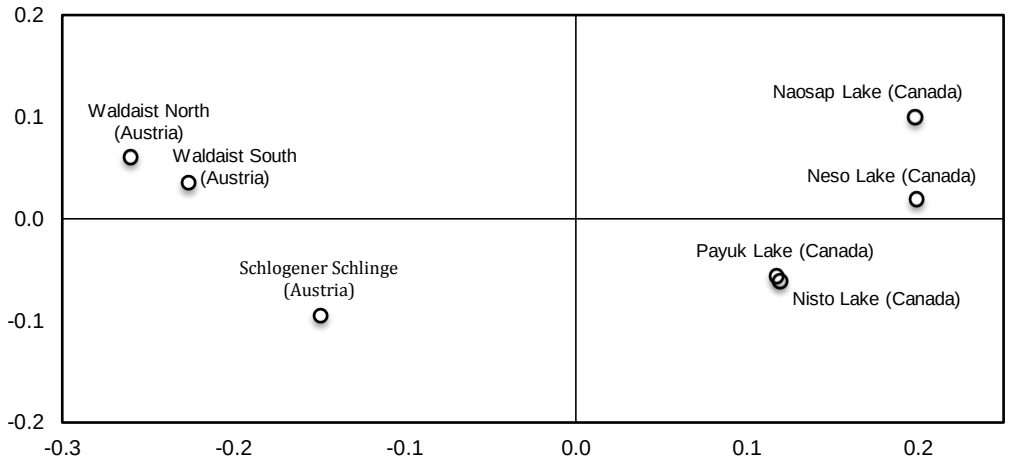
Categorization of Data	AMOVA Analysis	df	MS	Variation	% of total Molecular Variation	ϕ Stat	*P*-value	Summary of Total Molecular Variation
2 Regions 7 Populations	ϕRT	1	169.894	4.313	37	0.370	0.001	
	ϕPR	5	10.189	0.304	3	0.041	0.155	
	ϕPT	67	7.050	7.050	60	0.396	0.001	
2 Region 3 Populations	ϕRT	1	169.894	3.335	28	0.286	0.001	
	ϕPR	1	29.402	1.367	12	0.164	0.015	
	ϕPT	73	6.956	6.956	60	0.403	0.001	
1 Region 7 populations	ϕPR	6	36.807	2.844	29	-	-	
	ϕPT	67	7.050	7.050	71	0.287	0.001	
1 Region 2 populations	ϕPR	1	169.894	4.399	38	-	-	
	ϕPT	72	7.268	7.268	62	0.377	0.001	
MB Lakes vs. Waldaist Pop's	ϕPR	1	171.177	5.456	46	-	-	
	ϕPT	0	387.323	6.455	54	0.458	0.001	

Table 4.4 Pairwise PhiPT values (below diagonal) and associated *P* – values (above diagonal) representing the samples collected at each sites in Manitoba, Canada (Payuk, Nisto, Neso, and Naosap), and Upper Austria, Austria ($Waldaist_{1-10}$, $Waldaist_{11-22}$, and Schlogener Schlinge) derived from raw ITS rDNA sequence data. Significant *P* – values are highlighted.

	Payuk	**Neso**	**Nisto**	**Naosap**	**Waldaist 1-10**	**Waldaist 11-22**	**Schlogener Schlinge**
Payuk		0.194	0.228	0.043	0.008	0.003	0.014
Neso	0.059		0.314	0.180	0.004	0.004	0.003
Nisto	0.001	0.009		0.040	0.013	0.009	0.022
Naosap	0.099	0.038	0.109		0.002	0.002	0.006
Waldaist 1-10	0.312	0.448	0.307	0.469		0.283	0.108
Waldaist 11-22	0.391	0.517	0.383	0.542	0.001		0.075
Schlogener Schlinge	0.224	0.358	0.210	0.390	0.086	0.120	

Table 4.5. Pairwise PhiPT values (below diagonal) and associated *P* – values (above diagonal) representing the samples collected in Manitoba, Canada, and Upper Austria, Austria (Waldaist, and Schlogener Schlinge) derived from raw ITS rDNA sequence data. Significant *P* – values are highlighted.

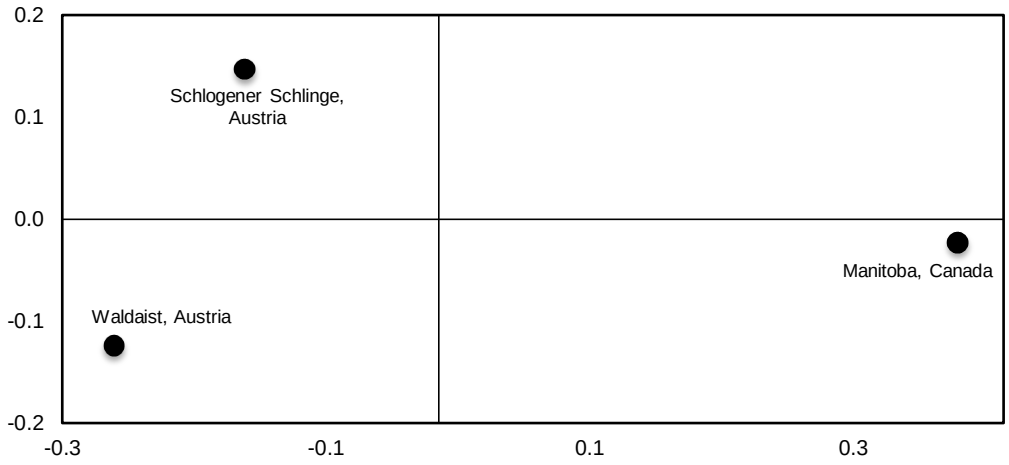
	Manitoba	**Waldaist**	**Schlogener Schlinge**
Manitoba		0.001	0.001
Waldaist	0.458		0.050
Schlogener Schlinge	0.358	0.115	



[Mantel Test for Isolation–by–distance (R_{XY}) = 0.90 ($p < 0.01$)]

Figure 4.4. Principal Coordinates Analysis (PCoA) constructed using standardized covariance of pairwise PhiPT values converted to eigen vectors representing each of the study sites in Manitoba, Canada (Payuk, Neso, Nisto, and Naosap Lakes) and Upper Austria ($Waldaist_{1\text{-}10}$, $Waldaist_{11\text{-}22}$, and Schlogener Schlinge) derived from raw ITS rDNA sequence data. PCoA axis one has an eigen value of 0.248 (81.77%), while PCoA axis two has an eigen value of 0.031 (10.28%).

Similarly, the PCoA ordination derived using the sites defined by Table 4.5 shows that the Austrian locations are oriented to the left side of the ordination, while Manitoba sites are oriented to the far right side. The two Austrian locations (Waldaist and Schlogener Schlinge) are separated more along PCoA axis two than they are along PCoA axis one (Figure 4.5). The two PCoA results (Figures 4.4, and 4.5) suggest the sites of Manitoba and those of Austria are genetically isolated–by–distance. Results of the Mantel test for isolation–by–distance are reported within each of the two ordination figures. Both strongly support isolation–by–distance with values ≥ 0.90. The *P* – values are: < 0.01 (Figure 4.4), and 0.169 (Figure 4.5).



[Mantel Test for Isolation–by–distance (R_{XY}) = 0.963 (p = 0.169)]

Figure 4.5. Principal Coordinates Analysis (PCoA) constructed using standardized covariance of pairwise PhiPT values converted to eigen vectors representing each of the study locations: Manitoba, Canada, and Upper Austria (Waldaist, and Schlogener Schlinge) derived from raw ITS rDNA sequence data. PCoA axis one has an eigen value of 0.162 (82.84%), while PCoA axis two has an eigen value of 0.038 (17.16%).

*The genetic structure of Diplosphaera chodatii **within** Upper Austria, Austria*

A total of 20 haplotypes in 38 *Diplosphaera chodatii* ITS rDNA gene sequences from Austrian samples were obtained (Table 4.1). Within the two Waldaist sites: $Waldaist_{1\text{-}10}$ (South; n = 13), and $Waldaist_{11\text{-}22}$ (North; n = 13) the number of haplotypes discovered totaled nine and six, respectively (Table 4.1). Two haplotypes are shared between the north and south collection sites making 13 haplotypes within this location of Upper Austria. Within the Schlogener Schlinge location nine haplotypes were discovered from 12 samples, with two of the haplotypes shared with the Waldaist location.

Although the sample size of Austrian actin, and combined actin with ITS data is very small, three genotypes were discovered from five samples for each data set (Table 4.1). The Waldaist samples (n = 3) each had the same genotype, while the two Schlogener Schlinge samples represented different haplotypes.

Most of the molecular variation in Austria is found within populations (Table 4.6). By defining the sampling locations of Upper Austria (Waldaist and Schlogener Schlinge) into two distinct regions and three populations [$Waldaist_{1\text{-}10}$ (South), $Waldaist_{11\text{-}22}$ (North), and Schlogener Schlinge] the AMOVA result displays 86% variation within populations, with 0% among populations, and 14% of the total variation among the regions. When Austria was classified as one region with two and three populations, the variation within populations was 89%, and 95%, respectively, and among populations was 11%, and 5%, respectively. Population genetic variation between the two Waldaist sites ($Waldaist_{1\text{-}10}$, and $Waldaist_{11\text{-}22}$) shows 100% of the variation is within populations (Table 4.6). The

highest PhiPT value is 0.115 between two populations, which is marginally significant, while the lowest is -0.049 between three populations. The Phi score presented when two regions are designated (PhiRT) was 0.145 which is also significant at $P = 0.05$.

The pairwise PhiPT score for Waldaist and Schlogener Schlinge in Table 4.5 was 0.115 (P =0.050), and the PCoA ordinations (Figures 4.4, and 4.5) show distance between the two Austrian locations. Pairwise PhiPT for $Waldaist_{1\text{-}10}$ and $Waldaist_{11\text{-}22}$ equals 0.001 (P = 0.296). Pairwise PhiPT comparison for these two site with Schlogener Schlinge reveals scores of 0.086 (P = 0.111), and 0.120 (P = 0.084), respectively (Table 4.7). Mantel test for isolation–by–distance between Waldaist and Schlogener Schlinge is reported within Table 4.7. Isolation–by–distance is strongly supported with a value of 0.964, and P = 0.165

Table 4.6. Analysis of molecular variance (AMOVA) based on ITS gene sequence data representing 38 *Diplosphaera chodatii* samples collected in Upper Austria, Austria. *P* – value estimates are based on 999 permutations. df = degrees of freedom, MS = mean squared deviations, and ϕ = Phi. ϕRT = Among Regions, ϕPR = Among Populations, and ϕPT = Within Population among all populations. Significant *P* – values are highlighted.

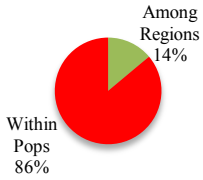
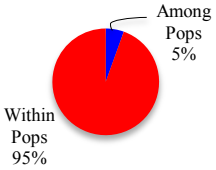
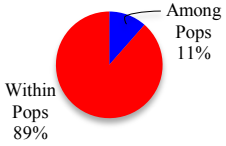
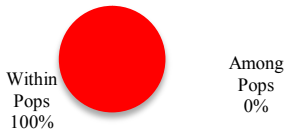
Categorization of Data	AMOVA Analysis	df	MS	Variation	% of total Molecular Variation	ϕ Stat	*P*-value	Summary of Total Molecular Variation
2 Regions 3 Populations	ϕRT	1	29.402	1.542	14	0.145	0.035	
	ϕPR	1	3.769	0.000	0	-0.049	0.733	
	ϕPT	35	9.551	9.551	86	0.103	0.067	
1 Region 3 Populations	ϕPR	2	16.586	0.556	5	-	-	
	ϕPT	35	9.551	9.551	95	0.055	0.134	
1 Region 2 Populations	ϕPR	1	29.402	1.219	11	-	-	
	ϕPT	36	9.390	9.390	89	0.115	0.048	
Waldaist $_{1-10}$ .vs. Waldaist$_{11-22}$	ϕPR	1	3.769	0.000	0	-	-	
	ϕPT	24	9.487	9.487	100	-0.049	0.757	

Table 4.7. Pairwise PhiPT values (below diagonal) and associated *P* – values (above diagonal) representing ITS rDNA sequence data from *Diplosphaera chodatii* collected in Upper Austria, Austria ($Waldaist_{1-10}$, $Waldaist_{11-22}$, and Schlogener Schlinge).

