Studies on the variability in reindeer lichens: the evolution of common North American species and thallus resynthesis in *Cladonia rangiferina*

By

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ABSTRACT

Cladonia is one of the largest and widely distributed genera of lichen-forming fungi and some form the main winter diet for northern caribou and dominate the northern peatlands. Therefore, they are an ecologically important group of lichens. Ongoing climate change of the northern regions, impose challenges for lichen growth and algal interactions in these species. As in other organisms, genetic variability and phenotypic plasticity in the resynthesis of the lichen thallus will be important for their continued survival. The goal of my thesis is to better understand the variability of the reindeer lichens in an evolutionary and ecological context. Phylogenetic reconstruction (Chapter 2) together with genetic polymorphic studies with RAPD (Chapter 3) showed a lack of monophyly for 12 of 18 species of *Cladonia* and significant intraspecific genetic variation within the group. Genetic variation in the obligate fungal partner must also rely on the success of the symbiont interaction, which was further studied by resynthesis experiments. *In vitro* resynthesis experiments of *C. rangiferina* (Chapter 4) examined recognition- and defense-related genes of the symbionts of *C. rangiferina* using quantitative Polymerase Chain Reaction (qPCR) (Chapter 5) and suggested that communication between partners occurs and the survival and reestablishment of lichens in nature is affected by the presence of the compatible algal pool. This emphasizes the importance of finding the compatible algal partner for successful lichenization and suggests that parasitism and short-term interaction with incompatible algae may be a strategy allowing the fungus to survive until it finds a compatible algal partner. These studies also make parallels between the lichen symbiosis and plant pathogenic systems and provide molecular evidence for the parasitic nature of the lichen symbiosis. The collapse of the interaction between symbionts of *C. rangiferina* under varying temperature and pH conditions (Chapter 6) suggests the potential effect of habitat change on the

reestablishment of lichens in nature. In summary, my Ph.D. research showed the importance of genetic and symbiotic versatility in an ecologically important species, using a model species in the group *Cladonia* and raised new questions for other lichen symbioses relevant to their life styles and habitats.

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DEDICATION

I dedicate this thesis to my past and the future; my loving parents and my two sons. My dear Dad, even though you could not stay longer to share this joy, your blessings are always with me. My dear Mom, whatever success I have in my life, that is because of you. My loving sons, whatever steps I take towards success in the future will be for you. My past and future are meant so much to me because of you all.

The publication status of each chapter of the thesis, and the role of each coauthor in relevant chapters

Chapter	Author(s)	Contribution of each author	Article status
1	Sarangi N.P. Athukorala	S.A. wrote the Introduction and Literature review and searched all the literature relevant to the chapter. This Chapter has not been submitted for publication.	Not applicable
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CHAPTER 1

1.1 Introduction

Fungi are heterotrophic microorganisms, which depend on external sources of fixed carbon. They mobilize fixed carbon as saprotrophs, parasites, or through mutualistic associations (symbioses) with other living organisms. Lichens represent one of the most successful and common nutritional strategies of fungi and more than 10% of physiologically limiting terrestrial ecosystems for vascular plants is lichen-dominated (Honegger 1998). A lichen is a mutualistic association of a fungus (mycobiont) with one or more photosynthetic partners (photobiont), which is either a green alga, a cyanobacterium or, sometimes both. The ecological fitness of both partners is believed to increase by forming a symbiotic state as a result of lichenization (Smith and Douglas 1987). Lichens play a significant role in many ecosystems. They have the ability to colonize bare rock and soil and serve as the pioneer species colonizing newly exposed surfaces, or they may be late successional species in a habitat forming the dominant species in climax forests such as the northern lichen woodlands. The lichens that incorporate nitrogen-fixing cyanobacteria within the thallus provide sources of nitrogen for newly exposed or disturbed surfaces. In winter, lichens may form some of the major food sources for reindeer and caribou. In addition, birds may use some lichens as nesting materials and small animals may use lichen surfaces for camouflage (Brodo et al. 2001). Furthermore, many lichens can be sensitive to air pollution and both direct thallus measurements and community compositions are used as air pollution indicators (Nash III 2010). Medicinal properties, perfume and cosmetic properties, colouring and dyes, and food are many practical uses of lichens, which are based on their secondary metabolite composition (Brodo et al. 2001).

Reindeer lichens form extensive ground cover in northern peatlands and woodlands, which cover 10% of the earth's surface north of 45 degrees north latitude. The continued success of the reindeer lichens is vital to preservation of many of the peatlands, the animals that use them, and the amelioration of climate change. An understanding of ecological and evolutionary diversity in the reindeer lichens would contribute to a better understanding of their role in northern ecosystems. While species evolution in the reindeer lichens has been studied in southern hemisphere species (Ruoss and Ahti 1989), little is known about the evolutionary relationships of the more common northern hemisphere species or their genetic variability and reproductive success. Genetic variability and phenotypic versatility would be important for the continued survival of these and other species. The reindeer lichens can regenerate through thallus fragments but dispersal may rely largely on wind-blown fungal spores. The spores must come into contact with a compatible algal cell before formation of the obligate lichen association. Investigations of algal choice by the fungal spore have been examined in an evolutionary and ecological context (Piercey-Normore 2004; Piercey-Normore 2006; reviewed in Werth 2010) showing that the fungal and algal genera were specific to one another but there was no species specificity. An understanding of the cellular interactions began with Ahmadjian (1962) and others, who showed that the mechanisms of the interactions may be species specific. Few studies have shown the consequences of incompatible algal interactions where these have resulted in the absence of a symbiosis (Joneson and Lutzoni 2009) or parasitism (Jacobs and Ahmadjian 1971; Ahmadjian et al. 1978; Ahmadjian and Jacobs 1983; Athukorala et al. 2014). Some genes involved in the interaction have also been examined (Trembley et al. 2002; Joneson et al. 2011; Miao et al. 2012; Junttila et al. 2013). Controlled lab experiments have begun to investigate these interaction mechanisms but the development of the symbiosis in nature is exposed to a vast array of

microhabitat conditions. An investigation of the development of the symbiosis in dominant and ecologically important species, such as the reindeer lichens, is needed to better understand their success.

The general goal of this Ph. D. project was to better understand the variability and success of the reindeer lichens in an evolutionary and ecological context. This Ph. D. project reconstructed phylogenetic relationships among the more common northern species in three taxonomic sections of *Cladonia*, it examined whether morphological and genetic diversity of the fungal partner of two species can explain their success and wide distribution in northern ecosystems, and it investigated how algal availability and environmental conditions may affect the initial stages of establishment of the lichen association, which will ultimately affect the success, survivability, and the distribution of the species. The literature review begins with an overview of symbioses and coevolution and then more specific descriptions of the lichen symbiosis, the morphology of lichens, their symbionts, and the cellular interaction. It then focuses on the study group, its taxonomy, the genetic diversity of lichen forming fungi, and research advancements in isolation techniques for the two symbiotic partners. The chapter concludes with research goals and specific objectives of the Ph. D. thesis.

1.2 Literature Review

1.2.1 Symbiosis and evolution

The prolonged and intimate interaction between individuals of two (or sometimes more) different species is described as a symbiosis (Honegger 1998). Symbioses may be comprised of a range of different types of interactions where mutualism and parasitism are two extreme lifestyles of the symbiotic continuum and considered "extremely specialized" conditions (Thompson 1994). Specialization refers to the restriction of a species to a narrow range of resources resulting from evolutionary processes (Ferry-Graham et al. 2002). Specialization between two species in a parasitic interaction may develop if the symbionts can withstand physiological changes from defense responses that allow the parasite to coevolve with the host (Thompson 1994). Specialization and coevolution may also be extended to those symbionts in a mutualistic interaction. A long-term interaction between two species along the symbiosis continuum producing reciprocal evolutionary changes is termed "coevolution" (Brown and Wilson 1956; Thompson 1994).

Although no inherent direction in the evolution of mutualistic interations has been postulated, mutualism appears to have originated from parasitism (Thompson 1982; Lutzoni et al. 2001). The evolution of increased defences developed by the host, and/or other traits developed by the parasite that are not detrimental to the host, might have reduced the degree of parasitism between two species. As documented for many symbioses, the ecological outcome of the interaction spans a continuum between mutualism and parasitism over time and space depending on the traits of the symbionts and the ambient environmental condition (Thompson 1988; Cushman and Whitham 1989; Cushman and Addicott 1991; Bronstein 2001). Frequent transitions between different lifestyles by symbionts occurred during evolution and similar

genetic machinery can be used to mediate parasitism and mutualism (Gargas et al. 1995; Ewald 2004; Sachs and Simms 2006). According to those studies, the fitness of the two interacting species not only depends on its own genotype (trait) but also on the distribution of genotypes (traits) in the other species. As a result, a change in a particular genotype in one species triggers a subsequent adaptive change in the traits in the other species, which is a genotype-genotype interaction (Wade 2003). For plant host-parasite systems, Flor (1955) proposed the "gene-for-gene" model, where for each resistant gene in the host; the presence of an avirulent gene in the pathogen was hypothesized. Compatibility between corresponding genes results in resistance, and incompatibility results in the disease. Another system, which is responsible for pathogenesis in some groups of fungi, is referred to as host-specific toxins (HST), In HST the gene product of the host is the direct (or indirect) receptor of the toxin produced by the fungus (Wolpert et al. 2002; Friesen et al. 2008). Here, the compatibility between products of the corresponding genes results in the disease while incompatibility results in resistance. Freeman and Rodriguez (1993), and Redman et al. (1999, 2001) showed that a disrupted virulence gene in the plant pathogenic fungus *Colletotrichum* produced a series of non-pathogenic, symbiotic mutants that express either mutualistic, commensal, or intermediate mutualistic lifestyles. Similarly, the matching allele-model proposed a "self-non-self recognition" mechanism in animal immune systems (Grosberg and Hart 2000), and infection occurs when alleles of the parasite match with corresponding alleles of the host (Agrawal and Lively 2002).

In addition, nonreciprocal selection exerted by the environment on an interaction (genotype-by-genotype-by-environment), and by the environmental conditions on each symbiont (genotype-by-environment) can alter the strength and direction of the coevolution (Thompson 1994, 2005; Thrall et al. 2006; Wolinska and King 2009). Nonreciprocal adaptations of species

in a particular interaction for both biotic and abiotic environmental factors have been demonstrated for both mutualistic (Bohrer et al. 2003; Saenz-Romero et al. 2006) and parasitic (Thomas and Blanford 2003; Mitchell and Read 2005) interactions. The rate, type of environmental change (biotic and/or abiotic) (Wolinska and King 2009), intensity, and biotic complexity (Thrall et al. 2006) can also affect the strength and direction of evolution. Thrall et al. (2006) suggested a shift from mutualism to parasitism with increasing environmental intensity. For example, the increase of nitrogen fertilizer facilitated the proliferation of less beneficial rhizobial and mycorrhizal strains (Johnson 1993; Corkidi et al. 2002; Denison and Kiers 2004), and increased disease incidence and severity (Smiley et al. 1996; Long et al. 2000). A dominant grass, *Andropogon gerardii,* was highly responsive to mycorrhizal fungi when derived from nutrient poor sites where those from nutrient-rich sites showed less response to the mycorrhizal association (Schultz et al. 2001). High food availability was suggested to either favour parasitism by increasing the host virulence (Forde et al. 2008), or reduce parasitism by increasing host defences as observed in rhizobia and mycorrhizal systems (Schwartz and Hoeksema 1998). An increase in temperature was thought to be responsible for an increase in parasitism (Thomas and Blanford 2003). In terms of biotic complexity, higher pathogen diversity is predicted to increase the virulence of the pathogen (de Roode et al. 2002), while higher symbiont diversity is thought to reduce the strength of the mutualism (Dwyer et al. 1990; Bever and Simms 2000). Furthermore, interaction between barley growth and aphid reproduction was affected by genotypes of the partners, and also by the presence of *Rhizobacterium* in the soil (biotic environmental factor) (Tétard-Jones et al. 2007).

Internal physiological conditions and life histories of symbionts (influenced by host age, reproductive status, gender) along with previous environmental selection (Mitchell and Read

2005; Little et al. 2007), and epidemiological dynamics (host density, efficiency of transmission) can also affect the strength and direction of the interaction. For example, several resistant genes in plants are only expressed at certain developmental stages (Kus et al. 2002), and many systems exhibit male, or female-biased infection rates (Sheridan et al. 2000). Low transmission rates, and strictly vertical transmission, appeared to favour less virulent parasite genotypes, whereas high transmission rates and horizontal transmission favoured more virulent parasite genotypes (Anderson and May 1982; Ewald 1983). The effect of reciprocal and nonreciprocal selection in the evolution of a particular interaction depends on the trait under investigation (Laine 2007). Some fitness traits might be more important than others in a given system of interacting symbionts (Wolinska and King 2009). Further evidence for a transition between mutualism and parasitism is provided by a comparison of phylogenetic trees as the population and community structure of specific lineages changes over evolutionary time (Pellmyr and Thompson 1992; Thompson and Pellmyr 1992). Phylogenetic comparisons have included different nutritional strategies of fungi showing a number of transitions between the strategies and some leading to lichenized lineages (Gargas et al. 1995; Lutzoni et al. 2001, 2004).

1.2.2 The lichen symbiosis

Lichens are mutualistic associations formed between fungi (mycobiont), green algae and/or cyanobacteria (photobiont). The formation of a lichen (lichenization) is one of the most successful nutritional strategies of fungi, with about 20% of all known species of fungi being lichenized (Hawksworth 1988). Lichens are taxonomically classified as fungi and most of the lichen forming fungi belong to the Ascomycetes with a few lichenized Basidiomycetes (Hawksworth et al. 1995). There are more than 15000 species of lichen fungi, which belong to

about 1000 genera (Kirk et al. 2008). About 98% of these species are ascomycetes (Honegger 2008). The species name of the lichen is the species name of the mycobiont involved (Greuter 1988) as most of the lichen thallus is made of fungal tissue. Lichen-forming fungi are a polyphyletic and taxonomically heterogenous group of nutritional specialists (Smith 1978; Hawksworth 1988; Honegger 1991a; 1996; Gargas et al. 1995; Nash III 2010). Most of the mycobionts are ecologically obligate and physiologically facultative biotrophs and can be cultured (Honegger 1998). Lichenized fungal hyphae do not cytologically differ from non-lichenized fungi (Honegger and Bartnicki-Garcia 1991), but they differ from all other fungi in the formation of complex, persistent vegetative thalli. In pure culture, the mycobiont forms thallus-like colonies, which do not demonstrate any similarity to its naturally occurring symbiotic phenotype. The colony is usually composed of cartilaginous, conglutinate hyphal masses in the centre and filamentous growth with aerial hyphae forming at the periphery (Ahmadjian 1973; Honegger and Bartnicki-Garcia 1991; Stocker-Wörgötter 1995).

The photobiont has its own name according to algal taxonomy and is included in algal phylogenies. About 40 genera and 100 species of lichen photobionts have been described (Tschermak-Woess 1988; Büdel 1992; Nyati et al. 2007; Werth 2011). About 85% of lichen-forming fungi are associated with green algae and are called “chlorolichens”, while about 10% of lichen-forming fungi are associated with cyanobacteria and are called “cyanolichens”. About 4-5% of lichens has both green algae and cyanobacteria and are called “cephalodiate” lichens. The cyanobacterial partner, in such cases, is located in structures called cephalodia. The heterocysts produced by cyanobacteria are capable of fixing nitrogen giving the lichens containing them the ability to fix nitrogen. Green algal photobionts have been identified to species level in only 5% of lichens (Friedl and Büdel 2008) and the identity of cyanobacteria at the species level has been

even more uncommon. All green algal photobionts known at present belong to the division Chlorophyta, and more specifically, the Trebouxiophyceae and Ulvophyceae, which are phylogenetically distantly related. Most of the green algal photobionts are reported from Trebouxiophyceae, within which green algal photobionts of lichens have been postulated to arise from at least six independent origins (Friedl and Büdel 2008). The most common photobiont genera include *Trebouxia, Trentepohlia* and *Nostoc,* while species of *Trebouxia* (which previously included *Asterochloris*) are most common (Tschermak-Woess 1988). The green algal genus *Trebouxia* s.*l*. is associated with about 20% of lichen fungi (Nash III 2010). The morphology of the lichen thallus is thought to be directly influenced by the photobiont (Armaleo and Clerc 1990). Studies on the diversity of photobiont species associated with a genus or lichenized ascomycete family have shown a limited number of species of *Trebouxia* or *Asterochloris*. However, the algal genetic diversity associated with a single fungal species has been considerable (Dahlkild et al. 2001; Piercey-Normore and DePriest 2001; Opanowicz and Grube 2004; Cordeiro et al. 2005; Hauck et al. 2007). The genus of photobiont was used as a taxonomic marker in delimiting suborders and families within the fungal order Leconorales (Peršoh et al. 2004) but the photobiont species is widely shared among many fungal species (Dahlkild et al. 2001; Piercey-Normore and DePriest 2001; Opanowicz and Grube 2004; Cordeiro et al. 2005; Hauck et al. 2007). An ecological advantage of sharing different photobionts is choosing the alga that is best adapted for a habitat. Photobiont sharing would suggest that horizontal transfer of algal species between fungal species occurred throughout evolutionary history.

1.2.3 Lichen evolution and climate change

The evolutionary origins of lichenization have not been unidirectional and are hypothesized to have occurred and been lost multiple times within fungi (Gargas et al.1995; Lutzoni and Vigalys 1995; Lutzoni et al. 2001, 2004; James et al. 2006; Schoch et al. 2009). Gorbushina et al. (2005) and Brunauer et al. (2007) showed that rock-inhabiting fungi can develop cellular contacts with algal cells isolated from lichen thalli in culture, which suggested that some lineages of rock-inhabiting fungi may have evolved lichenization with free-living algae. The origin of rock-inhabiting fungi and their allies might have occurred by loss of lichenization in an ancestral lineage in the early evolutionary history of *Chaetothyriales*, with limited abilities for symbiotic interactions with algae (Lutzoni et al. 2001). Specific traits gained by rock inhabiting ancestors that lived under extreme environments might be shared by descendants, and explain several independent lifestyle transitions between saprotroph, parasitism, and mutualism (Gueiden et al. 2008). Haustoria and appressoria observed in lichen thalli (Ahmadjian and Jacobs 1983) also support a hypothesis of transition between lifestyles. Deeply penetrating haustoria were observed in less organized thalli (intracellular), while in well organized thalli with complex tissues they were limited to the algal cell wall (intraparietal) (Webber and Webber 1970), which could be interpreted as a modification of a parasitic interaction through evolution with stabilization of the interaction in the more complex and derived thalli organized into tissues.

The effect of climatic change has been postulated on lichen diversity and distribution. A decrease in the extent and biomass of fruticose lichens has been revealed over recent decades in north-western Alaska, which may be representative of changes in other areas in the Arctic (Shaver and Jonasson 1999; Joly et al. 2009). Climate change has been suggested to be one of

the primary factors that cause lichen decline (Joly et al. 2009). Jandt et al. (2008) reported a decline in lichen cover from 20% to 6% on transects where no other disturbances to lichens such as fires and intense caribou grazing were observed. Inferences on climate change and experimental studies predict that atmospheric warming and drying will negatively affect lichens and mosses (Chapin et al. 1995; Robinson et al. 1998; Cornelissen et al. 2001; van Wijk et al. 2003; Epstein et al. 2004; Hollister et al. 2005; Walker et al. 2006; Wiedermann et al. 2007). One negative effect may be evident in the symbiosis where Peksa and Skaloud (2011) suggested that the occurrence of ecological guilds and the algal distribution in *Asterochloris*-associating lichens is independent of the mycobiont, but dependent on the biotic (lichen community) and abiotic (substrate type and climate) environmental factors. The environment is also suggested to influence the morphology of the lichen thallus by exerting different selective pressures (Pintado et al. 1997). Although lichens are pioneers of harsh environments, in the harshest of these environments where the rainfall is less than 120 mm per year, lichen biodiversity and spatial density is heavily reduced (Golubkova and Zogt 1974). In such cases, free-living fungal communities are more frequently observed than lichens on rock surfaces and within the upper rock layers (Krumbein and Jens 1981, Staley et al. 1982, Taylor-George et al. 1983). Gorbushina et al. (2005) suggests that only specifically adapted xerophilic free-living fungi can survive in the harshest environments while lichens cannot be formed due to the rapid abortion of the propagules before the symbiotic contact. Although the role of the environment in the success of the association has been postulated (Gorbushina et al. 2005), experimental studies to understand the mechanisms of how environmental factors affect the establishment of the lichen association are lacking. Ahmadjian (1987) reported that lichens grown under suboptimal growth conditions resulted in deeply penetrated haustoria and underdevelopment of lichen thalli under certain

nutritional and environmental conditions due to unbalanced growth of either symbiont. Temperatures and pH levels were observed to affect photosynthetic pigments, chlorophyll degradation and cell membrane integrity of the photobiont (Hájek et al. 2006; Pisani et al. 2007) and photobiont growth (Bačkor et al. 1998). The effect of culture conditions on secondary metabolite production has also been observed to differ depending on the growing conditions (Stocker-Wörgötter 2008).

Studies on the evolutionary origin, gene regulation, and factors affecting the formation of a lichen are rare, scattered over a different geographical areas and sometimes their conclusions are inconsistent. There are many gaps in knowledge, and outcomes vary with the species under investigation. Since the role of environmental change has been repeatedly shown in other symbiotic systems (Bohrer et al. 2003; Thomas and Blanford 2003; Mitchell et al. 2005; Saenz-Romero et al. 2006), the environmental effect on development of the lichen symbiosis should be investigated. The reindeer lichens are very successful in northern regions and are ecologically important species in habitat preservation and the food chain. An understanding of their biology and the factors affecting their survival may facilitate their use as indicators of climate change since they are thriving species in the northern hemisphere where the effects of climate change are first experienced (Symon et al. 2005).

1.2.4 Morphology and anatomy of the symbiotic phenotype

Approximately 45% of lichen-forming fungi form a morphologically and anatomically distinct phenotype when they associate with an algal partner, which is very different from their aposymbiotic form growing alone in culture (Ahmadjian 1967; Honegger and Bartnicki-Garcia 1991). The thallus is mainly composed of the mycobiont (Nash III 2010). Based on the thallus

appearance, lichens are divided into three morphological growth forms, namely, crustose, foliose and fruticose. Thalli of crustose lichens are firmly attached to the substrate over the entire thallus resulting in no lower side. Foliose lichen thalli are generally leaf-like with an upper and lower surface that is attached to a substrate by specialized attachment organs. Fruticose lichens have hair-like, strap-shaped, stalked, or shrubby thalli, which are round in section and are attached to the substrate at the base. The mycobiont and the photobiont are arranged in the thallus so that the photosynthetic efficiency of the photobiont and growth rate of the lichen is maximized (Nash III 2010). Based on the arrangement of the symbionts, there are two types of thalli, a homoiomerous (nonstratified) and heteromerous (stratified) thallus. In homoiomerous thalli the mycobiont and the photobiont are evenly distributed. This type can be found in thin crustose lichens, gelatinous crustose and foliose lichens. Stratified thalli are organized into an upper cortex, photobiont layer, medulla, and sometimes a lower cortex. The upper cortex contains densely arranged hyphae of the mycobiont. The major part of the thallus is occupied by the medulla, which contains loosely interwoven hyphae with numerous airspaces. The photobiont layer is found in the upper part of the medullary layer. Thick-walled conglutinated hyphae, which form the supporting tissue, can be found in the medullary layer of some fruticose lichens. This tissue can consist of irregularly arranged hyphal strands, a central cylinder, or a central, thread-like elastic strand (Nash III 2010). A lower cortex is also found in many foliose lichens (Nash III 2010). The complexity and diversity of the thallus structure is the result of the interaction between the mycobiont and photobiont for each species.

1.2.5 Interaction in the lichen symbiosis

The effect a particular organism in a community exerts on another organism in the community may be defined as "biological interactions". From an ecological perspective, biological interactions may occur between individuals of the same species (intraspecific), or between species (interspecific). The prolonged and intimate interactions between individuals of two (or sometimes more) species is described as a symbiosis (Honegger 1998), which is one of the major interspecific interactions that drive ecological function and evolutionary processes in a community. The outcome of a symbiosis may be parasitism, commensalism, or mutualism. In parasitic interactions, one organism benefits and reduces the fitness of the other, whereas in mutualistic interactions, both species benefit (Thompson 1994; Thrall et al. 2006). In commensalism one of the species benefits while the other is unaffected. Despite the contrasting outcome, symbiotic interactions exhibit common features such as asymmetries in body size of partners, deep phylogenetic separation of the interacting species, and high degrees of genetic specialization (Thompson 1994). Specialization refers to the restriction of a species to a narrow range of resources influenced by evolutionary processes (Graham et al. 2002) and resulting from a long-term interaction between two species producing reciprocal evolutionary changes, which are termed "coevolution" (Brown and Wilson 1956; Thompson 1994). The symbiosis that forms the lichen has been hypothesized to have coevolved (Ahmadjian 1987).

The separation of the alga and fungus from the natural lichen association in pure cultures and the subsequent recombining of the two symbionts is called resynthesis of the lichen. Studies on the resynthesis between lichen symbionts in culture showed that re-lichenization occurred only with certain photobionts. Other photobionts and non-lichenized algae were killed during the interaction (Ahmadjian and Jacobs 1981) or showed no change in their fitness (Joneson and

Lutzoni 2009; Athukorala et al. 2014). Fitness traits in the lichen association, including reduced virulence of the fungus, loss of sexuality, and slow growth of the symbionts and the absence of free-living symbionts, suggest a long-term coevolution in the system (review Ahmadjian 1987). However, Piercey-Normore and Depriest (2001) did not observe coordinated speciation in the symbionts of the genus *Cladonia*, and suggest a shift from specialization to generalization of symbionts through natural selection as described by Law's (1985) theory ("mutualistic environments tend to undergo evolutionary improvements that prevent host specificity"), while Werth and Scheidegger (2012) observed non-random association of fungal and algal genotypes in *Lobaria pulmonaria,* supporting coordinated adaptation between symbionts. Studies show varied results where coevolution is supported by some genera but not by others. Studies on the symbionts within the genus *Cladonia* suggest high levels of algal sharing among fungal species with some evidence to support the choice of photobiont based on several factors including habitat and genetic compatibility (Piercey-Normore 2004; Yahr et al. 2004; Piercey-Normore 2006).

*1.2.6 The study group, reindeer lichens (*Cladonia *sections* Crustaceae, Impexae, *and* Subtenuis*)*

The Family, Cladoniaceae, of the Order, Lecanorales, is one of the largest and diverse families of lichen-forming fungi with about 500 currently recognized species (Ahti 2000; Stenroos et al. 2002). *Cladonia* is a widely distributed genus of lichen-forming fungi, consisting of slightly more than 400 species, and the former genus "Cladina" (the reindeer lichens) has been subsumed within *Cladonia* (Ahti and DePriest 2001). Reindeer lichens are most abundant in continental cold climates (Miller 1955). They are abundant in boreal and austral regions, that is, in the circumpolar coniferous belt of the Northern Hemisphere and in the *Nothofagus* regions in

the Southern Hemisphere. In addition, they can be found in iceless Arctic and Antarctic regions. However, they may be locally common in the lowlands of the temperate regions and subtropical and tropical regions. They cannot efficiently compete with mosses and vascular plants, and therefore, they are rare in rich moist soils (Ahti and Hepburn 1967). The identification of reindeer lichens in *Cladonia* sections *Cladina, Impexae,* and *Subtenuis* (Ahti 1993) is primarily based on morphological characters such as having a crustose primary thallus (which is not observed in many species since it disappears as the thallus develops podetia; Ahti 2000) and lack of a cortex on the podetia. The thallus of podetia is richly branched and the branching pattern has a seasonal rhythm where a new ramification is produced each year (Stenroos et al. 2002). The branched upright thallus is called a podetium, and each branching podetial tip contains a pycnidium or a tiny apothecium.

1.2.7 Taxonomy of the Genus Cladonia

The status of *Cladina* was controversial until 2001 when it was subsumed into the genus *Cladonia*. In the Americas, Asia, Australasia, and Russia, *Cladina* was treated as a genus, while in Europe it was considered as a subgenus within *Cladonia* (Ruoss and Ahti 1989; Stenroos et al. 1997). Hyvönen et al. (1995), Stenroos et al. (1997), De Priest et al. (2000) and Ahti and DePriest (2001) demonstrated the paraphyly of *Cladina* relative to *Cladonia*. *Cladina* was included in the section *Perviae*, together with *Unciales* (Mattick 1938, 1940). Later studies revealed it should be grouped with members of the *Unciales* (Ahti 1984; Ruoss and Ahti 1989). According to Ahti (1993) there are three sections within the genus *Cladina*, which are *Cladina*, *Tenues*, and *Impexae*. The delimitation of these groups has been done based on chemical characters, such as perlatolic acid (*Impexae*), or a red pigment in the conidiomata (*Tenues*, Ahti

1984; Huovinen and Ahti 1986). However, with the increase of the addition of new species, only a very few attempts have been made with molecular methods to understand the infrageneric classification of the genus *Cladonia* s. lat. including *Cladina,* until the study by Stenroos et al. (2002) was performed. In this study, *Cladina* appeared as a monophyletic group within *Cladonia*. Furthermore, it is related to groups of the current *Uniciales* and more distantly to *Perviae* and *Coccifereae*. Stenroos et al. (2002) identified two major clades inside *Cladina*, one encompassing members of the *Impexae* plus *Cladonia delavayi* (a species that is morphologically similar to *Cladina*, but was placed in the *Unciales*). The other clade contained the species representing sections *Cladina* and *Tenues*. However, neither of these two sections was monophyletic since their members are intermixed. Even though this study used only 1 to 3 specimens for each species, it was not able to solve relationships at the termini of the tree or differentiate between *Cladonia arbuscula* and *C. mitis*. Furthermore, *C. densissima* was intermixed with the *C. mitis*-*C. arbuscula* group. Stenroos et al. (2002) also stated that *C. rangiferina* is highly variable within a small number of specimens, i.e. two species. In order to examine the level of variability present within some of these species, there is a need to re-examine this group with larger sample sizes and more variable markers.

1.2.8 Genetic diversity of Cladonia

The species circumscription of *Cladonia* was traditionally based on morphological characters and the application of molecular techniques has revealed that a direct correlation between morphological and molecular-based species delimitation is not always present (Myllys et al. 2003; Kotelko and Piercey-Normore 2010; Piercey-Normore et al. 2010; Pino-Bodas et al. 2012a, b). In addition, incongruence between different gene phylogenies created for a particular

species is also common (Myllys et al. 2003; Fontaine et al. 2010; Steinová et al. 2013). This incongruence between morphology and molecular data has been explained by significant intraspecific variation within a species in response to varying environmental conditions or recombination (Fontaine et al. 2010; Kotelko and Piercey-Normore 2010), pseudogenes, gene paralogy, horizontal gene transfer, incomplete lineage sorting (ILS), and hybridization (Steinová et al. 2013). The presence of more than one individual or species in the same podetium was also used to explain some of the variation (Kotelko and Piercey-Normore 2010). Intraspecifc genetic variation in lichens has been reported by some recent molecular studies (Zoller et al. 1999; Lindblom and Ekman 2006; Fernández-Mendoza et al. 2011). Although molecular phylogenetic analyses may resolve some of the taxonomic problems, they provide little information about the genetic variation within a single population (De Priest 1993; Beard and De Priest 1996; Robertson and Piercey-Normore 2007). Combination of both phylogeny and genetic structure within a species will provide broader insights into the genetic diversity of the species and will facilitate interpretation of the incongruence observed in gene phylogenies. Knowledge of the genetic structure of a species will further enhance our understanding of the adaptive potential of a particular species in changing environments (Mitton and Grant 1984; Wildt et al. 1987; Hedrick and Miller 1992).

Even though DNA-based molecular taxonomy and phylogeny of lichen-forming fungi has become frequent, molecular studies to examine the genetic structure at the population level are still uncommon (review Werth 2010). Among the techniques used for population genetic studies in lichen fungi, universal fingerprinting techniques such as Inter Simple Sequence Repeats (ISSR) (Cassie and Piercey-Normore 2008), Random Amplified Polymorphic DNA (RAPD) (Murtagh et al. 1999; Dyer et al. 2001), or Restriction Fragment Length Polymorphisms

(RFLP) (Piercey-Normore 2006) have been common. Fungal specific PCR-based markers that have been used for both phylogeny and population genetic studies on lichen-forming fungi are suggested as potential barcoding markers which include the small and large subunits, the internal transcribed spacer (ITS) and the intergenic spacer (IGS) of nuclear ribosomal DNA, the mitochondrial cytochrome c oxidase subunit 1, two subunits of RNA polymerase II, a maintenance protein, and the elongation factor (Printzen et al. 2003; Opanowicz and Grube 2004; Piercey-Normore 2004, 2006; Yahr et al. 2006; Robertson and Piercey-Normore 2007; Kelly et al. 2011; Schoch and Seifert 2012; Pinos-Bodas et al. 2013). Simple sequence repeats (i.e. microsatellites and minisatellites) seem to be promising markers for population genetics of lichenized fungi (Walser et al. 2005; Werth et al. 2006, 2007) due to their high polymorphism (review Werth 2010). Genetic diversity studies have been performed on only a small number of lichen-forming fungal species (review Werth 2010) and entire transcriptomes have been sequenced in *Cladonia rangiferina* (Juntilla and Rudd 2012). Axenic cultures of the fungus are needed for more reliable data since DNA isolated from the thallus also contains DNA from the algal partner and other microorganisms living in and on the thallus (reviewed in Werth 2010). Isolation and maintenance of the fungal cultures is a tedious process and when it involves a large number of cultures especially for a population genetic study, it becomes even more challenging.

1.2.9 Controlled parasitic interactions between lichen symbionts

In most natural lichen thalli, each algal cell is connected to a fungal hypha by appressoria and hasutoria to facilitate the mobilization of soluble carbohydrates of the alga to the fungus, to provide water and dissolved mineral nutrients to the alga by passive apoplastic transport and the movement of algal cells within the thallus for optimal gas exchange and illumination (Honegger

1985, 1991a, b). The morphology of the fungal-algal interface can vary depending on the type of lichen. In the majority of lichens including *Cladonia*, the fungal hypha does not pierce the cell wall of the alga and are called intraparietal haustoria (Honegger 1985, 1986, 1991a). "Wall-to-wall adhering is found in micro- and macrolichens with the alga belonging to the genus *Coccomyxa, Elliptochloris* (Brunner and Honegger 1985; review Honegger 1991b), *Myrmecia* and *Dictyochloropsis* (reviews Honegger 1990, 1991b, 1992). The relatively primitive crustose lichens contain transparietal (intracellular) haustoria, which pierce the cell wall and are in contact with the plasma membranes of the algal cells (reviews Honegger 1984, 1986, 1993).

Compatibility and selectivity/specificity are thought to determine the success of a lichenization between two symbionts. The specificity is the "taxonomic range of the acceptable partners". The selectivity is the "frequency of association between compatible partners" (Rambold et al. 1998; Yahr et al. 2004). Varying degrees of selectivity have been shown by lichen fungi, which includes selection for specific *Trebouxia* strains (Blaha et al. 2006; Guzow-Krzemińska 2006) but restricted selection of one or two strains (Ohmura et al. 2006; Hauck et al. 2007) have been reported. *In vitro* resynthesis of lichens has been successful only with the symbionts isolated from the same lichen from which they were originally isolated, and algae obtained from other lichens and sources were killed (Webber and Webber 1970; Jacobs and Ahmadjian 1971; Ahmadjian et al. 1978; Ahmadjian and Jacobs 1981; Ott 1987b; Ott et al. 1995; Schaper and Ott 2003; Meeβen et al. 2013), which suggested that there should be a compatibility between two partners for the lichenization to occur. Both resynthesis studies (Ahmadjian and Jacobs 1981) and field observations (Ott 1987b) suggested that the germ tubes and other free hyphae of the fungal partner can survive by forming a pre-thallus with

incompatible algal cells until they find a compatible alga. However, further development of the thallus requires a compatible alga (Honegger 1993).

