EVOLUTION AND EXPRESSION OF POLYKETIDE SYNTHASE GENE IN THE LICHEN-FORMING FUNGAL FAMILIES CLADONIACEAE AND RAMALINACEAE

By

Brinda Adhikari Timsina

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Department of Biological Sciences

University of Manitoba

Winnipeg Manitoba

Canada

ABSTRACT

 Fungal polyketides are synthesized by polyketide synthases (PKS) encoded by PKS genes. The function of many PKS genes is unknown and the number of PKS genes exceeds the number of polyketides in many genomes. The lichen-forming fungi, *Cladonia* and *Ramalina* have chemical variants separated by habitat suggesting that environmental conditions may influence polyketide production. The goal of this thesis was to examine evolutionary relationships as a framework to investigate PKS gene function in the lichen-forming fungal families Cladoniaceae and Ramalinaceae. A phylogenetic analysis of the genus *Ramalina* (Chapter 2) using nuclear and mitochondrial ribosomal DNA sequences showed monophyly for seven species and included three species, which were not examined in phylogenies prior to this study. One monophyletic species, *R. dilacerata* was chosen for further tests of the effect of growing conditions on PKS gene expression (Chapter 3). Growth media containing yeast extracts produced the largest colony diameters and the fewest number of polyketides. A significant negative relationship occurred between colony diameter and number of secondary metabolites. Expression of two types of PKS genes was correlated with pH-level and media conditions that produced larger numbers of secondary products in *R. dilacerata*. A PKS gene phylogeny was constructed for 12 paralogs detected in members of the *C. chlorophaea* complex (Chapter 4) and gene selection was inferred using dN/dS estimations. The gene phylogeny provided evidence for independent origins and purifying and positive selection of PKS paralogs. This research provided insight into the evolution of PKS genes in the *C.*

chlorophaea complex and identified potential genes that produce non-reduced

polyketides present in *C. chlorophaea*.

This thesis provided evidence for diversification of both morphological and

chemical species and monophyly of previously unstudied *Ramalina* species. This

research also supported theories of secondary metabolite synthesis based on growing

conditions of *R. dilacerata*, and it revealed that PKS genes under selection in the

Cladonia chlorophaea group provide the lichen with the adaptive capacity to survive

under variable conditions. Knowledge of the ecological function of fungal polyketides

can be valuable for conservation management and policy makers; and for understanding

the potential pharmaceutical roles of these natural products.

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Thesis, the publication status of chapters, and the role of each coauthor in each chapter

Chapter	Author	Contribution of each author	Article status
1	Brinda A. Timsina C. Deduke, and M. Piercey-Normore	B. T. wrote the Introduction and Literature Review for the thesis. I wrote the portions included in the book chapter and did all the literature searching for those parts of the chapter.	Parts of this chapter are included in a published book chapter. Open Access and online. ISBN: 978-953-307-815-1, InTech.
2	Brinda A. Timsina	B. T. performed all the lab work, data analysis, wrote the manuscript and served as a corresponding author.	Manuscript is published in the journal, *Botany*. *Botany*, 2013, 90(12): 1295-1307.
	Elfriede Stocker-Wörgötter	E. S-W. performed HPLC on 14 out of 50 samples to confirm my results and she edited the paper before submission to the journal.	
	Michele D. Piercey-Normore	M. P-N. served as my supervisor and provided funding.	
3	Brinda A. Timsina	B. T. performed all the lab work, analysis and wrote the manuscript and served as the corresponding author for the manuscript.	Manuscript available online, (http://dx.doi.org/10.1016/j.funbio.2013.09.003) *Fungal Biology*..
	John Sorensen	J. S. provided guidance on how to do HPLC and its interpretation; and he edited the paper before submission to the journal.	
	Dirk Weihrauch	D. W. provided guidance on how to do Real Time PCR and the interpretation; and he edited the paper before submission to the journal.	
	Michele D. Piercey-Normore	M. P-N. served as my supervisor and provided funding.	
4	Brinda A. Timsina	B. T. performed all the lab work, the data analysis, and wrote the manuscript; and B. T. is a corresponding author for the manuscript.	Manuscript is in review in the *American Journal of Botany*. Date of submission: July 4, 2013.
	Georg Hausner	G. H. provided guidance on how to analyse data using dN/dS estimations and interpretation; and he edited the paper before submission to the journal.	
	Michele D. Piercey-Normore	M. P-N. served as my supervisor and provided funding.	
5	Brinda A. Timsina	I wrote the Final Discussion and Conclusions for the thesis. This chapter has not been submitted for publication.	