	Waldaist 1-10	**Waldaist 11-22**	**Schlogener Schlinge**
$Waldaist_{1-10}$		0.296	0.111
$Waldaist_{11-22}$	0.001		0.084
Schlogener Schlinge	0.086	0.120	

[Mantel test for isolation–by–distance (R_{XY}) = 0.964 *P* – Value 0.165]

*Genetic structure of Diplosphaera chodatii **within** Manitoba, Canada*

The ITS data set was analyzed alone in two different ways: 1) the whole data set (n = 36) was used, and 2) only 16 samples were used (reduced data set) because the actin and ITS sequence data were analysed together. The number of haplotypes discovered within Manitoba totaled 20 for the whole data set, and10 from the reduced data set (Table 4.1). The numbers of haplotypes from each collection site are as follows (with the reduced data set number in parentheses): Payuk Lake 11 (5), Neso Lake 8 (5), Nisto Lake 5 (1), and Naosap Lake 4 (3). Four haplotypes were shared among all four sites. Payuk Lake shared 3 (1) haplotypes with Neso Lake, 3 (0) with Nisto Lake, and 2 (1) with Naosap Lake. Neso also shared 2 (1) haplotypes with Nisto Lake, and 2 (2) with Naosap Lake. Lastly, Nisto Lake shared 1 (1) haplotype with Naosap Lake (Table 4.1).

There were six actin haplotypes of 16 samples observed within Manitoba (Table 4.1). Payuk Lake had 2 haplotypes, Neso Lake, and Nisto Lake both had 1, while Naosap had 3 haplotypes. All Payuk and Naeosap haplotypes are unique to those lakes, while the single haplotype observed in Neso Lake, and Nisto Lake is the same.

The combined ITS and actin data set revealed 13 haplotypes from 16 individuals. Payuk Lake displays 5 haplotypes, Neso Lake 5, Nisto Lake 1, and Naosap Lake 3 (Table 4.1). Of the 13 haplotypes, one is shared between Neso Lake and Nisto Lake.

The AMOVA of Manitoba ITS rDNA was performed with each lake representing a population. The total molecular variation within populations was 96%, and 4% among populations (Table 4.8). Low–level differentiation is observed with a PhiPT score of 0.041 (P = 0.167). A pairwise PhiPT comparison of the four Manitoban lakes yields low–differentiation values ranging from 0.001 to 0.109 (Table 4.9). Statistically, these PhiPT score are not well supported, with only two of six values display significance (< 0.05).

The PCoA ordination (Figure 4.6) displays Payuk Lake and Nisto Lake to be equally positioned along PCoA axis 1 (77.53%), but isolated from one another along PCoA axis 2 (22.47%). Neso Lake is positioned more or less in the middle of the ordination along axis 1, and “low” along axis 2. Naosap Lake is positioned to the far right side of the ordination, and about half the distance “up” axis 2 as Payuk Lake. Mantels test for isolation–by–distance for the whole ITS data set provides a score of 0.539 (P = 0.211), revealing that population structure is influenced by distance (Figure 4.6).

Table 4.8. Analysis of molecular variance (AMOVA) based on ITS gene sequence data representing 36 *Diplosphaera chodatii* samples collected in Manitoba, Canada. *P* – value estimates are based on 999 permutations. df = degrees of freedom, MS = mean squared deviations, and ϕ = Phi. ϕPR = Among Populations, and ϕPT = Within Population among all populations.

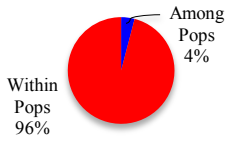
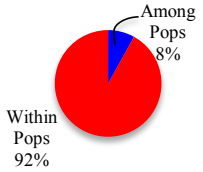
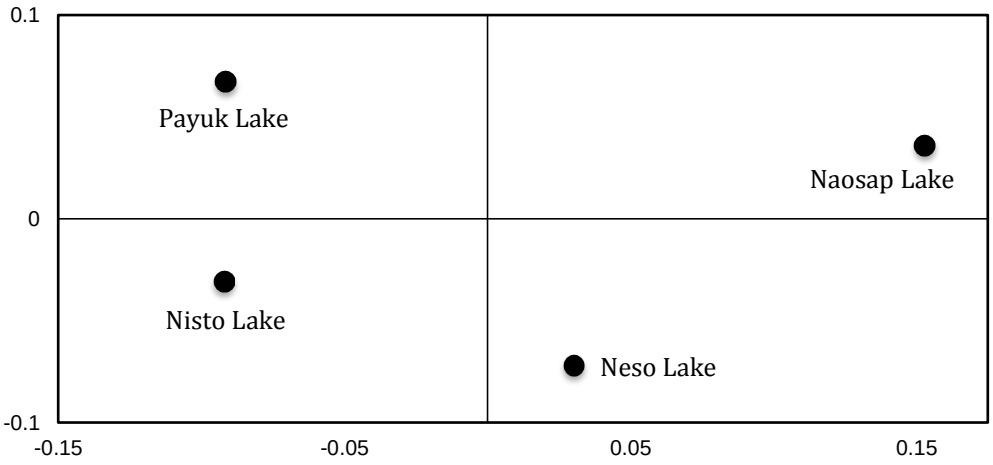
Categorization of Data	AMOVA Analysis	df	MS	Variation	% of total Molecular Variation	ϕ Stat	*P*-value	Summary of Total Molecular Variation
1 Region 4 Populations	ϕPR ϕPT	3 32	5.925 4.315	0.183 4.315	4 96	- 0.041	- 0.167	
1 Region 4 Populations (reduced to 16 samples)	ϕPR ϕPT	3 12	5.842 4.383	0.385 4.383	8 92	- 0.081	- 0.255	

Table 4.9. Pairwise PhiPT values (below diagonal) and associated *P* – values (above diagonal) representing the samples collected in Manitoba, Canada (Payuk, Neso, Nisto, and Naosap Lakes) derived from raw ITS rDNA sequence data.

	Payuk	**Neso**	**Nisto**	**Naosap**
Payuk		0.193	0.264	0.035
Neso	0.059		0.322	0.194
Nisto	0.001	0.009		0.035
Naosap	0.099	0.038	0.109	



[Mantel test for isolation–by–distance (R_{XY}) = 0.539 *P* – Value 0.211]

[Mantel test for isolation–by–distance (R_{XY}) = 0.105 *P* – Value 0.459 (reduced data set n = 16)]

Figure 4.6. Principal Coordinates Analysis (PCoA) constructed using standardized covariance of pairwise PhiPT values converted to eigen vectors representing each of the study locations in Manitoba, Canada (Payuk, Neso, Nisto, and Naosap Lakes) derived from raw ITS rDNA sequence data. PCoA axis one has an eigen value of 0.041 (77.53%), while PCoA axis two has an eigen value of 0.012 (22.47%). Note: there is no significant change to the layout of the ordination when the sample size is reduced to 16 samples.

Analysis of the actin data set (n = 16) representing Manitoban samples by NJ, and UPGMA methods reveals different associations than observed with the ITS rDNA data set (Figures 4.1, and 4.2). For instance, the NJ phenogram of the actin data strongly supports, by 100% bootstrap, the pairing of Payuk 28-1 with Naosap 15-1, and Naosap 20-1 (Figure 4.7A). In the ITS phenogram, Payuk 28-1 is distantly isolated from the Naosap 15-1, and Naosap 20-1 (Figure 4.1). The Neso Lake, and Nisto Lake samples form a clade that is well supported by 100% bootstraps (Figure 4.7A). Likewise, the dendrogram (Figure 4.7B) reveals the identical cluster of Neso Lake, and Nisto Lake samples. Also presented in the dendrogram is the clustering of Payuk Lake and Naosap Lake samples. The small cluster of Naosap 15-1, and Naosap 20-1 is represented in the phenogram supported by 87% bootstrap support (Figure 4.7A). Each of the Payuk Lake samples (four of them) that nicely cluster in the dendrogram (Figure 4.7B) with Naosap Lake 1-1, display negative branch lengths, and a dispersed placement in the NJ phenogram (Figure 4.7A). Naosap 1-1 is positioned most closely with the Neso Lake, and Nisto Lake clade with 98% bootstrap support.

The inclusion of five Austrian samples to the data set caused differences between the NJ, and UPGMA trees (Figures 4.8A, and Figures 4.8B, respectively). The Neso Lake and Nisto Lake samples remained together in a clade at the lower portion of the phenogram with high bootstrap support of 100% (Figure 4.8A). In the dendrogram (Figure 4.8B), the Neso Lake, and Nisto Lake samples form a stong cluster. The placements differ by a switch of the Payuk Lake/Naosap Lake and Waldaist clade (99% bootstrap support) with the Neso

Lake/Nisto Lake clade in the UPGMA dendrogram. Placement of the five Upper Austrian samples reveals similarity in the data set with Manitoban samples from Payuk Lake, and Naosap Lake.

Negative branch lengths seen in Figure 4.7A were not at all resolved in Figure 4.8A. However, analysis of the combined actin and ITS data sets nearly resolved the negative branch lenghts seen in Figure 4.7A and 4.8A, only the Payuk 19-1 sample still showed negative distance (Figure 4.10A). The other samples that showed negative branch lengths in Figures 4.7A and 4.8A are better positioned and have better bootstrap supports. Naosap 1-1 is no longer positioned in a clade with the Neso/Nisto Lake clade (Figures 4.10A and 4.10B), which better agrees with both UPGMA dendrogams using only actin data (Figures 4.7B, and 4.8B).