The process by which the mycobiont recognizes or selects the compatible photobiont or the characteristics of a photobiont, which make it compatible, are not yet fully understood in the lichen system and are under intense investigation. Many past and recent studies have demonstrated the role of lectins i.e. arginase or ariginase-like proteins produced by the mycobiont and glycosylated urease produced by the photobiont in the recognition process of compatible partners (Molina et al. 1995, 1998; Legaz et al. 2004; Sacristán et al. 2007). Urease, deposited on algal cell walls, acts as a receptor (ligand) for the mycobiont derived lectin molecules preventing lectins from entering the algal cell (Millanes et al. 2004). Incompatible algal cells lack urease on their cell walls; and therefore, lectins enter the algal cell. The entry of the lectin increases the concentration of putrescine, which disorganizes the chloroplast structure and activates glucanases. Glucanases hydrolyse cell walls and release the algal protoplast, leading to a parasitic interaction. Urease on cell walls of compatible algal cells confines lectins to cell walls leaving the concentration of putrescine unchanged. This results in the survival of algal cell (Molina et al. 1995, 1998) leading to a mutualistic interaction. Therefore, recognition between symbionts via arginase-like proteins and urease receptors can be identified as one of many factors which determines the type of interaction (whether a mutualism/controlled parasitism or parasitism as in a pathogenic interaction) of a particular mycobiont when in contact with a certain photobiont. Symbiont recognition might actually turn on an array of defense reactions in the photobiont, which limit the mycobiont to the cell wall (Ahmadjian and Jacobs 1983). This model provides chemical evidence for the parasitic nature of the interaction observed in the lichen association.

The current model describing the early events in host-pathogen interactions identifies the recognition between pathogen derived chemical compounds (elicitors) and host derived compounds (receptors) as the most important early event that determines the type of plant-pathogenic interaction (Ebel and Cosio 1994). Under proper recognition of the pathogen by the host, the host turns on defense responses, which kill or limit the growth of the pathogen. In the absence of defense responses, the pathogen continues to invade and kill the host. The role of genetic factors (gene-for-gene interactions) in pathogenicity has been documented for many plant-pathogenic systems (Dixon and Lamb 1990; Keen 1990; van Kan 1991). However, the study of genes and gene expression of lichen systems is in its infancy. Only few recent studies have attempted to understand the molecular mechanisms underlying the interaction in lichens *Baeomyces rufus* (Trembley et al. 2002), and *Cladonia grayi* (Joneson et al. 2011). These studies observed the differential gene expression patterns of the mycobiont in its symbiotic and aposymbiotic stages but they did not focus on recognition and defense gene expression in particular. The studies also showed that the resynthesis stages have different time periods for each species and are specific to laboratory conditions. In this regard, the resynthesis and identification of different stages of lichenization of the study species is the preliminary step before subsequent investigations can be performed.

1.2.10 Isolation of symbionts and in vitro resynthesis of lichen thalli

Isolation and *in vitro* culturing of fungal and algal partners from lichen thalli began as early as the 1880s (Bonnier 1887, 1889; Stahl 1877). Ahmadjian (1966, 1973) later developed advanced culturing techniques, especially for the fungal partner, where he introduced the "spore-rain method", where isolation of the fungus could be achieved from apothecia (Ahmadjian

1973). The isolation of cultures from other structures such as soredia (Armaleo and May 2009) and thallus fragments (Yamamoto et al. 1985; Armaleo 1991; Yamamoto et al. 1993) were developed much later. With the advancement of culturing techniques, there have been many reports of the culturing of both fungal and algal symbionts (Oliver et al. 1989; Jahns 1993; Crittenden et al. 1995; Stocker-Wörgötter 2001, 2002; Joneson et al. 2009; Sangvichien et al. 2011; McDonald et al. 2013). However, culturing lichen symbionts still remains a difficult task. Culture conditions that have been optimized for symbionts from one lichen (Stocker-Wörgötter1991; Crittenden et al. 1995; Bubrick 1998; Sangvichien et al. 2011) may not be suitable for another lichen since each species has different nutrient requirements and specific incubation conditions (McDonald et al. 2013). Pure cultures are very difficult to obtain since endolichenic fungi and external fungal and bacterial contaminants cannot be eliminated easily (Arnold et al. 2009; U'Ren et al. 2010). In addition, with the obligate nature of the lichen symbiosis, some lichen fungi are not possible to culture under laboratory conditions (McDonald et al. 2013). The lichen fungi are slow growing in culture and are easily overgrown by faster growing contaminants. Maintenance of cultures is difficult since they should be physically disrupted to stimulate the growth (Ahmadjian 1973) after they enter into a static state. This process increases the chance of introducing contaminants (McDonald et al. 2013). While the culturing of species of *Cladonia* requires precautions as with other lichens, it is not as difficult as physiologically obligate species in genera such as *Peltigera* or some cyanobacterial lichens.

An understanding of the cellular events that occur during the formation of the lichen thallus requires *in vitro* and *in situ* thallus resynthesis (Ahmadjian et al. 1980; Bubrick et al. 1985; Ott 1987b; Galun and Garty 1988; Stocker-Wörgötter and Türk 1991; Jahns 1993; Kon et al. 1993; Stocker-Wörgötter 1995; Shaper and Ott 2003; Joneson and Lutzoni 2009) of the

symbionts after the lichen symbionts have been separated in culture. As a result, five basic stages in species of *Xanthoria* and *Cladonia* that lead to a stratified lichen thallus have been described as follows; "pre-contact" where the symbionts are not in physical contact but are in close proximity allowing them to share extracellular excretions, the "contact stage" where two symbionts are starting to make physical contacts by appresoria and/or haustoria, the "growth together stage" where the symbionts are growing together to form cellular masses containing both symbionts, the "transitional pre-thallus stage" to form an undifferentiated thallus-like structure, and finally the stratified thallus (Ahmadjian et al. 1978; Galun 1988; Armaleo 1991).

1.2.11 Cladonia rangiferina *as a model system to study the nature of the interaction of the symbionts in the lichen association*

Cladonia rangiferina (L.) Weber ex F.H.Wigg, the gray reindeer lichen, is a fruticose lichen distributed in the northern ecosystems. Its fungal partner is *C. rangiferina* and the algal partner is *Asterochloris* sp. *C. rangiferina* has a relatively fast growth rate (5.2 mm/year (Andreev 1954)), and abundant apothecia, making it suitable for laboratory studies. In addition, recognition that *C. rangiferina* and other reindeer lichens, as winter food for caribou and reindeer, necessitates a better understanding of its biology in order to develop conservation strategies since significant effects of climate change on species of northern ecosystems have been identified. The use of a particular lichen species as a model system to study the interaction between symbionts requires the development and optimization of isolation and resynthesis conditions for each species. There have been two previous attempts to resynthesize *C. rangiferina* under *in vitro* conditions (Jahns 1993; Stocker-Wörgötter 1995) but resynthesis stages were not documented using the same terminology and timing of stages as more recent

resynthesis studies on other species. Jahns (1993) observed "algae containing lumps of tissues" during the first 7 weeks, which might resemble the "contact" and/or "growth together" stage as described by Joneson et al. (2011). In addition, Stocker-Wörgötter (1995) reported that isolation experiments with *C. rangiferina* were not as successful as they were with other species studied. Resynthesis was performed on undefined media such as clay (Jahns 1993) or media amended with soil extracts (Stocker-Wörgötter 1995). These media are not suitable for reproducibility of experiments since the media composition may change depending on the source of clay and soil. Therefore, resynthesis stages for *C. rangiferina* should be defined before further experiments can be conducted.

1.3 Goal and Objectives

To examine variability among the reindeer lichens in an effort to understand their survivability, this research project incorporated five objectives which contribute to five chapters in the thesis. The specific objectives of each chapter and a brief description of the research approaches are:

1. To reconstruct the phylogenetic relationships among selected species in *Cladonia* sections *Crustaceae*, *Tenues,* and *Impexae* to examine species delimitations, specifically those in northern North America. To achieve this objective, we propose to perform pylogenetic and haplotype analyses of fungal ITS rDNA and mt*SSU* sequences from 36 speciens obtained in this study (belonging to 11 species) and 58 accessioned DNA sequences retrieved from NCBI GenBank.
2. To further examine growth and genetic variation among two common and apotheciate North American reindeer lichen species, *Cladonia arbuscula s. l.* and *C. rangiferina.* For

this purpose, we compare genetic variation and colony growth among colonies grown from ascospores of the same apothecia, different apothecia, and different specimens of *C. arbuscula s.l.* and *C. rangiferina.*

3. To further explore the mechanisms of the interaction in *C. rangiferina*, one of the non-monophyletic, widespread and successful species. We achieve this objective by:
    a. isolating pure cultures of two symbionts from *C. rangiferina*,
    b. defining the first three stages of resynthesis, and
    c. comparing the first three stages of resynthesis between *C. rangiferina* and each of *Asterochloris glomerata/irregularis* (native lichen alga; compatible interaction), *Coccomyxa peltigerae* (native non-lichen alga; incompatible interaction), and *Chloroidium ellipsoideum* (foreign non-lichen alga; incompatible interaction).
4. To compare the difference in recognition- and defense-related gene expression in response to culture extracts (containing soluble elicitors) from compatible and incompatible interactions at each of three resynthesis stages. We achieve this objective by using quantitative PCR to examine whether early recognition in the compatible interactions will initiate defense responses.
5. To investigate how different growing conditions affect the algal and fungal symbionts and thereby the establishment of the lichen, *Cladonia rangiferina.* For this purpose we determine the effect of different temperature and pH conditions on the two symbionts and the success of their interaction under laboratory conditions during the resynthesis of *C. rangiferina.*

CHAPTER 2

Phylogenetic relationships among reindeer lichens of North America

2.1 Abstract

Cladonia is one of the largest lichen-forming ascomycete genera, which was formerly divided into ten sections, including *Crustaceae* (*Cladina*), *Tenues*, and *Impexae*. While previous studies elucidated the relationships among species and sections, they assumed monophyly of many species, so usually only one or a few specimens of each species were included in the analysis. While monophyly may suggest low levels of intraspecies variation, the absence of monophyly may suggest speciation or interbreeding among species. This study examined monophyly of some members of sections *Crustaceae, Tenues*, and *Impexae*, using the internal transcribed spacer region of the nuclear ribosomal DNA (ITS rDNA) and mitochondrial small subunit gene of the mitochondrial ribosomal DNA (mtSSU). Haplotype networks and AMOVA were also used to examine non-monophyletic species pairs. The phylogenetic tree contained four clades, two representing those belonging to section *Impexae*, one representing species that belong to sections *Crustaceae* and *Tenues*, and one clade basal to these clades. Five of 18 species; *C. pycnoclada*, *C. stellaris*, *C. evansii, C. cilliata* and *C. subtenuis* showed monophyly in the phylogenetic tree. The thallus branching pattern was interpreted as a heritable character using the mtSSU network. Three pairs of paraphyletic species were further examined using haplotype networks and AMOVA analysis. The results for the species pairs showed some mixing of haplotypes but the AMOVA analysis provided support for species differentiation within the pairs. While evidence supports distinct species to some extent, further study is needed to conclusively show separate species in these pairs.

2.2 Introduction

Cladonia is the largest genus in the lichen-forming fungal family Cladoniaceae consisting of about 515 accepted species (Ahti Jan 2014, unpubl.). The genus *Cladonia* was divided into seven taxonomic sections and three sections were recognized in the segregate genus *Cladina* (Ahti 2000; Ahti and DePriest 2001). *Cladina* is most abundant in continental cold climates but they are also abundant in the coniferous belt of the Northern hemisphere and in the *Nothofagus* regions in the Southern hemisphere. There are few records in warm temperate, hot or desert climates (Ahti 1961) where they cannot efficiently compete with mosses and vascular plants and are rare in rich moist soils (Ahti and Hepburn 1967). While this lack of competitive ability with plants is well known, the reindeer lichens have adapted to the niches uninhabited by vascular plants and bryophytes. Some species, such as *C. arbuscula, C. rangiferina, C. stygia*, and *C. stellaris,* are important components of northern ecosystems, where they provide vast areas of ground cover (Auclair and Rencz 1982; Shaver and Chapin 1991), and form a major component of the winter food for caribou and reindeer (Svihus and Holand 2000; den Herder et al. 2003). Knowledge of their species status, would inform ecosystem management and maintenance of biodiversity.

In recent times, the genus *Cladina* has not been recognized by most authors, because in phylogenetic studies it is nested within *Cladonia* (Stenroos et al. 2002). The group has been referred to as the reindeer lichens, i.e., *Cladonia*, sections *Crustaceae* (= *Cladina* section *Cladina*), *Tenues,* and *Impexae*, which typically have highly branched ecorticate podetia and have a disappearing crustose primary thallus. Phylogenetic relationships among all sections of *Cladonia* have been examined previously (Stenroos et al. 1997; Stenroos et al. 2002), but usually only one to a few specimens of each species were included in the analysis, making it necessary to

assume that most species were monophyletic. Indeed, Stenroos et al. (2002) recommended studying a larger number of specimens for each species, and while earlier studies were focused on species identification (Ruoss 1987a, b; Ruoss and Ahti 1989) recent studies show relationships among distant geographic collections of *Cladonia arbuscula* in the broad sense (Myllys et al. 2003; Piercey-Normore et al. 2010). Monophyly and diagnosability are considered to be important criteria for species delimitation (e.g. Bacon et al. 2012) but divergence of species may not be complete for many recognized species. While monophyly may suggest low levels of intraspecies variation, the absence of monophyly may suggest speciation, incomplete lineage sorting, or interbreeding among species (Funk and Omland 2003).

The goal of this study was to reconstruct the phylogenetic relationships among selected species in *Cladonia* sections *Crustaceae, Tenues,* and *Impexae* to examine species delimitations.

2.3 Materials and Methods

2.3.1 Lichen and mycobiont material

Lichen specimens were collected from Canada, borrowed from herbaria, and additional sequences were obtained from NCBI GenBank (Table 2.1). All collected specimen samples were air dried for 12 hours and those used for obtaining cultures were stored at 4 ˚C until processing within one month. Representative specimens are deposited in the University of Manitoba Herbarium (WIN). Other specimens used in the phylogenetic analysis were either obtained from the Botanical Museum, Finnish Museum of Natural History, Helsinki (H) or collected in North America (Table 2.1).

2.3.2 DNA extraction and amplification

For the phylogenetic analysis pieces of dry thalli (10 – 20 mg) from the apical region of each lichen sample were selected and visually inspected for contaminating debris. For samples Athukorala 7, Athukorala 12, Athukorala 13, Athukorala 17, Athukorala 22, Athukorala 24 and Normore 9468 three month old colonies of spore cultures (grown on malt yeast agar at 20 ˚C in the dark following Yoshimura et al. 2002) were used for DNA extraction. The DNA was isolated using a modified CTAB (hexadecytrimethylammonium bromide) protocol (Grube et al. 1995). The polymerase chain reaction (PCR) of fungal DNA on the Internal transcribed spacer 1 and 2 (ITS1 and ITS2) and the 5.8S of the nuclear ribosomal DNA (rDNA) was performed using the primers 1780F-5' (Piercey-Normore and DePriest 2001) and ITS2KL-3' (Lohtander et al. 1998), and on the mitochondrial small subunit (mtSSU DNA) using the primers mrSSU2 and mrSSU3R (Zoller et al. 1999). Where there were problems amplifying across both ITS regions for some samples, the primers, ITS1F, ITS2, ITS3 and ITS4 (White et al. 1990) were used to amplify the ITS region in two fragments. PCR reaction mixtures (20 µl) contained 20 ng of template DNA, 1X PCR buffer (50 mM KCl, 20 mM Tris), 0.5 µM of each forward and reverse primer, 3.0 mM of $MgCl_2$ (2.0 mM $MgCl_2$ for mtSSU), 200 mM of each dNTP (Invitrogen Life Technologies, CA, USA), and 0.1 U of Taq polymerase (Invitrogen Life Technologies, CA, USA). Amplification was carried out in a Biometra® TGradient thermocycler (American Laboratory Trading, Inc., CT, USA). The PCR conditions were as follows: initial denaturation at 95 ˚C for 5 min; 30 cycles of denaturation at 95 ˚C for 1 min, annealing at 54 ˚C for 1 min, and extension at 72 ˚C for 1 min 30 sec for all primers. For samples, which had difficulties with PCR amplification, a touchdown cycle was used as follows: initial denaturation at 95 ˚C for 5 min; 1

Table 2.1 Collection numbers, location information, and accession numbers for species of *Cladonia* and *Cladia* used in this study.

Section and species	Source: collection location, year, and collection number	Analysis: (Accession no. for ITS and mtSSU)
Genus *Cladia*		
Cladia aggregata s. lat.	New Zealand (Stenroos et al. 2002)	ITS: AF453268
Genus *Cladonia*		
Section *unknown*		
C. wainioi	Canada, Newfoundland; 2009, Normore 8805 (WIN)	ITS:KP001177
C. wainioi	Canada, Newfoundland; 2009, Normore 8807 (WIN)	ITS:KP001178
Section *Crustaceae*		
C. arbuscula	Canada, Manitoba; 2009, Athukorala 9 (WIN)	mtSSU:KP001224
C. arbuscula	Canada, Manitoba; 2009, Normore 9461 (WIN)	ITS: KP001204 mtSSU:KP001225
C. arbuscula	Canada, Manitoba; 2005, Normore 5073 (WIN)	mtSSU:KP001229
C. arbuscula	Canada, Manitoba; 2009, Athukorala 13 (WIN)	ITS :KP001208
C. arbuscula **(mixed with *C. mitis)***	Canada, Manitoba; 2009, Athukorala 12 (WIN)	ITS :KP001209
C. arbuscula	Canada, Manitoba; 2009, Athukorala 7 (WIN)	ITS :KP001207
C. arbuscula	Finland (Myllys et al. 2003)	ITS: AY170789
C. arbuscula	Argentina, Tierra del Fuego (Myllys et al. 2003)	ITS: AY170787
C. arbuscula	USA, Georgia (Myllys et al. 2003)	ITS: AY170773
C. arbuscula	Finland; Myllys 208 (TUR) (Myllys et al. 2003)	ITS: AY170771
C. arbuscula	Finland; 1999, Stenroos 5203 (TUR) (Stenroos et al. 2002)	ITS: AF458293
C. arbuscula **subsp *beringiana***	USA, Alaska, Talbot and Solomeshch (WIN) (Piercey-Normore et al. 2010)	ITS: GU169280
C. arbuscula **subsp *beringiana***	USA, Alaska, Talbot and Schofield (WIN) (Piercey-Normore et al. 2010)	ITS: GU169281
C. arbuscula **subsp *beringiana***	Canada, British Columbia (WIN) (Piercey-Normore et al. 2010)	ITS: GU169283
C. arbuscula **subsp *beringiana***	USA, Alaska, Talbot and Schofield (WIN) (Piercey-Normore et al. 2010)	ITS: GU169284
C. arbuscula **subsp *beringiana***	USA, Alaska, Talbot and Schofield (WIN) (Piercey-Normore et al. 2010)	ITS: GU169285
C. arbuscula **subsp *beringiana***	Finland, Stjernberg (H) (Piercey-Normore et al. 2010)	ITS: GU169223
C. arbuscula **subsp *beringiana***	Canada, Manitoba, Ahti 62904 (Piercey-Normore et al. 2010)	ITS: GU169224
C. mitis **(mixed with *C. arbuscula*)**	Canada, Manitoba; 2009, Normore 9468 (WIN)	ITS :KP001206
C. mitis **(mixed with *C. arbuscula)***	Canada, Newfoundland; 2009, Normore 8804 (WIN)	ITS:KP001205 mtSSU:KP001223
C. mitis	Canada, Manitoba; 2001, Normore 1155 (WIN)	mtSSU:KP001228
C. mitis	Argentina, Tierra del Fuego; Stenroos 5381 (TUR) (Myllys et al. 2003)	ITS: AY170764
C. mitis	Argentina, Tierra del Fuego; Stenroos 5501 (TUR) (Myllys et al. 2003)	ITS: AY170759
C. mitis	Finland; Myllys 218 (TUR) (Myllys et al. 2003)	ITS: AY170792
C. mitis	Argentina, Tierra del Fuego; Stenroos 5307 (TUR)	ITS: AY170767

	(Myllys et al. 2003)	
C. mitis	Argentina, Tierra del Fuego; Stenroos 5331 (TUR) (Myllys et al. 2003)	ITS: AY170768
C. mitis	Argentina, Tierra del Fuego; Stenroos 5342 (TUR) (Myllys et al. 2003)	ITS: AY170769
C. mitis	Argentina, Tierra del Fuego; Stenroos 5336 (TUR) (Myllys et al. 2003)	ITS: AY170783
C. mitis	Canada, British Colombia (WIN) Piercey-Normore et al. 2010)	ITS: GU169274
C. mitis	Canada, Manitoba Ahti 62905 (WIN) Piercey-Normore et al. 2010)	ITS: GU169228
C. mitis	Canada, British Columbia, Wells Gray (WIN)	ITS: GU169266
C. mitis	Greenland Hansen Exs. 855 (H) Piercey-Normore et al. 2010)	ITS: GU169222
C. mediterranea	Portugal 2006, Burgaz (MCAB) (H)	mtSSU:KP001242
C. rangiferina s. lat.	Ahti 62933	mtSSU:KP001252
C. rangiferina s. lat	Canada, Manitoba; 2009, Normore 9469 (WIN)	ITS:KP001199
C. rangiferina s. lat	Canada, Manitoba; 2006, Normore 5278 (WIN)	mtSSU:KP001251
C. rangiferina s. lat	Canada, Manitoba; 2009, Normore 9462 (WIN)	ITS:KP001192
C. rangiferina s. lat	Canada, Ontario; 2006, Normore 6597 (WIN)	ITS:KP001194
C. rangiferina s. lat	Canada, Ontario; 2006, Normore 6767 (WIN)	ITS:KP001195
C. rangiferina s. lat	Canada, New Brunswick; 2006, Normore 6888 (WIN)	ITS:KP001191
C. rangiferina s. lat	Canada, Ontario; 2006, Normore 7202 (WIN)	ITS:KP001198
C. rangiferina s. lat	Canada, Ontario; 2006, Normore 7307 (WIN)	ITS:KP001193
C. rangiferina s. lat	USA, Alaska; 2006, Kotelko 1043 (WIN)	ITS:KP001197
C. rangiferina s. lat	Canada, Manitoba; 2009, Athukorala 17 (WIN)	ITS :KP001202
C. rangiferina s. lat	Canada, Manitoba; 2009, Athukorala 22 (WIN)	ITS :KP001200
C. rangiferina s. lat	Canada, Manitoba; 2009, Athukorala 24 (WIN)	ITS :KP001201
C. rangiferina s. lat	China, CH050184 (Wei et al. 2008)	ITS: EU266113
C. rangiferina s. lat	USA, Washington (Smith et al. 2012)	ITS: JQ695918
C. rangiferina s. lat	USA, Mima Mounds, Class 2, McCune 31122 (Smith et al. 2012)	ITS: JQ695920
C. rangiferina s. lat	Finland; Stenroos 5173 (TUR) (Myllys et al. 2003)	ITS: AF458306
C. rangiferina* s. *lat	Guyana; 1997, Stenroos 4867 (TUR) (Stenroos et al. 2002)	ITS: AF458307
C. rangiferina s. lat	Sweden; Wedin 5081 (BM) (Lumbsch et al. 2004)	mtSSU: AY300881
C. rangiferina s. lat	India; 2000, Sinha 1643 (H)	ITS:KP001190
C. stygia	Canada, Manitoba; 2009, Normore 9466 (WIN)	mtSSU:KP001249
C. stygia	Canada, Manitoba; 2009, Normore 9465 (WIN)	mtSSU:KP001248
C. stygia	Canada, Newfoundland; 2009, Normore 8810 (WIN)	ITS:KP001186
C. stygia	Canada, Newfoundland; 2009, Normore 8811 (WIN)	ITS:KP001187 mtSSU:KP001250
C. stygia	Canada, Newfoundland; 2006, Normore 7079 (WIN)	ITS:KP001196
C. stygia	Canada, Nova Scotia, Normore 6905 (WIN)	ITS:KP001188
C. stygia	Canada, Newfoundland; 2006, Normore 7111 (WIN)	ITS:KP001185
C. stygia	Canada, Manitoba; 2007, Normore 7674 (WIN)	ITS:KP001184
C. stygia	Canada, Yukon, 2006, Kotelko 1038 (WIN)	ITS:KP001182
C. stygia	USA, Alaska, Kotelko 1044 (WIN)	ITS:KP001183
C. stygia	USA, Alaska, Kotelko 1090A (WIN)	ITS:KP001189
C. stygia	Finland (Stenroos et al. 2002)	ITS: AF458308
C. submitis	USA, New Jersey; 2004, Lendemer1803 (PH) (H)	ITS:KP001218 mtSSU:KP001230
C. argentea	Guyana, 1997 Stenroos 4918 (TUR) (Stenroos et al. 2002)	ITS: AF458305

C. dendroides	Guyana, 1997 Stenroos 4897 (TUR) (Stenroos et al. 2002)	ITS: AF458295
C. rotundata	Guyana, 1997 Stenroos 4854 (TUR) (Stenroos et al. 2002)	ITS: AF457913
C. densissima	Guyana, 1997 Stenroos 4899 (TUR) (Stenroos et al. 2002)	ITS: AF458294
Section *Impexae*		
C. confusa	Bolivia; 2004, Flakus 4568 (KRA) (H)	mtSSU:KP001234
C. confusa	Bolivia; 2004, Flakus 4645 (KRA) (H)	mtSSU:KP001235
C. confusa	Brazil, Minas Gerais,; 1997, Stenroos 5091 (TUR) (Stenroos et al. 2002)	ITS: AF458296
C. delavayi	Bhutan; 1998, Sùchting 8498 (H) (Stenroos et al. 2002)	ITS: AF458304
C. evansii	USA, Georgia; 2009, Lendemer 21090 (NY) (H)	ITS:KP001203 mtSSU:KP001232
C. evansii	USA, Georgia; 2009, Lendemer 21623 (NY) (H)	mtSSU:KP001233
C. evansii	USA, Georgia; 2010, Lendemer 22296 (NY) (H)	mtSSU:KP001231
C. evansii	USA, Georgia; 1999, Ahti 58302 (Stenroos et al. 2002)	ITS: AF458303
C. evansii	USA, Florida; (Yahr et al. 2004)	ITS: AY753590
C. portentosa	Denmark; 2007, Corfixen, Chjristensen, Hansen Lich. Danici Exs. No. 511 (H)	ITS:KP001216 mtSSU:KP001221
C. portentosa	Lithuania; 2008, Ahti 68573 (H)	ITS:KP001214 mtSSU:KP001222
C. portentosa	Spain, 2008, Burgaz s. n. 6 (MACB) (H)	ITS:KP001215 mtSSU:KP001220
C. portentosa	Spain, 2008, Burgaz s. n. 7 (MACB) (H)	ITS:KP001213 mtSSU:KP001219
C. portentosa	USA, Washington (Smith et al. 2012)	ITS: JQ695923
C. portentosa	USA, Washington (Smith et al. 2012)	ITS: JQ695922
C. portentosa	Germany; 1999, Thell 9930 (UTR) (Stenroos et al. 2002)	ITS: AF458302
C. portentosa	USA, Washington (Smith et al. 2012)	ITS: JQ695921
C. pycnoclada	Chile 1999; Feuerer 60275 (Stenroos et al. 2002)	ITS: AF458298
C. pycnoclada	Chile 1999; Feuerer 60296 (Stenroos et al. 2002)	ITS: AF458299
C. pseudoevansii	USA, Alaska; 2008, Talbot and Schofield ADA127-X-01 (UBC) (AKFWS) (H)	mtSSU:KP001243
C. stellaris	Canada, Manitoba; 2009, Normore 9402 (WIN)	ITS:KP001210 mtSSU:KP001241
C. stellaris	Canada, Manitoba; 2009; Normore 9463 (WIN)	ITS:KP001211 mtSSU:KP001239
C. stellaris	Canada, Newfoundland; 2009, Normore 8808 (WIN)	ITS:KP001212 mtSSU:KP001238
C. stellaris	Canada, Manitoba; 2006, Normore 6496 (WIN)	mtSSU:KP001240
C. stellaris	Finland; 1997, Stenroos 5102 (TUR) (Stenroos et al. 2002)	ITS: AF458301
C. stellaris* var. *aberrans	USA, Alaska, Ahti 63717 (Piercey-Normore et al. 2010)	ITS: GU169229
C. stellaris	Canada, Manitoba; Ahti 63128a (Piercey-Normore et al. 2010)	ITS: GU169230
Section *Tenues*		
C. ciliata* var. *ciliata	Spain, 2008, Burgaz s. n. 1 (MACB) (H)	mtSSU:KP001247
C. ciliata* var. *tenuis	Portugal, 2006, Burgaz s. n. 3(MACB) (H)	mtSSU:KP001244
C. ciliata* var. *tenuis	Spain, 2006, Burgaz s. n. 2 (MACB) (H)	mtSSU:KP001245

C. ciliata* var. *tenuis	Spain, 2008, Burgaz s. n. 5 (MACB) (H)	mtSSU:KP001246
C. ciliata* var. *ciliata	USA, Washington (Smith et al. 2012)	ITS: JQ695926
C. ciliata* var. *tenuis	USA, Washington (Smith et al. 2012)	ITS: JQ695917
C. ciliata* var. *ciliata	USA, Washington (Smith et al. 2012)	ITS: JQ695924
C. ciliata* var. *tenuis	USA, Washington (Smith et al. 2012)	ITS: JQ695915
C. ciliata* var. *tenuis	Portugal; 1997, Ahti and Burgaz 55883 (H) (Stenroos et al. 2002)	ITS: AF458311
C. ciliata* var. *ciliata	Ireland; 2000, Rikkinen s.n. (TUR) (Stenroos et al. 2002)	ITS: AF458310
C. subtenuis	USA, Georgia; 2010, Lendemer 21941 (NY) (H)	ITS:KP001217 mtSSU:KP001237
C. subtenuis	USA, South Carolina; 2010, Lendemer 22118 (NY) (H)	mtSSU:KP001236
C. subtenuis	USA, Florida; 2001, Yahr 3695 (Yahr et al. 2006)	ITS: DQ482684
C. subtenuis	USA, Florida; 2002, Yahr 4087 (Yahr et al. 2006)	ITS: DQ482690
C. subtenuis	USA, South Carolina; 2003, Brown 1021987 (Yahr et al. 2006)	ITS: DQ482710
C. subtenuis	USA, South Carolina; 2003, Brown 1021989 (Yahr et al. 2006)	ITS: DQ482711
C. subtenuis	Canada, Nova Scotia; 1999, Ahti 57068 (H) Stenroos et al. (2002)	ITS: AF457911
C. subtenuis	USA, Missouri; 2002, Harris 46489 (Yahr et al. 2006)	ITS: DQ482696
C. subtenuis	USA, Pennsylvania; 2003, Yahr 4784 (Yahr et al. 2006)	ITS: DQ482706
C. terrae-novae	Canada, Newfoundland; 2009, Normore 8806 (WIN)	ITS:KP001180 mtSSU:KP001227
C. terrae-novae	Canada, Newfoundland; 2009, Normore 8809 (WIN)	ITS:KP001181 mtSSU:KP001226
C. terrae-novae	Canada, Newfoundland; 1999, Ahti and Scott 56942 (H) Stenroos et al. (2002)	ITS: AF458300
C. terrae-novae **(mixed with *C. confusa*)**	Bolivia; 2004, Flakus 4567 (KRA) (H)	ITS:KP001179

cycle of denaturation at 95 ˚C for 1 min, annealing at 60 ˚C for 1 min, and extension at 72 ˚C for 1 min 30 sec. Annealing temperature of the following 3 cycles was dropped by 2 ˚C at each cycle (58, 56, 54) followed by 26 cycles with an annealing temperature of 52 ˚C.

2.3.3 DNA sequencing and alignment

Four to six identical 50 μL reaction volumes of PCR product were pooled for DNA sequencing and gel purified using the Wizard ® SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA) following the manufacturer's instructions. Cycle sequencing reaction volumes were 20μl, containing 60 to 70 ng of purified DNA, BigDye V3.1 (Applied Biosystem, CA, USA) and the same PCR primers that were used for sequencing. Reactions were cleaned using the ethanol/EDTA precipitation Method (Applied Biosystem Handbook) according to the manufacturer's instructions. The dried product was dissolved in 20μL formamide and loaded into a 96-well plate for sequencing on a 3130 Genetic Analyser (Applied Biosystems, CA, USA). The sequences were edited using Sequencher® version 4.6 (Gene Codes Corporation, MI, USA). In addition, 55 accessioned DNA sequences were retrieved from NCBI GenBank, and were included in the phylogenetic analysis. All sequences were automatically aligned using the ClustalX (Jeanmougin et al. 1998) program and manually edited.

2.3.4 Data analysis

The fungal ITS1 and ITS2 rDNA phylogeny was inferred from 36 sequences from specimens obtained in this study (belonging to 11 species) and 58 accessioned DNA sequences retrieved from NCBI GenBank (Table 2.1) for a total of 94 sequences and 14 ingroup species. All nucleotide sequences generated in this study have been deposited in NCBI GenBank (Table

2.1). *Cladia aggregata* was assigned as the outgroup taxa because of its close phylogenetic relationship to *Cladonia* (Stenroos and DePriest 1998) but falling outside the genus *Cladonia*. *C. wainioi* was included in the ingroup because it is a member of the genus *Cladonia* but was shown to be basal by Stenroos et al. (2002). Aligned sequences were subjected to phylogenetic analyses using PAUP* 4.0b10 (Swofford 2003) and MrBayes v3.1.2 (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003). Phylogenetic determinations were based on maximum parsimony (MP) and Bayesian analyses. MP analyses were performed using tree bisection and reconnection branch swapping, heuristic searches with 1000 random addition replicates and bootstrap searches of 1000 resamplings (Felsenstein 1985). Bootstrap was performed using the MP option in PAUP, and values greater than 70 are reported in the phylogenies. For the Bayesian analysis, a six parameter hLRTs (Hierarchial Likelihood Ratio Tests) model (SYM+G) was applied with a gamma-shaped parameter and uniformly distributed proportion of invariable sites. The best model was estimated using Modeltest 3.7 (Posada and Crandall 1998). The Bayesian analysis was performed using 5,000,000 generations. The run was terminated when the standard deviation of split frequencies fell below 0.01. Haplotype analysis was performed using 38 mtSSU sequences belonging to 15 species and one mtSSU sequence obtained from GenBank with the TCS program version 1.21 (Clement et al. 2000) to construct a parsimony network. The parsimony probability criterion (Templeton et al. 1992) with gaps coded as a fifth character state was used in the analysis. Haplotype analyses using the same procedure were also conducted on three pairs of species (*C. rangiferina – C. stygia; C. arbuscula – C. mitis; and C. portentosa – C. terrae-novae*) using the ITS rDNA alignments with 472, 564 and 560 of aligned positions, respectively. An analysis of molecular variance (AMOVA) was conducted in GenAlEx ver. 6.5 (Peakall and Smouse 2012) with 999 permutations to determine the extent of gene flow among

species pairs. The same ITS rDNA alignments used for the haplotype networks were also used for AMOVA analysis.