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from the *Cladonia chlorophaea* complex based on the amino acid

sequence alignment deduced from the DNA sequence of the KS

domain. This is one of 422 most parsimonious trees, which is

consistent with the Bayesian tree (not shown). Major clades are

indicated by brackets and circled letters (A to F). The assigned

outgroup is ORS-PKS. Numbers above branches represent node

support values greater than 70% based on maximum parsimony

analysis and thickened lines indicate that the posterior

probabilities are greater than 90% based on Bayesian analysis. The

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contain a known gene structure from NCBI GenBank. The gain of

ME domain early within the evolution of the PKS genes and the

eventual loss of the ME domain in the potential ancestor of the

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analysis. Clades discussed in the text are indicated by circled

letters (A and B) and sub-clades are indicated by brackets (Roman

numerals i to vi).

CHAPTER 1

General Introduction and Literature Review

1.1. INTRODUCTION

The lichen is a symbiotic association between a fungal partner (mycobiont) and one or

more photosynthetic partners (photobiont), which may be either a green alga or a

cyanobacterium (Nash 1996). The name of the fungal partner is the same name that is

given to the whole lichen and there are more than 13,500 described species, which

include almost one-fifth of all known fungi (Kirk et al. 2001). In most lichens the

mycobiont comprises the largest portion of the lichen thallus while the photobiont forms

a small component as part of the medullary layer in the thallus (Figure 1A & B). The

thallus is comprised of a loose network of fungal hyphae forming the medulla and

sometimes a conglutinated layer of densely arranged fungal hyphae forming a cortex,

which is exposed to the outer atmosphere. Other thallus layers and morphological

features may also be present on the thallus to aid in protection, attachment, or water

retention, depending on the species and the habitat in which the lichen grows. One of the

protective features of the lichen thallus is the wide array of secondary metabolites

produced by the mycobiont. These secondary metabolites may be differentially produced

in the thallus cortex or the medulla depending on the function of the metabolite. Cortical

metabolites are hypothesized to play a role in light screening or herbivory (Stocker-

Wörgötter 2008, Gauslaa 2005) while medullary metabolites are thought to play a role in

water relations for algal photosynthesis (Lawrey 1986, Rundel 1978). Although the

secondary metabolic pathways are entirely present within the mycobiont (Culberson and

Armaleo 1992), the photobiont is also thought to play a role in their biosynthesis

(Brunauer et al. 2007, Yamamoto et al. 1993)

Lichenized fungi synthesize a diversity of secondary metabolites (Huneck 1999)

and most lichen substances are small aromatic polyketides synthesized by a polyketide

synthase (PKS) in the fungal partner of the symbiosis (Schmitt et al. 2007, Culberson and

Armaleo 1992;). In fungi, most polyketides are catalysed by iterative Type I PKS

multidomain enzymes that catalyse multiple carboxylic acid condensations (Keller et al.

2005). After fungal polyketides are formed, they usually undergo modifications

(reductions, oxygenations, esterifications, etc.), which are catalysed by enzymes other

than the PKS enzymes (Proctor et al. 2007). The genes encoding the PKS and modifying

enzymes are located adjacent to each other in gene clusters. The genes in a cluster are co-

regulated with transcription of all the genes being repressed or activated simultaneously

(Keller and Schwab 2008). A single fungal genome may contain more than one PKS gene

and each species of fungi can produce more than one polyketide or polyketide family

(Proctor et al. 2007). Members of the lichen family Parmeliaceae have been shown to

have six paralogs (Opanowicz et al. 2006) and the genome of *Cladonia grayi* contains at

least 12 paralogs (Armaleo et al. 2011), but little is known about the function of the

paralogs. Studies on PKS genes from different lichen groups suggest a high level of gene

paralogy (Grube and Boustie 2005). A large number of paralogous PKS genes are also

expected to be present in the genomes of some *Ramalina* species because they are rich in

diverse phenolic compounds (LaGreca 1999, Culberson et al. 1990). Based on a

phylogeny of European species of *Ramalina*, Stocker-Wörgötter et al. (2004)

hypothesized that polyketide-poor species are ancestral to polyketide-rich species of

Ramalina. Species that are thought to be closely related, such as *R. farinacea* and *R. intermedia*, differ by a single polyketide and subtle morphological features, but it is not known if some of these species form monophyletic groups. Studies on gene evolution at high taxonomic levels suggests that the paralogs are monophyletic and under selection pressure (Kondrashow et al. 2002). After a duplication event, one gene copy may be under selection pressure for a specific function but the other copy might not be subject to selection pressure. Alternatively, both of the duplicated genes may undergo a period of relaxed selection, and accumulate deleterious or beneficial mutations (Kondrashow et al. 2002).