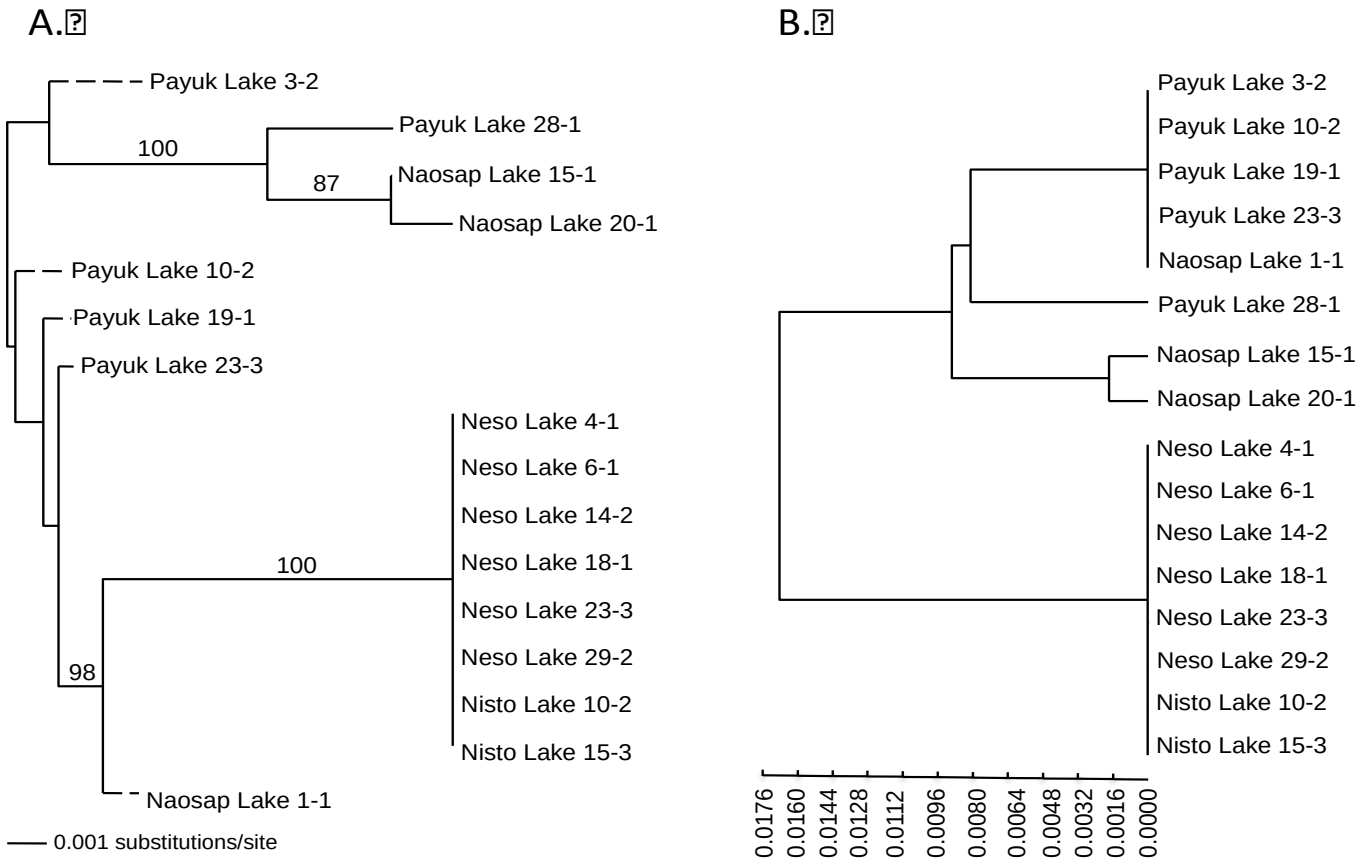


Figure 4.7. A. Midpoint rooted neighbour joining phenogram; **B.** UPGMA dendrogram. Both are constructed using 16 Canadian *Diplosphaera chodatii* actin gene sequences; 5 from Payuk Lake, Manitoba, 6 from Neso Lake, Manitoba, 2 from Nisto Lake, Manitoba, and 5 from Naosap Lake, Manitoba. Pairwise genetic distances were calculated using a maximum likelihood parameter. Bootstrap values ≥ 50% are shown above the branch internodes in the phenogram. Collection site and collection number represents each of the 16 *D. chodatii* samples in the phenogram and dendrogram.

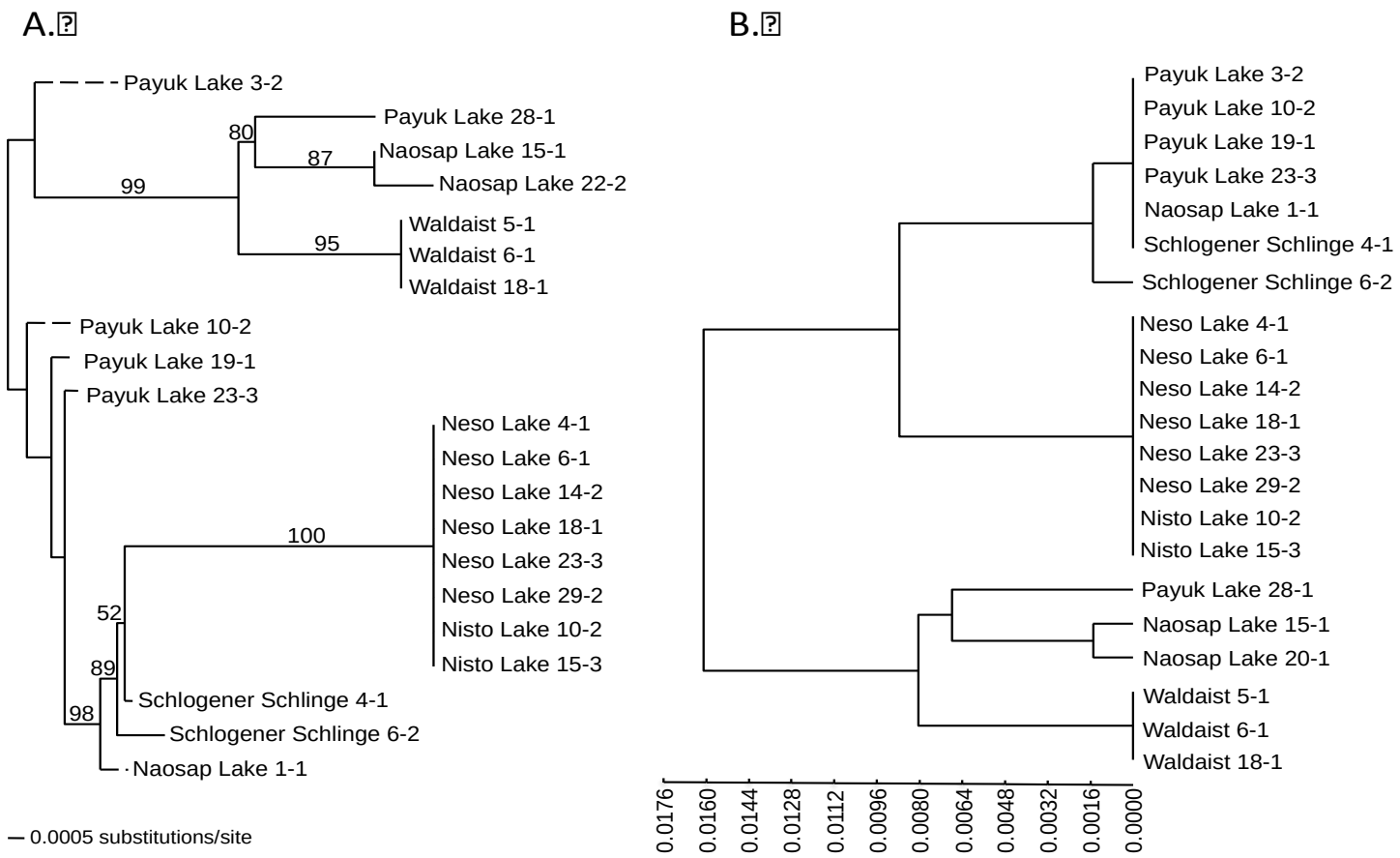


Figure 4.8. A. Midpoint rooted neighbour joining phenogram; **B.** UPGMA dendrogram. Both are constructed using 16 Canadian and 5 Austrian *Diplosphaera chodatii* actin gene sequences; 5 from Payuk Lake, Manitoba, 6 from Neso Lake, Manitoba, 2 from Nisto Lake, Manitoba, and 5 from Naosap Lake, Manitoba; Thee from Waldaist, Austria, and 2 from Schlogener Schlinge, Austria. Pairwise genetic distances were calculated using a maximum likelihood parameter. Bootstrap values ≥ 50% are shown above the branch internodes in the phenogram. Collection site and collection number represents each of the 21 *D. chodatii* samples in the phenogram and dendrogram.

Analysis of molecular variance and Phi statistics were calculated in two ways: 1) the actin data set for Manitoba samples (n = 16), in which each lake represents a population, and 2) the same actin data set for Manitoba samples with five Austrian samples added, in which two populations are defined (Manitoba and Austria).

For the first analysis, the AMOVA revealed that 80% of the actin molecular variation within Manitoba exists among populations and 20% of the actin molecular variation occurs within populations (Table 4.10). The PhiPT is 0.796 (*P* = 0.001), showing a high–level of differentiation with a significant *P* – value suggests that low level of gene flow is occurring between these populations. The analysis that includes the Austrian actin genes shows shows that the majority of the variation is within populations (77%), and 23% among populations (Table 4.10). The PhiPT score shows a high–level of differentiation (0.234; *P* = 0.034) suggesting low levels of gene flow.

Pairwise PhiPT scores for the Manitoba samples data set reveals high–level differentiation between each pairwise comparison with the exception of Neso Lake to Nisto Lake (0.001). The *P* statistics are only considered significant (< 0.05) for two pairwise comparisons (Table 4.11). A PCoA ordination (Figure 4.9) of the pairwise PhiPT scores shows Nisto Lake, and Neso Lake positioned to the far left of the ordination along PCoA axis 1 (86.55%), and only slightly separated along axis 2 (13.45%). Naosap Lake, and Payuk Lake are positioned to the far right of the ordination, where they are separated along axis 2. Differentiation is not attributed to isolation–by–distance according to the Mantel Test (Figure 4.9).

Table 4.10. Analysis of molecular variance (AMOVA) based on actin gene sequence data representing: **(A)** 16 *Diplosphaera chodatii* samples collected in Manitoba, Canada. **(B)** 16 *Diplosphaera chodatii* samples collected in Manitoba, Canada, and 5 *D. chodatii* samples collected in Upper Austria, Austria. *P* – value estimates are based on 999 permutations. df = degrees of freedom, MS = mean squared deviations, and ϕ = Phi. ϕPR = Among Populations, and ϕPT = Within Population among all populations.

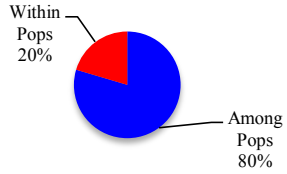
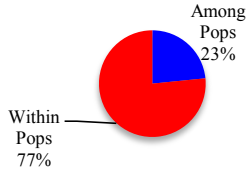
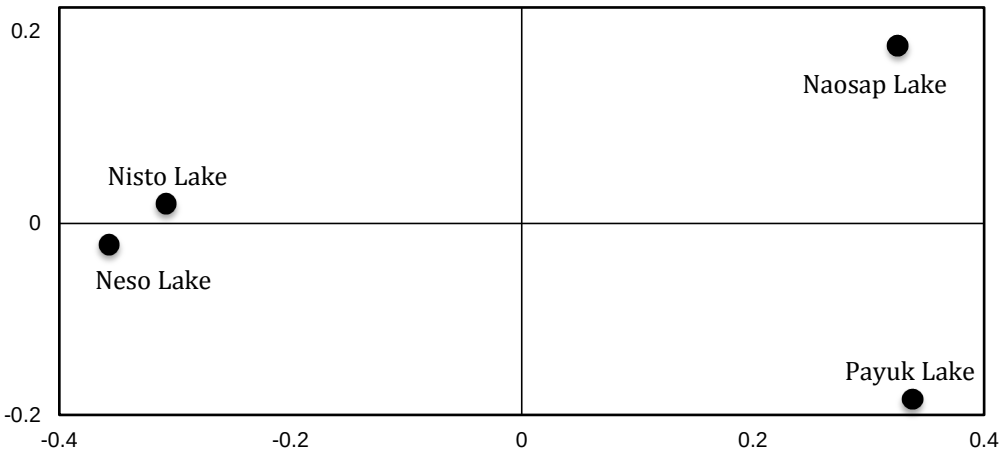
Categorization of Data	AMOVA Analysis	df	MS	Variation	% of total Molecular Variation	ϕ Stat	*P*-value	Summary of Total Molecular Variation
(A)								
1 Region 4 Populations	ϕPR	3	24.200	5.978	80	-	-	
	ϕPT	12	1.533	1.533	20	0.796	0.001	
(B)								
1 Region 2 Populations (n=16 + 5)	ϕPR	1	19.019	1.746	23	-	-	
	ϕPT	19	5.716	5.716	77	0.234	0.034	

Table 4.11. Pairwise PhiPT values (below diagonal) and associated *P* – values (above diagonal) for the reduced actin gene sequence data set totaling 16 samples of *Diplosphaera chodatii* collected in Manitoba, Canada (Payuk, Neso, Nisto, and Naosap Lakes).