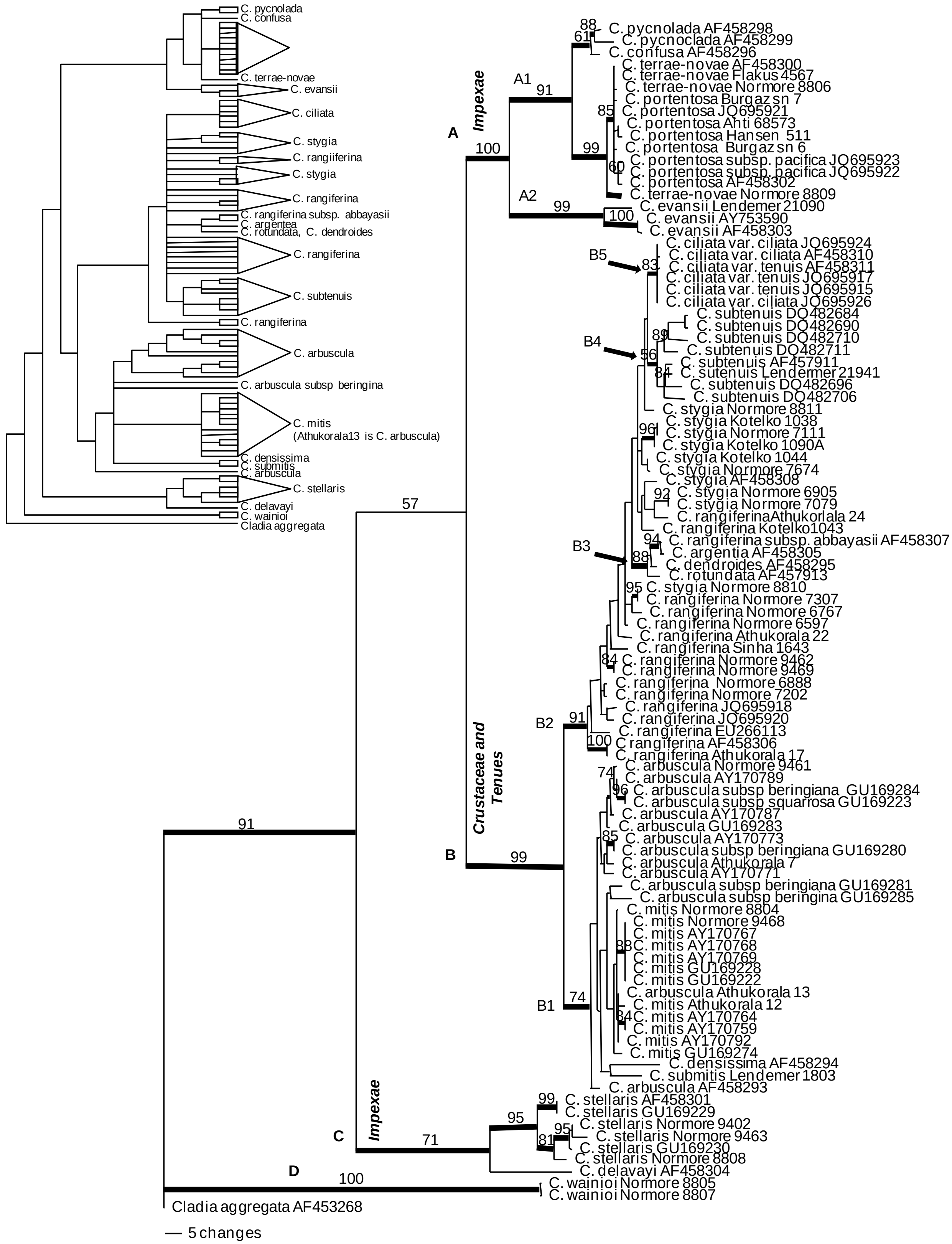
2.4 Results

2.4.1 Phylogenetic analysis

The ITS rDNA maximum parsimony analysis generated 100 most parsimonious trees. Ninty-nine taxa used in the analysis produced 638 aligned sites from which 178 were parsimony informative. The consistency index (CI) and retention index (RI) were 0.6081 and 0.8838, respectively. Bayesian analyses produced a tree with a topology that was consistent with that of the MP tree shown.

The phylogeny shows four major clades A, B, C, and D. Clades A and C represent the section *Impexae* where clade A is highly supported with bootstrap value of 100% and clade C has 71% bootstrap support (Fig. 2.1). Both clades A and C have more than 0.95 posterior probability support. Clade B represents sections *Crustaceae* and *Tenues* together, and is highly supported with 99% bootstrap and more than 0.95 posterior probability support. Clade D represents the single species *Cladonia wainioi*, which has 100% bootstrap support, and is basal to clades A and B. In clade A there are highly supported subclades with subclade A1 representing *C. pycnoclada*, *C. confusa, C. terrae-novae,* and *C. portentosa*. *C. pycnoclada* is the only species in this subclade that is monophyletic with 88% bootstrap support. *Cladonia evansii* alone forms subclade A2 with greater than 0.95 posterior probability and 99% bootstrap support, which has earlier been named section *Bicornutae*. Subclades A1 and A2 are sister clades with greater than 0.95 posterior probability 100% bootstrap support. Clade C contains a monophyletic species, *C. stellaris* (representing a group earlier named sect. *alpestris*), which is sister to the

Figure 2.1 One of 100 most parsimonious trees based on the ITS rDNA sequences showing relationships among species of *Cladonia* in sections *Crustaceae, Tenues*, and *Impexae*. Clades and subclades are discussed in the text. Values on the branches are bootstrap values greater than 70% and the thickened branches have posterior probability values greater than 0.95. The tree inserted in the upper left hand corner is the strict consensus tree showing the placement of each clade.


C. pycnolada
C. confusa
C. terrae-novae
C. evansii
C. ciliata
C. stygia
C. rangiiferina
C. stygia
C. rangiferina
C. rangiferina subsp. abbayasii
C. argentea
C. rotundata, C. dendroides
C. rangiferina
C. subtenuis
C. rangiferina
C. arbuscula
C. arbuscula subsp beringina
C. mitis
(Athukorala13 is C. arbuscula)
C. densissima
C. submitis
C. arbuscula
C. stellaris
C. delavayi
C. wainioi
Cladia aggregata
A
Impexae
A1
A2
B
Crustaceae and Tenues
B1
B2
B3
B4
B5
C
Impexae
D
C. pycnolada AF458298
C. pycnoclada AF458299
C. confusa AF458296
C. terrae-novae AF458300
C. terrae-novae Flakus 4567
C. terrae-novae Normore 8806
C. portentosa Burgaz sn 7
C. portentosa JQ695921
C. portentosa Ahti 68573
C. portentosa Hansen 511
C. portentosa Burgaz sn 6
C. portentosa subsp. pacifica JQ695923
C. portentosa subsp. pacifica JQ695922
C. portentosa AF458302
C. terrae-novae Normore 8809
C. evansii Lendemer 21090
C. evansii AY753590
C. evansii AF458303
C. ciliata var. ciliata JQ695924
C. ciliata var. ciliata AF458310
C. ciliata var. tenuis AF458311
C. ciliata var. tenuis JQ695917
C. ciliata var. tenuis JQ695915
C. ciliata var. ciliata JQ695926
C. subtenuis DQ482684
C. subtenuis DQ482690
C. subtenuis DQ482710
C. subtenuis DQ482711
C. subtenuis AF457911
C. sutenuis Lendermer 21941
C. subtenuis DQ482696
C. subtenuis DQ482706
C. stygia Normore 8811
C. stygia Kotelko 1038
C. stygia Normore 7111
C. stygia Kotelko 1090A
C. stygia Kotelko 1044
C. stygia Normore 7674
C. stygia AF458308
C. stygia Normore 6905
C. stygia Normore 7079
C. rangiferinaAthukorlala 24
C. rangiferina Kotelko1043
C. rangiferina subsp. abbayasii AF458307
C. argentia AF458305
C. dendroides AF458295
C. rotundata AF457913
C. stygia Normore 8810
C. rangiferina Normore 7307
C. rangiferina Normore 6767
C. rangiferina Normore 6597
C. rangiferina Athukorala 22
C. rangiferina Sinha 1643
C. rangiferina Normore 9462
C. rangiferina Normore 9469
C. rangiferina Normore 6888
C. rangiferina Normore 7202
C. rangiferina JQ695918
C. rangiferina JQ695920
C. rangiferina EU266113
C rangiferina AF458306
C. rangiferina Athukorala 17
C. arbuscula Normore 9461
C. arbuscula AY170789
C. arbuscula subsp beringiana GU169284
C. arbuscula subsp squarrosa GU169223
C. arbuscula AY170787
C. arbuscula GU169283
C. arbuscula AY170773
C. arbuscula subsp beringiana GU169280
C. arbuscula Athukorala 7
C. arbuscula AY170771
C. arbuscula subsp beringiana GU169281
C. arbuscula subsp beringina GU169285
C. mitis Normore 8804
C. mitis Normore 9468
C. mitis AY170767
C. mitis AY170768
C. mitis AY170769
C. mitis GU169228
C. mitis GU169222
C. arbuscula Athukorala 13
C. mitis Athukorala 12
C. mitis AY170764
C. mitis AY170759
C. mitis AY170792
C. mitis GU169274
C. densissima AF458294
C. submitis Lendemer 1803
C. arbuscula AF458293
C. stellaris AF458301
C. stellaris GU169229
C. stellaris Normore 9402
C. stellaris Normore 9463
C. stellaris GU169230
C. stellaris Normore 8808
C. delavayi AF458304
C. wainioi Normore 8805
C. wainioi Normore 8807
Cladia aggregata AF453268
5 changes

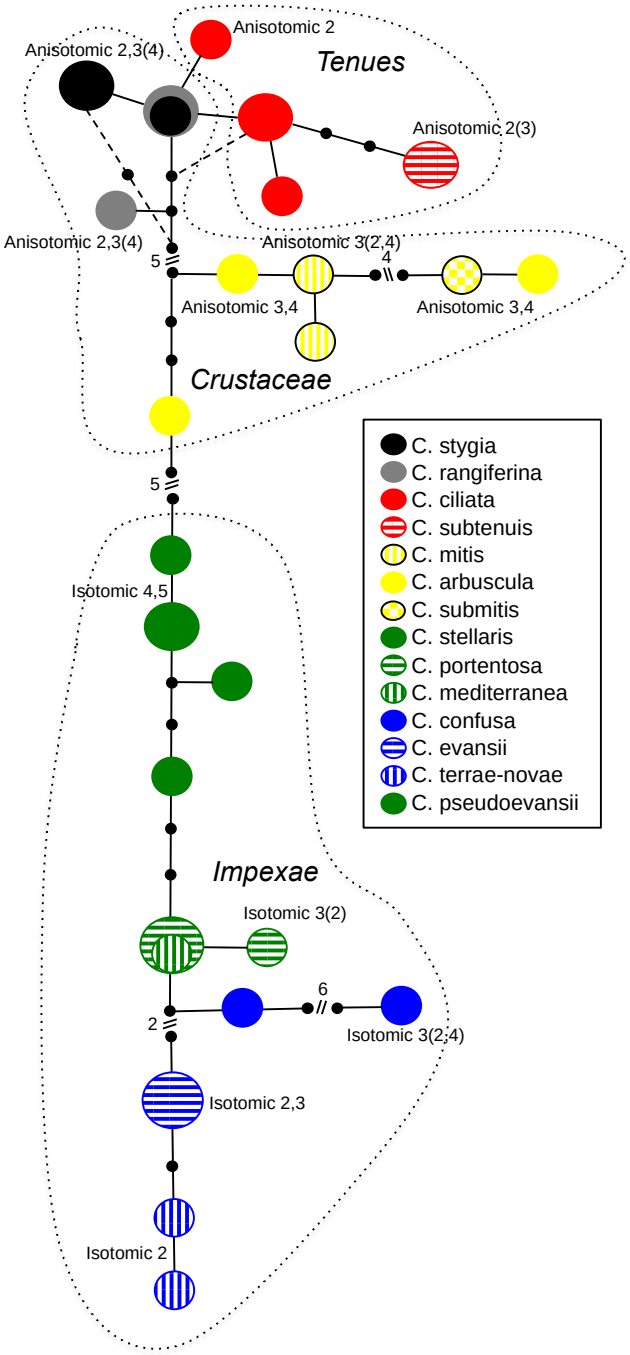
only specimen of *C. delavayi*. *C. delavayi* (Himalayan) is very different from *C. stellaris* in morphology and chemistry, which is supported by the ITS rDNA sequence difference.

Clade B supports species from both sections, *Crustaceae* and *Tenues*, with bootstrap support of 99% and is further divided into subclades B1 and B2 with greater than 0.95 posterior probability and 74% and 91% bootstrap support, respectively. Subclade B3 represents four species of section *Crustaceae* taken from GenBank. Section *Tenues* is represented by two monophyletic groups within subclades B4 and B5; subclade B5 contains six sequences from *C. ciliata*, with greater than 0.95 posterior probability and 83% bootstrap support; and subclade B4 contains eight sequences from *C. subtenuis* with greater than 0.95 posterior probability and only 56% bootstrap support. *C. rangiferina* and *C. stygia* are in subclade B2 but they do not support monophyletic species with the ITS rDNA. Similarly, subclade B1 consists of sequences representing *C. arbuscula, C. submitis*, *C. densissima*, and *C. mitis* with greater than 0.95 posterior probability and bootstrap support of 74%, but they do not represent monophyletic species. It is remarkable to observe that another chemically and morphologically distinct segregate, *C. submitis*, as well as *C. densissima* nested inside *C. arbuscula* in addition to *C. mitis*..

2.4.2 Haplotype network analysis

The mtSSU haplotype network produced 24 haplotypes from 35 sequences. Three sections of *Cladonia* form lineages in the network, where section *Crustaceae* linked section *Impexae* with section *Tenues* (Fig. 2.2). In this network, haplotypes were mixed between *C. arbuscula* and *C. mitis* further supporting the non-monophyly observed in the ITS phylogeny. One haplotype was shared between *C. rangiferina* and *C. stygia*. All other haplotypes

Figure 2.2 Haplotype network of mtSSU sequences showing relationship among *Cladonia* taxa (see legend) with thallus branching pattern indicated beside each species. Small solid black dots indicate genetic changes between haplotypes and the size of circles represents the number of haplotypes (1 to 4 haplotypes).



are represented by a single species each. The species in sections *Crustaceae* and *Tenues* have anisotomic complex branching patterns with *Tenues* having fewer branch numbers than *Crustaceae* (Fig. 2.2). Species in section *Impexae* have isotomic branching patterns with branch numbers ranging from 2 to 5 branches.

The haplotype networks of species pairs are shown in Fig. 2.3. The haplotype network of *C. portentosa – C. terrae-novae* produced 11 haplotypes from 14 sequences and no haplotypes were shared between species (Fig. 2.3a). The haplotype network of *C. arbuscula - C. mitis* produced 21 haplotypes from 26 sequences, and two haplotypes are shared between species (Fig. 2.3b). Twenty-seven sequences of *C. rangiferina – C. stygia* produced 23 haplotypes with one haplotype shared between two species (Fig. 2.3c).

2.4.3 Gene flow

The AMOVA analysis showed low to moderate levels of gene flow between species pairs (Table 2.2). Gene flow supported separate species for each of *C. arbuscula, C. mitis, C. rangiferina,* and *C. stygia*, but not for *C. portentosa* and *C. terrae-novae*.

2.5 Discussion

2.5.1 Species delimitation

This study showed that five of 18 species examined in sections *Crustaceae, Impexae* and *Tenues* are monophyletic, seven species were represented by single sequences, and six species were paraphyletic based on the MP tree and the strict consensus tree of the ITS rDNA nucleotide sequences (Fig. 2.1). Two major groups corresponding to sections *Impexae* and both *Crustaceae* and *Tenues* together (Fig. 2.1) were consistent with the recommendation of

Figure 2.3 Haplotype networks of ITS rDNA sequences showing relationship between three pairs of species, (*a*) *C. portentosa* and *C. terrae-novae*, (*b*) *C. arbuscula* and *C. mitis*, and (*c*) *C. rangiferina* and *C. stygia*. Small solid black dots indicate genetic changes between haplotypes and the size of the circles represents the number of haplotypes (1 to 3 haplotypes).

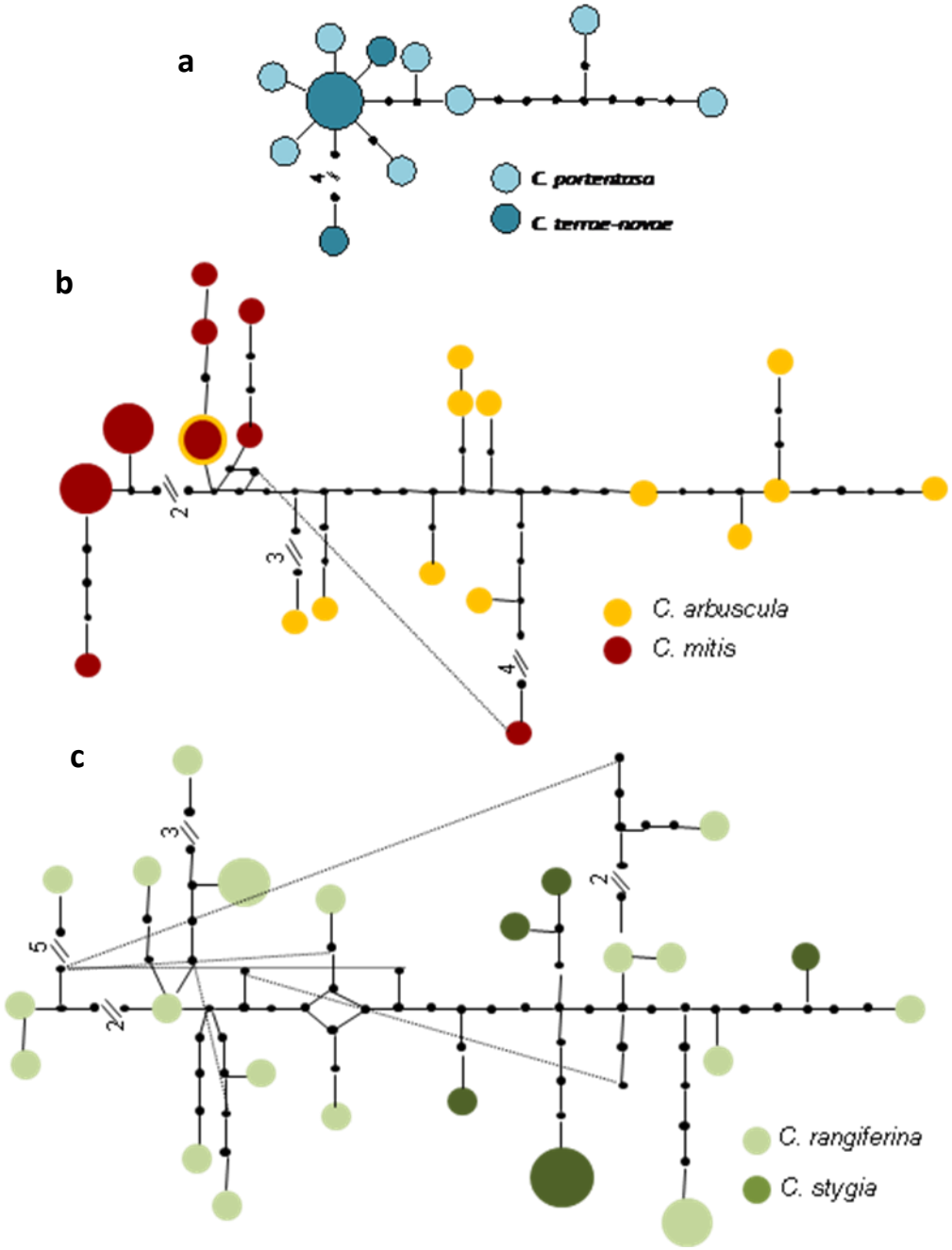


Table 2.2 Gene flow between and within species for each pair of paraphyletic species, C. *rangiferina* - *C. stygia*, *C. arbuscula* – *C. mitis*, and *C. portentosa* – *C. terrae-novae*.

Species comparison		**d.f.**	**SS**	**MS**	**Est. variation**	**%**	**Phi**	**P**
C. rangiferina-*C. stygia*	Between species	1	9.195	9.195	0.414	8	0.078	0.034
	Within species	25	122.471	4.899	4.899	92		
C. arbuscula-*C. mitis*	Between species	1	23.955	23.955	1.459	23	0.228	0.001
	Within species	25	123.341	49.34	49.34	77		
C. portentosa-*C. terrae-novae*	Between species	1	4.042	4.042	0.216	7	0.070	0.188
	Within species	10	28.875	2.888	2.888	93		

monophyly by Stenroos et al. (2002). However, section *Impexae* was split into two clades, clades A and C, in the phylogenetic tree but formed a single lineage in the mtSSU haplotype network. The haplotype network also supported the non-monophyly of two pairs of species (*C. rangiferina* and *C. stygia;* and *C. mitis* and *C. arbuscula*). While some morphological characteristics were not useful in elucidating the evolutionary relationships within certain subdivisions of *Cladonia* (Stenroos et al. (2002), the thallus branching pattern showed a heritable trend in species discrimination in this study. The anisotomic (unevenly divergent) branching was common to both sections *Crustaceae* and *Tenues* and the isotomic (evenly divergent) branching was present in section *Impexae*. The basal position of *C. wainioi* (section *Ascyphiferae*) in the phylogenetic tree is also supported by Stenroos et al. (2002) where it is in an isolated position in the phylogenetic tree and represents an unnamed single species section of its own. *C. wainioi* grows in a similar habitat as the reindeer lichens but the branching system and chemistry are different. Moreover, the findings of this study support the synonomy of sections *Crustaceae* (*Cladina*) and *Tenues* (Stenroos et al. 2002).

2.5.2 Monophyly in section Impexae

Within section *Impexae, C. portentosa* (8 sequences) and *C. terrae-novae* (4 sequences) are morphologically similar to one another with the thallus branches in groups of three and sometimes groups of two and both grow in a boggy habitat, but *C. terrae-novae* produces atranorin and *C. portentosa* produces perlatolic acid and usnic acid (usnic acid is sometimes lacking (Orange 1993). The two species are closely related and they are geographically separated from one another in North America where *C. portentosa* is distributed along the west coast and *C. terrae-novae* is along the east coast. Allopatry may encourage divergence between these

species but if the period of time has not been sufficient for complete divergence they would show incomplete genetic divergence and an absence of monophyly. The AMOVA analysis suggested moderate levels of gene flow between the two species, implying they are not genetically or reproductively isolated species. However, the sample size for this species pair was low which may have biased the analysis (Fitzpatrick 2009). Monophyly of *C. portentosa* was reported by Smith et al. (2012) but they did not include *C. terrae-novae* in their analysis for comparison.

C. stellaris is strongly monophyletic and *C. delavayi* is basal to *C. stellaris*, which is consistent with Stenroos et al. (2002). While *C. stellaris* is basal in this study (Fig. 2.1), it is a derived species in the evolution of the Cladoniaceae (Stenroos et al. 1997). The basal position of *C. delavayi* supports the postulation by Choisy (1928) that *C. stellaris* originated from ancestors of *Cocciferae* and *Perviae.* While *C. delavayi* was originally thought to be a member of *Unciales* (sensu Ahti 2000) it was proposed to be moved to supergroup *Crustaceae* and group *Cladinae* because of this affiliation.

The two species examined within section *Tenues*, *C. ciliata* (6 sequences) and *C. subtenuis* (8 sequences) are both monophyletic and are similar in their branching pattern. Even though *C. subtenuis* is monophyletic in this study, Yahr et al. (2006) reported a low level of population structure and inferred recombination to be occurring within this species. Monophyly of *C. ciliata* was also reported by Smith et al. (2012); however, the two colour variants *C. ciliata* var. *ciliata* (= f. *ciliata*) and *C. ciliata* var. *tenuis* (= f. *flavicans*) were not resolved. *Cladonia ciliata* var. *tenuis* contains usnic acid giving it a yellow-green colour, while *C. ciliata* var. *ciliata* does not contain usnic acid giving it a gray-green colour (Litterski and Ahti 2004), suggesting that the colour results from the expression of an usnic acid synthase gene which may evolve independently from the ITS rDNA.

2.5.3 Evolution in section Crustaceae

The finding that *Cladonia arbuscula s.l.* and *C. mitis* (together with *C. submitis* and *C. densissima*) formed a highly supported clade, sister to the clade containing other members of the sections *Cladina* and *Tenues*, was also consistent with Stenroos et al. (2002). Difficulty in separating the larger number of specimens of *C. arbuscula* and *C. mitis* in subclade B1 is consistent with Piercey-Normore et al. (2010). However, Smith (2012) showed these as monophyletic species with fewer numbers of specimens. Multiple gene phylogenies showed that *C. mitis* is supported as a monophyletic species when beta-tubulin, GAPDH, an intron, and the ITS rDNA are used in a phylogeny, but it is paraphyletic when the intron was omitted from the group of genes or when either gene was used alone in the analysis (Myllys et al. 2003). The reticulate nature of the haplotype analysis in this study may suggest interbreeding between these two species. However, the AMOVA analysis indicates a moderate level of gene flow and a low p-value suggesting the species are genetically different from one another. The diagnostic characters overlap between the species where *C. arbuscula* has denser branching of the apices with more browned and curved branch tips than in *C. mitis. C. mitis* produces usnic acid alone whereas *C. arbuscula* produces both usnic acid and fumarprotocetraric acid, but this feature can be variable (Ruoss 1987a; Ruoss and Ahti 1989). The close evolutionary relationship between the two species and their physical proximity in a similar habitat provides opportunities for gene flow to occur between them. Therefore, they may have had only a short history of reproductive isolation (Myllys et al. 2003) resulting in low resolution of *C. arbuscula* and *C. mitis* where sequence divergence (speciation) has lagged behind morphological evolution. In addition, recombination between these species (Robertson and Piercey-Normore 2007; Kotelko et al. 2008) may lead to reticulate tree patterns and gene flow cannot be ruled out. Evolutionary

processes may also have influenced the patterns observed in this study such as incomplete lineage sorting through speciation (Knowles and Carstens 2007). A coalescent-based approach using multiple loci will improve the resolution but may not remove the effects of incomplete lineage sorting depending on the extent of speciation (Knowles and Carstens 2007).

The position of *C. rotundata*, *C. argentea*, and *C. dendroides*, nested within a clade of *C. rangiferina* is consistent with Stenroos et al. (2002). *C. rangiferina* and *C. stygia* were shown to be closely related by Stenroos et al. (2002) but many studies often do not distinguish *C. stygia* from *C. rangiferina* making them more difficult to interpret. The absence of monophyly in these two species may be explained by their similar habitats but they are commonly separated by moist and dry habitats. The thallus branching pattern is the same but the stereome of *C. stygia* is black and it produces red pycnidial jelly, whereas the stereome of *C. rangiferina* is gray or brown and it produces colorless pycnidial jelly (Ahti and Huovinen 1985; Ruoss and Ahti 1989). Haplotype networks and AMOVA analysis imply frequent interbreeding and gene flow between *C. rangiferina* and *C. stygia*. Therefore, they may not be reproductively isolated from each other and in early stages of speciation. The close physical proximity in overlapping habitats and geographic distribution may encourage interbreeding and obscure genetic divergence between these species.

2.5.4 Phylogenetic signal

The paraphyly observed in *C. arbuscula* and *C. mitis*, and similarly in *C. rangiferina* and *C. stygia*, may also suggest there is insufficient phylogenetic signal in the ITS region to resolve the morphological differences among these species. Among the several gene regions proposed for species discrimination in fungi (Taylor et al. 1999, 2000; Myllys et al. 2003), Pino-Bodas et

al. (2013) concluded that the best combination for barcoding in *Cladonia* is *rpb2* and ITS rDNA. The ITS rDNA region was also supported by Schoch et al. (2012) as a potential barcoding marker for fungi. While the ITS rDNA is widely used, species discrimination using ITS rDNA has previously shown to be a challenge with some members of *Cladonia* (Fontaine et al. 2010; Kotelko and Piercey-Normore 2010; Kelly et al. 2011; Pino-Bodas et al. 2011). The greater intraspecific variation in the ITS rDNA observed with species of *Cladonia* such as *C. arbuscula, C. mitis, C. rangiferina* and *C. stygia,* may also suggest that evolutionary processes such as incomplete lineage sorting in a recent divergence may obscure species delimitation (Knowles and Carstens 2007).

In conclusion, the current study supported monophyly for five of 18 species in *Cladonia* sections *Crustaceae, Tenues,* and *Impexae* and was consistent with the segregation of sections proposed by Stenroos et al. (2002) using a phylogenetic analysis of the ITS rDNA and a haplotype network of the mtSSU gene. The mtSSU network also illustrated a morphological trend of the thallus branching pattern in these lichens where members of section *Impexae* have isotomic branching and *Tenues* and *Crustaceae* have anisotomic branching. Incomplete lineage sorting, recombination, gene flow, and recent divergence were raised as explanations for the reticulate nature of the haplotype networks of the six non-monophyletic species. These results emphasize the importance of examining the non-monophyletic species using multiple loci for a coalescence-based approach. In addition, further investigation of gene flow and recombination between and within the species pairs reported in this study, may reveal the evolutionary status of these species.

CHAPTER 3

Comparison of *in situ* growth and genetic polymorphism in *Cladonia arbuscula* and *C. rangiferina*

3.1 Abstract

Cladonia arbuscula and *C. rangiferina* are two reindeer lichen species, which are widely distributed in northern climates. Previous studies imply diversity within these species, which is not readily apparent in phylogenies with small numbers of individuals per species. The current study examined the colony growth and genetic diversity of mycobiont ascospore colonies within and between apothecia of two species, *C. rangiferina* and *C. arbuscula s. l.* using Randomly Amplified Polymorphic DNA (RAPD)-PCR and colony growth rates. *Cladonia rangiferina* showed higher rates of growth than *C. arbuscula* but more variability in growth between apothecia was observed in *C. arbuscula* than *C. rangiferina*. Both species showed levels of genetic variation within and among apothecia that was consistent with heterothallism and recombination. It is hypothesized that higher genetic variation in *C. arbuscula* than in *C. rangiferina* may give *C. arbuscula* an adaptive advantage over *C. rangiferina*.

3.2 Introduction

The genus *Cladonia* is a large genus of terricolous lichen-forming fungi including the highly branched reindeer lichens, which are known from the segregate genus *Cladina* (Ahti 2000; Ahti and DePriest 2001). Species of *Cladina* cover large portions of northern areas forming the main source of winter food for northern caribou and reindeer (Rominger et al. 1996). The success of these mat-forming reindeer lichens may result from thallus fragmentations, which

produce clonal mats of each species. The annual divergent growth increments of the highly branched thallus double the thallus biomass each year during early growth phases (Ahti 1959). But low levels of genetic diversity in these clonal mats may reduce the capacity of the population to adapt to changing climate (Pauls et al. 2013). While Beard and DePriest (1996) reported low levels of variation in mats of *C. subtenuis*, they reported higher levels of genetic variation within *C. chlorophaea* but the variation between apothecia from the same thallus mat has not been examined. Since *Cladonia arbuscula* (Wallr.) Flotow and *C. rangiferina*, (L.) F. H. Wigg. are widely distributed in northern ecosystems, and both species were have been reported to produce apothecia frequently in Manitoba (Piercey-Normore 2004; Robertson and Piercey-Normore 2007; Athukorala et al. 2014), we chose to examine the variation using ascospore colonies isolated from apothecia and a sensitive multilocus technique.

The level of genetic variation in any organism will partially depend on the genetic marker examined, such as nucleotide sequence comparison (Myllys et al. 2003; Printzen and Ekman 2003; Piercey-Normore et al. 2010), presence or absence of group 1 introns (Robertson and Piercey-Normore 2007), Restriction Fragment Length Polymorphism (RFLP; Beard and DePriest 1996), and Randomly Amplified Polymorphic DNA (RAPD; Dyer et al. 2001). Methods such as RAPDs that use random priming of multiple loci within the genome offer a sensitive method to elucidate variation, but RAPD requires careful lab practices in order to maintain reproducibility of results (Weising et al. 1995) and cultivation of the fungal partner to separate the genome from other symbionts or contaminating organisms. Two groups of genetic variants were distinguished by comparing RAPD variation among 59 single spore cultures of *Xanthoria parietina* (L.) Th. Fr. (Honegger et al. 2004) and polymorphism among RAPD loci was reported for single spore isolates of some species within the Parmeliaceae, Ramalinaceae and Physciaceae (Honegger and

Zippler 2007). RAPD and Amplified Fragment Length Polymorphism (AFLP) variation was also compared showing genetic polymorphism within single apothecia of three *Cladonia* species (Seymour et al. 2005) but the reindeer lichens were not examined. This study provides preliminary information on growth and genetic variation among colonies grown from ascospores of the same apothecia and different apothecia, for two common North American reindeer lichen species, *Cladonia arbuscula s. l.* and *C. rangiferina*.

3.3 Materials and Methods

Single ascospores were isolated from five samples of *C. rangiferina* and four samples of *C. arbuscula* (Table 3.1) according to the method described by Athukorala et al. (2014), grown on 1.5% Malt Yeast extract agar (MYA: 20 g malt extract, 2 g yeast extract, 15 g agar, 1 L distilled water), and incubated at 20 $^{\circ}$C in the dark. Five apothecia were placed in each plate where spore clusters are generally ejected in a narrow radius relative to the spacing of the apothecia. Large gaps of media surface were devoid of any spores between apothecia preventing the mixture of apothecial spores. To confirm the cultured mycobiont was the same as the natural lichen, total cellular DNA was extracted from each colony and the lichen using a modified protocol of Grube et al. (1995). Amplification and sequencing of the Internal Transcribed Spacer region of nuclear ribosomal DNA (ITS rDNA) followed Athukorala et al. (2014). The Random Amplified Polymorphic DNA (RAPD) of the colonies was conducted using primers, UBC 31 (ccg gcc ttc c), UBC 34 (ccg gcc cca a), UBC 60 (ttg gcc gag c) and UBC 184 (caa acg gca c) on 24 colonies of *C. rangiferina* (SA22); and using UBC 31, UBC 60, UBC 90 (ggg ggt tag g) and UBC 122 (gta gac gag c) on 22 colonies of *C. arbuscula s. l.* (Nomore 9468, Athukorala 7, Athukorala 13). Amplification was carried out on 20 µl PCR reactions contained 1X PCR buffer

(50 mM KCl, 20mM Tris), 1µM primer (1.1 µM for UBC 184), 2.0 mM of $MgCl_2$, 200 mM of each dATP, dCTP, dGTP and dTTP (Invitrogen Life Technologies, California, USA), 0.15 U of Native Taq polymerase (Invitrogen Life Technologies, California, USA), and 0.4 to 2.0 ng DNA. The RAPD-PCR cycle contained an initial denaturation step of 2 min at 94 ˚C, followed by 38-40 cycles of 94 ˚C for 1 min, 38 ˚C (UBC 31), UBC 60), 40 ˚C (UBC 34), 30 ˚C (UBC 184) for 30 s, and 72 ˚C for 45 s (20 s for UBC 184) before a final elongation step of 72 ˚C for 10 min. Amplified products were run on 1 % agarose gels and detected by staining with ethidium bromide (0.5 µg/µl) using a 1 Kb DNA plus ladder (Invitrogen Life Technologies, California, USA) and the bands that were greater than 4 ng (compared to the 1650 bp fragment) were used in scoring. Primer amplifications were repeated by two authors (SA and JD), on different thermal cyclers (T100^{TM} thermal cycler, Bio-Rad Laboratories, Hercules, California, USA and.a Biometra T-gradient thermal cycler, Thistle Scientific, Glasgow, UK), and with different amounts of template DNA (2.0 – 10 ng, 0.4 – 2.0 ng, 0.2 – 1 ng). The banding patterns produced under different conditions were identical but some of the bands were weaker than others. The RAPD bands were scored as present or absent (1 or 0, respectively), representing binary data sets. Percent polymorphism of colonies within each apothecium was calculated manually using the formula: number of polymorphic loci/total number of amplified loci x 100. Polymorphism was calculated using each RAPD primer matrix separately and using the combined matrix. Comparisons were made between specimens for colony growth and all comparisons were shown for percent polymorphism to avoid effects of psuedoreplication. The number of haplotypes recorded for each apothecium was determined with regards to each primer matrix and combined matrix of both species using the Haplotype Analysis version 1.04 program (Eliades and Eliades 2009).

Table 3.1 Species and collection location for samples used in the study showing the number of colonies from each specimen in parentheses. The GenBank accession numbers obtained for ITS rDNA sequences for representative colonies are in the far right column.