The regulation of fungal secondary metabolism to some extent is thought to depend upon the chromosomal organization of biosynthetic genes in addition to general environmental factors such as carbon and nitrogen sources, temperature, light, and pH (Shwab and Keller 2008). With regard to two *Cladonia* species, *C. pocillum* and *C. pyxidata*, it is thought that pH may be a driving environmental factor that creates the diagnostic difference between the two morphotypes (Kotelko and Piercey-Normore 2010, Gilbert 1977). Members of the *Cladonia chlorophaea* complex have been found to share virtually identical morphologies but different secondary metabolites (Culberson et al. 1988, Culberson 1986). *Cladonia grayi* and *C. merochlorophaea* grow at lower pH levels than *C chlorophaea sensu stricto* or other members of the species complex. If the pH is one factor that is regulating production of the polyketides, which are diagnostic of these chemospecies, the pH may also drive the morphological species difference. Although members of the *Cladonia chlorophaea* complex produce only one to six polyketides (Huovinen and Ahti 1982), they may have up to 12 known PKS gene paralogs (Armaleo

et al. 2011) implying that some of the paralogs are not functional or have undetected functions. Identity of the functional paralogs may initiate further studies on the biosynthesis of these metabolites, their ecological function, economic value, and it may provide insight into the species differences in this complex.

Biosynthesis of polyketides has been reviewed for non-lichenized fungi (Keller et al. 2005, 1997, 1994) and much of our knowledge of polyketide synthesis in lichen-forming fungi has come from studies on the non-lichenized fungi (Proctor et al. 2007, Verdas and Sorensen 2003, Bingle et al. 1999). Lichens have been studied for more than two centuries as morphological entities but experimental lichenology remained a nearly unexplored field because of the slow growing nature of the mycobiont in culture. Thomas (1939) reported the first successful resynthesis of *Cladonia pyxidata*, and Ahmadjian (1961), and Stocker-Wörgötter (2002) refined the culturing techniques making pure cultures of the mycobiont and the photobiont more easily obtainable to a larger number of researchers. DePriest (1993) was the first to conduct studies on the genus *Cladonia* using small subunit ribosomal DNA data to investigate the diversity of separate lichen mats composed of members from the *Cladonia chlorophaea* species complex. Grube and Kroken (2000) reviewed the concept of species and species complexes in lichen-forming fungi using a molecular approach. Following these studies, molecular studies became more frequent for lichen symbionts, influencing our knowledge of phylogeny and taxonomy in lichen associations. While knowledge of phylogenetic relationships, polyketide function, and the expression of lichen-forming fungal genes has grown exponentially, investigation of the effect of environmental conditions on PKS gene

expression is in its infancy and the link between the gene and the polyketide product has not been made for lichen-forming fungi.

1.2. THESIS GOALS AND OBJECTIVES

The main goal of this dissertation is to estimate evolutionary relationships as a framework to investigate environmental influence and function of PKS genes in members of the lichen-forming fungal families Cladoniaceae and Ramalinaceae. There are three research projects that represent three chapters in the thesis and each project has more specific objectives:

1. To infer the phylogeny of the polyketide-rich genus *Ramalina* and develop insights into PKS gene function by:

a) examining the phylogenetic relationship of North American species of *Ramalina*, and

b) screening nine species for the presence of PKS gene paralogs and infer function from PKS gene sequences.

2. To investigate the environmental influence on spore development and polyketide production relative to PKS gene expression in *Ramalina dilacerata* by:

a) examining morphological variability in early colony growth of *R. dilacerata*.

b) testing the effects of environmental changes on growth and polyketide production of *R. dilacerata* using different growth media and pH, and

c) comparing PKS gene expression in *R. dilacerata* using quantitative PCR for colonies grown in different pH and media conditions.

3. To examine the gene evolution of PKS paralogs in members of the *Cladonia chlorophaea* species complex by:

a) investigating the number of PKS genes and the number of polyketides in some members of the *Cladonia chlorophaea* complex.

b) studying PKS gene evolution in selected members of the *Cladonia chlorophaea* complex.

1.3. LITERATURE REVIEW

1.3.1. The lichen association

Lichen symbioses are considered to be biotrophic associations involving a single type of photobiont and a mycobiont (DePriest 2004, Richardson 1999, Honegger 1998, Smith 1973). Many researchers refer to lichens as mutualistic associations while others regard them as associations that result from controlled parasitism. The interaction is considered to be controlled because the photobionts grow more slowly than in the free-living state, cell structure is often deformed, sexual reproduction does not occur when they are in a lichen association; and the mycobiont seems to get the maximum benefits from the symbiosis (Ahmadjian 1993). However, the lichen symbiosis is a successful association since both mycobiont and photobiont inhabit a wider range of habitats while in the lichen thallus, where individually they would not be capable of surviving (Nash 2008). Most free-living green algae and cyanobacteria are found either in aquatic or moist terrestrial habitats but as part of the lichen thallus algae may also occur in drier habitats. Photobionts are adversely affected by high light intensity (Demmig-Adams et al. 1990) but in the lichen association, the fungus can reduce the intensity of light by producing various thallus features or by secondary product synthesis thus protecting the algae (Ertl 1951).