	Payuk	**Neso**	**Nisto**	**Naosap**
Payuk		0.003	0.054	0.107
Neso	0.880		0.990	0.012
Nisto	0.792	0.001		0.101
Naosap	0.235	0.880	0.739	



[Mantel test for isolation–by–distance (R_{XY}) = 0.003 *P* – Value 0.495]

Figure 4.9. Principal Coordinates Analysis (PCoA) constructed using standardized covariance of pairwise PhiPT values converted to eigen vectors representing each of the study locations in Manitoba, Canada (Payuk, Neso, Nisto, and Naosap Lakes), derived from raw actin gene sequence data. PCoA axis one has an eigen value of 0.442 (86.55%), while PCoA axis two has an eigen value of 0.069 (13.45%).

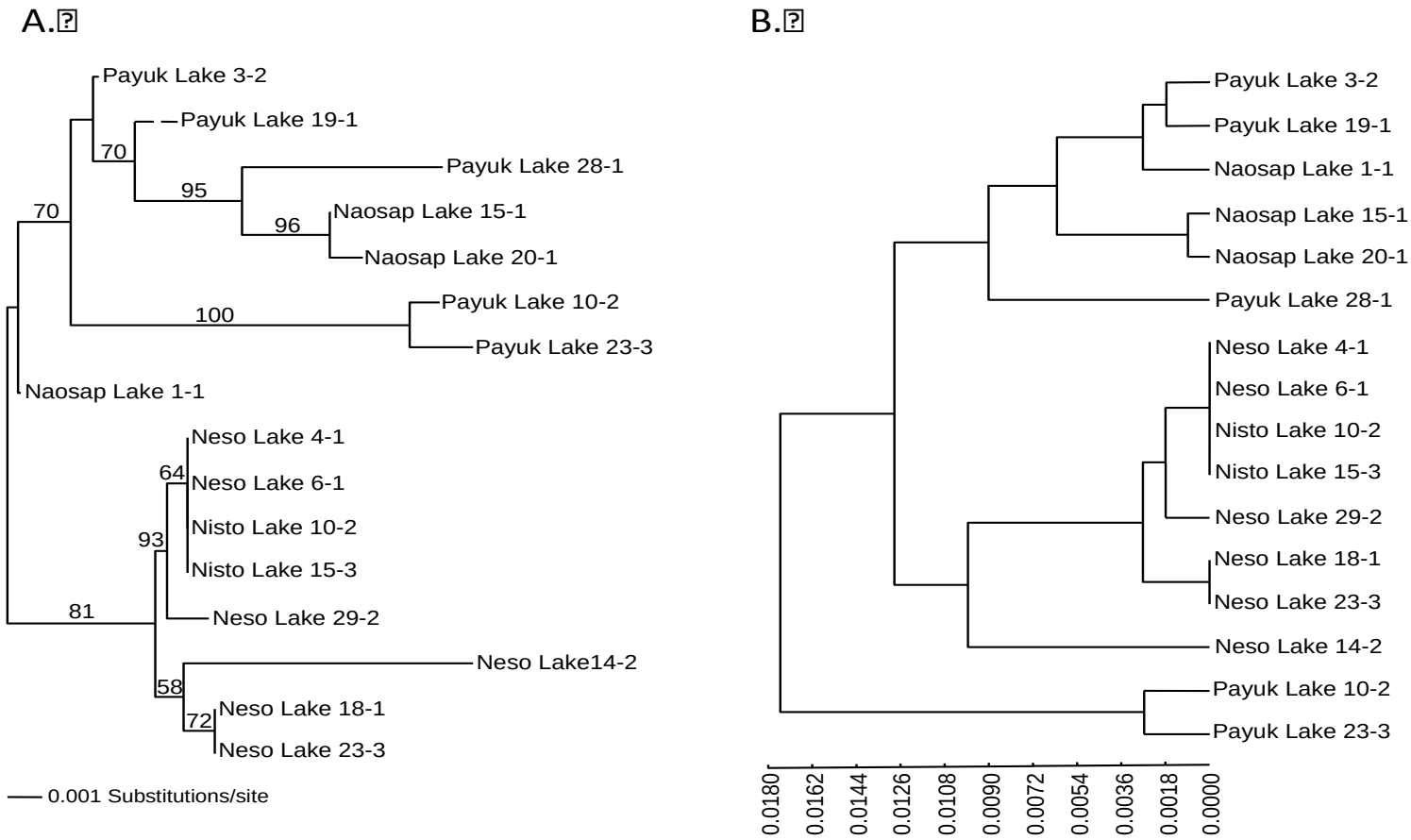


Figure 4.10. A. Midpoint rooted neighbour joining phenogram; **B.** UPGMA dendrogram. Both are constructed using 16 Canadian *Diplosphaera chodatii* combined ITS rDNA and actin gene sequences; 5 from Payuk Lake, Manitoba, 6 from Neso Lake, Manitoba, 2 from Nisto Lake, Manitoba, and 5 from Naosap Lake, Manitoba. Pairwise genetic distances were calculated using a maximum likelihood parameter. Bootstrap values ≥ 50% are shown above the branch internodes in the phenogram. Collection site and collection number represents each of the 16 *D. chodatii* samples in the phenogram and dendrogram.

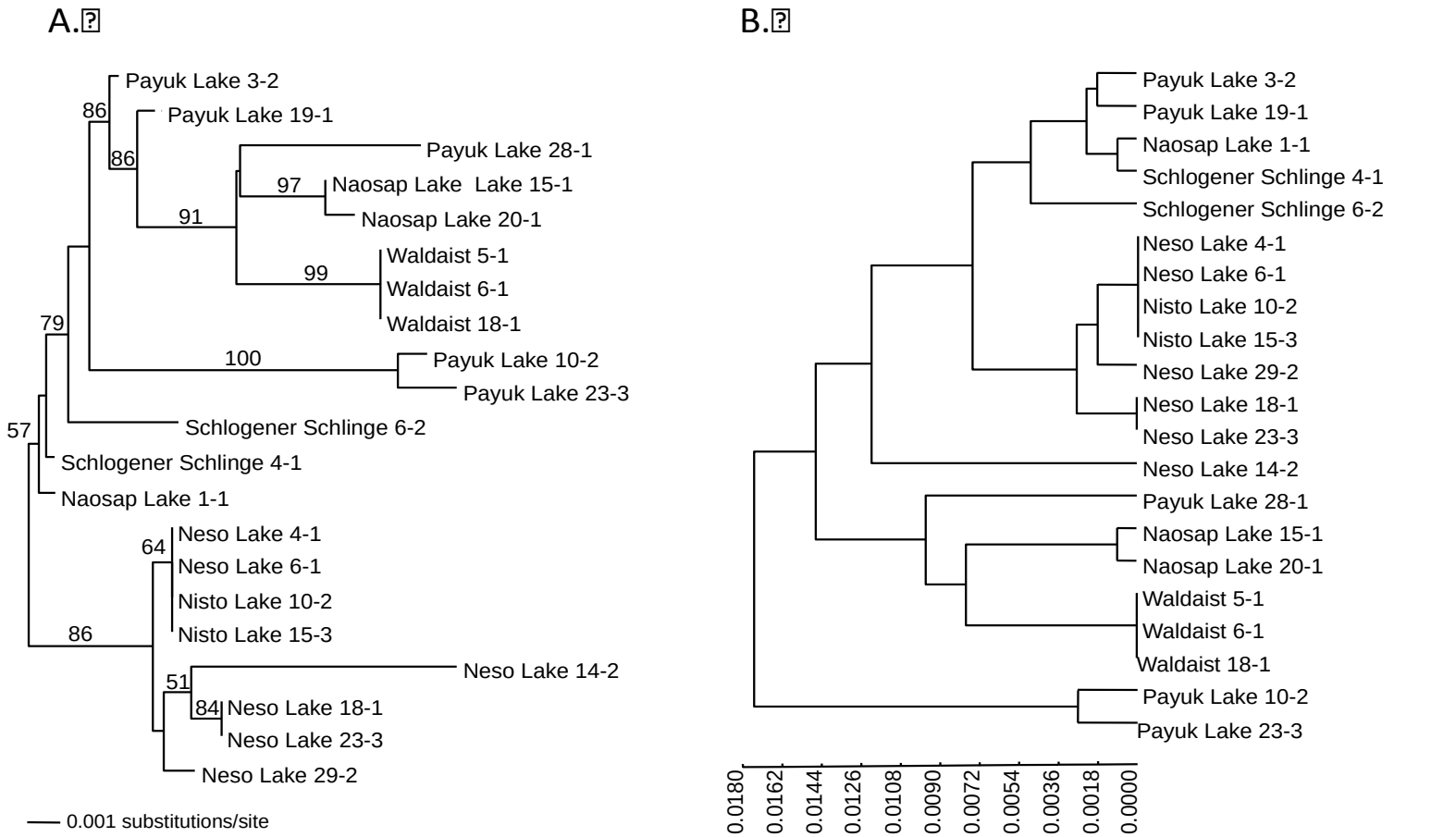


Figure 4.11. A. Midpoint rooted neighbour joining phenogram; **B.** UPGMA dendrogram. Both are constructed using 16 Canadian and 5 Austrian *Diplosphaera chodatii* cobined ITS rDNA and actin gene sequences; 5 from Payuk Lake, Manitoba, 6 from Neso Lake, Manitoba, 2 from Nisto Lake, Manitoba, and 5 from Naosap Lake, Manitoba; 3 from Waldaist, Upper Austria, and 2 from Schlogener Schlinge, Upper Austria. Pairwise genetic distances were calculated using a maximum likelihood parameter. Bootstrap values ≥ 50% are shown above the branch internodes in the phenogram. Collection site and collection number represents each of the 21 *D. chodatii* samples in the phenogram and dendrogram.

Table 4.12. Analysis of molecular variance (AMOVA) based on combined ITS rDNA and actin gene sequence data representing: **(A)** 16 *Diplosphaera chodatii* samples collected in Manitoba, Canada. **(B)** 16 *Diplosphaera chodatii* samples collected in Manitoba, Canada, and 5 *D. chodatii* samples collected in Upper Austria, Austria. *P* – value estimates are based on 999 permutations. df = degrees of freedom, MS = mean squared deviations, and ϕ = Phi. ϕPR = Among Populations, and ϕPT = Within Population among all populations.

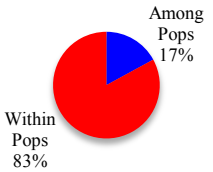
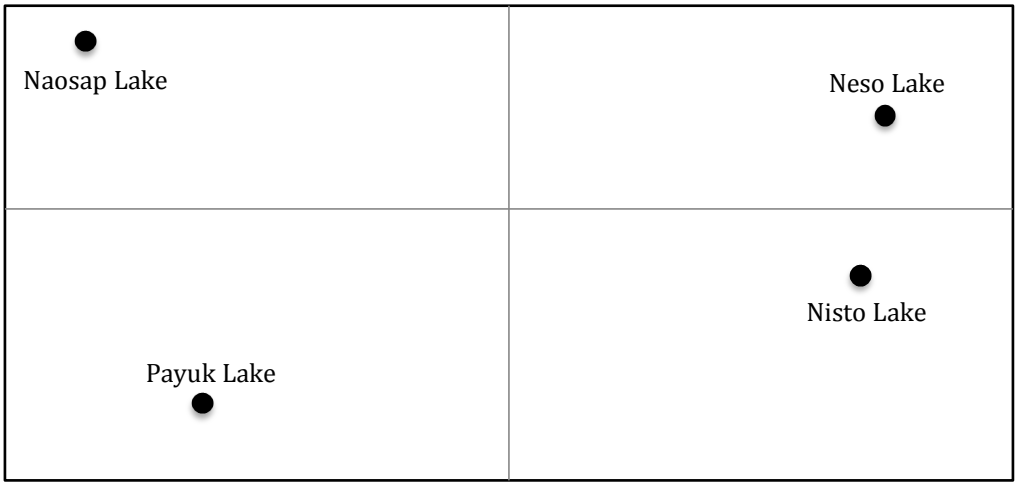
Categorization of Data	AMOVA Analysis	df	MS	Variation	% of total Molecular Variation	ϕ Stat	*P*-value	Summary of Total Molecular Variation
(A)								
1 Region 4 Populations	ϕPR	3	30.042	6.363	52	-	-	
	ϕPT	12	5.917	5.917	48	0.518	0.006	
(B)								
1 Region 2 Populations (n=16 + 5)	ϕPR	1	24.913	1.997	17	-	-	
	ϕPT	19	9.701	9.701	83	0.171	0.032	

Table 4.13. Pairwise PhiPT values (below diagonal) and associated *P* – values (above diagonal) for the reduced combined ITS rDNA and actin gene sequence data set totaling 16 samples of *Diplosphaera chodatii* collected in Manitoba, Canada (Payuk, Neso, Nisto, and Naosap Lakes).