Species	Collection information	ITS rDNA accession number
C. arbuscula	Canada, Manitoba, Spruce Woods, Normore 9642 (n=76)	KP031551
	Canada, Manitoba, near Leaf Rapids, Normore 9403 (n=68)	KP031550
	Canada, Manitoba, Sandilands Provincial Forest, Athukorala 7 (n=20)	KP001207
C. mitis	Canada, Manitoba, Sandilands Provincial Forest, Athukorala 12 (n=12)	KP001209
	Canada, Manitoba, between Ponton and Leaf Rapids, Normore 9468 (n=26)	KP001206
C. rangiferina	Canada, Manitoba, Sandilands Provincial Forest, Athukorala 16 (n=06)	KP031549
	Canada, Manitoba, Sandilands Provincial Forest, Athukorala 17 (n=05)	KP001202
	Canada, Manitoba, Sandilands Provincial Forest, Athukorala 22 (n=34)	KP001200
	Canada, Manitoba, Sandilands Provincial Forest, Athukorala 24 (n=06)	KP001201

3.4 Results and Discussion

The results showed that ITS rDNA sequences obtained from colonies in this study were identical between cultures and corresponding lichen samples, and showed a high level of similarity to GenBank accessions: Accession no. GU169281 (*C. arbuscula*) had 99% similarity and 0.0 e-score with SA7. Accession no. GU169249 and GU169228 (*C. arbuscula* subsp. *beringiana* and *C. mitis*) had 99% similarity and 0.0 e-score with Athukorala 12, Normore 9403, Nomore 9468 and Normore 9642. Accession no. DQ394367 (*C. rangiferina*) had 98% similarity and 0.0 e-score with Athukorala 24, Athukorala 22, Athukorala 17 and Athukorala 16. All sequences generated in the current study were deposited in GenBank and the relevant accession numbers are listed in Table 3.1.

Ascospores were released from apothecia of *C. arbuscula* and *C. rangiferina* within 21-28 days and germination occurred within 24 hours after spore discharge. Colony growth was observed within one week and they were between 2 to 5 mm in diameter after 1 month (Fig. 3.1). Colony diameter continued to increase after 4 weeks and differences between colonies and species became more evident after 8 to 12 weeks, where *C. rangiferina* had larger colony diameters than *C. arbuscula s. l.* and the growth of *C. mitis* was in between that of *C. arbuscula* and *C. rangiferina* (Fig. 3.1). There was an overall significant difference ($P<0.001$) in colony diameters between the three species at 12 weeks but colony growth in individual specimens overlapped. One *C. mitis* specimen (SA 12) was not significantly different from three of the four *C. rangiferina* specimens and one of the *C. arbuscula* specimens (Fig. 3.1). Average colony diameter of four specimens of *C. rangiferina* was not significantly different between apothecia ($P=0.7773$). However, average colony diameter of *C. arbuscula* was significantly different between specimens (Fig. 3.2).

Figure 3.1 Change in colony diameter for three specimens of *C. arbuscula* (dotted lines), two specimens of *C. mitis* (dashed lines), and four specimens of *C. rangiferina* (solid lines) from one to three months on malt yeast extract agar. The mean colony diameter and standard error bars are shown for each 4 week interval. Standard errors are shown for sample sizes of 5 to 76 colonies (see Table 3.1). Different lower case letters above bars indicate significant difference at P=0.05.

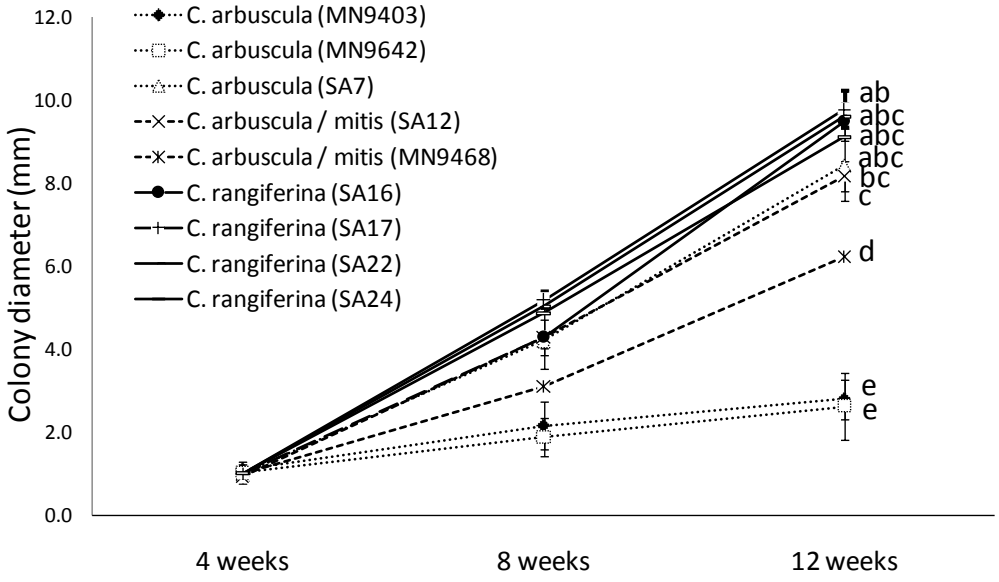
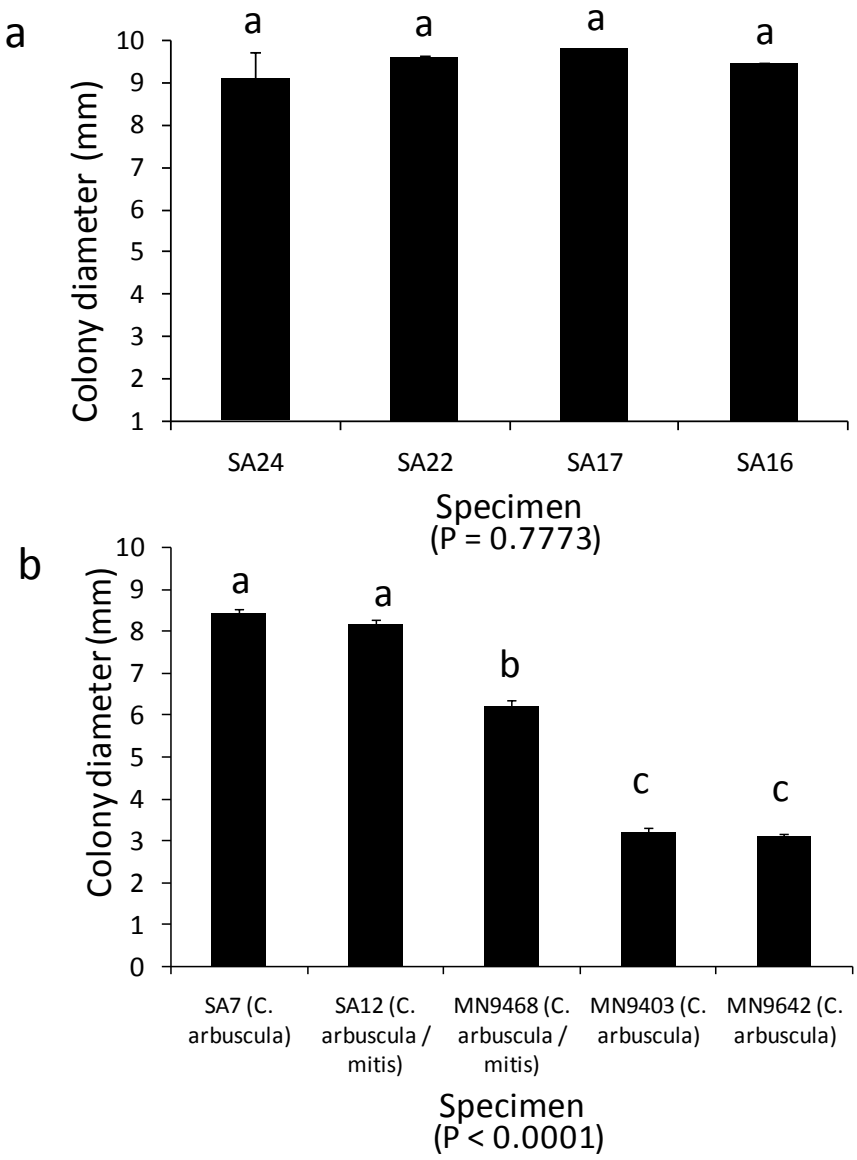


Figure 3.2 Comparison of average colony diameter between specimens of (*a*) *C. rangiferina* and (*b*) *C. arbuscula*, and *C. mitis*. Mean and standard error bars are shown for 5 to 76 replicates (see Table 3.1). Different lower case letters above bars indicate significant difference at P=0.05.



The percent polymorphism of four RAPD primers exhibited variability among spore cultures of the 23 colonies from *C. rangiferina*, 11 colonies from *C. mitis*, and 9 colonies from *C. arbuscula*. An example of a gel is shown in Fig 3.3. The eight primers (four primers for *C. rangiferina* and four primers for both *C. mitis* and *C. arbuscula*) amplified fragments with a range of 375–3000 bp in length resulting in a total of 47 fragments for *C. arbuscula* and *C. mitis* together, and 52 fragments for *C. rangiferina,* where each size of fragment is equivalent to a locus. There were 1 to 5 haplotypes of *C. rangiferina* and 1 to 8 haplotypes of *C. arbuscula* and *C. mitis* depending on the primer. The percent polymorphism ranged from 33 to 100% for *C. arbuscula* and 0 to 91% for *C. rangiferina* (Table 3.2).

This study showed comparable numbers of haplotypes between species (18 for one individual of *C. rangiferina* and 15 for 4 individuals of C. *arbuscula s. l.*). In another study, 10 intergenic spacer (IGS) and 16 ITS haplotypes were reported from 14 individuals of *Xanthoria parietina* (Lindblom and Ekman 2006). The markers used by Lindblom and Ekman (2006) were DNA sequence markers for two loci and those used in this study were multilocus banding patterns; therefore, direct comparisons are difficult to make. The large amount of variation within *C. rangiferina* is surprising since all four samples were collected within the same area whereas the specimens of *C. arbuscula/mitis* were collected from areas that were several hundreds of km apart. In both species, variation was shown to be greater than previously shown with a different marker (Beard and DePriest 1996) and comparable with that reported for other lichens (Honegger et al. 2004; Lindblom and Ekman 2006; Honegger and Zippler 2007). While the choice of marker depends on the question addressed in each study, variation may be compared if the marker is similar between studies.

Figure 3.3 RAPD-PCR gel image obtained from spore cultures of *C. rangiferina* with the primer UBC 31 showing an example of the results. The far left lane shows a 1Kb ladder with fragment lengths indicated in base pairs to the left of the image. Lanes 1 to 5 show banding pattern for five colonies from one apothecium of SA22, lanes 6 to 8 show results of three colonies from another apothecium of the same sample, and lanes 9 to 10 show results of two colonies from a third apothecium of SA22.

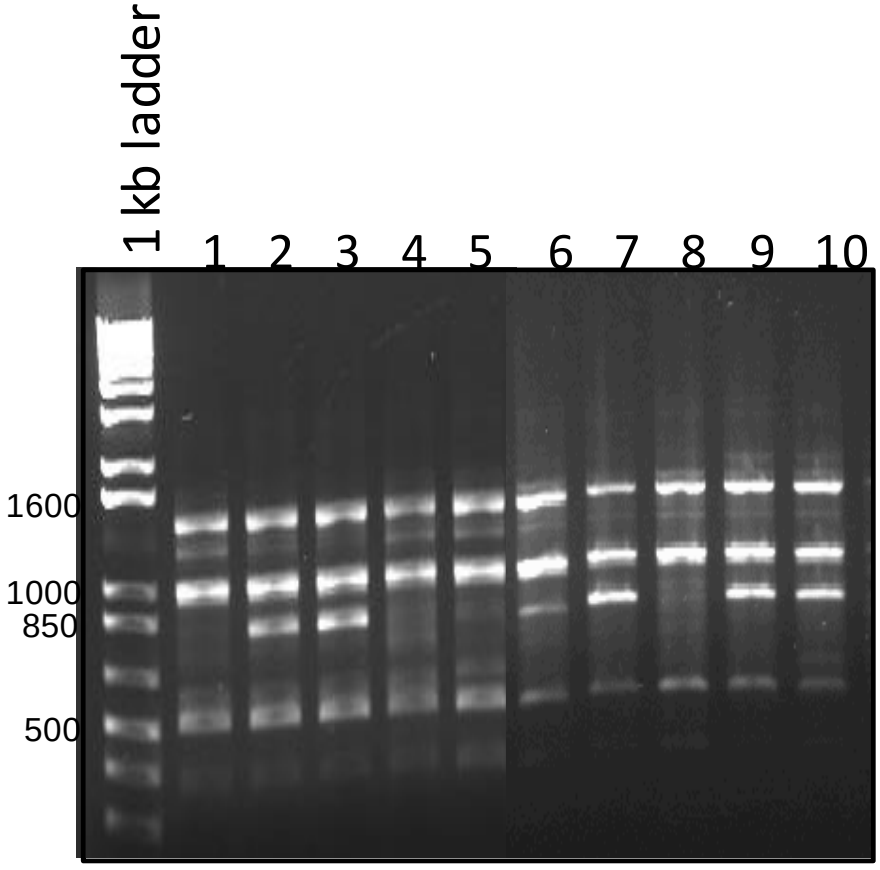


Table 3.2 Percent polymorphism of RAPD bands among apothecia from two species, *C. arbuscula* and *C. rangiferina*, showing both separate and combined results. SA represents collection by Athukorala and MN represents collection by Normore. Numbers in each column represent % polymorphism, (number of polymorphic loci/total number of loci), and number of haplotypes.

Species and collection no.	**Primer UBC 31**	**Primer UBC 60**	**Primer UBC 90**	**Primer UBC 122**	**Combined primers**
C. arbuscula					
SA7-I, n=3	33% (3/9) 3	0% (0/4) 1	No bands	No bands	54% (15/28) 3
SA13-I, n=6	100% (6/6) 5	No bands	60% (3/5) 2	100% (7/7) 6	100% (18/18) 6
MN9468-I, n=8	83% (5/6) 7	100% (10/10) 5	91% (10/11) 7	75% (6/8) 3	91% (29/32) 8
MN9468-II, n=3	80% (4/5) 3	No bands	80% (8/10) 2	No bands	80% (12/15) 2
C. rangiferina	**Primer UBC 31**	**Primer UBC 34**	**Primer UBC 60**	**Primer UBC 184**	**Combined primers**
SA22(1)-I, n=3	91% (10/11) 3	33% (2/6) 2	83% (5/6) 3	75% (3/4) 2	74% (20/27) 3
SA22(1)-II, n=4	14% (1/7) 2	70% (7/10) 4	83% (5/6) 4	33% (1/3) 2	52% (13/25) 4
SA22(1)-III, n=2	37.5% (3/8) 2	60% (6/10) 2	40% (2/5) 2	0% (0/4) 1	41% (11/27) 2
SA22(2)-I, n=3	37.5% (3/8) 3	29% (2/7) 2	75% (3/4) 3	67% (2/3) 3	45% (10/22) 3
SA22(2)-II, n=2	14% (1/7) 2	44% (4/9) 2	No bands	0% (0/4) 1	37.5% (9/24) 2
SA22(2)-IV, n=2	22% (2/9) 2	12.5% (1/8) 2	75% (3/4) 2	0% (0/4) 1	17% (4/24) 2
SA22(2)-V, n=5	14% (1/7) 2	17% (1/6) 2	100% (8/8) 5	75% (3/4) 5	52% (13/25) 5
SA22(2)-VI, n=2	14% (1/7) 2	57% (4/7) 2	50% (3/6) 2	0% (0/2) 1	36% (8/22) 2

* Numbers in the first column are the collection number, apothecium number (I-VI), and the number of colonies (n) tested. The *C. rangiferina* samples have an additional podetium number in parentheses (1 – 2) after the collection number.

The overall smaller colony diameters in *C. arbuscula* than *C. rangiferina* (Fig. 3.1) suggest that *C. rangiferina* grows faster than *C.arbuscula/mitis* in culture. This finding is consistent with literature findings in field studies. The growth rate of *C. rangiferina* observed in the current study fall within the same range (3.9-7.7 mm/yr) as described by Vasander (1981) for the same species. However, the growth rates were different (3.9-4.3 mm/yr for *C. rangiferina* and 3.0-3.5 mm/yr for *C. mitis*) as reported by Helle et al. (1983). These results are somewhat consistent with the faster growth of *C. rangiferina* in this study, recognizing that growth under laboratory conditions may not be representative of field conditions. The implications are that the relative growth rates among species may be comparable in culture when the growing conditions are constant.

In summary, the preliminary assessment was that *C. rangiferina* had faster colony growth and a larger number of haplotypes per individual than *C. arbuscula.* *C. rangiferina* was also more consistent in its growth between individuals and the range of colony growth was an average of 9.1 to 9.8 mm, but *C. arbuscula* varied among individuals and the range of average colony diameter was 3.0 to 8.4 mm (Fig.3.2). This study also showed that overall polymorphism was lower in *C. rangiferina* than *C. arbuscula/mitis.* While these differences between species are interesting and provide a preliminary assessment of the level of variation, they may have ecological implications that warrant further study with a larger number of samples.

CHAPTER 4

Identification and comparison of the three early stages of resynthesis for the lichen *Cladonia rangiferina*

4.1 Abstract

A lichen is an association between a biotrophic fungal partner and a green algal and/or cyanobacterial partner, which may be considered a "controlled" parasitic interaction. While controlled parasitism implies benefit to both interacting partners, a parasitism that is not controlled implies that one partner benefits to the detriment of the other partner. The objective of this study was to compare morphological development of the interaction between *Cladonia rangiferina* with its compatible algal partner (*Asterochloris glomerata/irregularis*) and incompatible algae (*Coccomyxa peltigerae* and *Chloroidium ellipsoideum*) at three early resynthesis stages. The fungus was co-inoculated with each alga separately and the stages of development were compared using quantitative measures. The first three stages of development of the lichen thallus were identified in the compatible interaction as the "pre-contact" stage (1 day post co-inoculation (PCI)), "contact" stage (8 days PCI) and "growth together" stage (21days PCI). Compatible interactions showed significantly shorter internode length, a larger number of new lateral hyphal branches, greater appressorial frequency, and no reduction in cell diameter of the algal cells, than incompatible interactions. At 21 days PCI, a parasitic interaction was observed between *C. rangiferina* and *Chloroidium ellipsoideum.* These findings support the importance of recognition between compatible partners for successful lichenization. This study also revealed a strategy that may explain the success of this species in northern habitats.

Identification of the resynthesis stages of *C. rangiferina* is required before expression of the proteins involved in recognition and defense can be understood.

4.2 Introduction

Lichens, associations between biotrophic fungal partners and photosynthetic partners, may be considered "controlled" parasitic interactions (Honegger 1998). Parasitism is supported by the presence of appressoria (Webber and Webber 1970; Ahmadjian et al. 1978; Ahmadjian and Jacobs 1981, 1983; Trembley et al. 2002; Joneson and Lutzoni 2009). Appressoria in fungal pathogens are flattened hyphal tips that bind to the host cell surface and initiate a penetration peg (Kirk et al. 2008). Lichen formation may occur only with certain algal partners, while others may be parasitized during the interaction (Jacobs and Ahmadjian, 1971; Ahmadjian et al. 1978; Ahmadjian and Jacobs 1983; Joneson et al. 2011). Morphological observations of both symbionts can be made during resynthesis experiments, which have been described previously (Ahmadjian et al. 1980; Bubrick et al. 1985; Ott 1987b; Galun and Garty 1988; Stocker-Wörgötter and Türk 1991; Jahns 1993; Kon et al. 1993; Stocker-Wörgötter 1995; Shaper and Ott 2003; Joneson and Lutzoni 2009; Muggia et al. 2011). Timing of the events during the first three stages of resynthesis is variable depending on the species, media, and incubation conditions. The first three developmental stages that have been described are: 1) the "pre-contact" stage where symbionts are in close proximity to share extracellular secretions but not physical contact, 2) the "contact" stage where two bionts begin to make physical contact by fungal appressoria, and 3) the "growth together" stage where two bionts grow together in a network to form cellular masses containing both bionts (Ahmadjian et al. 1978; Galun 1988; Armaleo1991; Joneson and Lutzoni, 2009). Quantitative comparisons of morphological characteristics of interacting symbionts are

rare (except see Meeβen and Ott 2013) but characteristics of the interaction have been shown to change throughout development.

There have been two attempts to resynthesize the lichen, *Cladonia rangiferina* (Jahns 1993; Stocker-Wörgötter 1995) by growing the two symbionts in culture but resynthesis stages as described above were not documented. *C. rangiferina* is a common lichen in northern ecosystems, it has a relatively fast growth rate (5.2 mm/year; Andreev 1954), and abundant apothecia, making it suitable for laboratory study. In addition, the recognition that *C. rangiferina* and other reindeer lichens, serve as winter food for caribou and reindeer, necessitates a better understanding of its biology. Stocker-Wörgötter (1995) reported that thallus resynthesis experiments with *C. rangiferina* were not as successful as they were with other studied species. Resynthesis was performed on undefined media such as clay (Jahns 1993) or media amended with soil extracts (Stocker-Wörgötter 1995), which are not suitable for reproducibility of experiments. Reproducibility requires the characterization of resynthesis stages with standard terminology and use of defined media for each lichen species before further experimental work can be performed.

The objectives of the present paper were 1) to isolate pure cultures of two symbionts from *C. rangiferina*, 2) to define the first three stages of resynthesis, and 3) to compare the first three stages of resynthesis between *C. rangiferina* and each of *Asterochloris glomerata/irregularis* (native lichen alga; compatible interaction), *Coccomyxa peltigerae* (native non-lichen alga; incompatible interaction), *Chloroidium ellipsoideum* (foreign non-lichen alga; incompatible interaction). This study will form the basis for comparing the expression of recognition and defense-related genes at early resynthesis stages.

4.3 Materials and Methods

4.3.1 Isolation of the fungal and the algal partners

The fungal partner was isolated from apothecia of *C. rangiferina* collected from Manitoba, Canada (SA24) according to Yoshimura et al. (2002) and Stocker-Wörgötter and Hager (2010). The spores were allowed to be ejected onto 1.5% water agar from apothecia attached to the Petri plate lid at 20 ˚C in the dark. Following spore ejection or germination, spores were transferred to 1.5% Malt Yeast extract agar (MYA) (Malt extract; 20g, yeast extract; 2g, agar; 15g in 1L distilled water). When fungal colonies were about 10 mm in diameter (3-4 months), colonies were ground with a sterile mortar and pestle and inoculated into liquid MY medium (Malt extract; 20g, yeast extract; 2g in 1L distilled water). Liquid cultures were maintained at 20 ˚C in the dark with continuous shaking at 120 rpm.

The native, compatible alga was isolated on Bold Basal Medium (BBM: Deason and Bold, 1960; Bischoff and Bold, 1963) from thallus fragments of *Cladonia rangiferina* as described by Yoshimura et al. (2002). Pure colonies were subcultured to liquid BBM. The native, incompatible alga was isolated from *Peltigera aphthosa* in the same way and kindly provided by M. Elshobary. A pure culture of a non-native, incompatible alga, *Chloroidium ellipsoideum* was obtained from the Phycology lab, Faculty of Science, Zagazig University, Egypt. All liquid algal cultures were maintained at 20 ˚C under continuous light (845 lumens) and shaking at 120 rpm.

4.3.2 Species confirmation by DNA sequencing

To confirm the identity of each of the cultured symbionts, DNA was extracted from thalli of *C. rangiferina* and *P. aphthosa*, as well as pure cultures of the fungal partner, *C. rangiferina*, and three algae with a CTAB method modified from Grube et al. (1995). The Internal

Transcribed Spacer 1 and 2 (ITS 1 and 2) region of the nuclear ribosomal RNA gene was amplified and sequenced with primers 1780-F (Piercey-Normore and DePriest 2001) and ITS2-KL (Lohtander et al. 1998) for the mycobiont and 1780A (Piercey-Normore and DePriest 2001) and ITS4 (White et al. 1990) for the photobiont. Twenty-µl PCR reactions for fungal ITS contained 20 ng of template DNA, 1X PCR buffer, 500 mM of each of forward and reverse primers, 2.5 mM of $MgCl_2$, 200 µM of each dATP, dCTP, dGTP and dTTP (Invitrogen Life Technologies, CA, USA), and 0.1 U of Taq DNA polymerase *(*Invitrogen Life Technologies, CA, USA). Amplification was performed in a Biometra® TGradient thermocycler (American Laboratory Trading Inc., CT, USA). The PCR reaction for the algal ITS contained reagents at the same concentration as for the fungal ITS region except the $MgCl_2$, concentration was 2.0 mM. The PCR cycle conditions for both fungal and algal ITS rDNA were as follows: initial denaturation at 95 ˚C for 5 min; 30 cycles of denaturation at 95 ˚C for 1 min, annealing at 54 ˚C for 1 min, and extension at 72 ˚C for 1 min 30 s. The DNA was purified by Wizard(R) SV gel and PCR clean-up system (Promega Corporation, WI, USA). The PCR product was sequenced using Big Dye terminator V3.1 (Applied Biosystems, CA, USA) on a 3130 Genetic Analyser (Applied Biosystems, CA, USA). The sequences were searched by BLASTn in NCBI GenBank to check for similarity with other sequences.

4.3.3 Co-inoculation experiments

The co-inoculation experiments were prepared by using sub-cultures of the isolated fungus, the native, compatible lichen alga (*Asterochloris glomerata/irregularis*), the native, incompatible lichen alga (*Coccomyxa peltigerae*), and the non-native, incompatible lichen alga (*Chloroidium ellipsoideum*) following a modified procedure from Joneson et al. (2011). The

subcultures were prepared by inoculating 100 ml of liquid MY (for the fungus) and BBM (for the lichen alga) one month prior to the start of the co-inoculation experiments. Flasks containing cultures were incubated at 20˚ C with continual shaking at 120 rpm in an incubator shaker (Innova® 40, New Brunswick Scientific, CT, USA). After one month, 50 ml of fungal culture was centrifuged in a Sorvall Legend X1R refrigerated centrifuge having a TX-400 swinging bucket rotor (Thermo Fisher Scientific Inc., MA, USA) at 8000 rpm for 10 min, drained, and the solid cellular material was ground with a mortar and pestle under sterile conditions, and resuspended in 50 ml of 99:1 medium (1 ml of MY added to 99 ml of BBM; Joneson et al. 2011) to obtain a homogeneous suspension. One hundred ml of lichen algal culture was centrifuged, at 8000 rpm for 10 min, drained, and the algal cell pellet was resuspended in 50 ml of 99:1 medium to obtain a concentrated suspension. Cultures of the non-lichen alga in 100 ml of BBM were initiated two weeks before the beginning of the experiment. Fifty ml of the culture was centrifuged under the same conditions as above and resuspended in 50 ml of 99:1 medium (growth rate of *Chloroidium ellipsoideum* was higher than that of the lichen algae). For the interaction between *C. rangiferina* and each of *A. glomerata/irregularis* and *Co. peltigerae*, 500 µl of the prepared *C. rangiferina* suspension was placed on a nitrocellulose membrane (25 mm diameter, 0.22 µm, Millipore GSWP02500) in the petri plate containing 99:1 solid medium and allowed to dry for 1 h under sterile conditions. After 1 h the same nitrocellulose filter papers containing *C. rangiferina* were inoculated with 500 µl one of *A. glomerata/irregularis, Co. peltigerae,* or *Chloroidium ellipsoideum* and dried for 1 h under sterile conditions. Four replicates were prepared for each of seven treatments: 1) *C. rangiferina + A. glomerata/irregularis,* 2) *C. rangiferina + Ch. ellipsoideum,* 3) *C. rangiferina + Co. peltigerae,* 4) *C. rangiferina* alone, 5) *A. glomerata/irregularis* alone, 6) *Co. peltigerae* alone, and 7) *Ch.*

ellipsoideum alone. Each set of interactions were prepared in two petri plates and each petri plate contained two (co)-inoculated membranes resulting in four replicates of each treatment.

4.3.4 Observations of resynthesis stages

Each resynthesis stage in this study was identified by referring to the terminology and characters already defined with other species for each of the four resynthesis stages (Ahmadjian et al. 1978; Ahmadjian and Jacobs 1983; Stocker-Wörgötter and Türk 1991; Trembley et al. 2002). Starting from 1d post co-inoculation (PCI), all cultures were observed and compared every following day using a dissecting microscope (Leica MZ6, Leica Microsystems (Schweiz) AG, Switzerland) to describe morphological development of cultures. Each observation was compared with descriptions of previous studies with other species. In addition, examination of the interacting symbionts was made by removing a small portion of the colony by an inoculation needle under sterile conditions and recording observations (over time and between treatments) under a compound light microscope (Nikon EclipseE200, Nikon Instruments Inc., USA). Observations were photographed using an Olympus SP-500 UZ digital camera.

All cultures were further characterized by using Scanning Electron Microscopy (SEM) at three early resynthesis stages defined for *C. rangiferina* and *A. glomerata/irregularis*. SEM was performed for each treatment on three small discs of nitrocellulose filter paper containing co-inoculated cultures. The filter papers were cut and fixed in a modified Karnovsy's fixative consisting of 2.5% gluteraldehyde, 1% paraformaldehyde, in a 0.1 M sodium cacodylate buffer at pH 7.2. After fixation (12 to 24 hours at 4 ºC) the cells were washed in 0.1 M sodium cacodylate buffer pH 7.2 with 4 changes over 2 h. Subsequently tissue was post fixed in a 1% solution of osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at 4 ˚C. After a brief

rinse in buffer, the tissues were rapidly dehydrated in an ascending series of -20 ˚C ethanol from 70% - 100%. After the tissues were placed in 100% ethanol, they were warmed to room temperature and an additional 4 changes at room temperature in 100% ethanol of 30 min each were performed. The ethanol was then replaced by anhydrous acetone with 2-3 changes over a 1 h period, Tissues were then transferred to a critical point dryer (Electron Microscopy Sciences, PA, USA) and routine critical point drying done. Dried samples were mounted with conductive carbon tape and examined in a Hitachi TM 1000 Table Top SEM equipped with a backscatter detector.

4.3.5 Quantitative measurements at three early stages of resynthesis

Four types of cell features were compared between treatments for each resynthesis stage (1d, 8d and 21d) and between resynthesis stages for each treatment. 1) The internode length of new lateral fungal hyphae and old hyphae immediately below new branches, or growing hyphae in the case where no or fewer new lateral branches were observed (in incompatible and *C. rangiferina* alone cultures) was calculated as the mean of the first 3 to 5 internodes from the apex of new lateral branches or growing branches. Measurements were made from 3 to 5 SEM images per treatment and they were the average of 7 to 20 internodes per treatment. 2) The number of new lateral branches produced by the fungal hyphae, which were visible as translucent hyphae arising from older hyaline fungal hyphae of *C. rangiferina* was counted in compatible, incompatible and *C. rangiferina* alone treatments from 100 X 100 µm areas selected from 5 – 8 SEM images per treatment. 3) The frequency of fungal appressoria was calculated as the number of algal cells in contact with fungal appressoria divided by the total number of algal cells present in a 100 X 100 µm area of 5 SEM images. An appressorium was defined where the fungal

hyphal tip produced an expansion against an algal cell. Appressorial frequency was calculated and compared in compatible and incompatible treatments and between resynthesis stages. 4) The algal cell diameter for each of the compatible and incompatible algae in co-inoculated and cultures of symbionts growing alone was measured. Algal diameter was the average of 20 matured algal cells selected randomly from five SEM images for each treatment. Two measurements of diameter were taken perpendicular to each other from each algal cell and the average of these two measurements was used for the analysis. Statistical analyses were performed using JMP software (SAS Institute Inc.) Version 11. Data were analysed by one way ANOVA of each of the measured symbiont features as the continuous variable, and the resynthesis stage and treatment were categorical variables. Means were compared using the Tukey HSD test. The current study used SEM images to measure and count the variables, which is different from the method used by Meeβen and Ott (2013) to statistically compare morphological characteristics such as hyphal growth and branching frequency of the lichen fungus *Fulgensia bracteata*.

4.4 Results

4.4.1 Isolated symbionts

Ejected and germinated fungal spores were observed either in clusters of 2-8 spores or occasionally as single spores on solid water agar after about 28 days. After transfer to MYA and 3 months of incubation, the fungal colonies were 10 mm in diameter. The growth of the lichen-forming alga from thallus fragments was visible as dark green colonies on BBM after 3–4 weeks. All pure cultures were maintained in liquid media (MYA or BBM). The liquid cultures of the non-lichen alga seemed to have a slightly faster growth rate than the lichen alga.

Species identity of the cultures was confirmed using ITS rDNA sequence comparisons with those of the lichen thallus and both sequences were subjected to comparisons with GenBank sequences. The five best matches were considered for identity to avoid matches with incorrectly named GenBank sequences. PCR from genomic DNA extracted from the fungal symbiont (in culture and lichen thallus) yielded a fragment that was 600 bp long and the sequence matched the nuclear ITS1, 5.8S, and ITS2 ribosomal DNA of *C. rangiferina* (DQ394367) with 97% identity and e-score of 0.0 over 99% query coverage. The amplified DNA of the algal symbiont (in culture and lichen thallus of *C. rangiferina*) yielded a fragment that was about 500 bp long, which matched the nuclear ITS1, 5.8S, and ITS2 ribosomal RNA gene of *Asterochloris glomerata/irregularis* (AM905998) and *A. irregulars* (AM905999) with 99% identity over 86-94 % bp coverage with an e-score of 0.0. Similarly, the amplified DNA of the algal symbiont from *Peltigera aphthosa* yielded a 500 bp product, which matched the ITS1, 5.8S, and ITS2 ribosomal DNA of *Coccomyxa peltigerae* (AY328522) with 100% identity over 88% bp coverage with an e-score of 0.0. The amplified DNA from the *Ch. ellipsoideum* culture yielded a fragment that was 700 bp long, which showed a 90% maximum identity to the nuclear ITS1, 5.8S, and ITS2 ribosomal RNA gene of *Chlorella emersonii* (FR865657). However, the culture was morphologically identified by M. Ismaiel as *Ch. ellipsoidea*, which was recently changed to *Chloroidium ellipsoideum* (Gerneck) Darienko, Gustavs, Mudimu, Menendez, Schumann, Karsten, Friedl, and Proschold (Darienko et al. 2010).

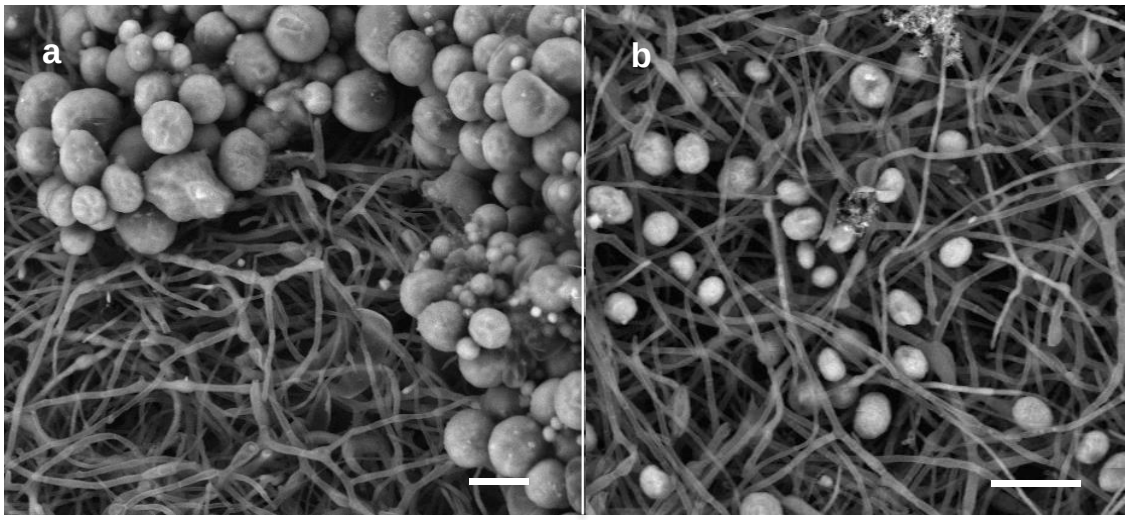
4.4.2 Developmental stages of resynthesis – morphological comparison

Growth of the algae and fungi on the nitrocellulose paper was recorded starting from 1d PCI. At 1d PCI, no apparent fungal or algal growth was observed in the compatible (Cr+As; Fig.