	Payuk	**Neso**	**Nisto**	**Naosap**
Payuk		0.001	0.107	0.160
Neso	0.585		0.275	0.015
Nisto	0.474	0.001		0.101
Naosap	0.134	0.692	0.704	



[Mantel test for isolation–by–distance (R_{XY}) = 0.159 *P* – Value = 0.288]

Figure 4.12. Principal Coordinates Analysis (PCoA) constructed using the standardized covariance of pairwise PhiPT values converted to eigen vectors representing each of the study locations in Manitoba, Canada (Payuk, Neso, Nisto, and Naosap Lakes), derived from the reduced raw combined ITS rDNA and actin gene sequence data. PCoA axis one has an eigen value of 0.442 (88.74%), while PCoA axis two has an eigen value of 0.069 (11.26%).

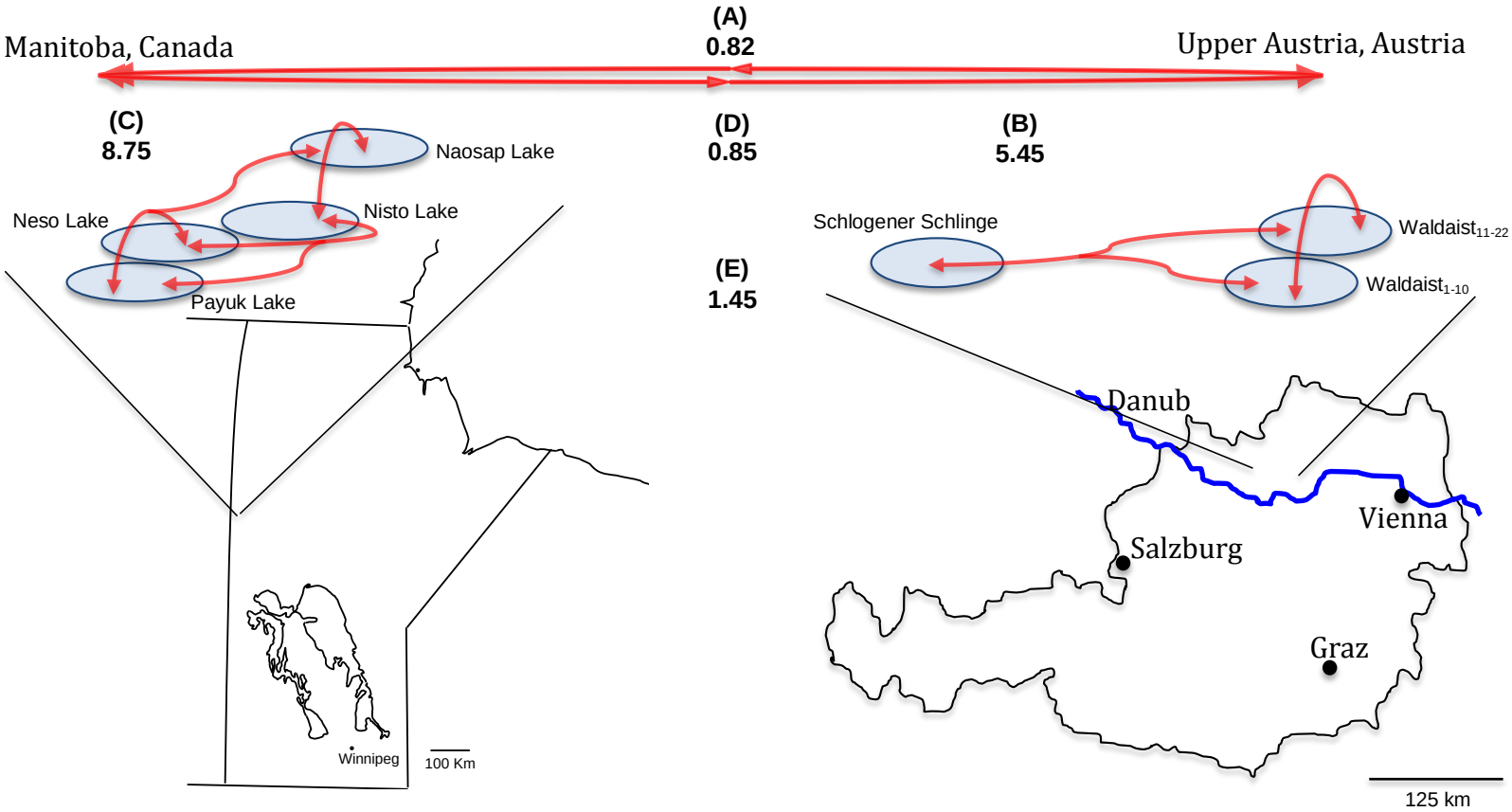


Figure 4.13. Geographical representation of average estimated migration rate within and between Manitoban and Austrian collection sites. **(A)** = migration between continental populations based on ITS data only; **(B)** = migration within Upper Austrian sites using ITS data only; **(C)** = migration between Manitoban sites based on ITS data only; **(D)** = migration within Manitoban sites based on actin data only; **(E)** = migration within Manitoban sites based on combined ITS/actin data. Averages were calculated from the Nm values from each of the tests based on the categorization of data by number of regions and associate populations as performed in Tables 4.3, 4.6, 4.8, 4.11, and 4.13, respectively.

Analysis of genetic structure in Manitoba using the NJ method with the combined ITS and actin sequence data produces a phenogram (Figure 4.10A) that places Payuk Lake and Naosap Lake samples together in the upper portion of the tree. This clade, excluding Naosap 1-1 is supported with 70% bootstrap support. Within this clade there is strong support for the placement of the samples, ranging from 70% to 100% bootstrap support. The lower portion of the phenogram is comprised of Neso Lake and Nisto Lake samples. The clade is supported by 81% bootstrap support. Comparison of the phenogram (Figure 4.10A) with the UPGMA dendrogram (Figure 4.10B) from the same data set reveals similar information. However, one attribute that really stands out is the placement of the cluster, Payuk 10-2 and Payuk 23-3, at the bottom of the dendrogram. In the phenogram (Figure 4.10A), this clade/cluster of two samples was positioned in the upper portion with 100% bootstrap support for the clade; within the 70% bootstrap supported upper clade of the phenogram.

The analysis of genetic diversity within Manitoba, using AMOVA, for the combined ITS and actin data reveals that 48% of the genetic variation is found within populations and 52% is found among populations (Table 4.12). The fixation index for this data set was found to be 0.518 (P = 0.006), which suggests very high–level differentiation (Table 4.12) and therefore low levels of gene flow. Pairwise PhiPT scores for the Manitoba Lakes (populations) confirm the high degree of differentiation between lakes (Table 4.13). The lowest pairwise PhiPT score of 0.001 (P = 0.275) is seen between Neso Lake and Nisto Lake, with Payuk Lake and Naosap Lake presenting a pairwise PhiPT score of 0.134 (P =

0.160). All other pairwise comparisons between lakes range between 0.474 and 0.704, where two of the four are significant ($P < 0.05$).

The PCoA analysis of the standardized pairwise PhiPT scores (Figure 4.12) displays Naosap Lake and Payuk Lake on the left side of the ordination along PCoA axis 1 explaining 88.74% of the variation, and distanced from one another along PCoA axis 2 explaining 11.26% of the variation. On the far right side of the ordination is Neso Lake and Nisto Lake that are also distanced from one another along PCoA axis 2. The Mantel test does not strongly support that differentiation is attributed to by isolation–by–distance (0.159; $P = 0.288$).

The AMOVA of the combined ITS/actin data set for Manitoba (n = 16) with the addition of Austrian samples (n = 5) reveals that 83% of the genetic variation is within populations and 17% is among populations (Table 14.13). The PhiPT score for this analysis suggests low to moderate–level differentiation (0.171, $P = 0.032$) suggesting low levels of gene flow.

Re-analysis of the combined data set via NJ and UPGMA methods while including the addition of five Austrian samples (Figures 4.11A, and 4.11B, respectively) merely replicated the same results seen in Figures 4.10A, and 4.10B. The three Waldaist samples were placed among the upper clade in the phenogram, and well supported by strong bootstrap supports (Figure 4.11A). In the dendrogram, the Waldaist cluster is positioned toward the bottom with Payuk 28-1, Naosap 18-1, and Naosap 20-1 (Figure 4.11B), which were in the upper portion of the phenogram (Figure 4.11A). The position of the two Schlogener Schlinge samples is poorly supported in the phenogram. In the dendrogram, the

positioning of the two Schlogener Schlinge samples is more defined, as are the placements of Payuk 3-2, Payuk 19-1, and Naosap 1-1 in the uppermost cluster (Figure 4.11B).

Estimated migration rates were calculated for all the different categorizations of data sets into regions and populations presented in the various AMOVA tables. The mean of all these estimated migration rates are presented in Figure 4.13. Migration values within Manitoba and Austria base on ITS alone are the highest at *Nm* = 8.75 and 5.45, respectively. Meanwhile, values for the actin and combined actin and ITS data set for Manitoba are considerably lower. The actin data alone for Manitoba reveals a migration rate of 0.85 while analysis of the combined actin and ITS data set provides an *Nm* value of 1.45. Between continental populations the migration rate is low *Nm* = 0.82 from analysis of the ITS data alone.

Discussion

Continental isolation of Diplosphaera chodatii populations

This study shows that at the intercontinental level, a low level of gene flow is inferred using genetic distance (UPGMA, NJ, and PCoA). AMOVA, fixation indices, and migration rates between continents show that there is significant genetic differentiation suggesting a negligible level of gene flow. While higher levels of gene flow are inferred within continents, only one haplotype is shared between the two continents. At least three possible scenarios may explain this outcome. 1) Genetic differentiation may be attributable to considerable genetic

drift as a result of isolation-by-distance (Wright 1943). 2) Recent gene flow may have occurred by a rare, but possible, long-distance dispersal event resulting in the sharing of one haplotype. 3) Alternatively, genetic mixing may have already been present from historical gene flow and the loss of shared haplotypes occurred over time through vicariance events. These scenarios are examined separately, but they are not necessarily mutually exclusive.

Isolation–by–distance of D. chodatii

Since the jet stream flows from west to east in the Northern Hemisphere (potential vector of long-distance dispersal) and the collecting sites were similar in lattitude, it is reasonable to suggest that long distance dispersal is possible for *D. chodatii*. However, the absence of all shared haplotypes except one ITS rDNA haplotype shared between Canada and Austria in this study may suggest differentiation between the continental populations with infrequent long distance dispersal and colonization events as was inferred in other studies (Högberg et al. 2002; Walser et al. 2005). The lack of shared haplotypes, between the two geographic regions, suggest that the two populations no longer share a common gene pool and the actin and ITS genes may be evolving or have evolved independently from historical founder events. *Diplosphaera chodatii* is a widely distributed free-living and symbiotic algal species that must have the potential for dispersal, but the exact mechanism of photobiont dispersal and availability of symbiotically suitable *D. chodatii* remains unknown. It is evident that gene flow is occurring within local ranges and there is implied gene flow by a single haplotype

between large geographic distances (Table 4.1), North America and Europe. Gene flow theoretically counteracts local selection pressures, which overall restricts genetic drift (Allendorf 1983; Lenormand 2002).