4.1a), both incompatible interactions (Cr+Ch and Cr+Co; Fig. 4.1b), or cultures of fungi and algae growing alone. The hyphal tips were not growing in the direction of any algal cells and there was no evidence of hyphal tip attachment. Cells of *A. glomerata/irregularis* were distributed on the filter paper in clusters of 20 - 200 cells (held together by mucilage) while single cells were seldom observed. Aplanospores were observed by SEM as cells of variable sizes (Fig. 4.1a). The cells of *Ch. ellipsoideum* (Fig. 4.1b) and *Co. peltigerae* were more evenly distributed over the filter paper, more commonly as single cells, than those of *A. glomerata/irregularis*. Since no growth of *C. rangiferina* was observed and no significant interaction was observed until 5-6 days post co-inoculation (PCI), the "pre-contact" stage was defined as 1 day PCI.

Rich branching of fungal hyphae with short internodes was observed at 5-6 days in some cases but more commonly after 8 days PCI in the treatment *C. rangiferina* and *A. glomerata/irregularis* (Cr+As; Fig. 4.2). Hyphal tips of the mycobiont were growing towards algal cells of *A. glomerata/irregularis*, and some of those which started to make contact with the algal cells, contained swollen tips (appressoria; see arrow in Fig. 4.2a). Hyphae were growing around single algal cells (Fig. 4.4.2b), and sometimes around a cluster of cells (Fig. 4.2c). Mucilage was frequently observed on *C. rangiferina – A. glomerata/irregularis* aggregations (see arrows in Fig. 4.2c). The interaction between *C. rangiferina* and *Co. peltigerae* (Cr+Co) showed a small number of lateral branches and the lateral branches were not growing towards cells of *Co. peltigerae*. Some *Co. peltigerae* cells were adjacent to fungal hyphae (see arrows in Fig. 4.2e and 2f); however, no appressoria or appresoria-like structures were observed as they were in the *C. rangiferina* and *A. glomerata/irregularis* interaction. The network of hyphae observed to be growing around *A. glomerata/irregularis* cells were not observed for cells of *Co.*

Figure 4.1 Scanning electron microscope view of co-inoculated cultures at 1 day post co-inoculation (PCI) in the compatible and incompatible interactions. (*a*) Coculture of the compatible interaction between *Cladonia rangiferina* and *Asterochloris glomerata*/*irregularis* and (*b*) coculture of the incompatible interaction between *C. rangiferina* and *Chloroidium ellipsoideum*. No interaction between *C. rangiferina* and either *A. glomerata*/*irregularis* or *C. ellipsoideum* had started. Scale bar = 15 µm.



peltigerae. *Coccomyxa peltigerae* cells were growing in clusters by 8d PCI. The interaction between *C. rangiferina* and *Ch. ellipsoideum* (Cr+Ch) showed significantly fewer lateral branches and the internodes of growing hyphae were longer than those in the interaction between *C. rangiferina* and *A. glomerata/irregularis* (Cr+As; Fig. 4.2d). The cells of *Ch. ellipsoideum* also continued to reproduce as suggested by the presence of different cell sizes. No mucilage was observed in *C. rangiferina* and *Ch. ellipsoideum* (Cr+Ch) or *C. rangiferina* and *Co. peltigerae* (Cr+Co) cultures. As a result of the extensive contact between *C. rangiferina* and *A. glomerata/irregularis*, 8 d PCI was defined as the "contact" stage.

Coordinated growth between *C. rangiferina* and *A. glomerata/irregularis* (Cr+As) was observed by 21d PCI where neither symbiont appeared to be overgrowing the other symbiont. The cells of *A. glomerata/irregularis* were integrated within a hyphal matrix of *C. rangiferina* (Fig. 4.3a). Hyphae were emerging through the middle of algal colonies and numerous hyphae formed hyphal networks within and between algal colonies (Fig. 4.3b). The fungal appressoria at 21d PCI were observed to have expansions at the hyphal tips (see arrows in Fig. 4.3c and 3d). Some cells of the *A. glomerata/irregularis* colonies had indentations of the cell wall (see arrow in Fig. 4.3e). *Cladonia rangiferina* and *Co. peltigerae* formed loosely arranged aggregations (Fig. 4.4a); however, no appresoria or appresoira-like contacts were observed. At this same time period (21d PCI), physical contact between fungal hyphae and the non-lichen alga, *Ch. ellipsoideum* was absent in most cultures (Fig. 4.4b) but was present in some cultures (Fig. 4.4c-g). In the culture of *Ch. ellipsoideum* showing physical contacts, some cells of *Ch. ellipsoideum* had indentations at the point of hyphal contact (Fig. 4.4g). The production of very few lateral branches by *C. rangiferina* was observed in these cultures (Fig. 4.4b), and otherwise lateral

Figure 4.2 Scanning electron microscope view of cocultures at 8 days post-co-inoculation (PCI) in the compatible and incompatible interactions. (*a*) Hyphae of *Cladonia rangiferina* growing around cells of *Asterochloris glomerata*/*irregularis* and swollen tips of the fungal hyphae in contact (arrow); (*b*) lateral branches from *C. rangiferina* growing around an individual *A. glomerata*/*irregularis* cell; (*c*) hyphae of *C. rangiferina* growing around cell clusters of *A. glomerata*/*irregularis* and mucilage (arrows); (*d*) cocultures of *C. rangiferina* and *Chloroidium ellipsoideum* at 8 days PCI, showing no visible contacts as observed between *C. rangiferina* and *A. glomerata*/*irregularis*; (*e*) adhesion of some *Coccomyxa peltigerae* cells to *C. rangiferina* (arrows); (*f*) a cluster of *C. peltigerae* cells covered by *C. rangiferina* mycelia. Scale bar = 15 µm.

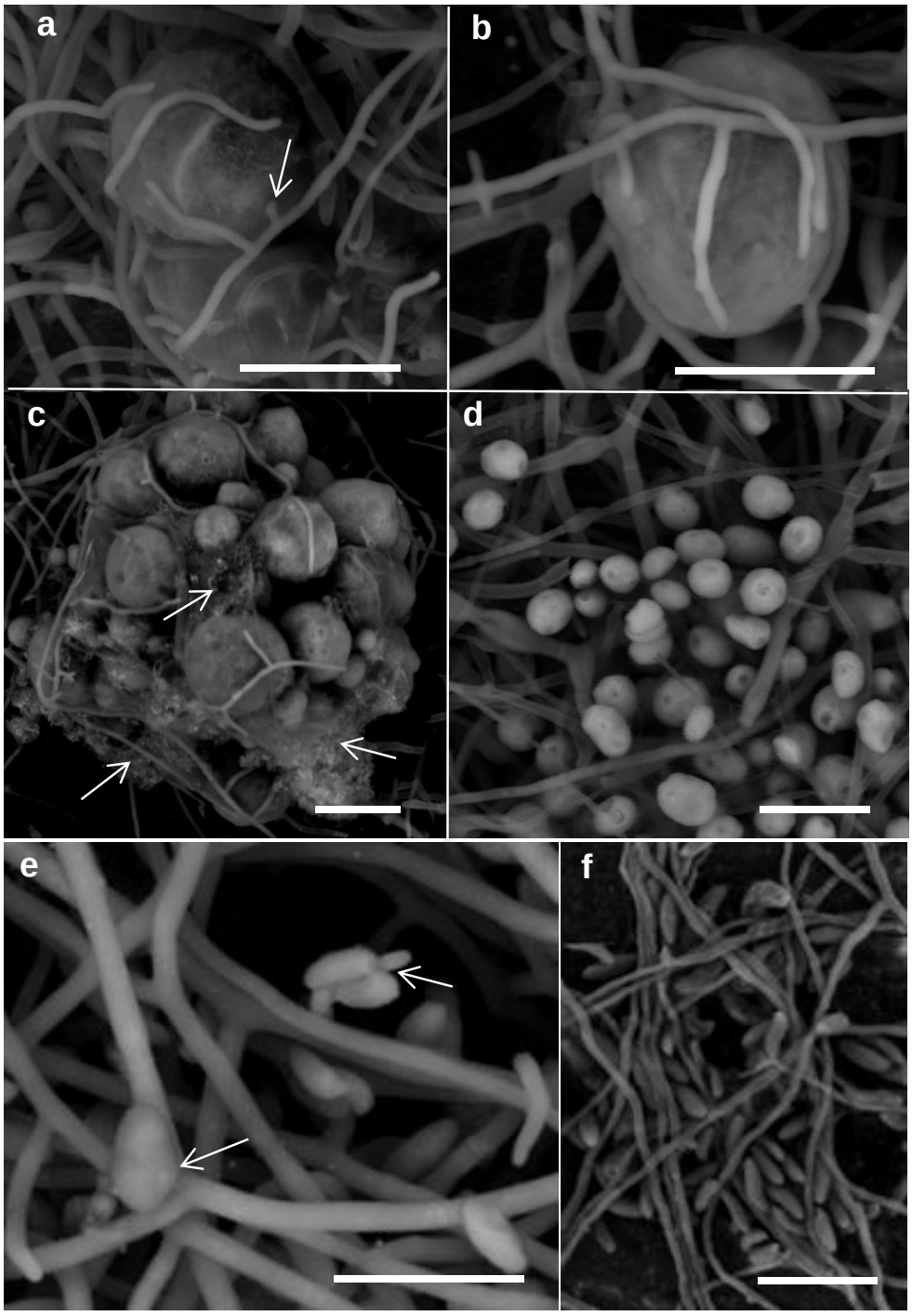
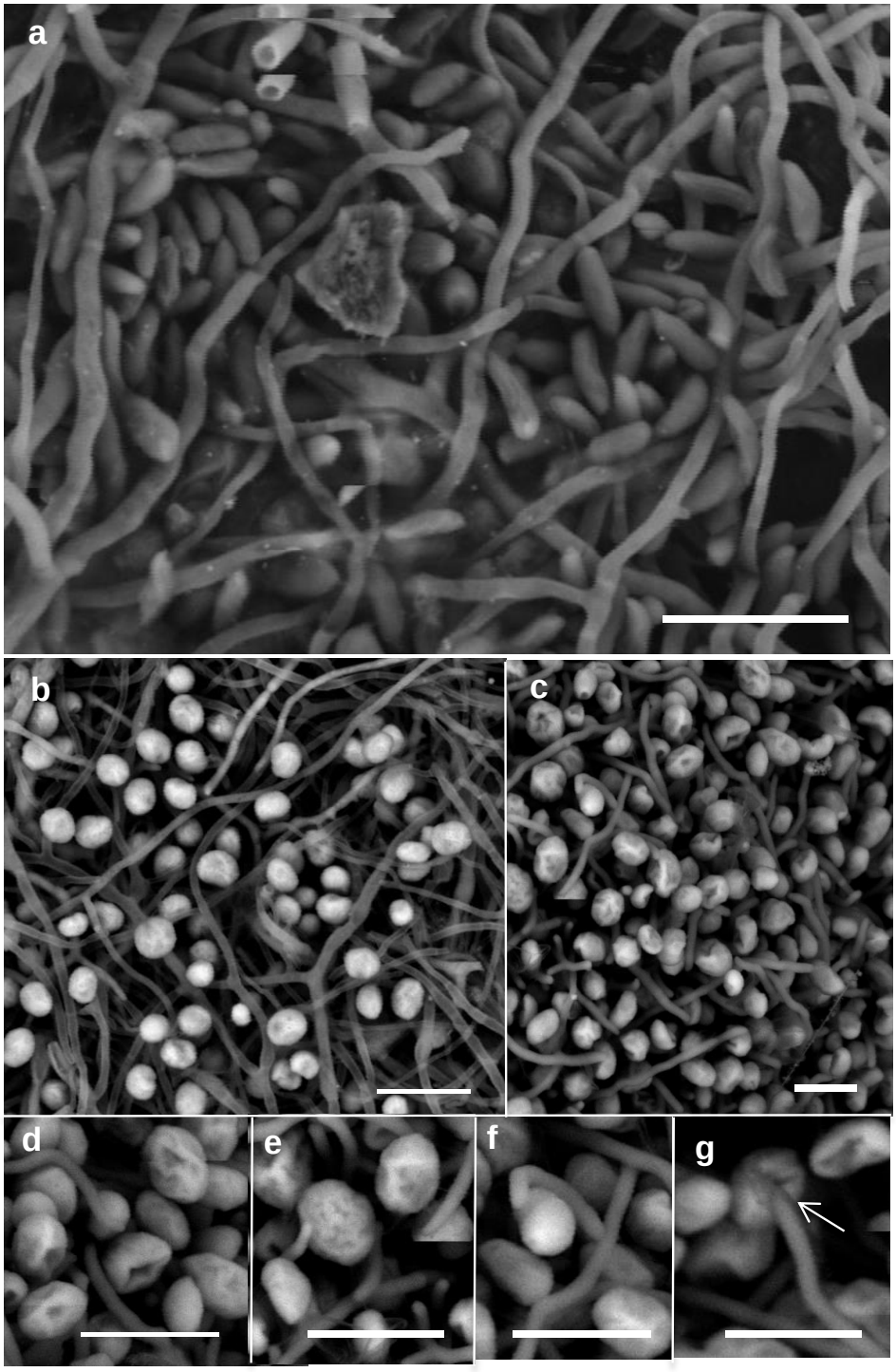

a
b
c
d
e
f

Figure 4.3 Scanning electron microscopic view of cocultures of *Cladonia rangiferina* and *Asterochloris glomerata*/*irregularis* at 21 days post co-inoculation. (*a*) Mycelia of *C. rangiferina* forming a network between colonies of *A. glomerata*/*irregularis* and mucilage (arrow); (*b*) lateral branches of *C. rangiferina* starting contacts (arrow) with new aplanospores of *A. glomerata*/*irregularis* (arrow); (*c*) expansion of fungal appressoria (arrow); (*d*) *C. rangiferina* forming more frequent and prominent appressoria with algal cells; (*e*) haustoria that seem to penetrate algal cells (arrow). Scale bar = 15μm.

Figure 4.4 Scanning electron microscopic view of cocultures of *Cladonia rangiferina* with *Coccomyxa peltigerae* and *Chloroidium ellipsoideum* at 21 days post co-inoculation. (*a*) Loose hyphal network of *C. rangiferina* and *C. peltigerae* cells; (*b*) *C. ellipsoideum* had further increased cell number but there was no or much less growth or lateral branches produced by *C. rangiferina* and no visible contact between partners; (*c*) prominent growth of *C. rangiferina* (arrows); however, no intense short lateral branches were observed; (*d*) *C. ellipsoideum* cells having a shrivelled and dead appearance; (*e*, *f*) *C. rangiferina* hyphae occasionally making contact (appressoria-like structures) with *C. ellipsoideum* cells (arrows); (*g*) *C. rangiferina* hyphae penetrating (arrow) *C. ellipsoideum* cells. Scale bar = 15 μm.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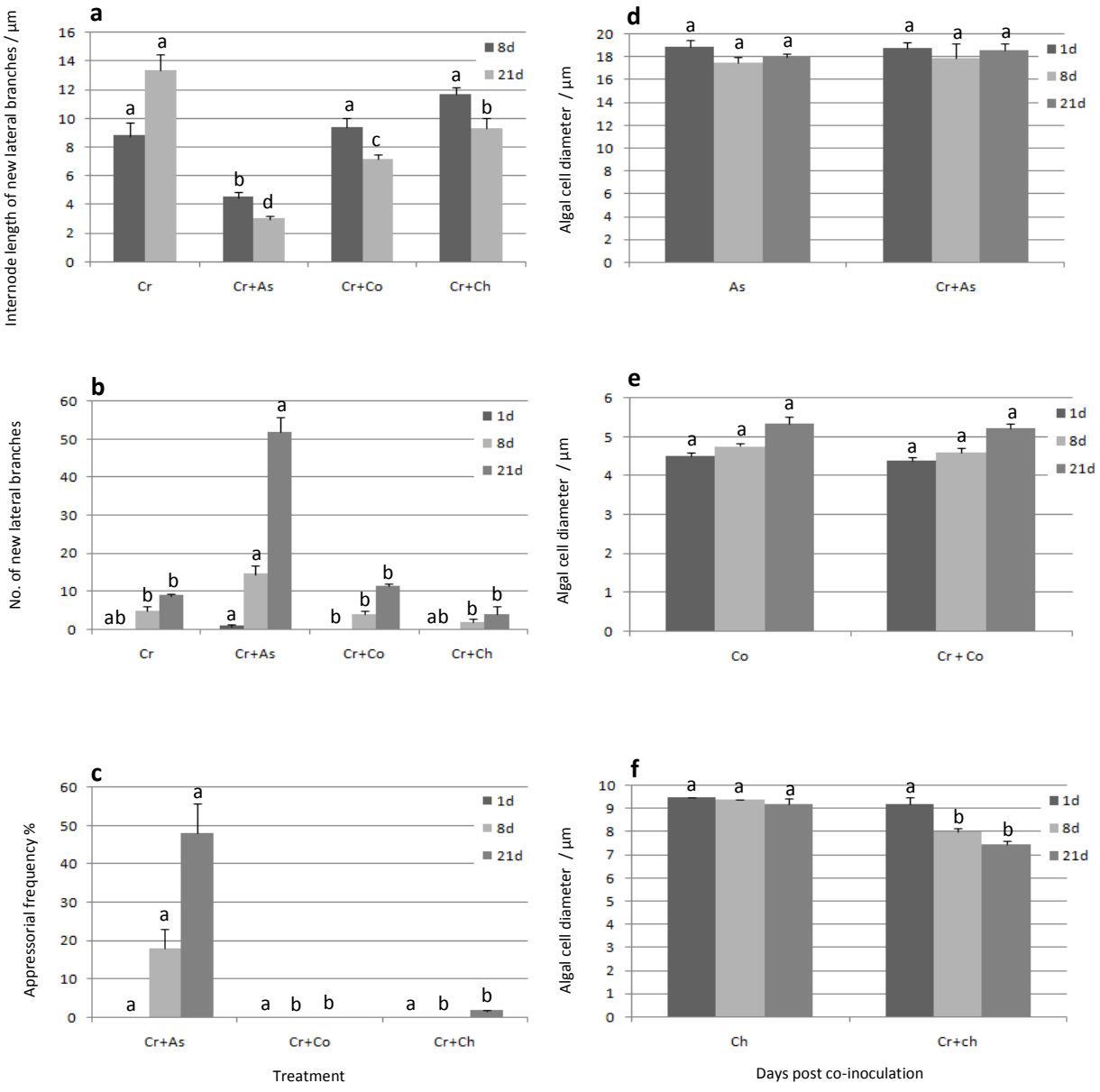
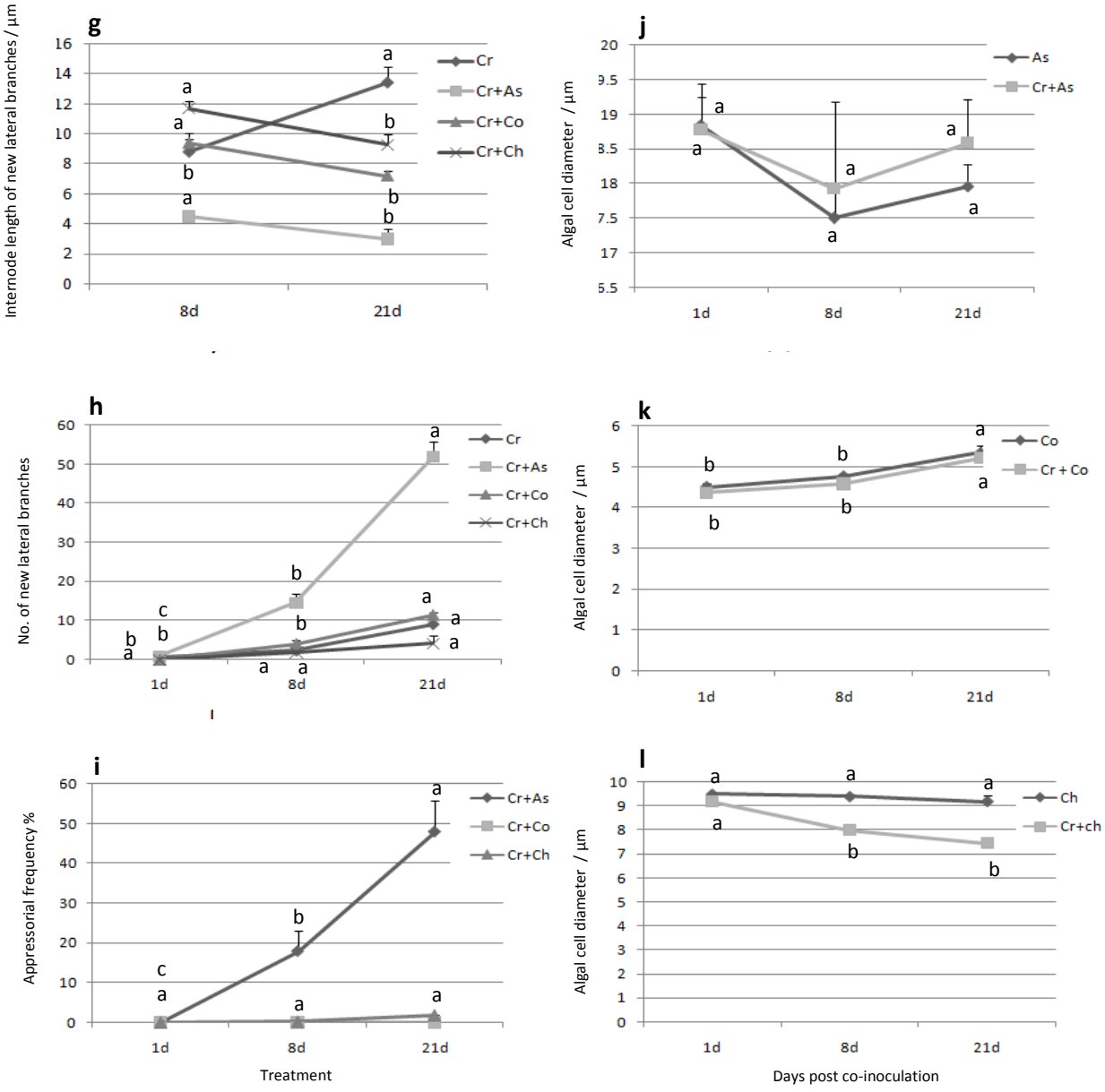
branch production by *C. rangiferina* growing with *Ch. ellipsoideum* was rarely observed (Fig. 4.4c). Mucilage was observed in *C. rangiferina* and *A. glomerata/irregularis* (Cr+As) (see arrow in Fig. 4.3a) but it was not observed in *C. rangiferina* and *Ch. ellipsoideum* (Cr+Ch) or *C. rangiferina* and *Co. peltigerae* (Cr+Co) cultures. Due to the coordinated growth observed between *C. rangiferina* and *A. glomerata/irregularis*, the 21 d PCI was defined as the "growth together" stage.

4.4.3 Quantitative measurements of interactions

The statistical comparisons between treatments are shown in Fig. 4.5a -f. The internode lengths of *C. rangiferina* growing with *A. glomerata/irregularis* (Cr+As) were significantly shorter ($P<0.0001$) compared to those growing alone (Cr) and those growing with *Co. peltigerae* (Cr+Co) and *Ch. ellipsoideum* (Cr+Ch) at both 8 d and 21 d PCI (Fig. 4.5a). At both 8 d ($P=0.0147$) and 21 d ($P<0.0001$) PCI, *C. rangiferina* growing with *A. glomerata/irregularis* (Cr+As) showed a significantly higher number of new lateral branches compared to those growing alone (Cr) and those growing with *Co. peltegerae* (Cr+Co) and *Ch. ellipsoideum* (Cr+Ch; Fig. 4.5b). The frequency of appressoria produced by *C. rangiferina* was significantly greater in the interaction with *A. glomerata/irregularis* (Cr+As) than with *Ch. ellipsoideum* (Cr+Ch; Fig. 4.5c), while no appresoria or appresoria-like contacts were observed between *C. rangiferina* and *Co. peltigerae* (Cr+Co). *Ch. ellipsoideum* cells growing with *C. rangiferina* (Cr+Ch) showed significantly smaller diameters in comparison to those growing alone (Ch; Fig. 4.5d), while there was no significant difference observed in cell diameter of *A. glomerata/irregularis* and *Co. peltigerae* growing with *C. rangiferina* (Cr+As and Cr+Co) and alone (As and Ch) at 8d and 21d PCI (Fig. 4.5e and 5f).

Figure 4.5 Changes in first 3 resynthesis stages (1, 8, and 21 days) and each treatment (Cr+As, *Cladonia rangiferina* + *Asterochloris glomerata*/*irregularis*; Cr+Co, *C. rangiferina* + *Coccomyxa peltigerae*; Cr+Ch, *C. rangiferina* + *Chloroidium ellipsoideum*; Cr, *C. rangiferina* alone; As, *A. glomerata*/*irregularis* alone; Co, *C. peltigerae* alone; Ch, *C. ellipsoideum* alone), with panels *a–f* showing comparison between treatments within resynthesis stages and panels *g–l* showing the variation and comparison of each parameter from early (1 day) to late (21 days) resynthesis stage for each treatment. Lowercase letters over bars show Tukey's mean comparison test (P= 0.05) comparing between treatments within resynthesis stages. Lowercase letters on data point on line charts show Tukey's mean comparison test (P = 0.05) comparing between resynthesis stages within treatments.


a
Internode length of new lateral branches / µm
8d
21d
Cr
Cr+As
Cr+Co
Cr+Ch
b
No. of new lateral branches
1d
8d
21d
c
Appressorial frequency %
Treatment
d
Algal cell diameter / µm
As
Cr+As
e
Co
Cr + Co
f
Ch
Cr+ch
Days post co-inoculation


g
Internode length of new lateral branches / µm
Cr
Cr+As
Cr+Co
Cr+Ch
8d
21d
j
Algal cell diameter / µm
As
Cr+As
1d
8d
21d
h
No. of new lateral branches
Cr
Cr+As
Cr+Co
Cr+Ch
1d
8d
21d
k
Algal cell diameter / µm
Co
Cr + Co
1d
8d
21d
i
Appressorial frequency %
Cr+As
Cr+Co
Cr+Ch
1d
8d
21d
Treatment
l
Algal cell diameter / µm
Ch
Cr+ch
1d
8d
21d
Days post co-inoculation

The statistical comparisons over time (1d, 8d, and 21d PCI) within each treatment are shown in Fig. 4.5g-l. Internode lengths of *C. rangiferina* when in culture with an alga decreased over time in contrast to *C. rangiferina* growing alone (from 8d to 21d PCI; Fig. 4.5g). A significant increase in number of lateral branches was evident in *C. rangiferina* growing alone (Cr), growing with *A. glomerata/irregularis* (Cr+As) and growing with *Co. peltigerae* (Cr+Co) over time (from 8 d to 21 d PCI; Fig. 4.5h) in comparison to *C. rangiferina* and *Ch. ellipsoideum* (Cr+Ch). There was a significant increase in ($P=0.0019$) appressorial frequency from 1 d to 21 d PCI between *C. rangiferina* and *A. glomerata/irregularis* (Cr+As) but not between *C. rangiferina* and *Ch. ellipsoideum* or between *C. rangiferina* and *Co. peltigerae* (Cr+Ch or Cr+Co; Fig. 4.5i). The cell diameter of *Ch. ellipsoideum* growing with *C. rangiferina* (Cr+Ch) was significantly smaller at 21d PCI than it was at 1 d PCI, while there was no significant change in algal diameter observed over time in *A. glomerata/irregularis* alone (As), *Ch. ellipsoideum* alone (Ch) and each of *A. glomerata/irregularis* and *Co. peltigerae* growing with *C. rangiferina* (Cr+As and Cr+Co; Fig. 4.5j - 5l).

4.5 Discussion

This study described the first three stages of resynthesis for *C. rangiferina* and *A. glomerata/irregularis* and statistically compared four quantitative measures of growth and development during the interaction of *C. rangiferina* with its native lichen alga (compatible), a native lichen alga (*Coccomyxa peltigerae*) isolated from a different lichen, *Peltigera aphthosa* (incompatible), and a foreign, non-lichen alga, *Chloroidium ellipsoideum* (incompatible). The interaction of a lichen fungus with its compatible algal partner triggered specific morphological differentiation which was not seen with incompatible algal partners.

4.5.1 Characteristics of the first three resynthesis stages in C. rangiferina

The "pre-contact" stage was defined as 1d PCI since no fungal growth toward algal cells or physical contact was observed in the compatible interaction. Trembley et al. (2002), reports that both symbionts of *Baeomyces rufus* were bound together by mucilage after 24 h PCI. Mucilage was not visible between partners in the current study, although cells of *A. glomerata/irregularis* appeared to be held within a cluster of cells by mucilage. In the current study, the growth of *C. rangiferina*, *A. glomerata/irregularis* and *Ch. ellipsoideum* was visible within the first few days of culture, but the initial physical contact between compatible organisms was observed at 8 days PCI. Therefore, "contact" stage was defined at 8 d PCI when frequent lateral branches with short internodes, hyphal growth towards compatible algal cells, and appressoria were observed. These were the characteristics that were also observed in other studies during the "contact" stage of resynthesis (Ahmadjian et al. 1978; Ahmadjian and Jacobs 1983; Stocker-Wörgötter and Türk 1991; Trembley et al. 2002; Joneson and Lutzoni 2009). *B. rufus* showed evidence for the "contact" stage after 12 days (Trembley et al. 2002) and Joneson and Lutzoni (2009) observed envelopment of algal cells through increased lateral hyphal branching of *C. grayi* at 21 days while other studies have not documented the number of days taken to reach the "contact" stage. The "growth together" stage was defined around 21 days PCI in the current study by the presence of fungal hyphae protruding through colonies of *A. glomerata/irregularis* as observed under the dissecting microscope and when coordinated growth between partners was observed by SEM. This was regarded as the "arachnoid-like" stage and "hyphal networks" between colonies described by Schuster et al. (1985) and Stocker-Wörgötter and Türk (1991), respectively. However, Joneson and Lutzoni (2009) observed aerial hyphae of *C. grayi* protruding from *Asterochloris* colonies around 10 days PCI under a dissecting

microscope. *Cladonia cristatella* showed squamule development as early as 14-21 days after inoculation (Ahmadjian and Jacobs 1981) while no squamule development was observed in this study. *Peltigera praetextata* formed soredia-like structures after about 3 months (Stocker-Wörgötter and Türk 1991). However, a well organized prethallus stage was not obtained in our study even after 3 months (90 days) PCI, a situation that was also experienced in a previous study (Joneson and Lutzoni 2009). An explanation for the absence of a prethallus stage may be the high levels of moisture in the petri plate created by condensation on the petri plate since wet conditions have been observed to interfere with lichenization (Ahmadjian and Jacobs 1983). High moisture content may increase the rate of reproduction of the photobiont making them less susceptible to fungal attack (Ahmadjian and Jacobs 1981) and therefore they would be slower to form a thallus. However, describing the later stages of resynthesis (stages 4 and 5) for the lichen *C. rangiferina* which will require specific media and growing conditions, was not an objective of the current study.

The extensive mucilage production observed in the compatible interaction only (Cr+As) has been suggested to be a vital characteristic during the resynthesis of *Xanthoria parietina* with its compatible photobiont (Ott 1987b) and six potential photobionts with the mycobiont *Fulgensia bracteata* (Schaper and Ott 2003). Mucilage is postulated to be important in specific biont recognition, successful lichenization and early lichenization (Meeβen and Ott 2013). The absence of mucilage in the incompatible interactions in this study supports the proposed function. Similarly, the formation of loosely interwoven networks (Ahmadjian and Jacobs 1983; Garty and Delarea 1987; Ott 1987b) that were covered with mucilage has been observed during compatible interactions (Schaper and Ott 2003; Meeβen and Ott 2013).

4.5.2 Dynamic nature of the compatible interaction

The significantly short internodes and increased lateral branching in the compatible interaction compared to the incompatible and control treatments indicate that a recognition mechanism may exist between the compatible partners which triggers significant growth responses as also suggested by previous studies (Molina et al. 1993; Legaz et al. 2004; Sacristán et al. 2007; Joneson et al. 2011; Meeβen and Ott 2013). The physical contact between hyphal tips and algal cell walls, consistent with other studies, is interpreted here to be appressoria on the algal cell. The number of hyphal attachments (appressoria) significantly increased in the compatible interaction from 1d to 21d PCI, reaching a maximum of 50% hyphal attachment in the compatible interaction compared to the incompatible interactions without apparent detrimental effect on algal cells. The absence of a detrimental effect in the compatible interaction is further evident by the continuous reproduction of algal cells. The colonies of *A. glomerata/irregularis* contain indentations of the cell wall where hyphal tips are found at 21d PCI. This may indicate possible appressorial penetrations into the algal cell during the interaction between *A. glomerata/irregularis* and *C. rangiferina*. Appressorial penetrations have also been observed in the resynthesis of *C. cristatella* (Ahmadjian et al. 1978; Ahmadjian and Jacobs 1981, 1983). The apparent penetration of appressoria of the parent cell may be interpreted as hyphae that were trying to attach to new cells (aplanospores), which is also supported by Greenhalgh and Anglesea (1979). On the other hand, the indentation on the algal cell wall may also suggest a shift from the "controlled parasitic" interaction between compatible partners to a parasitic interaction. The hypothesized shift in virulence may be explained by changes in micro environmental conditions in the medium or an artefact of SEM preparation. When comparing with previous studies, Durrell (1967) reported haustorial attachment frequency of 20% while

Ahmadjian and Jacobs (1981) reported haustorial frequencies of 57 to 61% in the natural lichen thallus of *C. cristatella*. In addition, Ahmadjian et al. (1978) observed a haustorial frequency of 40 % in *C. cristatella* resynthesis studies.

Differences in appressorial attachment and number of days to achieve the same resynthesis stages in different species are reported by various authors. These differences may suggest a dynamic nature of the interaction produced by microenvironmental culture conditions such as available nutrients and symbiont growth rates. This phenomenon may be even more pronounced in nature, resulting in a mosaic of different gradients of lichenizing populations. Frequent transitions between different lifestyles by symbionts have been suggested to occur during evolution (Gargas et al. 1995; Ewald 2004). As hypothesized for many symbioses, the ecological outcome of the interaction may span a continuum between mutualism and parasitism over time or space depending on the traits of the symbionts and the ambient environmental conditions (Cushman and Whitham 1989; Cushman and Addicott 1991; Bronstein 2001).

4.5.3 Interaction between C. rangiferina *and* Co. peltigerae

The interaction between *C. rangiferina* and *Co. peltigerae* showed no significant responses with regards to the measured variables in comparison to the compatible interaction (Cr+As) and controls. However, while the algal cell diameter in *Ch. ellipsoideum* treatment changed with time, it did not change in *Co. peltigerae* compared with the control suggesting that *C. rangiferina* did not have a negative effect on *Co. peltigerae* as it did on *Ch. ellipsoideum*. The intermediate position of the Cr+Co treatment, between Cr+As and Cr+Ch in hyphal internode length and number of lateral branches (Fig. 4.5), suggests that *Co. peltigerae* may be more likely to form a beneficial symbiosis with *C. rangiferina* than *Ch. ellipsoideum*. Loosely arranged

aggregations of fungal hyphae with cells of *Co. peltigerae* and no appressoria-like structures, were also observed by Muggia et al. (2011) in co-culture experiments between the facultative lichenized fungus *Schizoxylon albescens* and its photobiont *Coccomyxa* sp. The intermediate position of *Co. peltigerae* may also be support for the controlled parasitic nature of lichen associations. One explanation for the intermediate position in the types of interactions may be the evolutionary or ecological distance of the algal partners. While support exists for "*Chlorella*" in the broad sense to be more distantly related to both *Coccomyxa* and *Asterochloris* (Neustupa et al. 2011), closely related (ecologically or genetically) incompatible algal partners have been observed to induce phenotypic responses in the fungal partner that are similar to those induced by the compatible algal partners (Ahmadjian and Jacobs 1981; Meeβen and Ott 2013). This has been interpreted as due to the production of shared (Joneson and Lutzoni 2009) ubiquitous, basal or incomplete lichenization signals (Ahmadjian 1992; Meeβen and Ott 2013).