For lichens and other symbiotic organisms, geographical barriers may influence both symbionts independently and together in the symbiotic state. Geographic features influence the movement of genetic information (gene flow) within and between populations (Hanski 1997), altering the population structure by means of isolation–by–distance or environmental and biological bottlenecks (Buschbom 2007). Significant linkage between gene flow and geographic distance has been well documented within many groups of organisms constituting terrestrial and aquatic environments. If *D. chodatii*, which associates with *D. luridum* var. *luridum* is mainly distributed by water, the vast geographic barriers of land and salt water oceans between North America and Austria would limit its dispersal.

Isolation-by-distance was supported by photobiont populations of *Lobaria pulmonaria* in a geographic region of more than 500 km apart in British Columbia (Walser et al. 2005). A broader sampling strategy was performed by Fernández-Mendoza et al. (2011), in their study of the lichen *Cetraria aculeata*. The algal partner associated with *Cetraria aculeata, Trebouxia jamesii 'vulpinae',* showed evidence for genetic differentiation for the north polar, temperate and south polar study zones (Fernández-Mendoza et al. 2011). The temperate representatives of *T. jamesii 'vulpinae'* formed a monophyletic clade, but individuals from the north and south polar zones produced a similar pattern displayed by *Diplosphaera*

chodatii from Manitoba and Upper Austria. Domaschke et al. (2012) more recently reported that the diversity of the photobiont associated with *C. aculeata* is highest in temperate regions and decreases toward the polar regions, and suggested that the polar populations resulted from long-distance dispersal. Lichen photobiont species that are distributed between North America and Europe, include the *Trebouxia jamesii* photobiont associating with *Letharia vulpina* (Kroken and Taylor 2000), and *Dictyochloropsis reticulata* associating with *Lobaria pulmonaria* (Werth et al. 2006). Similar genetic patterns of fungal symbionts have been reported along the same spatial scales (Högberg et al. 2002; Buschbom 2007).

Historical gene flow and vicariance

The intercontinental genetic differentiation in *D. chodatti* may be explained by historical gene flow and loss of haplotypes through long time isolation from vicariance events (Walser et al. 2005; Galloway 2008). The continental drift of Pangaea separated North America from the European countries 175 mya during the Jurassic when there were also many environmental changes. It has been hypothesized that members of Trebouxiophyceae have been present in Antarctica 84 mya and survived in refugia while some lineages were estimated to precede the break-up of Gondwana (De Wever et al. 2009). Vicariance events were used to explain the genetic differentiation of a red alga (Hommersand and Fredericq 2003) and lichen fungi (Printzen et al. 2003). Other intercontinental studies in lichens focused mainly on the fungal partner (Palice and Printzen

2004; Walser et al. 2005; Widmer et al. 2010) with less known about lichen algae. Palice and Printzen (2004) reported a pattern of genetic variation in the fungal partner, which is similar to the algal genetic variation in this study where there was no overlap in haplotypes between continents but populations on both continents did not produce monophyletic groups where European haplotypes were closely related to haplotypes in Central America. They hypothesized an absence of long-distance dispersal and long-term isolation of the populations on each continent.

Gene flow of D. chodatii among populations within continents

Gene flow between algal populations within a continent was greater than that between continents (Figure 4.13). The gene flow between lakes in Manitoba suggests that dispersal of the algae is occurring and that the genetic diversity of *D. chodatii* within Manitoba is high. The evidence that supports gene flow is shown by high estimated migration rates between lakes, low phiPT values, and an absence of monophyletic lineages in the trees. This level of gene flow was also observed in *Ramalina menziesii* (Werth and Sork 2010) and *Evernia mesomorpha* (Piercey-Normore 2006). Similarly, the populations in Austria show no structure suggesting that gene flow is also occurring at that geographic scale. There is stronger gene flow occurring between the two Waldaist collection sites than there is between Waldaist and Schlogener Schlinge, an indication that isolation-by-distance is influencing this system. Genetic drift may be influencing the population structure within Upper Austrian populations of *D. chodatii,*

however as migration rates between sites are high, genetic drift is outcompeted by gene flow.

Gene flow in green algae has been assumed to follow the “everything is everywhere” hypothesis because of their small organismal size and common habitat (Finlay 2002). However, a view of a more restricted distribution of microbial eukaryotes has been gaining support (Luo et al. 2006; Foissner 2008; Evans et al. 2008) but less is known about terrestrial green algae (Rindi et al. 2009) and even less about lichen algae (Werth 2011). This study shows that at the intercontinental level, no strong/clear population genetic structure is inferred via genetic distance means (UPGMA, NJ, and PCoA), however AMOVA and fixation index scores suggested that there is significant genetic differentiation. There is only one shared haplotype from all the data sets revealed between the two continents, where a number of possible individual scenarios or better yet multiple interacting scenarios yield this outcome. Genetic differentiation is attributable to considerable genetic drift as a result of isoloation-by-distance; additional confirmation for this comes from the very low level estimated migration rate per generation between continental populations. The similar result has been documented for a number of other lichen photobiont species between North American and European populations, such as the *Trebouxia jamesii* photobiont associating with *Lepraria vulpina* (Kroken and Taylor 2000), and *Dictyochloropsis reticulata* associating with *Lobaria pulmonaria* (Werth et al 2005). It is all too common that collection sites are compared over immense geographic distances (eg. regional populations within North America and Europe). Seldom are

sampling regimes comprised of scattered populations spanning the area between two extreme sites. The best-known attempt at this using a lichen was performed by Fernández-Mendoza et al. (2011) in their study of *Cetraria aculeata*.

Within Manitoba, Canada the population structure assessments using genetic distance methods exhibited no clear structural differences. This time around, the AMOVA and fixation index scores support the genetic distance tree and ordination methods. There is minimal/low-level population differentiation between lakes/micropopulations suggesting genetic interaction. There is an indication that isolation–by–distance is influencing this system's differentiation. However, there is evidence that adequate gene flow is occurring between the lakes and that the genetic diversity of *D. chodatii* within Manitoba, or for this region of Manitoba. The evidence that supports gene flow comes from the considerably high estimated migration rate between lakes within this region.

Likewise, within Upper Austria, Austria, there is no indication of population genetic structure from the UPGMA, NJ and PCoA analyses, which reveals Waldaist and Schlogener Schlinge samples grouping together. Also, as observed with the Manitoba data set, the AMOVA and fixation index scores are low suggesting interaction of genetic information between the three sites. There is considerably stronger interaction or gene flow occurring between the two Waldaist collection sites than there is between Waldaist and Schlogener Schlinge. Genetic drift may be influencing the population structure within Upper Austrian populations of *D. chodatii,* however as migration rates between sites are high, genetic drift may be outcompeted by gene flow. Isolation-by-distance in

Lobaria photobiont was shown between populations >500 km apart in British Columbia (Walser et al. 2005).

Isolation–by–distance is a facet of population biology that is virtually always estimated using molecular data. This is the indirect method for assuming Isolation-by-distance because direct observational studies of dispersal and colonization events are not practical (Warren 2003). *Candelariella viellina* and *Placynthium nigrum* dispersal and their colonization of tombstones are the only known experiments where isolation–by –distance was studied with lichens using a direct method (Warren 2003).

Diplosphaera chodatii a well-distributed free-living and symbiotic algal species must have the potential to disperse immense distances using a series of vectors. However, the exact mechanism of photobiont dispersal and availability of symbiotically suitable *D. chodatii* remains unknown. It is evident that considerable gene flow is occurring within local ranges and there is implied movement between large geographic distances, between North America and Europe. Gene flow theoretically counteracts local selection pressures, which overall restricts adaptation. However, biologists have debated the role of gene flow in limiting adaptation. It is strongly believed, however that replenishment of population and genetic variation is achieved through gene flow thus providing a basis for evolution by natural selection (Lenormand 2002). Although recent studies are beginning to shed light on algal distributions, knowledge of algal dispersal is in its infancy (Rindi et al. 2009) with studies on other eukaryotic microbes showing varied results. Protein electrophoresis of North American and

European populations of a diatom suggested that clonal reproduction was evident within lakes but variation was present between lakes (Soudek and Robinson 1982). In contrast, microsatellite analysis of *Daphnia magna* from lakes in Europe suggested higher genetic diversity and that this more rapid rate of evolution was influenced by contemporary rather than historical processes (Walser and Haag 2012). Microsatellite studies of another diatom, *Pseudo-nitzschia pungens* showed no evidence for genetic differentiation suggesting high levels of gene flow preventing genetic drift in populations between water bodies with different degrees of connectivity (Casteleyn et al. 2009). Although algae may disperse by air or water, this study suggests that water may be an important mode of dispersal for *D. chodatii* and the geographic barriers between continents prevent genetic mixing.

Factors that influence population algal structure

Dermatocarpon luridum var. *luridum* is known to grow only on rock as a substratum. Two studies examined population structure in the photobionts of epiphytic lichens that grow on trees, *Trebouxia decolorans* (from *Ramalina menziesii*; Werth and Sork 2010) and *Dictyochloropsis reticulata* (from *Lobaria pulmonaria*; Walser et al. 2005). The findings were consistent with the regional analyses of *Diplosphaera chodatii* in this study. *T. decolorans* showed weak to no population structure difference between collection sites at a 2km distance. But, significant photobiont genetic diversity differences were revealed between the *T. decolorans* associated with *R. menziesii* growing on three different phorophyte

(host tree) species within the collection sites. This outcome suggested the importance of substrata ecology on the adaptability and availability of photobiont haplotypes within a local/small regional scale. The results of Yahr et al. (2006) also revealed that habitat is a primary factor for the differentiation of photobiont populations associating with *Cladonia subtenuis*. It would be interesting to investigate the degree to which substratum features, such as geological composition or water quality influences genotype availability and thus population structure of *Diplosphaera chodatii* in association with *D. luridum* var. *luridum*.

Even though *Dermatocarpon luridum* var. *luridum* and its aquatic community are considered rare in some areas (Glavich 2009), the alga is thought to be both lichenized and free-living. This study examined diversity of lichenized *D. chodatii* only. Algal diversity was high in a rare species, *Ramalina sinensis* (Francisco et al. 2012) over 400 km, but structured for a common species *Evernia mesomorpha* over a 4 km range (Piercey-Normore 2006). Piercey-Normore (2004) showed that algal diversity differed for three species of *Cladonia* distributed among the same habitats and locations suggesting that fungal species play a role in the distribution of lichenized algae. Similary, Doering and Piercey-Normore (2009) showed variable selectivity of the algae by lichen fungi growing on the same tree. Therefore, both habitat and fungal compatibility play important roles in determining the distribution of algal partners. The role that habitat plays in diversity of free-living *D. chodatii* in unknown.

Free-living photobiont species may express sexual recombination, a life history characteristic that has yet to be observed while in symbiotic lichen

associations. Sexually derived zoospores have been observed for photobionts outside of lichen thalli (Slocum et al. 1980). Now, although never observed in lichen thalli, Doering and Piercey-Normore (2009) still proposed that if zoospores are produced within the thallus they might quickly escape and colonize elsewhere shortly after (Slocum et al. 1980). Colonies established from zoospores are then available for germinating fungal spores to come in contact with, ultimately generating a lichen with a new genetic composition than any others in the population (Murtagh et al. 2000).

Mode of dispersal

The method of dispersal of the algal partners will also influence population structure (Yahr et al. 2004; 2006; Ohmura et al. 2006; Werth and Sork 2010; Peksa and Skaloud 2011). *Dermatocarpon luridum* var. *luridum* contains algae within the thallus, and the ability to disperse is potentially limited to one method, fragmentation. There is a possibility that the very common and widely distributed free-living *D. chodatii* is of a suitable strain or same strain as the photobiont of *Dermatocarpon luridum* var. *luridum.* If this is the case the potential for long-distance dispersal utilizing wind, animals and anthropogenic vectors greatly increases the potential for genetic exchange across large geographical scales (Buschbom 2007). Prevailing wind patterns have also correlated with large distribution and colonization patterns for lichens and bryophytes (Muñoz et al. 2004; Cassie and Piercey-Normore 2008). If the algal cells successfully make their way into the jet-stream, the question would be whether they could endure

the conditions and be dispersed to a suitable habitat for lichenization. A study performed on cultured *D. chodatii* cells that were extracted and dispersed from *Endocarpon pusillum* revealed that *Diplosphaera chodatii* is a physiologically hardy species that can withstand significant levels of desiccation and nutrient stress for periods up to and potentially exceeding eight months (Zhang and Wei 2011). Even though this suggests that long-distance dispersal is possible, this study shows evidence that long-distance dispersal is a rare event for lichenized *D. chodatii*.