4.5.4 Interaction of C. rangiferina *with* Ch. ellipsoideum

Cladonia rangiferina co-inoculated with *Ch. ellipsoideum* showed slow growth in most of the areas on the culture at 8d and 21d PCI, while cells of *Ch. ellipsoideum* cells were continuously growing and reproducing. This slow growth shown by *C. rangiferina* might have resulted from the high growth rate of *Ch. ellipsoideum* observed in culture (data not shown) compared to that of *C. rangiferina* and *A. glomerata/irregularis* and the slow growing mycobiont might have not have had the ability to compete for nutrients in the medium with a fast growing alga. This could also be interpreted as the absence of compatible algal-derived signals which are needed to trigger the growth of *C. rangiferina* and / or the presence of growth-abolishing signals produced by incompatible *Ch. ellipsoideum*. In vesicular-arbuscular mycorrhizas, host-derived

signals that abolish growth arrest are known (Siqueira et al. 1991; Giovanett et al. 1996). However, by 21d PCI the fungal hyphae showed growth and a few new lateral branches with significantly shorter internodes than those of the fungus growing alone (Fig. 4.5a). In addition, occasional physical contact between hyphal tips and algal cells suggested the presence of appressoria although the percentage of these between *C. rangiferina* and *Ch. ellipsoideum* was significantly lower than that between *C. rangiferina* and *A. glomerata/irregularis* at this stage.

Growth and physical contact between the alga and fungus were more common on the medium where there was prominent growth of *C. rangiferina* than in places on the medium with less growth of fungal hyphae and more *Ch. ellipsoideum* growth. An explanation may be that *C. rangiferina* was exhibiting parasitic behaviour on *Ch. ellipsoideum*. Significant reduction in cell diameter of *Ch. ellipsoideum* from 1d to 21d PCI co-inoculated with *C. rangiferina* provides further evidence in support of this hypothesis. *Chlorella* spp. are widely involved in symbiotic interactions, both beneficial (Karakashian 1975; Omura et al. 2004; Vu et al. 2010; Kodama and Fujishima 2011) and parasitic ones, especially in fishes (Hoffman et al. 1965). Watanabe et al. (2005) reported a symbiotic interaction (beneficial) between *Ch. sorokiniana* and the fungal strain CSSF-1 through direct adhesion to the cell surface of *Ch. sorokiniana*, which is similar to the observations in the current study. Ahmadjian and Jacobs (1981) also observed a number of lichenized and non-lichenized algal species that were apparently parasitized by the mycobiont *Cladonia cristatella*. The formation of aerial hyphae within colonies of algae or hyphae that are growing around algal cells were not observed between *C. grayi* and *Ch. vulgaris* in this study as they were by Joneson and Lutzoni (2009) and between the facultative lichenized fungus *S. albescens* and the *Chlorella*-like photobiont of *Flakea papillata* (Muggia et al. 2011). If growth rates influence parasitism, and incubation conditions influence growth rates, then this further

implies that consistency of incubation conditions may be very important to maintain for cross comparisons of resynthesis and degree of mutualism and parasitism in an interaction. The balanced growth between compatible partners is evident by no significant reduction in cell diameter of *A. glomerata/irregularis* co-inoculated with *C. rangiferina* in comparison with that of *Ch. ellipsoideum* co-inoculated with *C. rangiferina*.

This study establishes the timing of the three resynthesis stages for further studies on the interaction between *C. rangiferina* and *A. glomerata/irregularis*, forming the basis for comparing expression of recognition and defense-related genes at early resynthesis stages. The three stages of resynthesis were different from those of other studies and other species suggesting that growth conditions must be defined for each species. This study also reports quantitative evidence for the specific morphological differentiation that occurs during the interaction between compatible partners in a lichen thallus in comparison to those that occur between non-compatible partners. The production of mucilage in the interaction of *C. rangiferina* and *Asterochloris* suggests that mucilage production is an important part of lichen formation. The absence of such characteristics in *Ch. ellipsoideum* and *Co. peltigerae* but the presence of some of the features expected to be present in parasitic interactions, and the intermediate position of the Cr+Co features between Cr+As and Cr+Ch supports the controlled parasitic nature of the lichen association, which warrants further research. Further research on comparisons between species and well defined culture conditions may reveal effects of habitat on resynthesis, strategies of each of the alga and fungus for lichenization, and hence species success. While other studies exist that define resynthesis stages for other lichens (Joneson and Lutzoni 2009; Muggia et al. 2011), the establishment of the resynthesis stages for the *C. rangiferina – A. glomerata/irregularis* model

system in this study, may facilitate our understanding of ecological strategies leading to the success of this important lichen species.

CHAPTER 5

Recognition and defense-related gene expression at three resynthesis stages in lichen symbionts

5.1 Abstract

Recognition and defense responses are early events in plant-pathogen interactions and between lichen symbionts. The effect of elicitors on responses between lichen symbionts is not well understood. The objective of this study was to compare the difference in recognition and defense-related gene expression as a result of culture extracts (containing secreted water-soluble elicitors) from compatible and incompatible interactions at each of three resynthesis stages in the symbionts of *Cladonia rangiferina*. This study investigated gene expression by quantitative PCR in cultures of *Cladonia rangiferina* and its algal partner, *Asterochloris glomerata/irregularis* after incubation with liquid extracts from cultures of compatible and incompatible interactions at three early resynthesis stages. Recognition-related genes were significantly upregulated only after physical contact, demonstrating symbiont recognition in later resynthesis stages than expected. One of three defense-related genes, *chit*, showed significant downregulation in early resynthesis stages and upregulation in the third resynthesis stage demonstrating a need for the absence of chitinase early in thallus formation and a need for its presence in later stages as an algal defense reaction. This study revealed that recognition- and defense-related genes are triggered by components in culture extracts at three stages of resynthesis and some defense-related genes may be induced throughout thallus growth. The parasitic nature of the interaction reveals parallels between lichen symbionts and plant pathogenic systems.

5.2 Introduction

Lichens, which are associations between fungi and algae, may be considered a "controlled" parasitism (Ahmadjian and Jacobs 1983; Honegger 1998) rather than a mutualism (Smith 1980). In the controlled parasitism of lichens, the fungus is a biotrophic parasite on the alga and the recognition between partners allows the alga to turn on defensive strategies that limit fungal growth (Ahmadjian and Jacobs 1983; Molina et al.1993; Honegger 1998), which was biochemically examined by Legaz et al. (2004). While knowledge of controlled parasitism in lichens is in its infancy, the interaction between lichen symbionts seems to resemble early events that occur in plant-fungal pathogen systems, where recognition of the fungal pathogen by elicitors triggers plant defense mechanisms to control the fungus (reviews Crute 1994; Dixon et al. 1994; Benhamou 1996; Odjakova and Hadjiivanova 2001; Staskawicz 2001; Bogdanove 2002; Dodds and Rathjen 2010). The plant defense response may then trigger an anti-defense response in the fungus, but it is not known if an anti-defense response is triggered in the lichen fungus.

A compatible interaction between lichen symbionts is important for successful lichenization (Ott 1987b; Ott et al. 1995; Schaper and Ott 2003; Meeβen and Ott 2013; Meeβen et al. 2013). Previously, Athukorala et al. (2014) showed that compatible symbionts of *Cladonia rangiferina* (L.) F.H. Wigg. initiated an interaction with defined resynthesis stages, "the pre-contact" stage occurred 1 day post co-inoculation (PCI) of both symbionts, the "contact" stage occurred 8 days PCI, and the "growth together" stage occurred 21 days PCI, but the incompatible symbionts showed either no interaction or a late parasitic interaction. The consistent formation of defined stages in the compatible, but not in the incompatible interactions, suggests that compounds (elicitors) produced as a result of the compatible partners would facilitate recognition

between compatible partners. Subsequent formation and penetration of a fungal appressorium on the algal cell wall would trigger algal defense responses. Additionally, anti-defense responses by the fungus would be expected to occur at later stages. Early studies showed that fungal arginase-like proteins and algal urease act as an elicitor-receptor system to recognize compatible lichen symbionts (Bubrick and Galun 1980; Bubrick et al.1981; Galun and Bubrick 1984; Ahmadjian 1987; Kardish et al. 1991; Molina and Vicente 1995; Molina et al. 1998; Legaz et al. 2004). Urease on algal cell walls acts as a receptor for fungal elicitors (Millanes et al. 2004) confining elicitors to the cell walls and leaving the algal cell intact (Molina and Vicente 1995; Molina et al.1998). The absence of algal urease allows entry of the fungal elicitor into the algal cell (Molina and Vicente 1995) resulting in a lethal parasitic interaction (Molina et al. 1998).

Recent studies on gene expression in lichen symbiont interactions (Trembley et al. 2002; Joneson et al. 2011; Miao et al. 2012; Junttila et al. 2013) have provided the impetus needed for further progress in this field, but these studies were not focused on elicitor induced responses. The objective of this study was to compare the difference in recognition and defense-related gene expression in response to culture extracts (containing elicitors) from compatible and incompatible interactions at each of three resynthesis stages. The hypotheses were that 1) early recognition in the compatible interactions will initiate defense responses; and 2) the absence of early recognition in the incompatible interactions will result in no defense response.

5.3 Materials and Methods

5.3.1 Origin of the fungal and algal isolates

Axenic cultures of the fungal and algal partners isolated from *Cladonia rangiferina* (SA 24) as described by Athukorala et al. (2014) were used in the study. Fungal cultures were

maintained in 100 ml of liquid malt yeast extract medium (MY: 20g malt extract, 2g yeast extract, 1l distilled water) for 24 hour in the dark. The compatible alga, *Asterochloris glomerata/irregularis (*hereafter referred to as *Asterochloris)* and a non-native, incompatible alga, *Chloroidium ellipsoideum* (from the Phycology lab, Zagazig University, Egypt) were maintained in 100 ml of liquid Bold's basal medium (BBM: Deason and Bold 1960; Bischoff and Bold 1963) in 12 hour light (845 lumens) at 20 °C with continuous shaking at 160 rpm. *Asterochloris* was obtained from the lichen thallus as described in Athukorala et al. (2014)

5.3.2 Experimental design and culture conditions for test cultures

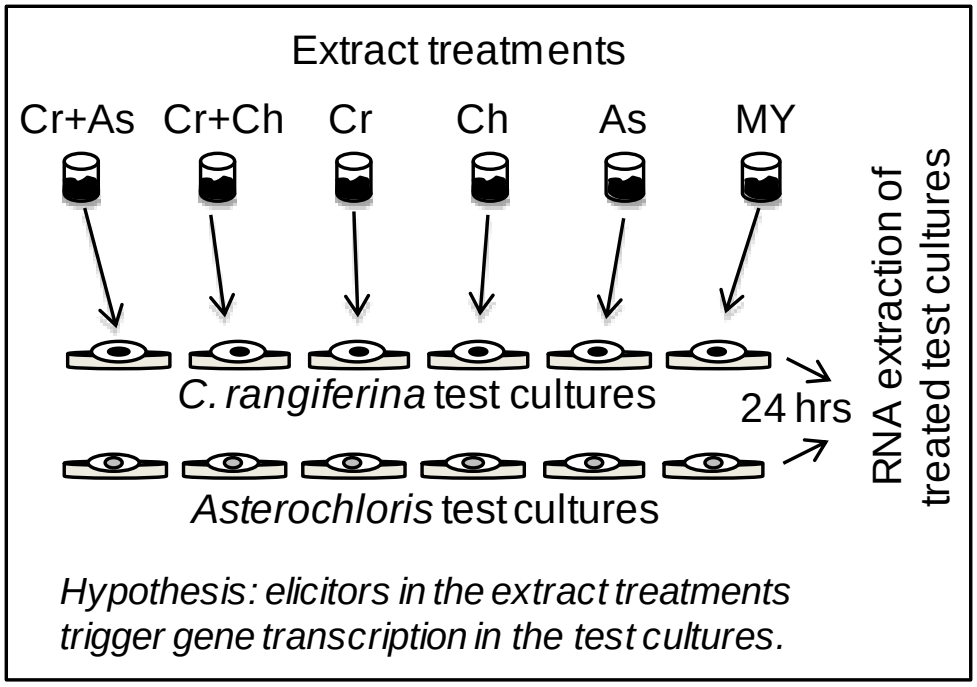
One ml of liquid cultures of SA24 from each of *Asterochloris* and *C. rangiferina* were inoculated onto separate nitrocellulose membranes (0.22 µm, Millipore GSWP02500, one membrane per 50 mm diameter petri plate) and placed on solid MY and BBM, respectively. Five replicates for each of the *C. rangiferina* and *Asterochloris* test cultures were incubated for four weeks at 20 °C in 24 hour (h) of dark (for *C. rangiferina*) and 12 h of light (for *Asterochloris*). These test cultures were exposed to liquid extracts from six treatments (previously prepared as described in next section), where a treatment refers to the liquid extraction from each of the following; 1) compatible interaction (*C. rangiferina* + *Asterochloris* [Cr+As]), 2) incompatible interaction (*C. rangiferina* + *Ch. ellipsoideum* [Cr+Ch]), 3) *C. rangiferina* only [Cr; control], 4) *Asterochloris* only [As; control], 5) *Ch. ellipsoideum* only [Ch; control], 6) control (medium only [MY; internal control]). After a 24 h incubation period (which was selected from a preliminary study (data not shown), each test culture of *Asterochloris* or *C. rangiferina* exposed to the six extract treatments was scraped from the membrane and collected in 1.5 µl micro centrifuge tubes. These test cultures were preserved in liquid nitrogen just after

harvesting and were stored at -80 °C until RNA was extracted using Trizol® (Invitrogen, Carlsbad, CA, USA) (Fig. 5.1).

5.3.3 Preparation of the six extract treatments

The six extract treatments in the previous paragraph (Cr+As; Cr+Ch; Cr; Ch; As; MY), were prepared from various combinations of pure liquid cultures of each of *C. rangiferina, Asterochloris*, and *Chloroideum ellipsoideum* grown alone. The pure cultures were centrifuged in a Sorvall Legend X1R refrigerated centrifuge with a TX-400 swinging bucket rotor (Thermo Fisher Scientific Inc., MA, USA) at 8 000 rpm for 10 min, and the supernatant drained away through filter paper in a funnel. The fungal pellets were gently ground with a mortar and pestle under sterile conditions to break up the colonies, and resuspended in 50 ml of 99:1 medium (1 ml of MY added to 99 ml of BBM; Joneson et al. 2011) to obtain a homogeneous suspension after grinding. Algal cells were not ground and most hyphal fragments continued to grow in the suspension. Three ml of each suspension were inoculated into liquid 99:1 medium to prepare the six extract treatments above. The set of six treatments was incubated for 1d (pre-contact), 8d (contact) or 21d (growth together) to represent the first three early stages of resynthesis of the lichen (Athukorala et al. 2014). Soluble elicitors were expected to be released in treatment cultures that are relevant to each of the three resynthesis stages and are hypothesized to trigger gene transcription in the *C. rangiferina* and *Asterochloris* test cultures. Incubation conditions were 12 h light at 20 °C. After the relevant incubation period (1d, 8d and 21d), cultures from all treatments were centrifuged under sterile conditions at 10 000 x g for 10 min and the supernatant was carefully pipetted without disturbing the cell pellets and filtered through sterile filter paper

Figure 5.1 Illustration of experimental design showing liquid extracts from six treatments (Cr+As, Cr+Ch, Cr, As, Ch, and MY) of three resynthesis stages (1d, 8d, and 21d PCI) applied to the test cultures of each of *Cladonia rangiferina* and *Asterochloris*. There were five replicates for each treatment. RNA was extracted from each of the test cultures after 24 hrs incubation to determine whether soluble elicitors in the extract induced gene expression. See methods for more details.



to a new 50 ml volume centrifuge tube. Two ml of each filtrate were then used for treating the test cultures of *C. rangiferina* and *Asterochloris*, and incubated as described in the previous section.

5.3.4 Candidate gene selection for quantitative PCR (qPCR)

Gene selection was based on findings of previous studies both on lichen and fungal-plant pathogen systems. Arginase and urease genes were selected as two recognition-related genes because of the role of arginase-like proteins secreted by the lichen fungus and glycosylated urease located on cell walls of compatible algae as suggested by previous biochemical studies (Molina et al. 1993; Molina and Vicente 1995; Molina et al. 1998; Legaz et al. 2004; Millanes et al. 2004; Sacristán et al. 2007). Arginase is found in all groups of organisms and even within *Neurospora crassa* it can be differentially expressed for different roles (Marathe et al. 1998) making it difficult to determine the function of an *arg* sequence through BLAST comparisons. However, since the BLAST queries in GenBank failed to provide significant matches to homology of the urease gene, but it matched to proteins with intercellular adhesive properties and cell to cell recognition properties, the particular gene in the current study was named as a recognition-related gene1 (*RR1*; see results). The catalase B gene was selected as the defense-related fungal gene because of the known roles of fungal catalases during biotic and abiotic stress responses (Barbăneagră et al. 2011), as an anti-defense mechanism against plant defences (Mandell 1975; Buonario and Montalbini 1993; Garre et al. 1998; Lebeda et al. 1999), and in strengthening the fungal appressorial cell wall facilitating the entry into the host (Skamnioti et al. 2007). The GenBank homology to catalase B gene, however, was shown to have pathogenesis related and cell morphogenesis related properties; therefore, the particular gene in the current

study was named as the *Sog2* gene (see results). Chitinase is known to be one of the defense molecules or pathogenesis-related (PR) proteins produced by the host plant in its defense against fungal pathogens (Daugrois et al. 1990; Linthorst 1991; De Lorenzo et al. 2001) and the gene was reported to be upregulated in *Asterochloris* in *C. grayi* (Joneson et al. 2011). The longevity assurance gene product plays a role in biotic and abiotic stress reactions (Brandwagt et al. 2000; Ahmed et al. 2012) and it was also upregulated in *Asterochloris* (Joneson et al. 2011). Based on their potential roles in both plant pathogenic and lichen systems and the availability of the nucleotide sequences from a lichen system (Joneson et al. 2011), the chitinase and longevity assurance genes were selected as algal defense-related genes for the current study.

5.3.5 DNA / RNA extraction, cDNA synthesis, PCR and DNA sequencing

Five ml of 1 month old *C. rangiferina* culture in liquid MY and 10 ml of 1 month old *Asterochloris* culture in BBM were used for DNA/RNA extraction. For homogenization of *Asterochloris*, 10 glass beads (3mm, Fisher Scientific, ON, Canada) were added to the microcentrifuge tube with the *Asterochloris* pellet and vortexed. The *C. rangiferina* mycelium was homogenized using a micro pestle. The DNA from each of *C. rangiferina* and *Asterochloris* was extracted using a modified CTAB-based method (Grube et al. 1995, Athukorala et al. 2014), and RNA was extracted using the Trizol® reagent (Invitrogen Life Technologies, CA, USA) according to the manufacturer's instructions. One µg of extracted RNA was treated with Deoxyribonuclease I (Invitrogen Life Technologies, CA, USA) following the manufacturer's instructions to remove any DNA contamination present in the extracted RNA. PCR was subsequently performed with the relevant reference gene using 1 µl of Deoxyribonuclease treated RNA to confirm the absence of DNA contamination. The cDNA was synthesized using Maxima

First Strand cDNA synthesis kit for RT-qPCR (Thermo Fisher Scientific Inc., ON, Canada), according to the manufacturer's instructions. PCR of target genes (two *C. rangiferina* and three *Asterochloris*) was performed in 20 µl reactions containing 20 ng of template DNA, 1X PCR buffer, 500 mM of each of the forward and reverse primers (Table 5.1), 1.75 mM (actin), 2.0 mM (*chit*, *LAG1*), 2.5 mM (*arg*, *Sog2*, β-tubulin), or 3.0 mM (*RR1*), of $MgCl_2$, 200 µM of each dNTP (Invitrogen Life Technologies, CA, USA), and 0.1 U of Taq DNA polymerase (Invitrogen Life Technologies, CA, USA). Amplification was performed in a T100^{TM} Thermal Cycler (Bio-Rad, ON, Canada) using the temperature cycles as outlined in Table 5.1. For sequencing, PCR products obtained from 200 µl reactions were gel purified with the Wizard(R) SV gel and PCR clean-up system (Promega Corporation, WI, USA) and sequenced using the same primers with a Big Dye terminator version 3.1 (Applied Biosystems, CA, USA) on a 3130 Genetic Analyser (Applied Biosystems, CA, USA). PCR product for the *RR1* was cloned with pGEM Easy Vector System (Promega Corporation, WI, USA), according to manufacturer's instructions, before sequencing due to superimposed chromatographic peaks obtained with direct sequencing. BLASTn and BLASTx searches were carried out with the target gene sequences obtained primarily using NCBI GenBank database and secondarily with JGI database (http://genome.jgi.doe.gov/Clagr2/Clagr2.home.html). GenBank homologies obtained for each target gene are presented in Table 5.2. Conserved domains related to homologous area of the target gene sequences were identified by comparing them to those of matching protein sequences from GenBank.

Total of forty accessioned DNA sequences were retrieved from NCBI GenBank, and included in the phylogenetic analyses for each of five genes. Sequences were aligned using the ClustalX (Jeanmougin et al. 1998) program and manually edited. Aligned sequences were

Table 5.1 List of genes, primer names, the purpose of the primers, PCR cycle conditions, and the source of the primers used in the current study.

Gene and Primer Name	Sequence (5' – 3')	Purpose and PCR cycle conditions	Source/Reference
Arginase (*arg*)			
Arg_sar1F Arg_sar1R	ggagaccactccatcgcc tcaagagcatccacgtcaaa	Sequencing: 5 min 94 ˚C, 30 X (45sec 94 ˚C, 30 sec 55 ˚C, 1 min 30s 72 ˚C), 10 min 72 ˚C	This study (aligning *Coccidioides immitis* (L36550, XM_001244241), *Rattus norvegicua* (NM_019168), *Bombyx mori* (NM_001046640, *Homo sapiens* (NM_001172), *Mus muculus* (NM_009705) arginase sequences)
Arg_sar3F Arg_sar3R	ggaggagccgtgtttgagtt atatgtcccacgccatttcc	qPCR: 4 min 95 ˚C, 40 X (15 sec 95 ˚C, 20 sec 63 ˚C)	This study
Catalase B-like (*Sog2*) CATB-F CATB-R	ggtcaccgtccacaacaataatc tcagggcactgggtgtataagg	Sequencing: 5 min 94 ˚C, 30 X (1min 95 ˚C, 1 min 59 ˚C, 1 min 72 ˚C)	Skamnioti et al.2007
CAT_SAR2F CAT_SAR2R	cagtccttccggttcacaca tatcgggaggccactcatct	qPCR: 4 min 95 ˚C, 40 X (15 sec 95 ˚C, 20 sec 61 ˚C)	This study
Urease-like (Recognition-related gene1; *RR1*) Urealg-F Urealg-R	traarctdcaygargaytgggg tcaggwgtnggdatrctnscatt	Sequencing: 5 min 94 ˚C, 30 X (45 sec 94 ˚C, 45 s 54 ˚C, 45 sec 72 ˚C)	In consultation with G. Brunnauer (University of Salzburg, Austria)
Ure_sar5F Ure_sar5R	gcaccatacggtgtctcgcacc gcatagtgcggcattgggtcca	qPCR: 4 min 95 ˚C, 40 X (15 sec 95 ˚C, 30 sec 63 ˚C)	This study
Longevity assurance gene (*LAG1*) LAG1-1F LAG1-1R	ccctctgctgatggtgct cccatatcgtgaaggctgtt	Sequencing: 5 min 94 ˚C, 30 X (45sec 94 ˚C, 30 sec 55 ˚C, 1 min 30s 72 ˚C), 10 min 72 ˚C	Joneson et al. 2011 and this study
LAG_SAR1F LAG_SAR1R	gggtcctgctctgctgcagc caagcaaggcaccaccctgg	qPCR: 4 min 95 ˚C, 40 X (15 sec 95 ˚C, 30 sec 63 ˚C)	This study
Chitinase-like (*chit*) Chit-al-1F Chit-al-1R	acggtgtctcgcaccttctt atcgacactctgggcaacct	Sequencing: 5 min 94 ˚C, 30 X (45sec 94 ˚C, 30 sec 65 ˚C, 45 sec 72 ˚C)	Joneson et al.2011 and this study
Chit-al_sarF Chit-al_sarR	ctgcaactgcgtgaggaaac gttacccccctggaatgcaaa	qPCR: 4 min 95 ˚C, 40 X (15 sec 95 ˚C, 45 sec 58 ˚C)	This study

B-tubulin			
BT3LM BT10LM	gaacgtctacttcaacgag tcggaagcagccatcatgttctt	Sequencing: 2 min 94 ˚C, 30 X (45sec 94 ˚C, 30 sec 53 ˚C, 30s 72 ˚C), 10 min 72 ˚C	Myllys et al. 2001
BT-sarF BT-sarR	aatctggtgctgggaacaac ttcctccaccgagagagtgt	qPCR: 4 min 95 ˚C, 40 X (15 sec 95 ˚C, 20 sec 63 ˚C)	This study
Actin			
Act1T Act4T	cacacrgtrcccatctaygagg gttgaacagcacctcagggca	Sequencing: 2 min 94 ˚C, 1X (45sec 94 ˚C, 30 sec temperature residing from 62 - 58 ˚C, 30s 72 ˚C), and 27 X (45sec 94 ˚C, 30 sec 58 ˚C, 30s 72 ˚C), 10 min 72 ˚C	Kroken and Taylor 2000
Act-sarF Act-sarR	ccttttgccctcttttgtg gcctgcgagagggtactatg	qPCR: 4 min 95 ˚C, 40 X (15 sec 95 ˚C, 45 sec 58 ˚C)	This study

subjected to phylogenetic analyses using PAUP* 4.0b10 (Swofford 2003) and phylogenetic determinations were based on maximum parsimony (MP) using tree bisection and reconnection branch swapping, heuristic searches with 1000 random addition replicates and bootstrap searches of 1000 resamplings (Felsenstein 1985). Bootstrapping was performed using the MP option in PAUP, and values greater than 70 are reported in the phylogenies. Features of the aligned regions (size, base positions) of one reference sequence with the sequence of each target gene region are listed under dendrograms (Fig. 5.2) and blastX was used to determine the conserved domains found on aligned regions of each reference gene.

5.3.6 Quantitative PCR (qPCR)

Quantitative PCR for the arginase (*arg*), Sog2 (*Sog2*), longevity assurance gene (*LAG1*), recognition-related gene1 (*RR1*) and chitinase-like (*chit*) genes was performed on a MiniOpticon Real Time PCR system (Bio-Rad) using the SsoFasr EvaGreen Supermix (Bio-Rad). The primers were designed with the Primer3 online programme (http://sourceforge.net/projects/primer3/) based on the cDNA sequences generated in the current study (Table 5.1). All the primer pairs produced single bands of the predicted size after PCR and gel-visualization using PCR cycle conditions in Table 5.1. Messenger RNA expression levels were measured in replicates of five for each condition and the ß-tubulin (fungus) or actin (algal) genes were used as internal controls to normalize the expression. cDNA was reverse transcribed from 50ng of total RNA. Each quantitative PCR run contained non-template and distilled water controls to check contaminations.

5.3.7 Data analysis

Each sample was normalized against one reference gene for each of the fungi (β-tubulin) and algae (actin) and also for the control (medium only) using the Livak method (Real-time qPCR application guide, Bio-Rad, ON, Canada). The relative expression values were normalized to the growth medium in addition to the reference gene; hence, any effect on the relevant gene expression exerted by the growth medium was standardized in all treatments. Relative expression values of *C. rangiferina* resulted from the treatments Cr+As, Cr+Ch, Cr, As and Ch were divided by that resulted from the treatment Cr to obtain the fold change of fungal gene expression and also to determine whether the change was an upregulation or a downregulation. Similarly, relative expression values of *Asterochloris* resulted from the treatments Cr+As, Cr+Ch, Cr, As and Ch were divided by that resulted from the treatment As to obtain the fold change of algal gene expression and also to determine whether the change was an upregulation or a downregulation. The fold change of expression in each gene was compared between treatments for a particular resynthesis stage and between resynthesis stages for a particular treatment using one-way ANOVA with JMP software (SAS Institute Inc.) version 11. Comparisons that showed a two-fold change or more were considered for interpretations. Data points that lie outside ±1 from upper and lower confidence levels were eliminated from the analysis.

5.4 Results

5.4.1 Confirmation of target genes

Partial sequences of two *C. rangiferina* genes and three *Asterochloris* genes for qPCR were successfully generated in this study. The GenBank accessions obtained for these five gene sequences and two reference gene sequences (β-tubulin and actin) are KM878708 (*arg*),

KM878709 (*Sog2*), KM878710 (β-tubulin), KM878711 (*chit*), KM878712 (*LAG1*), KM878713 (*RR1*) and KM878714 (actin). The *C. rangiferina* gene, *arg*, and the *Asterochloris* genes, *LAG1* and *chit* showed significant similarities to sequences in NCBI GenBank (Table 5.2) suggesting these are homologous genes. The amino acid sequence obtained from the catalase B gene showed a 48% and 48% similarity to the cell morphogenesis protein *Sog2* and RAM signaling pathway protein rather than catalase B protein, respectively. Both regions are in the Sog2 superfamily of genes and involved in cell separation and cytokinesis with more than the cited 35% homology required for a significant match (Anderson and Brass 1998). Another database (JGI: http://genome.jgi.doe.gov/Clagr2/Clagr2.home.html data not shown), showed that each *C. rangiferina* gene sequence had significant matches with the *Cladonia grayi* genome confirming an origin in lichen fungi, but the proteins were not identified in the incompletely annotated genome. Although the *catB* gene in the current study was obtained from primers that were originally designed by other researchers from the non-lichenized fungus *Magnoporthe grisea* (Table 5.1), the BLAST query produced a significant match with the cell morphogenesis gene, *Sog2* in this study. Therefore, we feel confident that the gene is involved in early morphogenesis of *C. rangiferina*, and plays a role in specific morphological differentiation towards its compatible alga. Because of the low match of the gene to *catB*, and the significant match to the *Sog2*, along with a function of *Sog2,* consistent with expectations of a defense-related gene, we refer hereafter to this gene as *Sog2* in this study.

The cDNA sequence obtained from primers for the algal urease gene showed low sequence similarity to *Ostreococcus tauri UreABC*, Ni-dependant urease apoenzyme (IC) (Ot15g01750) mRNA, complete cds (XM_003083270), and the predicted protein intercellular

Table 5.2 Genes used in the current study and the cDNA (blastn) and protein (blastx) homology with GenBank accessions, putative matching domains and their functions.

Gene	Amplicon size (bp)	Significant Hit(s), (aligned nucleotide region of the amplicon)	Putative domain(s) in aligned region	Domain function(s)	Matching Accession No	Query cover (%)	Max identity (%)	E value
arg	550	*Ajellomyces capsulatus* NAm1 arginase (HCAG_00035) partial mRNA; cDNA, (233 – 541 bp)	-	-	XM_001542939	73	76	$5e^{-50}$
		arginase (*Coccidioides posadasii*); protein, (76 – 537 bp)	Arginase_HDAC superfamily	Control of cellular arginine level, signal transduction	AAA65960	84	61	$1e^{-56}$
catB (Sog2)	443	RAM signaling pathway protein [*Marssonina brunnea* f. sp. '*multigermtubi*' MB_m1]; protein, (30 – 431 bp)	Sog2 superfamily	Cell separation and cytokinesis	XP_007292967	90	48	$7e^{-26}$
		Similar to cell morphogenesis protein Sog2 [*Botryotinia fuckeliana*]; protein, (30 - 431 bp)	Sog2 superfamily	Cell separation and cytokinesis	CCD49653	90	48	$1e^{-27}$
ure (RR1)	276	*Ostreococcus tauri* UreABC, Ni-dependant urease apoenzyme (IC) (Ot15g01750) mRNA, complete cds; cDNA, (5 – 30 bp)	Urease_gamma	Use of urea as a nitrogen source	XM_003083270	15	92	8.8
		PREDICTED: *Sus scrofa* intercellular adhesion molecule 4-like (LOC100516594), mRNA; cDNA, (37 - 71 bp)	Ig2_ICAM-1_like Ig superfamily	May act as host receptor for viruses and parasites Components of immunoglobulin, neuroglia, cell surface glycoproteins and membrane glycoproteins	XM_003123246	12	86	8.8
		Hypothetical protein CHLNCDRAFT_143337 [*Chlorella variabilis*]; protein (179 – 256 bp)	PLDc_Tdp1_1 PLDc_SF superfamily	Catalytic activity	XP_005849969	28	67	0.29

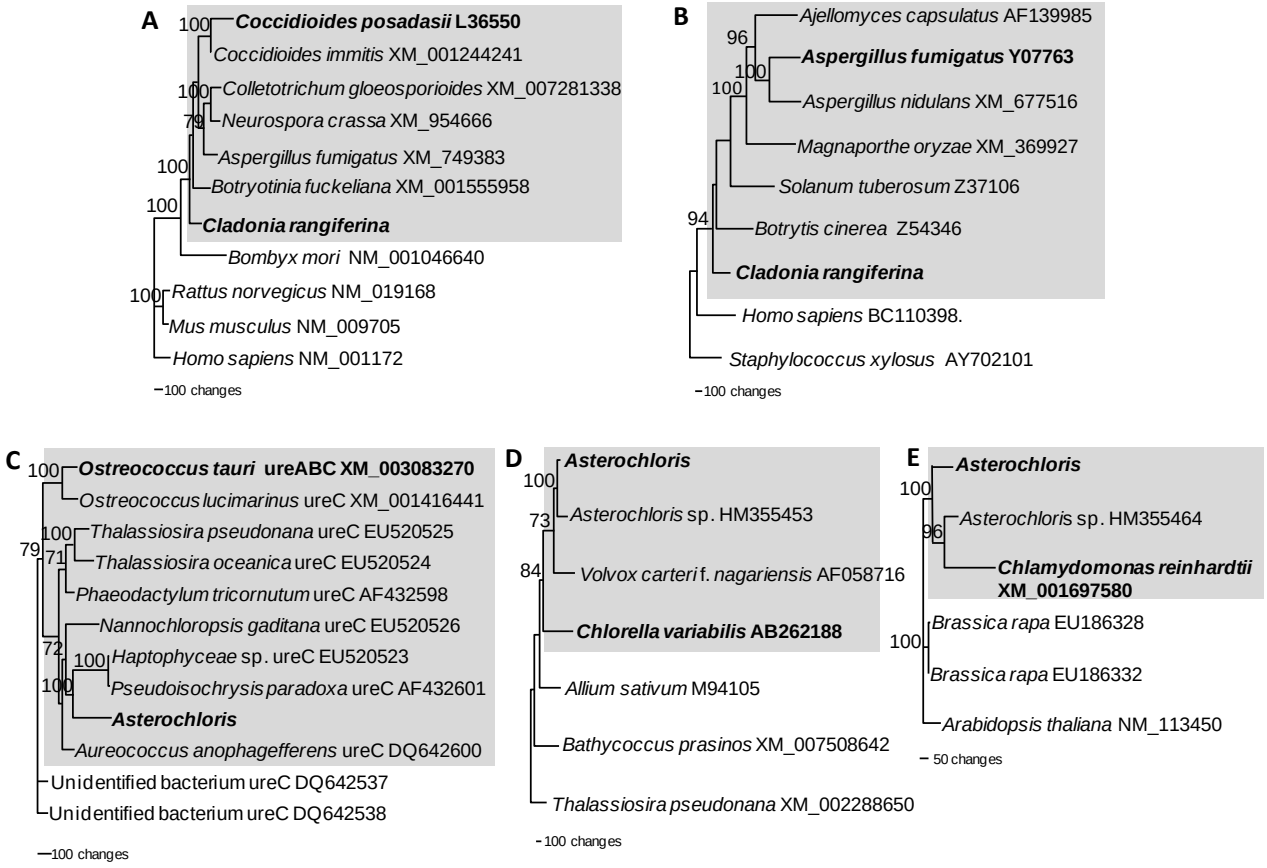
LAG1	252	*Asterochloris* sp. DA2 clone SLJC1grIV.433_o161_LAG1 hypothetical protein mRNA, partial cds; cDNA, (6 – 76 bp)	-	-	HM355464	27	90	$2e^{-06}$
		Hypothetical protein W97_08544 [*Coniosporium apollinis* CBS 100218]; protein, (4 – 168 bp)	REC superfamily	Receives the signal from the sensor partner in two-component systems	XP_007784503	65	69	$1e-^{14}$
chit	385	*Asterochloris* sp. DA2 clone SLJClgrIV433_0367_chit hypothetical protein mRNA, partial cds; cDNA, (1 - 193 bp)	-	-	HM355453	100	97	$5e^{-86}$
		Hypothetical protein [*Asterochloris* sp. DA2]; protein, (1 – 180 bp)	GH18_chitinase-like superfamily	Component of cell walls of fungi	ADP36947	94	98	$2e^{-33}$

Sog2 = RAM signaling pathway protein
PLDc_Tdp1_1 = Catalytic domain, repeat 1, of Tyrosyl-DNA phosphodiesterase
PLDc_SF = Catalytic domain of phospholipase D superfamily proteins
Ig2_ICAM-1_like = Second immunoglobulin (Ig)-like domain of intercellular cell adhesion molecule-1 (ICAM-1, CD54) and similar proteins
Ig = Immunoglobulin domain
REC = Signal receiver domain
GH18 = glycosyl hydrolase, family 18

adhesion molecule 4-like (LOC100516594), mRNA of *Sus scrofa* (XM_003123246) (Table 5.2). Sequence similarity to a predicted protein of *Chlorella vulgaris* that belongs to a catalytic domain of the phosphlipase D (PLD) superfamily proteins was slightly better and with higher query coverage (Table 5.2) but it did not produce a significant match. An immediate product of the action of PLD is phosphatidic acid, which is increasingly recognized as a signal molecule or intercellular messenger molecule. PLD is also thought to induce phospholipid changes in various cellular membranes and plays a role in vesicle trafficking and other membrane-related events (reviewed in Exton, 1997). Although the particular sequence did not give a significant match to the urease gene as expected, with its match to proteins with intercellular adhesive properties and cell signaling and messenger properties, we hypothesize this gene region we sequenced to have a role in cell-to-cell recognition, therefore, it was included as a recognition-related gene. Furthermore, alignment of urease genes from about 10 phytoplankton showed greater variability between nucleotide sequences (data not shown) suggesting greater sequence variability between species. The primers used in the study were degenerate primers, which are designed on a fairly conserved region after aligning several urease sequences. This also supports the variability of urease sequences across species. The sequence variability and the lack of GenBank accessions available from lichen algae may have also contributed to the low match observed. Therefore, as a result of the non-significant match to urease, yet a low match to proteins with intercellular adhesive properties and cell signaling and messenger properties, it was re-named as a "recognition-related gene1 (*RR1*)" in this study with emphasized caution in the interpretations.