Dispersal of *D. chodatii* may also be facilitated by exchange of algal partners from other lichens. Close relatives of *D. luridum* var. *luridum* associate with *Diplosphaera chodatii* (Thüs et al. 2011; Fontaine et al. 2012) making the potential of algal exchange between species fragments a feasible method for acquiring different haplotypes (Ohmura et al. 2006; Piercey-Normore 2006; Fernández-Mendoza et al. 2011). Some lichens (eg. *Endocarpon* sp., *Staurothele* sp., and *Verrucaria* sp) that also associate with *D. chodatii* are actively dispersing their spores with the suitable photobiont, which lives within the hymenial layer of the perithecia amongst the asci and paraphyses. This active dispersal of the photobiont with ascospores presents a ways for germinated *D. luridum* var. *luridum* spores or even fragments to commandeer the photobiont that has been dispersed to the environment (Fontaine et al. 2012).

Sampling for diversity

The decision to use a particular gene for population genetic studies depends on the degree of variation present in the gene for the species being studied. In the green alga *Diplosphaera chodatii* in this study, there was a higher proportion of haplotypes in the nuclear ITS rDNA than the actin gene, but both genes provided variation sufficient to make inferences on the population structure of the lichen alga. With 39 *Diplosphaera chodatii* ITS haplotypes identified from 74 samples (53%), there were many single haplotypes dispersed throughout all sampling areas, indicating that the number of haplotypes shared among geographic locations is low. This low level of sharing may underestimate the inferred level of gene flow between populations. Since the ITS rDNA is comprised of multiple copies arranged in tandem repeat it may also contain multiple paralogs which would inflate the appearance of haplotype diversity and may explain the single haplotypes that were not shared among populations. However, the ITS rDNA has been used as a population marker in studies on lichen algae (Kroken and Taylor 2000; Yahr et al. 2004; 2006; Piercey-Normore 2006; Ohmura et al. 2006; Doering and Piercey-Normore 2009; Werth and Sork 2010; Fernández-Mendoza et al. 2011; Domaschke et al. 2012). The actin gene is a single copy nuclear gene that contains variation in its intron. There were only three haplotypes from five actin gene sequences (60%) from Austria. Within Manitoba the actin gene showed six haplotypes from 16 gene sequences (38%) indicating lower diversity in the actin gene than the ITS region.

Both the ITS rDNA and the actin genes are suitable markers for revealing population genetic structure of *Diplosphaera chodatii*, the photobiont of the sub-aquatic lichen *Dermatocarpon luridum* var. *luridum*. The use of multiple alleles/genes of haploid organisms helps confirm individual gene results, which may over, or underestimate population structure/differentiation and movement of genetic information. Although sample size varied between the ITS and actin sequences, evolutionary hypotheses of these two genes comprising the *D. chodatii* genome is evident from the numerous analyses performed. The use of genetic information from the rDNA gene has been shown to be a valid way to assess population structure of lichen photobionts at the local scale (Doering and Piercey-Normore 2009), of a photobiont at a larger level (Yahr et al. 2006; Piercey-Normore 2006; Ohmura et al. 2006; Robertson and Piercey-Normore 2007; Werth and Sork 2010), and of a photobiont over considerable distances (Kroken and Taylor 2000; Yahr et al. 2004; Fernández-Mendoza et al. 2011; Domaschke et al. 2012).

The sampling regime of this project was comparable to other photobiont population genetic inquiries, and the haplotype diversity capacity could have been obtained or as shown here requires greater sampling effort. Werth and Sork (2010) showed that a sample size of 72 yielded 32 ITS haplotypes; this was sufficient enough to show genetic congruence between sites but also the genetic variability of photobionts associated with particular phorophytes. Lindblom (2009) suggested that a minimum number of 20 lichen thalli would be required from a population in order to obtain adequate haplotype diversity or even haplotype

saturation (plateau) of either symbiont (Lindblom 2009). Domaschke et al. (2012) paraphrased the idea of “stopping rules”, which basically indicate the point at which additional sampling is unnecessary. However this rule is suitable for studying species diversity (Magurran 2004) but is not feasible for inquiring about genetic diversity (Domaschke et al. 2012). Werth (2010) applied rarefaction methods to a microsatellite data set to determine the number of samples per population, across a larger geographic region, and the number of populations needed to adequately assess the allelic diversity in a species. She reported that 20 fungal and slightly more alga samples were needed per population, 300-400 samples in total and 25-30 populations would provide an assessment of most of the allelic diversity. This study examined about 20 samples per population, which should cover most of the diversity present within populations. However, the sampling of 72 specimens in total and six populations in this study may under-represent the worldwide diversity present in *D. chodatii*.

In conclusion, this study provided evidence to support gene flow within and between populations of lichenized *Diplosphaera chodatii* on the same continent but not between continents. The greatest intensity of gene flow occurred within populations, and less gene flow occurred between the most distant populations supporting the isolation-by-distance theory. Since the distribution of *D. chodattii* is widespread across the Northern Hemisphere, and this study shows two geographically isolated populations, there may be multiple gene pools of *D. chodatii* within the temperate zone of the Northern Hemisphere that are evolving more or less independently from one another. This study also

provided evidence that higher levels of gene flow occurs within populations suggesting that the algae may disperse predominantly by water rather than by air. Based on the propagules this may be from fragments of the lichen thallus or as free living algae. The presence of ITS and actin haplotypes suggests that *D. chodatii* may have potential for adaptation to changing environmental conditions either natural or anthropogenic.

Chapter 5

Significance and future contributions

General significance of the studies

The sub-aquatic lichen *Dermatocarpon luridum* var. *luridum* is a globally distributed lichen of temperate watercourses, lakes, rivers, and streams that experience regular water level fluctuations, periodically inundating the lichen for extended periods of time. Even though this lichen is widely distributed, populations are frequently located very distant from one another, which suggests that *D. luridum* var. *luridum* may require specific ecological requirements for survival. Evidence suggesting habitat specificity was illustrated by attempting to culture the symbionts of *D. luridum* var. *luridum* collected in Manitoba and Austria using two conventional isolation techniques and 11 standard growth media for the cultivation of lichen symbionts. The “spore rain” approach, although resulting in both bacterial and fungal contamination, revealed the ability of discharged spores to germinate within a short time period; however, germinated spores did not continue to grow. Cultivation of the mycobiont using the “Yamamoto method” was unsuccessful, whereas cultivation of the photobiont was successful.

The inability to identify optimal culture conditions for the cultivation of the mycobiont, *D. luridum* var. *luridum* presents more speculation than answers about the ecological requirements of this particular fungus and other sub-aquatic and aquatic lichen fungi. Optimal culture conditions for the cultivation of the photobiont; however, seems to be consistent with the growth conditions used for culturing free-living and lichenized algae. Modified BBM+S medium, a nutritionally deprived medium commonly used to cultivate lichen photobionts proved to be an optimal growth medium for successful isolation of multiple

replicates with reduced contamination frequencies. However, WHM medium, a universally used algal growth medium proved optimal for rapid cultivation of the photobiont once transferred to liquid WHM medium, at the cost of reduced success due to the increased frequency of contamination.

An optimal culturing practice for the isolation of axenic colonies of the photobiont from *D. luridum* var. *luridum* would follow an initial isolation using BBM+S medium until suitable size for sub-culturing is reached. At such time, sub-culturing to liquid WHM medium would accelerate colony growth enabling sooner utilization for scientific study.

Speculation of several photobiont genera associated with *D. luridum* var. *luridum* has long been postulated and morphological characteristics of the cultured photobiont provided an opportunity to confirm identification of the photobiont. Two algal species, *Diplosphaera chodatii* and *Elliptochloris bilobata* were identified from the cultures. Both species are known as free-living species as well as photobionts associated with lichen fungi of the Verrucariaceae. *E. bilobata* however was isolated from one of the six thalli of *D. luridum* var. *luridum* and one thallus of *D*. *luridum* var. *decipiens* cultured. Based on the number of thalli investigated, it was thought that *D. luridum* var. *luridum* has the potential to associate with more than one photobiont simultaneously or independently. Alternatively, the *E. bilobata* culture could have been the result of contamination from algal cells on the thallus surface that were not eliminated prior to inoculation during the wash procedure. The occurrence of two photobionts and the isolation of the same photobiont from *D*. *luridum* var. *luridum* and *D*. *luridum* var.

decipiens suggested that *Diplosphaera chodatii* might be shared between different *Dermatocarpon* species.

Phylogenetic examination of mycobiont and photobiont ITS rDNA gene sequences obtained from three different sub-aquatic *Dermatocarpon* species (and one subspecies) and one terrestrial relative provided insight about the ancestral relationships between the species and the potential likelihood for photobiont sharing. *Dermatocarpon luridum* var. *luridum* is a monophyletic species, however specimens from different continents do not form monophyletic clades. The classification of the other sub-aquatic lichens is an artificial classification because of the polyphyly of the tree. *Dermatocarpon rivulorum* was the most basal species while the other sub-aquatic species, *D. arnoldianum* and *D. luridum* var. *decipiens* may be perceived as species or subspecies of *D*. *miniatum*. To date none of the sub-aquatic or aquatic lichen fungi are have been classified as *D*. *miniatum,* a species designation so far representing only terrestrial lichen fungi.

The photobionts of these lichens, with the exception of *D. rivulorum,* were revealed, by the ITS sequences as *Diplosphaera chodatii*, confirming the morphological identification of the cultures. *Dermatocarpon rivulorum* appears to associate with a photobiont species more phylogenetically similar to a *Stichococcus sp.,* which is currently considered to be in the genus *Diplosphaera chodatii*. Additionally, distantly related mycobiont species of *Dermatocarpon* associating with *D. chodatii* may be sharing the same photobiont strains (haplotypes) with each other. The photobionts of these lichens are not easily

dispersed from the thalli; therefore the photobiont must be readily available in the habitat as the result of the free-living co-nature of *D. chodatii*. One of the hypotheses put forward suggests that lichens, such as *Staurothele* sp. and *Endocarpon pusillum* that co-disperse their photobiont (*Diplosphaera* sp.*)* with ascospores are supplying suitable photobionts for other mycobiont species, such as *Dermatocarpon luridum* var. *luridum.* The free-living nature of *D. chodatii* along with the sharing potential of the photobiont by multiple lichen fungi suggests that the photobiont may have the potential to disperse readily within and between populations of *Dermatocarpon luridum* var. *luridum*.

The dispersal range along with regional and long-range geographic population structure of *D. chodatii* is unknown. It was hypothesized that long-range gene flow between the Canadian and Austrian populations is low and that regional gene flow is high. This hypothesis has been supported by AMOVA, showing that the Canadian population is genetically differentiated from the Austrian populations, where only one common haplotype between populations was observed. Isolation-by-distance and genetic drift are identified as significant contributors to the population differentiation. Migration of individuals within the two regional populations was found to be sufficiently high enough to indicate that gene flow is adequate to counter genetic drift, even in the event that isolation-by-distance within the Austrian population is exerting some influence. Long-distance dispersal of *D. chodatii* is rare as supported by the occurrence of the single shared haplotype between the Canadian and Austrian *D. chodatii* associating with *Dermatocarpon luridum* var. *luridum*. A limitation of this study is that only

lichenized samples of *D. chodatti* were selected for this study. If the species occurs free-living, then the movement among populations of free-living *D. chodatii* may show a different level of gene flow.

Future contributions

The outcomes of this research have provided insight about the current state of lichen culturing practices, the distribution, sharing potential and population structuring of *Diplosphaera chodatii* as they relate to the lichenized condition, in particular with *Dermatocarpon luridum* var. *luridum*. The suggestion proposed by Stocker-Wörgötter and Hager (2008) that lichen fungi are no longer challenging to culture in vitro may have some weight if it refers to terrestrial lichen fungi only. This study and other studies have shown that sub-aquatic and aquatic lichen-fungi are still difficult to culture. Future research may focus on identifying and implementing an optimized strategy for the successful cultivation of axenic *Dermatocarpon luridum* var. *luridum*. A first step would be to better understand the natural ecological conditions sustaining colonization and growth of *D. luridum* var. *luridum.* Once culturing success is accomplished, the population structure of *D. luridum* var. *luridum* can be investigated using genome spanning molecular markers, for example microsatellites, to accompany the results of the current population genetic assessment of the photobiont, *Diplosphaera chodatii.* Further cultures of the photobiont should be obtained and identified to confirm for sure if *D. luridum* var. *luridum* can associate with more than one photobiont simultaneously or independently. In order to avoid large

numbers of culture plates, the use of in situ hybridization could shed light on the location of the photobiont in the thallus and whether there is more than one photobiont, as has been carried out on bacterial communities of lichen thalli.

The current research investigated the population genetic structure of *D. chodatii* over an exceptionally large geographic distance. The distance between populations was complicated with population barriers that may have prevented the movement of genetic information. Future work might scale down the geographic distance separating populations in both North America to focus on gene flow within and between watersheds. A better understanding of the dispersal vectors such as water, wind, or animals, and the ability for dispersal would result from such inquiries.

Lastly, the algal sharing of *D. chodatii* between different *Dermatocarpon* species implies that there may be an abundance of free-living *D. chodatii* within the habitat, assuming that the free-living members of D. chodatii are capable of lichenizing. It would be interesting to test the hypothesis that the lichens which actively disperse their photobiont with their spores, for example *Staurothele fissa* a sub-aquatic lichen commonly co-occurring within the same community as *D. luridum* var. *luridum,* may be providing a source of algae for other species in the community and neighboring communities of *D. luridum* var. *luridum*. The impact of such a finding would essentially signify that the algal distributors are keystone species contributing to the adaptability and survival of multiple lichens.

Significance

It is known that *D. luridum* var. *luridum* is environmentally sensitive to increased metal concentrations, and anthropogenic pollution associated with the watercourses it inhabits. Continued climate change will alter the state of the environment further subjecting *D. luridum* var. *luridum* and other organisms within the community to increased stress. Identification of *D. chodatii* as the principal photobiont species associated with *D. luridum* var. *luridum,* a common photobiont of other lichen fungi within the Verrucariaceae and a sustainable free-living alga, suggests that this species is hardy and capable of adapting to adverse conditions. The population assessment supports this claim that *D. chodatii* is a hardy species with a dynamic gene pool. The sub-aquatic lichen fungus, *D. luridum* var. *luridum* may be too demanding and thus unable to adapt to adverse conditions. *Dermatocarpon luridum* var. *luridum* is on the Red List of threatened species within an array of countries in Europe (personal communications with Dr. E Stocker-Wörgötter and Dr. R. Türk – University of Salzburg, Salzburg Austria). In Manitoba, Canada, increased mining activity and hydroelectric damn alteration of watercourses and their associated landscapes threatens *D. luridum* var. *luridum* and other sub-aquatic and aquatic lichens, like the already threatened lichen *Leptogium rivulare*. From this work, it is hoped that land management and conservation officials will act to preserve the watercourses where *D. luridum* var. *luridum* and *L. rivulare* coexist.

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APPENDIX A: ***Recipes for mycobiont culture media***

1) Malt-Yeast Agar Medium (MY) (Yamamoto 1990)

Ingredients for 1L medium (g/L)	
Malt extract	20.0
Yeast	2.0
Agar	20.0

Note: For 1.0% or 0.5% mannitol add 10.0 g or 5.0 g for 1L medium, respectively. Combine all ingredients to an Erlenmeyer flask and top up to 1 L with ddH_2O. Autoclave at 121°C for 20 minutes; place in refrigerator for storage.

2) Lilly and Barnett Medium (LBM) (Lilly and Barnett 1951)

Ingredients for 1L medium (g/L)	
D-Glucose	40.0
L-Asparagine	2.0
$MgSO_4 \bullet 7\ H_2O$	0.5
KH_2PO_4	1.0
Agar	15.0
$Fe(NO_3)_3 \bullet 9\ H_2O$	0.0002
$ZnSO_4 \bullet 7\ H_2O$	0.0002
$MnSO_4 \bullet H_2O$	0.0001
Vitamins (per L)	
Thiamine-HCL (Vit.B_1)	100 µg
Biotin	5 µg

Combine all ingredients to an Erlenmeyer flask and top up to 1 L with ddH_2O. Autoclave at 121°C for 20 minutes; place in refrigerator for storage.

3) Murashinge and Skoog Medium (MS) pH 6 (Stocker-Wörgötter 2001)

Ingredients for 1L medium (g/L)

Malt extract	2.0
Caseine hydrolysate	2.0
Mannitol	20.0
Sucrose	40.0
Agar	18.0
Murashige mineral salts	0.001

Note: Sigma-Aldrich sells numerous murashige mineral salt mixtures.
Combine all ingredients to an Erlenmeyer flask and top up to 1 L with ddH_2O.
Autoclave at 121°C for 20 minutes; place in refrigerator for storage.

4) Sabouraud 2% and 4% Glucose (Dextrose) Agar Media (Stocker-Wörgötter and Hager 2008)

Ingredients for 1L medium (g/L)

Polypeptone (50:50 meat and cheese)	10.0
Glucose	20.0
Agar	18.0

Note: For 4.0% add 40.0 g of Glucose for 1L medium.
Sigma-Aldrich product numbers: Fluka 84086 (2%) and Fluka 84088 (4%).
Combine all ingredients to an Erlenmeyer flask and top up to 1 L with ddH_2O.
Autoclave at 121°C for 15 minutes; place in refrigerator for storage.

5) Potato Dextrose Agar Medium (PDA) (Stocker-Wörgötter and Hager 2008)

Ingredients for 1L medium (g/L)	
PDA (sigma-Aldrich 2182)	39.0

Pour PDA into an Erlenmeyer flask and top up to 1 L with ddH_2O. pH may be adjusted to alternative pH.
Autoclave at 121°C for 15 minutes; place in refrigerator for storage.

6) Georg Modified Lilly and Barnett Medium (G-LBM) [Modified by Georg Brunauer from Lilly and Barnett (1951) personal communication 2011]

Ingredients for 1L medium (g/L)	
D-Glucose	35.0
Mannitol	5.0
L-Asparagine	1.6
$MgSO_4 \bullet 7\ H_2O$	0.4
KH_2PO_4	0.8
CaCl	0.2
Cu-Acetate	0.2
Agar	14.0
NaCl	0.004
$Fe(NO_3)_3 \bullet 9\ H_2O$	0.0002
$ZnSO_4 \bullet 7\ H_2O$	0.0002
$MnSO_4 \bullet H_2O$	0.0001
Vitamins (per L)	
Thiamine-HCL (Vit.B_1)	100 µg
Biotin	5 µg

Combine all ingredients to an Erlenmeyer flask and top up to 1 L with ddH_2O.
Autoclave at 121°C for 20 minutes; place in refrigerator for storage.

Appendix B: ***Recipes for photobiont culture media***

1) Modified Woods Hole MBL (WHM) (Modified from Nichols 1973)

Macronutrients (g/L)	
$CaCl_2 \bullet 2\ H_2O$	36.76
$MgSO_4 \bullet 7\ H_2O$	36.96
$NaHCO_3$	12.60
K_2HPO_4	8.71
$NaNO_3$	85.01
$Na_2SiO_3 \bullet 9\ H_2O$	28.42
Micronutrients (g/L)	
$Na_2 \bullet EDTA$	4.36
$FeCl_3 \bullet 6\ H_2O$	3.15
$CuSO_4 \bullet 5\ H_2O$	0.01
$ZnSO_4 \bullet 7\ H_2O$	0.022
$CoCl_2 \bullet 6\ H_2O$	0.01
$MnCl_2 \bullet 4\ H_2O$	0.18
$Na_2MoO_4 \bullet 2\ H_2O$	0.006
Vitamins (per L)	
Thiamine • HCL	0.1 mg
Biotin	0.5 µg
Cyanocobalamin	0.5 µg

Note: Must make each macronutrient, micronutrient and vitamin individually (1L or fraction of 1L) then autoclave and place in refrigerator for storage.

For making 600 mL of media in Erlenmeyer flask add:

9.0 g of agar
0.6 mL of each macro- and micronutrient
0.6 mL Thiamine • HCL
1 drop of both Biotin and Cyanocobalamin
Top up flask to 600 mL with ddH_2O

Autoclave at 100°C for 20 minutes and place in refrigerator for storage.

2) Modified Bolds Basal Medium (BBM; *BBM + Soil Extract) (Modified from Deason and Bold 1960)

Macronutrients (g/400mL; sonicate to mix)	
$NaNO_3$	10.0
K_2HPO_4	3.0
$CaCl_2 \bullet 2\ H_2O$	1.0
$MgSO_4 \bullet 7\ H_2O$	3.0
KH_2PO_4	7.0
NaCl	1.0
Micronutrients (g/L)	
KOH + EDTA	50.0 + 31.0
$FeSO_4 \bullet 7\ H_2O$	4.98
- the water used for $FeSO_4 \bullet 7\ H_2O$ must be acidified with 1 mL H_2SO_4/L	
H_3BO_3	11.42
Salts (g)	
$ZnSO_4 \bullet 7\ H_2O$	8.82
MoO_3	0.71
$Co(NO_3) \bullet 6\ H_2O$	0.49
$MnCl_2 \bullet 7\ H_2O$	1.44
$CuSO_4 \bullet 5\ H_2O$	1.57

Notes: each of the salts are combined to the same flask or bottle along with 1L of H_2O.

Must make each macronutrient and micronutrient individually then autoclave and place in refrigerator for storage.

For making 600 mL of media in Erlenmeyer flask add:

9.0 g of agar

6.0 mL of each macronutrient

0.6 mL of each micronutrient

Top up flask to 600 mL with ddH_2O (* for BBM + Soil extract medium add 24 mL of soil extract before toping off ddH_2O)

Autoclave at 121°C for 20 minutes then place in refrigerator for storage.

3) Optimal Haematococcus Medium (OHM) (Fábregas et al. 2000)

Macronutrients (mg/L)	
KNO_3	410.00
Na_2HPO_4	30.00
$MgSO_4 \bullet 7\ H_2O$	246.50
$CaCl_2 \bullet 2\ H_2O$	110.90
Micronutrients (mg/L)	
$FeC_6H_5O_7 \bullet 5H_2O$	2.620
$CoCl_2 \bullet 6\ H_2O$	0.011
$CuSO_4 \bullet 5\ H_2O$	0.012
Cr_2O_3	0.076
$MnCl_2 \bullet 4H_2O$	0.989
$Na_2MoO_4 \bullet 2H_2O$	0.120
SeO_2	0.005
Vitamins (µg/mL)	
Thiamine	17.5
Biotin	25.0
B_{12}	15.0

Note: Must make each macronutrient, micronutrient and vitamin individually (1L or fraction of 1L) then autoclave and place in refrigerator for storage.

For making 600 mL of media in Erlenmeyer flask add:

9.0 g of agar
0.6 mL of each macro- and micronutrient
0.6 mL Thiamine
0.6 µL of both Biotin and B_{12}
Top up flask to 600 mL with ddH_2O

Autoclave at 100°C for 20 minutes and place in refrigerator for storage.