The target genes were also confirmed using phylogenetic analyses with similar matches from GenBank. Most parsimonious trees generated for *arg* (2602 steps, CI = 0.7848, RI =

Figure 5.2 Most parsimonious trees of (*a*) *arg*, (*b*) *Sog2*, (*c*) *RR1*, (*d*) *chit* and (*e*) *LAG1* sequences, showing relationships of each gene to respective gene sequences of different origins. Species from the current study and the reference gene sequence used to describe the features of the aligned region are bolded. Monophyly of *C. rangiferina* and *Asterochloris* sequences with respective sequences of fungal origin and algal origin are shown by shaded areas. The table shows the features of aligned region of the reference gene for each target gene.



Gene tree	Size of aligned region	Approximate base position of the reference sequence in the aligned region	Domain(s) in aligned region
A	670	480 - 1150	Arginase-like and histone-like hydrolase superfamiliy
B	445	720 - 1165	Catalase-like superfamily
C	285	1595 - 1880	Urease alpha subunit
D	450	590 - 1040	GH18_Chitinase-like superfamily
E	335	275 - 605	TRAM, LAG1, CLN8 homology domain

0.6884), and *Sog2* (4185 steps, CI = 0.7527, RI = 0.4623) showed monophyly with the respective GenBank sequences of fungal origin (Figs. 5.2a, b shaded areas) with 100% and 94% bootstrap support, respectively. Trees generated for *RR1* (2293 steps, CI = 0.7619, RI = 0.6232), *chit* (2895 steps, CI = 0.8121, RI = 0.3422) and *LAG1* (789 steps, CI = 0.9759, RI = 0.8908) showed monophyly with those of algal origin (Figs. 2c, d, e shaded areas) with bootstrap support of 79%, 84%, and 100% support, respectively. The aligned region of *RR1* gene with the sequence of *Ostreococcus tauri ureABC* (XM_003083270) obtained with phylogenetic analysis matched to the alpha subunit of the urease gene (Fig. 5.2), which is different from that obtained from the blastn search (Table 5.2).

5.4.2. Relative expression of genes

The expression of each gene was compared between treatments at each of the three growth stages to test whether the extract components may influence gene expression. The change in the expression levels of each gene was also compared between the three growth stages for each of the treatments.

The comparison of gene expression between treatments showed there was no upregulation of the fungal *arg* from 1d (pre-contact) compatible or incompatible extracts. However, the fungal *arg* was significantly (P=0.0024) upregulated by the extracts from the *Ch. ellipsoidium* culture at the pre-contact (1d) stage (Fig. 5.3). A significant (P=0.0349) upregulation of *arg* was observed with 8d (contact) compatible extracts. There was no upregulation of *arg* with 21d compatible extracts. The fungal *Sog2* gene showed no significant difference between treatments at any stage of resynthesis (Fig. 5.3). There was no upregulation of the algal *RR1* gene from 1d (pre-contact) or 8d (contact) compatible or incompatible extracts.

Figure 5.3 Comparison of the effect of culture extracts on the relative expression of genes in *C. rangiferina* at each incubation period (resembling resynthesis stages of the lichen). Graphs a, b, and c represent the expression of *arg* while graphs d, e, and f represent the expression of *Sog2*. Statistical comparisons were made within each incubation period (1d; graphs a and d, 8d; graphs b and e, and 21d; graphs c and f) separately. The black bars represent the gene expression in *C. rangiferina* treated with extracts of the compatible and incompatible interactions. The gray bars represent gene expression in *C. rangiferina* treated with extracts of the controls in the study. The y-axis in the graphs demonstrate the fold change of relative expressions with respect to that of the treatment Cr alone, which is shown by the dotted line across the graph. Bars denoted by different letters are significantly different at P=0.05 with Turkey's mean comparison test. Error bars represent standard error of the mean (SEM).

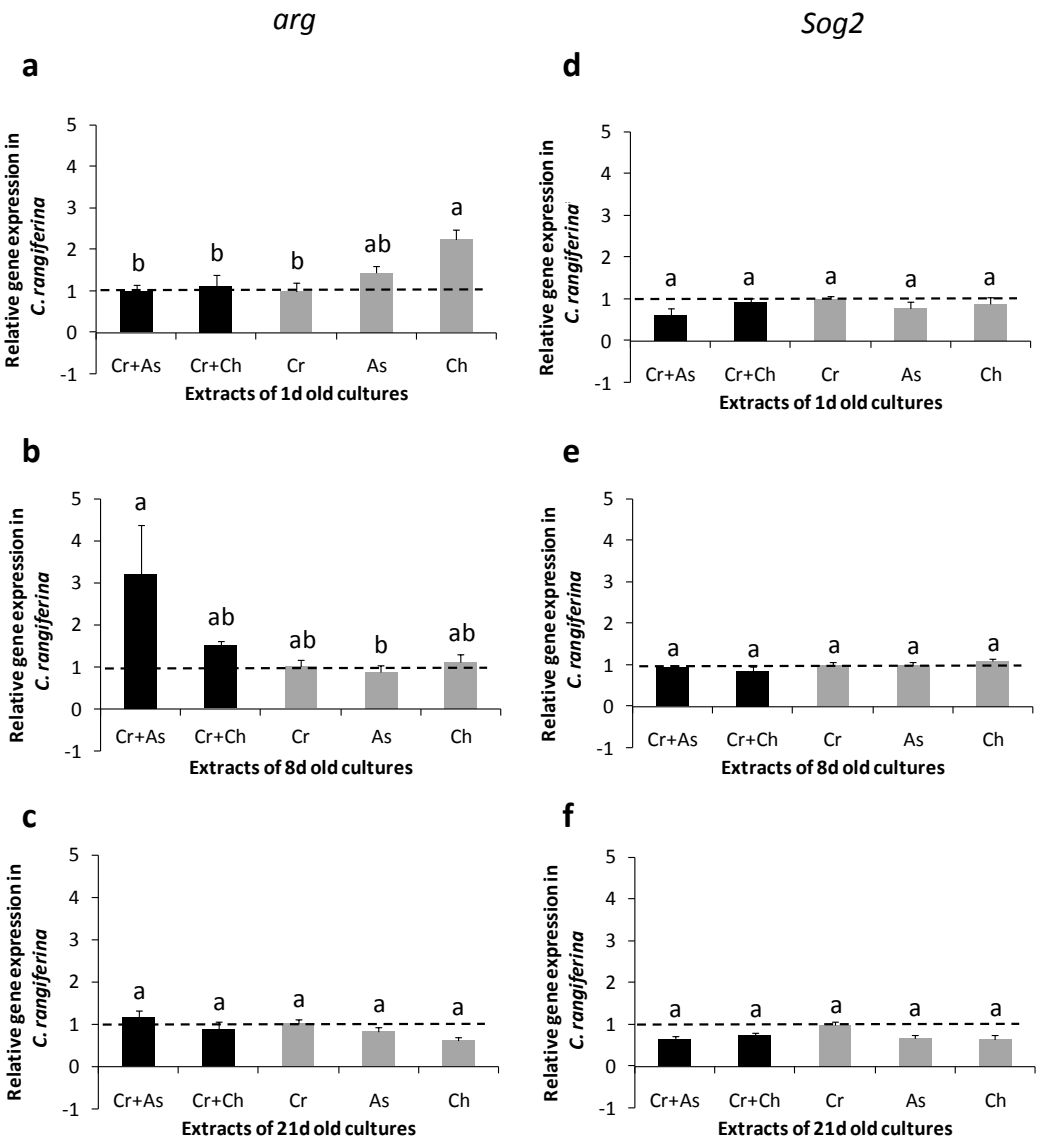
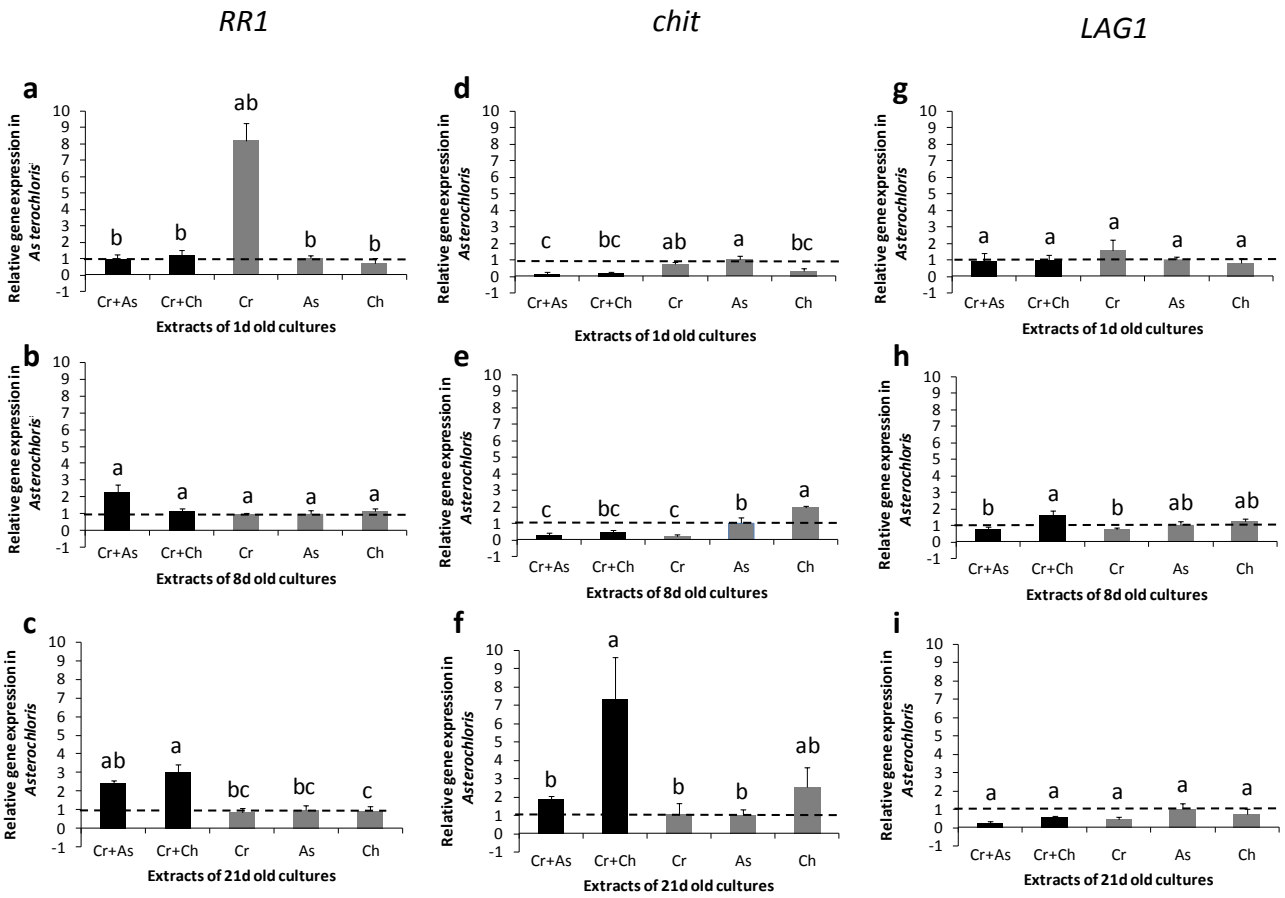

arg
Sog2
a
b
c
d
e
f
Relative gene expression in C. rangiferina
Cr+As
Cr+Ch
Cr
As
Ch
Extracts of 1d old cultures
Extracts of 8d old cultures
Extracts of 21d old cultures

Figure 5.4 Comparison of the effect of different culture extracts on the relative expression of genes in *Asterochloris* at each incubation period (resembling resynthesis stages of the lichen). Graphs a, b, and c represent the expression of *RR1;* graphs d, e, and f show the expression of *chit,* and graphs g, h, and i represent the expression of *LAG1*. Statistical comparisons were made within each incubation period (1d; graphs a, d and g, 8d; graphs b, e and h, and 21d; graphs c, f and i) separately. The black bars represent the gene expression in *Asterochrois* treated with extracts of the compatible and incompatible interactions. The gray bars represent gene expression in *Asterochloris* treated with extracts of the controls in the study. The y-axis in the graphs demonstrates the fold change of relative expressions with respect to that of the treatment As alone, which is shown by the dotted line across the graph. Bars denoted by different letters are significantly different at P=0.05 with Turkey's mean comparison test. Error bars represent standard error of the mean (SEM).


RR1
chit
LAG1
a
b
c
d
e
f
g
h
i
Relative gene expression in Asterochloris
Cr+As
Cr+Ch
Cr
As
Ch
Extracts of 1d old cultures
Extracts of 8d old cultures
Extracts of 21d old cultures

However, the response to the 8d Cr+As extract was marginally significant (P=0.0567) (Fig. 5.4). The algal *RR1* gene was significantly upregulated from both 21d (growth together) compatible and incompatible extracts (P=0.0004). The algal *chit* was downregulated at two of the early resynthesis stages from extracts of both compatible and incompatible interactions (1d P=0.0011; 8d P<0.0001). *Chit* was upregulated at the third resynthesis stage from the incompatible extract only (P=0.0132) (Fig. 5.4). The algal *LAG1* showed significant upregulation (P=0.0235) in response to 8d extracts of the incompatible interaction only (Fig. 5.4).The comparison of genes between resynthesis stages within each treatment showed that the fungal *arg* expression significantly decreased from 1d to the 8d and 21d stages in response to extracts of the compatible alga (As) and incompatible alga (Ch) alone, but not in response to extracts from either of the interactions (Table 5.3). The fungal *Sog2* gene and the algal *LAG1* gene showed no change in expression level for any treatment over the 21d time period (Table 5.3). The algal *RR1* gene expression increased from 1d to 21d in response to extracts from the incompatible interaction (Cr+Ch) (P=0.0066) and decreased in response to extracts of *C. rangiferina* (Cr) (P=0.0.0426) (Table 5.3). The algal *chit* expression changed depending on the treatments from 1d to 21d. The increase in gene expression was significant (P<0.0001) only in the extracts of the compatible (Cr+As) and the incompatible (Cr+Ch) interactions. The expression significantly decreased from 1d to 8d and then increased to 21d in response to the *C. rangiferina* (Cr) extract (Table 5.3).

5.5 Discussion

This is the first study to follow expression of recognition and defense-related genes through three early resynthesis stages of a lichen and it demonstrates the effect of water soluble compounds from culture extracts on the induction of gene expression during the first three stages of resynthesis. The findings suggest that each of the fungal (*C. rangiferina*) and algal

Table 5.3 Comparison of the effect of incubation periods (1d, 8d, and 21d) resembling resynthesis stages of the lichen) of each culture or treatment on the relative expression of the fungal (*C. rangiferina*) and the algal (*Asterochloris*) genes.

Treatment	**Incubation time (growth stage)**	**Relative gene expression in Cr culture* ± SEM**		**Relative gene expression in *Asterochloris* culture* ± SEM**		
		arg	***Sog2***	***RR1***	***chit***	***LAG1***
Cr+As	**1d**	1.00 ± 0.14^{a}	$0.61 + 0.16^{a}$	$0.94 + 0.27^{a}$	$0.94 + 0.27^{a}$	$0.91 + 0.49^{a}$
	8d	3.21 ± 1.16^{a}	$0.93 + 0.05^{a}$	$2.24 + 0.50^{a}$	$2.24 + 0.50^{a}$	$0.75 + 0.13^{a}$
	21d	$1.17 + 0.15^{a}$	$0.63 + 0.09^{a}$	$2.41 + 0.17^{a}$	$2.41 + 0.17^{a}$	$0.24 + 0.08^{a}$
	P	0.1243	0.0546	0.0426	0.0426	0.2571
Cr+Ch	**1d**	$1.13 + 0.26^{a}$	$0.94 + 0.07^{a}$	$1.15 + 0.35^{b}$	$0.18 + 0.08^{b}$	$0.98 + 0.32^{a}$
	8d	$1.50 + 0.12^{a}$	$0.86 + 0.09^{a}$	$1.16 + 0.17^{b}$	$0.44 + 0.11^{b}$	$1.43 + 0.27^{a}$
	21d	$0.88 + 0.18^{a}$	$0.74 + 0.07^{a}$	$3.00 + 0.39^{a}$	$7.32 + 2.31^{a}$	$0.51 + 0.06^{a}$
	P	0.1159	0.2338	0.0066	0.0013	0.1485
As/Cr	**1d**	$1.44 + 0.15^{a}$	$0.77 + 0.16^{a}$	$8.16 + 1.10^{a}$	$0.72 + 0.14^{a}$	$1.57 + 0.62^{a}$
	8d	$0.89 + 0.14^{b}$	$0.98 + 0.08^{a}$	$0.98 + 0.07^{b}$	$0.22 + 0.10^{b}$	$0.74 + 0.12^{a}$
	21d	$0.83 + 0.09^{b}$	$0.68 + 0.07^{a}$	$0.84 + 0.26^{b}$	$0.52 + 0.10^{ab}$	$0.41 + 0.15^{a}$
	P	0.0093	0.1257	<0.0001	0.0451	0.1874
Ch	**1d**	$2.23 + 0.24^{a}$	$0.88 + 0.16^{a}$	$0.35 + 0.12^{a}$	$0.31 + 0.16^{a}$	$0.79 + 0.28^{a}$
	8d	$1.12 + 0.18^{b}$	$1.09 + 0.07^{a}$	$1.16 + 0.16^{a}$	$1.93 + 0.10^{a}$	$1.22 + 0.19^{a}$
	21d	$0.62 + 0.07^{b}$	$0.65 + 0.10^{a}$	$0.93 + 0.27^{a}$	$2.50 + 1.09^{a}$	$0.74 + 0.25^{a}$
	P	0.0003	0.1575	0.0568	0.1579	0.3428

*demonstrates the fold change of relative expressions with respect to that of Cr or As alone culture respectively.
Relative gene expression with response to each treatment was statistically analyzed separately.
Relative expression values of resynthesis stages followed by different letters are significantly different at P=0.05 with Turkey's mean comparison test.
SEM represents standard error of mean, P is the probability obtained for each comparison by ANOVA.

(*Asterochloris*) cultures are responding to elicitors released from the activation cultures. The presence of elicitor molecules in liquid culture extracts and their importance in turning on recognition and defense processes was supported by upregulation of recognition and defense-related genes.

5.5.1 Expression of recognition-related genes

This study provides evidence to support the hypothesis that recognition-related genes, such as those encoding arginases, secreted by the lichen fungus and *RR1* (e.g. urease) of compatible algae are involved in the recognition between algae and fungi in the lichen association (Molina et al. 1993; Molina and Vicente 1995; Molina et al. 1998; Legaz et al. 2004; Millanes et al. 2004; Sacristán et al. 2007). Initiation of *arg* and *RR1* expression seemed to be specific to the compatible interaction when contact was made (Fig. 5.2b and 3b), where recognition is critical for the controlled interaction between lichen symbionts. The expression of *arg* and *RR1* genes beyond the precontact stage, and the necessity for the fungus to make algal cell contact during production of aplanospores by *Asterochloris* (Athukorala et al. 2014), suggests that the recognition process may occur between compatible partners throughout thallus development.

Significant upregulation of fungal *arg* in response to extracts from *Ch. ellipsoideum* (Ch) at the 1d pre-contact stage, indicates that compounds were present in the extracts that triggered *C. rangiferina arg* expression. Enhanced expression of fungal arginase genes have also been observed in response to stress (Chen et al. 2004; Asselbergh et al. 2007; Hruz et al. 2008) in tomato plants with increased resistance to insect larvae (Chen et al. 2005) and in the interaction between *Botrytis cinerea* with *Arabidopsis* plants, where they were less susceptible with

increased *arg* expression, but were more susceptible by silencing *arg* (Brauc et al. 2012). Induction of *arg* by *Ch. ellipsoideum* extracts (Fig. 5.3a) may be explained if *C. rangiferina* is recognizing *Ch. ellipsoideum* as a pathogen. The absence of upregulation by extracts containing the compatible alga (Cr+As and As) may be explained if elicitors were not present in adequate concentrations after 1d of incubation. Since *Ch. ellipsoideum* has higher growth rates than *Asterochloris*, extract of Ch at 1 d PCI may have contained elicitors in sufficient concentrations to induce *arg*.

The *RR1* upregulation in response to the extracts of 21d PCI incompatible interaction (Cr+Ch) may suggest an additional role of the *RR1* gene during incompatible interactions. Athukorala et al. (2014) showed initiation of a parasitic interaction between Cr+Ch at 21d PCI. If the arginase secreted by the fungus is a "parasitic attack" (Molina et al. 1998), the high expression levels of *RR1* observed at 21d may be a response to the high concentration of arginase present in the Cr+Ch extract. Alternatively, since a related species of *Chlorella* is known to form lichen associations with species of *Trapeliopsis* (Brodo et al. 2001), high expression levels of *RR1* may be triggered by the 21d Cr+Ch extract to recognize a suitable partner for the lichen association.

5.5.2 Expression of defense-related genes

Components from the extracts of the first three resynthesis stages did not induce expression of the fungal *Sog2,* a cell morphogenesis gene. The catalase B (*cat-B*) gene in *Magnaporthe grisea*, another cell-morphogenesis-related gene, was shown to play a role in strengthening the fungal appressorial cell wall, which facilitates forceful entry into the host (Skamnioti et al. 2007). Pathogenic fungi produce catalases as an anti-defense mechanism

against plant defenses (Mandell 1975; Buonario and Montalbini 1993; Garre et al. 1998; Lebeda et al. 1999). Catalase is also postulated to play a role in regulating reactive oxygen species (ROS) (Nakano and Asada 1981), where production of ROS in fungi may increase in response to biotic and abiotic stress (Barbăneagră et al. 2011). Genes of the RAM signaling pathway (including *Sog2*) have been shown to regulate pathogen adaptation and influence its host range in pathogenic fungi (Walton et al. 2006; Magditch et al. 2012). When considering the known roles of catalase, catalase-like, and *Sog2* genes, the gene in this study was expected to be upregulated in response to culture extracts of the compatible interaction after the contact stage. However, these results do not support the role of this gene in the first three stages of *C. rangiferina* resynthesis. Explanations may be that the elicitors to trigger *Sog2* expression are not released from the cells or are in insufficient concentration to trigger a reaction within the first three resynthesis stages. Furthermore, *Sog2* in *C. rangiferina* introduced in the current study might not have a significant role in the early stages of symbiont interactions.

The downregulation of the algal *chit* gene in response to extracts of both compatible and incompatible interactions at the precontact and contact stages is supported by the hypothesis proposed by Trembley et al. (2002) that defense genes may be among the suppressed genes during the establishment of lichen interactions. This highlights the importance of reducing the expression level of certain genes. If the alga produces chitinase in response to appressorial attachment, it would not be needed until after the contact stage, supporting the findings in this study. Athukorala et al. (2014) observed a potential parasitic response of *Ch. ellipsoidea* towards *C. rangiferina*. If *Ch. ellipsoidea* released elicitors in the extract that were detected by *Asterochloris*, the expression of the gene by *Asterochloris* may have been triggered. However, upregulation of the *chit* gene may also be in response to the fungal cell components present in

this extract by the activity of chitinases from *Ch. ellipsoidea*. Since chitinase is known to be one of the defense molecules produced by host plants against fungal pathogens (Daugrois et al. 1990; Linthorst 1991; De Lorenzo et al. 2001), expression of chitinase was speculated to have a defensive role in both early and late stages of the infection process (Punja and Zhang 1993). Viral infections caused chitinase expression in *Chlorella* (Hiramatsu et al. 1999; Ali et al. 2007), and fungal cell wall components hydrolysed by plant chitinases (Kasprzewska 2003) may also act as elicitors for the synthesis of defense molecules (Repka 1993; review Grover 2012). Although it is not known whether *chit* is responsible for all chitinases (Joneson et al. 2011), significant changes in its expression depending on the type of extract in the 21d resynthesis stage indicates a possible role of this gene in the controlled parasitism between *C. rangiferina* and *Asterochloris*.

A significant upregulation of the *LAG1* observed in response to extracts with the incompatible alga during the contact stage suggests a possible defensive role of this gene. The defensive roles against stress and apoptosis (Jazwinski and Conzelmann 2002) is also supported in tomato plants where the longevity assurance gene was involved in resistance against toxins produced by the pathogen *Alternaria alternata* f.sp. *lycopersici* (Brandwagt et al.2000) and abiotic stresses in *Brassica* (Ahmed et al. 2012). Therefore, one interpretation is that upregulation of *LAG1* in *Asterochloris* in response to extracts of incompatible algae may serve as a method of defense against elicitors produced by *Ch. ellipsoideum*. Joneson et al. (2011) observed expression of the *LAG1* in *C. grayi* only at the pre-contact stage of the compatible interaction, but explanations were not provided.

The addition of extracts from three resynthesis stages to cultured lichen symbionts provided an opportunity to examine the effect of elicitors external to the symbiont cells on

expression of both recognition and defense-related genes. The use of culture extracts to examine the effect of elicitors on the expression of defense and pathogenesis related genes has been a common practice in plant pathology. For example, El-Bebany et al. (2011) used potato root extracts to elicit pathogenicity-related genes in the fungus *Verticillium dahliae*. Benouaret et al. (2014) reported that early recognition, defence reactions, and cell death were elicited by grape marc extract. However, one of the limitations of the current study was the unknown elicitor type or concentration in the culture extracts potentially resulting in variation in the final transcription results in this study. Non-significant changes in expression of certain genes (e.g. *RR1*) may be due to shared (Joneson and Lutzoni 2009) ubiquitous, basal or incomplete lichenization signals (Ahmadjian 1992; Meeβen and Ott 2013) present in extracts or inadequate concentrations of signal molecules. The retesting of biological repeats to detect this variation might be useful for future studies. Now that we know recognition and defense genes respond to culture extracts, future studies may further assess gene expression using concentrated crude extracts and chemical profiling of extracts to purify and identify compounds.

In conclusion, the current study examined the expression of two recognition-related genes and three defense-related genes from three resynthesis stages of the lichen *C. rangiferina*. The finding that the recognition-related genes (*arg* and *RR1*) are expressed in response to a compatible interaction at the contact stage and also beyond the precontact stage in resynthesis has significant implications that these recognition genes may play a role throughout thallus growth and development. Throughout thallus development the fungus needs to recognize new daughter algal cells in order to continue the symbiosis without collapsing into an uncontrolled parasitic interaction. While this study also provides evidence for induction of defense-related genes at the contact and growth together stages, the strongest *chit* expression occured at the

growth together stage where appressoria have already formed and algal defense may be needed. Furthermore, this study suggests that pre-contact communication may occur even with non-lichenizing algae, where *Ch. ellipsoideum,* showed evidence of an interaction in a previous study (Athukorala et al. 2014), supporting communication between incompatible partners. The results of this study are consistent with those expected in plant-pathogen systems suggesting the interaction between lichen symbionts is parasitic in nature. However, the interaction also minimizes the extent of parasitism by timely recognition and defense that limit the outcome to the mutualistic or controlled parasitic relationships commonly referred to as lichen associations.

CHAPTER 6

Effect of temperature and pH on the resynthesis of compatible partners of the lichen *Cladonia rangiferina*

6.1 Abstract

Reindeer lichens (such as *Cladonia rangiferina*) are important winter forage for caribou and reindeer and are widely distributed in northern ecosystems. Widespread lichen communities may be explained by dispersal from thallus fragments or by fungal ascospores interacting with algal cells. Since three early stages of resynthesis between symbionts of the lichen *C. rangiferina* have already been established, this study investigated the effect of three temperatures (5 ˚C, 20 ˚C, and 35 ˚C) and pH levels (4.5, 6.5, and 8.5) on the early interaction of *C. rangiferina* by quantifying morphological differences for three fungal (internode length, number of lateral branches, number of appressoria) and one algal (cell diameter) characters using Scanning Electron Microscopy. The results showed that the fungal characters were significantly altered by the extreme temperatures (5 ˚C and 35 ˚C) and the pH level produced differences in the fungal characters at pH 8.5. The alga was more tolerant of the wide temperature range than the fungus while the fungus was more tolerant of pH changes than the alga. An interaction effect by temperature and pH on the symbiont characters was also observed. The study raises questions regarding the range of conditions tolerated by other species of lichens and their symbionts.

6.2 Introduction

The outcomes of symbiotic interactions span a continuum between mutualistic and parasitic lifestyles over evolutionary time and geographic space (Thompson 1988; Cushman and

Whitham 1989; Cushman and Addicott 1991; Bronstein 2001). Transitions between symbiotic lifestyles were postulated to have occurred during evolution (Gargas et al. 1995; Ewald 2004; Sachs and Simms 2006) where environmental conditions altered the strength and direction of the symbiotic interaction (Thompson 1994, 2005; Thrall et al. 2006; Wolinska and King 2009). Lichen regeneration often involves vegetative propagules, which are fragments of the thallus for *Cladonia rangiferina* (L.) F. H. Wigg. A new thallus can also be formed by the union of an ascospore with a compatible alga, resulting in the potential for each germinating fungal ascospore to associate with different algal partners. The successful union of an algal and fungal partner may be affected by growing conditions, which may indirectly act on the ability of the interaction to develop into a lichen thallus. The symbiont interaction revealed structural alterations at the mycobiont-photobiont interface under drought stress (Honegger et al. 1996) and an increase in the degree of fungal parasitism in high temperatures (Thomas and Blanford 2003; Upchurch and Ramirez 2011). Additionally, a review by Ahmadjian (1987) suggested that fungal haustoria may penetrate the photobiont to different depths depending on growing conditions. A previous study established the growing conditions and symbiont phenotype for three early stages of the interaction (resynthesis) between symbionts of the lichen *C. rangiferina* (Athukorala et al. 2014). Resynthesis here refers to the resynthesis of the interaction between the two symbionts that originally formed the lichen thallus. The fungal symbiont showed distinct phenotypic responses such as short hyphal internodes, an increased number of new lateral branches, and appressorial contacts with its compatible alga *Asterochloris glomerata/irregularis*, which were consistent with observations in other studies (Joneson and Lutzoni 2009; Meeßen and Ott 2013) where early stages of resynthesis were examined. If distinct phenotypic responses (Joneson and Lutzoni 2009; Meeßen and Ott 2013; Athukorala et al. 2014) occur between compatible partners

under a given set of growing conditions, then deviation from these phenotypes would be expected under adverse growing conditions.

The goal of the current study was to investigate how different growing conditions (pH and temperature) affect the phenotype of the algal and fungal symbionts of the lichen, *Cladonia rangiferina*, during early stages of their interaction in culture. Laboratory experiments under controlled conditions may be an alternative strategy to field experiments (with cautious interpretation), to determine the range of conditions under which a successful interaction may occur.

6.3 Materials and Methods

6.3.1 Source of specimen and experimental design

Axenic cultures of the fungal and algal partners isolated from the lichen *Cladonia rangiferina* (Athukorala 24) as described by Athukorala et al. (2014) were used in this study. The sample was collected from Sandilands Provincial Forest, Manitoba, Canada (N49° 22' 37", W96° 6' 31") on May 24, 2009. The Sandilands Provincial Forest is an open forest on glacial till dominated by Jack pine trees and an understory of white birch and aspen.

The experimental design consisted of three biological replicates for each of 27 treatments. The experimental treatments included: *C. rangiferina* + *Asterochloris* (Cr+As) grown at each of three temperatures (5 ˚C, 20 ˚C, and 35 ˚C), and each of three pH levels (4.5, 6.5, and 8.5) for nine treatments. The temperature and pH levels were chosen based on previous studies (Harrison et al. 1986; Bačkor et al. 1998; Marmor and Randlane 2007; Pisani et al. 2007) and to encapsulate the more extreme conditions often found in nature. The remaining 18 control

treatments included *C. rangiferina* grown alone (Cr) and *Asterochloris* grown alone (As) at each of the same conditions as the experimental treatments.

6.3.2 Co-inoculation of symbionts

Preparation of subcultures of the isolated fungus (*C. rangiferina*) and its compatible alga (*Asterochloris glomerata/irregularis*; hereafter called *Asterochloris*), the co-inoculation of *C. rangiferina* and *Asterochloris* onto cellulose acetate membranes (25 mm diameter, 0.45 µm, Sartorius AG, Germany) on 99:1 solid medium (Joneson and Lutzoni 2009), and the incubation of cultures were performed according to the procedure described by Athukorala et al. (2014).

6.3.3 Morphological observations and quantitative measurements

General morphological observations including fungal and algal cell appearance were recorded after 21 days post co-inoculation (PCI). The quantitative measurements of each of the fungal and algal symbionts included 1) the average internode length of 5-8 internodes of fungal branches behind the hyphal tip, 2) the number of lateral branches produced by the fungal hyphae, 3) the number of fungal appressoria appearing as contacts between fungal hyphae and algal cells, and 4) the algal cell diameter, using the method described by Athukorala et al. (2014). Briefly, the measurements were made using the SEM micrographs and a mm ruler and conversion to µm using the scale bar were made depending on the phenotypic character measured. These characters were specific morphological responses exhibited by the interaction between *C. rangiferina* and its compatible algal partner at 21 days PCI (Athukorala et al. 2014) and therefore were selected for quantitative analyses. The number of appressoria and new lateral branches was counted from each of five images (per replicate), which resulted in 15 measurements per treatment. In the case of internode length, the number of measurements made from each image

varied from 2 to 15, depending on the availability of the phenotypes within the image, which resulted in 30 – 150 measurements per treatment. Ten algal cell diameter measurements were taken from each image, which resulted in 150 measurements per treatment (10 cells per image x 5 images x 3 replicates).

6.3.4 Scanning Electron Microscope (SEM) preparation

For each treatment the discs of cellulose acetate membrane were processed for SEM using a modified method of Athukorala et al. (2014) as described below. The membranes were fixed in a modified Karnovsy's fixative consisting of 2.5% gluteraldehyde, 1% paraformaldehyde, in a 0.1 M sodium phosphate buffer at pH 7.2. After fixation (12 to 24 hours at 4 ˚C) the cells were washed in 0.1 M sodium phosphate buffer pH 7.2 with 4 changes over 2 h. Subsequently, the tissue was post fixed in a 1% solution of osmium tetroxide in 0.1 M sodium phosphate buffer for 30 min at 4 ˚C. After a brief rinse in 0.1 M sodium phosphate buffer, the tissues were rapidly dehydrated in an ascending series of -20 ˚C ethanol from 70% - 100%. Tissues were then transferred to vials containing hexamethyldisilazane (HMDS) and left to evaporate under a fume hood. After three days, dried samples were mounted with conductive carbon tape and examined in a Table Top SEM (Hitachi TM 1000, Germany) equipped with a backscatter detector. All chemicals were purchased from Electron Microscopy Sciences (PA, USA) unless otherwise stated.

6.3.5 Data analysis

The number of lateral branches and the internode length of the fungus inoculated with the alga (Cr+As) were compared with those from the same treatments of the fungus inoculated alone (Cr). The number of appressoria of *C. rangiferina* in Cr+As could not be compared with a

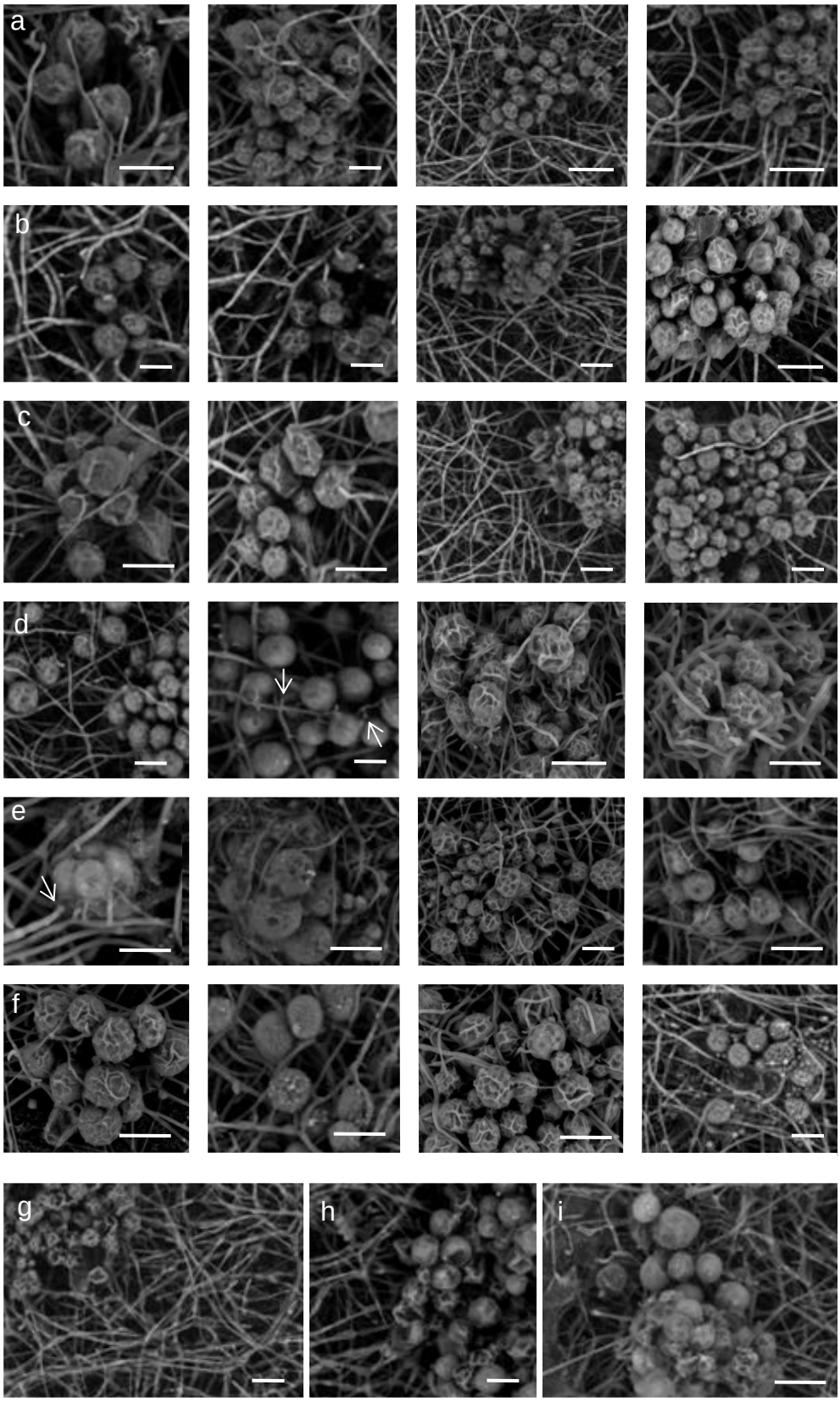
control so it was compared among experimental treatments only. Algal cell diameter in the interaction (Cr+As) was compared with the cell diameter of *Asterochloris* growing alone (As), and comparisons were made among all treatments. To test the hypothesis that temperature and pH level interact to influence the success of the symbiosis, data were analysed by two-way ANOVA for each of the measured symbiont features as each of the continuous (response) variables, and the treatments (temperature and pH) as the independent categorical variables. The response variables and their residuals were tested for normal distribution. The number of lateral branches and the internode length were transformed by log (1+x) transformation. Means were compared using the Tukey-Kramer Honest Significant Difference (HSD) test for three or more variables or by the Student t-test where only two variables were compared. Statistical analyses were performed using JMP version 11 (SAS Institute Inc.).

6.4 Results

6.4.1 Effect of temperature and pH on the morphology of the fungus and alga

The morphology of the each of the symbionts varied among temperature and pH treatments (Fig. 6.1). At 5 ˚C and all three pH levels, the fungal hyphal tips were in contact with algal cells, the hyphae appeared to be segmented, and the algal cells were forming clusters (Fig. 1 a-c). At 20 ˚C, and pH 4.5 and 6.5, the hyphal tips were in contact with algal cells and swollen tips could be seen in some images (Fig. 6.1 d, e; see white arrows). The hyphae had lateral branches and the algal cells were in clusters. At 20 ˚C and pH 8.5, there were not as many lateral branches or appressoria than were present at pH 4.5 and 6.5 (Fig. 6.1f). At 35 ˚C and all three pH levels, the fungal hyphae did not have lateral branches or appressoria; however, the algae were in clusters. Some of the algal cells and fungal hyphae appeared to be collapsed (Fig. 6.1g-i).

Figure 6.1 SEM images of the interaction between *C. rangiferina* and *Asterochloris* at 21 days PCI grown under three temperature and three pH conditions showing the hyphal tip connections with algal cells (appressoria) (column 1 and 2), new lateral hyphal branches (column 3 and 4), the internode length (all columns), and the diameter of algal cells (all columns). The swollen tips of *C. rangiferina* at the point of contact of *Asterochloris* are shown with arrows. Images are shown for (*a*) 5 ˚C, 4.5 pH; (*b*) 5 ˚C, 6.5 pH; (*c*) 5 ˚C, 8.5 pH; (*d*) 20 ˚C, 4.5 pH; (*e*) 20 ˚C, 6.5 pH; (*f*) 20 ˚C, 8.5 pH; (*g*) 35 ˚C, 4.5 pH; (*h*) 35 ˚C, 6.5 pH; (*i*) 35 ˚C, 8.5 pH. Scale bars represent 10 µm.

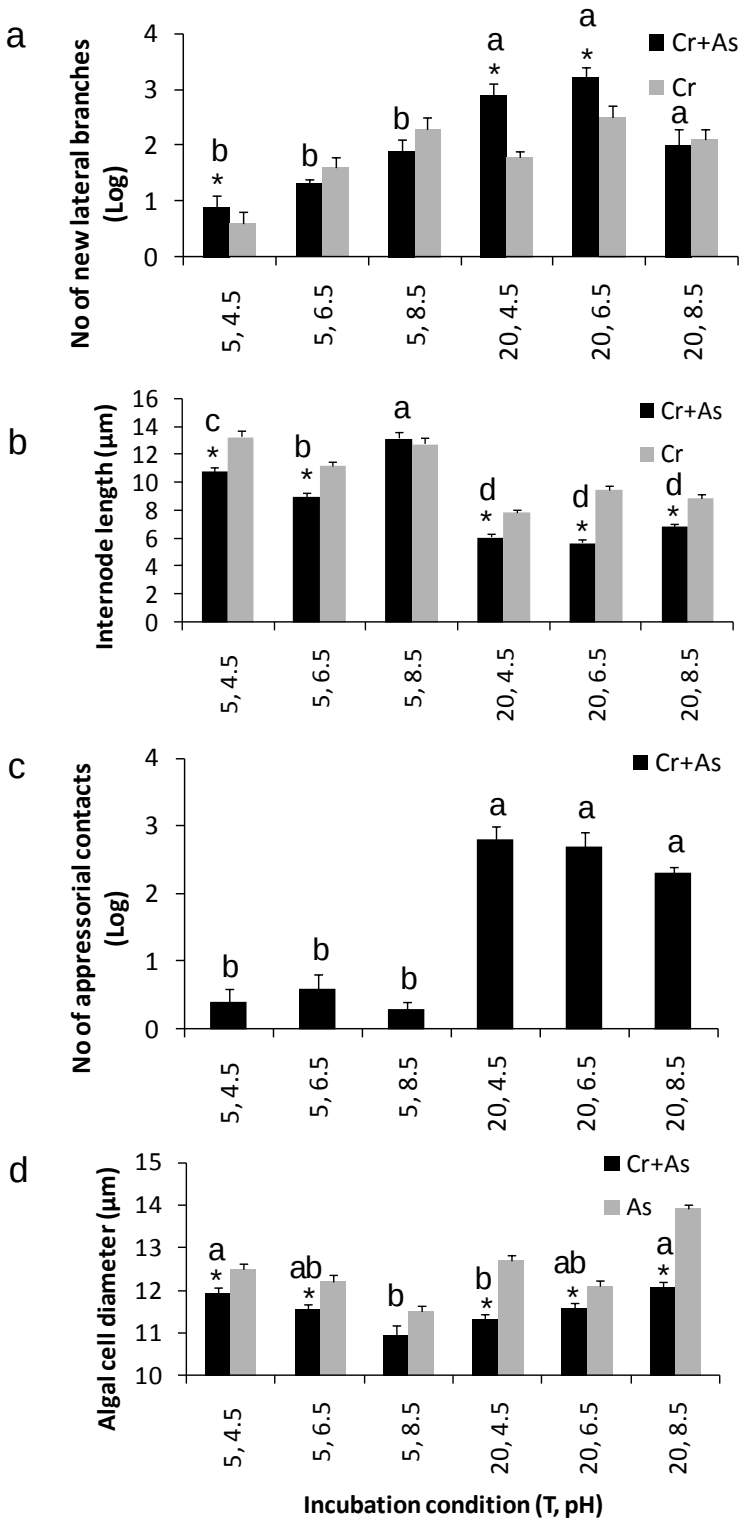

a
b
c
d
e
f
g
h
i

6.4.2 Quantitative comparison of phenotypic features

The differences in phenotypic characters between the interaction and the control treatments (indicated by an asterisk) were variable (Fig. 6.2). The number of lateral branches was significantly higher ($P<0.001$) in the compatible interaction (Cr+As) than in the control (Cr alone) in cultures incubated at 20 ˚C, 4.5 pH, 20 ˚C, 6.5 pH, and 5 ˚C, 4.5 pH while the difference was not significant in other conditions (Fig. 6.2a). The internode length of the fungus in Cr+As was significantly shorter ($P<0.001$) than that in Cr for all pH conditions at 20 ˚C, and for 4.5 and 6.5 pH conditions at 5 ˚C (Fig. 6.2b). Lateral branch numbers and internode lengths were not observed at the 35 ˚C treatment because the hyphae were fragmented with no evidence of lateral branch growth. The diameter of *Asterochloris* cells in the interaction (Cr+As) was significantly smaller than those in As alone under all conditions except at 5 ˚C, 8.5 pH, which was marginally significant ($P=0.052$) (Fig. 6.2d).

Overall, the fungal phenotype showed a larger number of significant differences among temperature than pH level (Fig. 6.2a, b, c), while the alga showed a larger number of significant differences between the pH levels than the temperature (Fig. 6.2d) (indicated by lower case letters). The number of lateral branches in Cr+As was significantly higher ($P<0.0001$) for those incubated at 20 ˚C in comparison with 5 ˚C (Fig. 6.2a). The internode lengths were significantly shorter ($P<0.001$) in the fungal hyphae at 20 ˚C in comparison to those at 5 ˚C except at pH 8.5. There was a significant difference in the internode length between pH levels at 20 ˚C (Fig. 6.2b). There was no significant difference in appressoria number between pH levels for both 5 ˚C and 20 ˚C, but the number of appressoria was significantly higher at 20 ˚C than at 5 ˚C ($P<0.001$) (Fig. 6.2c). Algal cell diameter was significantly smaller at 5 ˚C and pH 8.5 ($P<0.0001$) and 20

Figure 6.2 Comparison of each phenotypic character between the compatible interaction (Cr+As; black bars) and the control (Cr or As alone; gray bars) with an asterisk to indicate significant differences at P=0.05, except the number of appressoria. The graphs also compare the growing conditions (temperature and pH) by indicating significance levels using lower case letters. Different lower case letters within each graph indicate significant differences between growing conditions. The graphs show (*a*) the number of fungal lateral branches, (*b*) the fungal internode lengths, (*c*) the number of fungal appressoria, and (*d*) the algal cell diameter in each of the different temperature and pH conditions except the 35 ˚C conditions. Measurements were not made at 35 ˚C since the phenotypic features were not present. Error bars represent standard error of the mean (SEM).


a
No of new lateral branches (Log)
Cr+As
Cr
b
Internode length (µm)
Cr+As
Cr
c
No of appressorial contacts (Log)
Cr+As
d
Algal cell diameter (µm)
Cr+As
As
5, 4.5
5, 6.5
5, 8.5
20, 4.5
20, 6.5
20, 8.5
Incubation condition (T, pH)

˚C and pH 4.5 ($P<0.0001$) than other growing conditions (Fig. 6.2d). An interaction effect between temperature and pH level was present in all of the two-way ANOVA tests except the number of appressoria which was affected by temperature only (Table 6.1).

6.5 Discussion

The interaction between *C. rangiferina* and *Asterochloris* in higher (35 ˚C) and lower (5 ˚C) temperatures showed phenotypic changes that differed from those at temperatures more close to optimal (20 ˚C), suggesting it was less successful than at 20 ˚C. Optimal temperature for *C. rangiferina* in terms of respiration and photosynthesis rates was recorded to range from 10 – 15 ˚C in temperate regions (Bliss and Hadley 1964; Adams 1971). Both symbionts showed some growth at the extreme temperatures indicating that each of the symbionts could survive under those conditions but the conditions may not have been sufficient for them to form an interaction. Temperature had a greater effect than pH level on the lichen fungus where the number of lateral branches and appressorial contacts were reduced at extreme temperatures. These findings are not inconsistent with other studies where it has been shown that growing conditions, such as certain nutritional conditions may influence haustorial development (reviewed in Ahmadjian 1987). Nutrient conditions have also been shown to influence hyphal length (Paustian and Schnürer 1987). Lateral branches of the hyphae allow the fungus to increase its surface area to extract nutrients from the media (St John et al. 1983). The finding that less extreme conditions in this study produced hyphae with many lateral branches, may be explained if the lateral branches are triggered by the alga where culture extracts containing lichen algae have been shown to turn on recognition and defense related genes in the lichen fungus (Athukorala and Piercey-Normore 2015), or the hyphae are searching for nutrients produced by the alga (Lallemant and Bernard,

Table 6.1: Two-way ANOVA showing interactions between temperature and pH response variables in Cr+As for each of the morphological indicators used in this study.

	df	Sum of Squares	F ratio	Prob > F
Internode length (n= 541)				
Temperature	1	882.78	113.87	<0.0001
pH	2	802.12	51.73	<0.0001
Temperature * pH	2	197.47	12.74	<0.0001
Number of appressoria (n=90)				
Temp	1	46.60	154.27	<0.0001
pH	2	0.83	1.37	0.2600
Temperature * pH	2	0.56	0.92	0.4026
Number of branches (n=90)				
Temp	1	29.04	45.20	<0.0001
pH	2	6.41	4.99	0.0090
Temperature * pH	2	16.52	12.86	<0.0001
Algal cell diameter (n=806)				
Temp	1	5.12	1.95	0.1633
pH	2	1.60	0.30	0.7384
Temperature * pH	2	78.16	14.86	<0.0001

1977; Meeßen and Ott 2013; Meeßen et al. 2013).

While this study showed that temperature had a greater effect on the lichen fungus, the alga was affected more by substrate pH than by temperature since there were no differences in algal cell diameter between 5 ˚C and 20 ˚C. In this study, many algal cells were collapsed at 35 ˚C, but there were clusters of algal cells present, suggesting that growth had occurred in the alga at least for a short period of time at this temperature. Growth at 35 ˚C may give the alga an advantage for survival over short periods of extreme temperatures. Temperature tolerance of *Trebouxia* spp. has previously been documented (Ahmadjian 1993; Casano et al. 2011) but the differential effect on compatible symbionts has not been previously reported. The effect of pH changes on the alga is consistent with findings of lichen distribution where substrate pH changes due to air pollutants have been shown to limit lichen diversity and distribution (Hallingback and Kellner 1992; Marmor and Randlane 2007; Öztürk and Oran 2011). Sulfur dioxide (SO_2) affected the algal partner while the fungus resisted the SO_2 oxidative stress in the lichen *Xanthoparmellia mexicana* (Kong et al. 2002). Additionally, Bačkor et al. (1998) reported that the growth of *Trebouxia irregularis* was inhibited due to a change in pH of the growth medium.

This study showed that a change in growing conditions has an effect on lichen symbionts to establish successful early interactions resulting in significant changes in symbiont phenotypic characters. These interactions have been identified as necessary characteristics for successful resynthesis of compatible lichen symbionts that are thought to lead to the formation of a lichen thallus (Ahmadjian et al. 1980; Bubrick et al. 1985; Ott 1987; Galun and Garty 1988; Stocker-Wörgötter and Türk 1991; Jahns 1993; Kon et al. 1993; Stocker-Wörgötter 1995; Schaper and Ott 2003; Joneson and Lutzoni 2009; Muggia et al. 2011; Athukorala et al. 2014). Changes in temperature and pH levels have also been shown to affect the production of photosynthetic

pigments, chlorophyll degradation and cell membrane integrity of the lichen photobiont (Hájek et al. 2006; Pisani et al. 2007) and photobiont growth (Bačkor et al. 1998). Culture extracts of lichen fungi have been postulated to contain elicitors produced by the interaction between compatible partners (Joneson et al. 2011; Athukorala and Piercey-Normore 2015). If changes in growing conditions affect algal physiology and the production of elicitors that serve as recognition and defense related molecules, the development of specific morphological responses may also be affected. Signal molecules such as arginase and urease, may be greatly affected by temperature and pH changes (Bornside et al. 1952; Erisir et al. 2005). A cell signalling molecule (Sutton and Hoegh-Guldberg 1990; Grant et al. 1997) was shown to modify carbon metabolism in the coral symbiosis (Grant et al. 2013). Therefore, modification of signal molecules and elicitors of one partner may lead to changes in carbon metabolism or the production of special phenotypic features of the other partner, which can affect the interaction.

In conclusion, the current study showed that the specific phenotypic responses of the lichen fungus towards its compatible algal partner are altered by changes in temperature and pH. The presence of fungal hyphae and algal cells at 35 ˚C indicated some growth of both symbionts and the potential for both symbionts to remain alive at least for a short period of time. However, the symbiosis itself (by the inability to form phenotypic features) was sensitive to the high temperature. This study also showed that temperature and pH affect each of the two symbionts differently. While the alga was more tolerant of the wide temperature change than the fungus, the fungus was more tolerant of pH changes than the alga at 5 ˚C and 20 ˚C. The temperatures and pH levels were chosen to represent a broad range of conditions often found in nature but further research is needed to define the range of conditions tolerated by this and other species more accurately. The study also showed that temperature and pH were not independent of one another

in producing an effect on the interaction between the symbionts as shown by the two-way ANOVA. The effect of more refined growing conditions between those tested in this study as well as additional abiotic conditions such as moisture and light levels also need further examination to better understand their effect on the *in vitro* symbiont interaction.

CHAPTER 7

General Discussion and Conclusions

Vast areas of land in northern regions of the world are covered with just a few species of *Cladonia,* which serves as insulation for underground permafrost and provides a winter food source for caribou and reindeer. The goal of this dissertation was to investigate the variability of reindeer lichens in an evolutionary and ecological context in an effort to understand their survivability in northern ecosystems. The widespread success of *Cladonia arbuscula, C. rangiferina, C. stygia,* and *C. mitis* may partially be explained by the evolutionary versatility exhibited by paraphyly (Chapter 2) suggesting that they are undergoing speciation with many lineages incompletely resolved. Upon further examination with multilocus markers, two of these species showed high variability in polymorphism within and among apothecia (Chapter 3) suggesting that sexual reproduction may be common. These species can reproduce by fragments and by sexual reproduction providing both a colonizing ability and recombination, which provides the necessary genetic variation within a single thallus for adaptation to different habitats. Fungal adaptation to habitats must also involve a compatible interaction, which is limited to a small range of algal partners (Piercey-Normore 2004; Yahr et al. 2004). However, further studies on *C. rangiferina* showed that the fungal symbiont can interact with other algae such as *Chlorella*. Regardless of whether the interaction is parasitic or mutualistic (Chapter 4), general recognition and defense-related genes of the fungal symbiont are triggered by culture extracts containing *Chlorella*. This suggests the survival of the fungal symbiont even if the compatible alga is not immediately available by communicating with other algae and potentially feeding on their resources (Chapter 5). Furthermore, the resynthesis capacity of the symbionts under a large temperature and pH range (Chapter 6)), supports the versatility of fungal lineages

alone and within the symbiosis further linking the evolutionary paraphyly reported in Chapter 2. Together, the dissertation provides novel findings to better understand the success of caribou and reindeer lichens; it raises intriguing new hypotheses to further study the interacting symbionts, and provides possible explanations for their survivability despite the obligate nature of the symbiosis.

While species delimitation within *Cladonia* has been mainly based on phenotypic (morphological and chemical) characteristics for over a century, the lack of correlation between phenotypic and molecular characters as observed in many studies (Stenroos et al. 2002; Myllys et al. 2003; Kotelko and Piercey-Normore 2010; Piercey-Normore et al. 2010; Pino-Bodas et al. 2012a, b) may be interpreted as intraspecific morphological (Ahti 2000; Osyczka et al. 2007; Osyczka and Rola 2013) and genetic variation influenced by changes in different environmental conditions or through recombination (Fontaine et al. 2010; Kotelko and Piercey-Normore 2010). Genetic variation within a population can also be generated from mutation and further modified by genetic drift, natural selection and gene flow (Loveless 1984) and depends on various factors including taxonomy, reproductive mode, life history, mating system and habitat parameters (Zoller et al. 1999; Lindblom and Ekman 2006; Dyer and Murtagh 2001; Crespo et al. 2002; Osyczka et al. 2007). High genetic variation provides the species an adaptive advantage and enhances the survival probability (Wildt et al. 1987; Hedrick and Miller 1992; Lynch 1996). Phylogenetic relationships between the most common northern species of *Cladonia* in sections *Impexae, Crustaceae* and *Tenues* showed that only five species of 18 species examined were monophyletic, further spporting the genetic variation present within species. There can be several factors affecting the monophyly of a species such as pseudogenes, gene paralogy, horizontal gene transfer, incomplete lineage sorting or hybridization (Myllys et al. 2003; Stenová et al.

2013). Therefore, the intraspecific variation in the fungal partner of one selected species pair was further investigated in Chapter 3.

While the fungus makes up the largest proportion of the thallus, the initial thallus development must rely on a successful union between the alga and the germinating fungal ascospore in nature. Therefore, the goal of the Chapter 3 was to examine the morphological and genetic variation of ascospore cultures within and among apothecia in each of two species (*Cladonia arbuscula* and *C. rangiferina*) that did not show monophyly in the phylogeny. The polymorphism observed in both species was interpreted to suggest sexual reproduction in both species. The variation was higher in *C. arbuscula* than in *C. rangiferina* suggesting that *C. rangiferina* may use vegetative reproduction through thallus fragmentation as a more common means of reproduction than sexual reproduction. Thallus fragments ensure the new thallus has the same symbionts and the same fungal genotype as the parent thallus. However, frequently observed apothecia in *C. rangiferina* also indicate the presence of sexual reproduction, which would result in some intraspecific genetic variation by shuffling of alleles, large dispersal distances, and recoupling of the lichen symbionts. Higher genetic variation (percent polymorphism) observed in *C. arbuscula* in comparison to *C. rangiferina* may benefit its survival by having a larger number of alleles, which facilitates adaptation to changes in the environment. While the polymorphic fungal partner may be equipped to withstand the environment, it is also important to understand how the hyphae emerging from the germinating ascospores may survive and resynthesize with both compatible and incompatible algal partners in nature. The most common compatible photobiont species (*Trebouxia* s. l.) are rare in the aposymbiotic (free-living) state (Tschermak-Woess 1988) making the chance for a dispersed ascospore to find a compatible algal partner in nature also very rare (Piercey-Normore 2004;

Yahr et al. 2004). Therefore, knowledge of these challenges requires a better understanding of how a lichen fungus survives, relichenizes and becomes widely distributed. The next three chapters focus on a better understanding of the resynthesis (*in vitro* lichenization) of one species, *C. rangiferina*.

Chapter 4 provided novel insights into the resynthesis capacity of *C. rangiferina* with its compatible algae and other two incompatible algae. The quantification of three fungal characteristics and one algal characteristic showed that *C. rangiferina* exhibited specific morphological responses towards the compatible alga, which were significantly different than those exhibited toward the incompatible algae. Three early resynthesis stages were identified for *C. rangiferina* with its compatible algal partner. The morphological responses, shown at the later stage of resynthesis by the interaction between the non-lichenized incompatible alga, *Chlorella*, and *C. rangiferina*, were interpreted as parasitism. Previously some lichen fungi were shown to parasitize incompatible algae (ranging from *Trebouxia* to *Trentepohlia* sp.) (Jacobs and Ahmadjian 1971; Ahmadjian et al. 1978; Ahmadjian and Jacobs 1983; Joneson et al. 2011; Athukorala et al. 2014), which is consistant with the finding of the current study. The hyphae of *C. rangiferina* occasionally adhered to *Coccomyxa peltigerae*, which is an alga that commonly forms a lichen with distantly related fungi to *C. rangiferina* and is also commonly found free-living in nature. "Wall-to-wall" adhering of fungal and algal partners is characteristic of *Coccomyxa*-containing macrolichens (Brunner and Honegger 1985; review Honegger 1991a). Therefore, the interaction of *C. rangiferina* with *C. peltigerae* might be a short-term interaction, a strategy that the fungus may use in nature to survive until it finds the compatible alga. Some lichen fungi are known to steal photobionts from other lichens and successfully establish a new thallus (Peolt and Doppelbauer 1956; Friedl 1987), and germ tubes of lichen fungi have been

reported to colonize the photobiont of adjacent pre-thalli, thalli or soredia (Ott 1987 a,b) until they find a compatible alga to establish their own thallus (Ott 1987a, b). These previous findings together with the findings of the Chapter 4 indicate the versatility of lichen fungi toward a range of algae, where the type of symbiotic interaction may depend on the degree of compatibility between the two partners. Since the type of alga was shown to have an effect on the outcome of the interaction (Chapter 4), Chapter 5 further investigated the genetic factors involved in the communication between partners, which may determine the outcome of the interaction.

Considering the fact that recognition and defense are two important early events that control the outcome of the interaction between a plant host and its fungal pathogen (reviews Crute 1994; Odjakova and Hadjiivanova 2001; Dodds and Rathjen 2010), Chapter 5 quantified the expression of recognition-related genes and defense-related genes. Expression of these genes was examined for symbionts of *C. rangiferina* in response to culture extracts of compatible and incompatible interactions at the three early resynthesis stages described in Chapter 4 to test whether these genes are differentially expressed depending on the type of culture extract and the timing of the defined resynthesis stages (Chapter 4). This study revealed that recognition- and defense-related genes are specifically triggered by culture extracts containing *Asterochloris* when and after the symbionts make contact and some defense-related genes are induced throughout all three resynthesis stages. This implies a role for recognition and defense processes in the interaction between two symbionts in the lichen association that are known to occur between host plant and parasitic fungus. Therefore, the findings support the hypothesis that the lichen fungus is a biotrophic parasite on the alga and the recognition between partners allows the alga to turn on defensive strategies that limit fungal growth (Ahmadjian and Jacobs 1983; Molina et al. 1993; Honegger 1998). The expression of genes in response to extracts containing the

incompatible algae further supports the fact that communication also occurs between incompatible partners, which does not result in a mutualistic interaction.

Different morphological responses (mutualism to parasitism) and differential gene expression shown by *C. rangiferina* to different algae depending on their compatibility support the apparent variability of the symbiosis, where symbiosis is thought to span between mutualism and parasitism (Thompson 1988; Cushman and Addicott 1991; Cushman and Whitham 1991; Bronstein 2001). Theories of the evolution of mutualism and hypotheses on the origin of lichenization suggest that mutualism evolved from parasitism (Thompson 1982; Lutzoni 2001) and lichenization (mutualism) arose multiple times during evolution of the Ascomycetes as a nutritional strategy in response to varying environmental conditions (Gargas et al. 1995; Lutzoni and Vigalys 1995; Lutzoni et al. 2001, 2004; James et al. 2006; Schoch et al. 2009). If the algal pool at the vicinity of the geriminating spore of a lichen fungus is considered as its biological environment, Chapter 4 and Chapter 5 showed the importance of the biological environment for the success of relichenization of a lichen fungus. Chapter 6 was designed to explore the effect of physical (external) environmental factors on the outcome of the lichen symbiosis. Two environmental parameters, temperature and pH, were varied under controlled lab conditions and their effect on the *in vitro* resynthesis capacity of the lichen *C. rangiferina* was examined. Comparison of three characters of *C. rangiferina* and one character of *Asterochloris* that were significant in building the interaction and resynthesis with its compatible alga showed that the fungal characters are significantly altered by temperatures 5 ˚C and 35 ˚C, and pH 8.5, implying that the resynthesis is less successful at these conditions. The fungus and alga were affected differently. The effect of temperature was prominent on the fungus while that of pH was

prominent on the alga. The results showed that the outcome of the interaction is affected by the growing conditions even in the presence of the optimal compatible alga.

In summary, the phylogenetic analysis of the more common species of reindeer lichens from North America showed that some species are not monophyletic raising questions about their intraspecific variation. Intraspecific variation was further examined by studies carried out on two paraphyletic species, *C. rangiferina* and *C. arbuscula*, providing evidence that the paraphyly may be due to selection against monophyly (Chapter 2 and 3). If monophyly were selected against, the other common and widespread species of reindeer lichens should also be paraphyletic. Larger numbers of samples are needed to address this question. Intraspecific variation may be a strategy for adaptation and survival in varying environmental conditions related to their wide distribution and it was further strengthened by the effect that the environment (both biological and physical) shown to have on the interaction of symbionts (Chapter 4 and 6). This study also established parallels between the lichen (mutualistic) system and the plant pathogenic (parasitic) system (Chapter 5). This is the first study to follow expression of recognition and defense-related genes through three early resynthesis stages of a lichen and it demonstrates the effect of soluble compounds from culture extracts on the induction of gene expression during the first three stages of resynthesis.This study provides evidence to support the hypothesis that recognition related genes such as arginases secreted by the lichen fungus and *RR1* (e.g. urease) of compatible algae are involved in the recognition between algae and fungi in the lichen association (Molina et al. 1993; Molina and Vicente 1995; Molina et al. 1998; Legaz et al. 2004; Millanes et al. 2004; Sacristán et al. 2007). This dissertation collectively highlighted the versatility of the lichen association by demonstrating that lichenization of *C. rangiferina* is a complex process, which is governed by internal factors such as genetic

variability of the fungal symbiont alone (Chapter 2 and 3) and together with the variability of the alga (Chapter 4, 5, and 6) and external factors such as growing condition, which affects not only the interaction but also the ability of the symbionts alone to survive (Chapter 6).

Further research directions may include phylogenetic and population genetic studies with larger sample sizes over a larger geographic distribution and with more species belonging to this group. It will be necessary to examine whether the paraphyly observed with the three species pairs in the current study is affected by the number of samples and their distribution to further strengthen the hypothesis that the paraphyly is a result of sexual reproduction, or is inherent in the lineage and maintained to enhance allelic variation. The effect of type of fungus on the symbiosis will be an important question for future studies since this dissertation accounted for the algal diversity only. Similarly, intraspecific genetic variation of the algal partner needs to be explored. The primers developed in the current study can be used as the preliminary step to characterize the recognition- and defense-related genes of other lineages of *C. rangiferina* and *Asterochloris* as well as in symbionts of other lichens species to test their variation. Since it was observed that the liquid extracts of compatible and incompatible interactions differentially induce expression of genes, further research on expression studies with concentrated crude extracts, isolation and identification of compounds from extracts using chromatographic techniques will enable the identification of signal and elicitor molecules involved in the symbiont interaction. It will also be important to carry out protein profiling to determine the functional aspects of the recognition- and defense-related genes. Furthermore, the effect of environmental parameters on the expression of the above genes and on the production of relevant proteins would also be interesting to examine to understand the mechanisms of the effect of environment on the interaction. The effect of more refined growing conditions between those

tested in this study as well as additional abiotic conditions such as moisture and light levels also need further examination to better understand their collective effect on the *in vitro* symbiont interaction, so that more accurate interpretations can be made on the effect of phenomena such as global warming on lichen communities. Therefore, the molecular techniques and experimental procedure developed during my Ph.D. research can be used to further test a vast array of hypotheses, including those related to functional genomics and systems biology, hypotheses relevant to mechanisms of symbiont interaction in lichens, and those that may assess their survivability in the future.

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APPENDIX 1

Apothecial number, the number of spore cultures used to compare culture morphology, the number of spore cultures used in RAPD and RAPD primers used for species *C. rangiferina* and *C. arbuscula.* SA represents collection by Athukorala and MN represents collection by Normore.

Species (sample)	**Primers used for RAPD**	**Apothecium no. (no. of spore cultures – no. of spore culture used for RAPD)**
Cr-SA24 (1)		I(4), II(2)
Cr-SA22 (1)	31, 34, 60, 184	I**(3),** II**(4),** III**(2)**
Cr-SA22 (2)	31,34, 60, 184	I**(**6-**3**), II(9-**2**), III(3), IV(**2**), V(10-**5**), VI(3-**2**), VII(**1**)
Cr-SA17 (1)		I(5)
Cr-SA16 (1)		I(6)
Ca-SA7 (1)	31, 60, 90, 122	I(3), II(3), III(6-**3**), IV(4-**1),** V(4)
Ca-SA12 (1)		I(2), II(4), III(3)
Ca-SA13 (1)	31, 60, 90, 122	I(**6**)
Ca-MN9403 (1)		I(8), II(6), III(7), IV(7), V(7), VI(8), VII(8), VIII(8), IX(8), X(8)
Ca-MN9468 (1)	31, 60, 90, 122	I**(**10-**8**), II(8-**3),** III(8)
Ca-MN9642 (1)		I(8), II(8), III(8), IV(8), V(8), VI(8), VII(4), VIII(8), IX(8), X(7)

*The number of spore cultures used for RAPD PCR is bold within parentheses.