All the known lichen-forming fungi are associated with either green algae (phycobiont) or cyanobacteria (cyanobiont) as the photobiont. It has been reported that about 85% are bipartite associations where the lichen-forming fungus associates with a phycobiont and about 10% associate with a cyanobiont. The remaining 5% of lichen-

forming fungi form a tripartite association containing both phycobiont and cyanobiont within the same thallus (DePriest 2004, Rikkinen 1995). The tripartite thallus forms specialized structures called cephalodia where the cyanobionts are confined within a compartment on the thallus and undergo nitrogen fixation (Richardson 1999, Büdel and Sdheidegger 1996, Rikkinen 1995, Ott 1988). Different combinations of green algae and cyanobacteria may allow the lichen-forming fungus to live in photobiont-specific habitats where photosymbiodemes may have different thallus morphology depending on whether the same fungal species associates with a cyanobacterium or a green alga. On the other hand, a single species of mycobiont can associate with two different species of green algae and form morphologically identical thalli. For example, the lichen-forming fungus *Parmelia saxatilis* associates with two morphologically distinct species of *Trebouxia* (*T. arboricola* and *T. jamesii*; Friedl 1989b) but it lives in the same habitat. In the lichen association the photosynthetic partner is retained within the thallus of the lichen-forming fungus providing carbon to the mycobiont (Nash 1996). The lichen is known by the name of the fungal partner but the photobionts are given their own separate names. Sometimes lichenicolous fungi (Lawry and Diederich 2003) and bacteria (Bates et al. 2011, Cardinale et al. 2006) may associate with the lichen thallus making the association more complex. Lichenicolous fungi are different from the dominant mycobint and they may be mutualistic, saprophytic, parasitic, or commensalistic to the lichen association (Rambold and Triebel 1992). The lichen hosts may be neither benefitted from nor damaged by the presence of secondary lichenicolous fungi. One parasitic fungus, *Diploschistes muscorum*, is reported to develop in the thallus squamules and podetia of species of

Cladonia and damage the host, in order to associate with *Trebouxia irregularis*, the photobiont of the host (Friedl 1987, Ott 1987a).

1.3.2. Classification and taxonomy

Most of the known lichen-forming fungi belong to the class Ascomycota (following Kirk et al. 2008). About 13,500 species (98%) of lichenized fungi are Ascomycetes that comprise 42% of all Ascomycetes. Fifty species (i.e. 0.4%) belong to the class Basidiomycota and about 200 species (1.5%) belong to Deuteromycota (DePriest 2004, Kirk et al. 2001, Hawksworth and Honegger 1994, Honegger 1992). Approximately 100 species of photosynthetic partners belonging to 40 genera from five different classes: Cyanophyceae (prokaryotic), Tribophyceae, Phaeophyceae, Chlorophyceae and Pleurastrophyceae (eukaryotic); are lichenized (Friedl and Büdel 1996). Of the 40 genera of lichenized algae, *Trebouxia* is the most common genus (Honegger 1998, Ahmadjian 1970) among the eukaryotic classes. *Nostoc vaucher* is the most common species from the Cyanophyceae (Rikkinen 1995). Only about 10% of all lichen species contain a cyanobacterium as the primary photobiont (Nash 2008). One of the basic physiological features in lichens, the uptake of CO_2, varies depending on whether the photobiont is a prokaryote or eukaryote. The eukaryotic photobiont, green algae, release polyol (a polyhydric sugar alcohol) to the mycobiont whereas cyanobacteria release glucose (Feige and Jensen 1992). The transfer of photosynthate from the autotrophic photobiont to the heterotrophic mycobiont also depends upon the photobiont involved. The release of glucose by cyanobacteria is thought to be mediated through the surrounding gelatinous polysaccharide sheath (Richardson and Smith 1996). The subsequent formation of

mannitol in the mycobiont increases with an increase in water content of the cyanolichen (MacFarlane and Kershaw 1982). In the case of green-algal lichens, alternating periods of dry and wet conditions may be required for carbon transfer in the symbiosis, which may be facilitated by haustoria (Farrar 1976b). In both cases, the carbohydrate taken up by the mycobiont is rapidly and irreversibly metabolized into mannitol by the pentose phosphate pathway (Lines et al. 1989). Carbohydrates are important for general metabolism of the fungus and they are thought to provide the starting carbon units for polyketide synthesis (Nash 2008).

A publication by Carolus Linnaeus in 1753 entitled "*Species Plantarum*" included some of the beginnings of lichen nomenclature, but the classification of lichens was based mainly on gross morphology and in part on anatomical characters (Tehler 1996). Names given to lichens apply to their fungal components; therefore, characters derived from the fungus are regarded as the most important characters taxonomically (Purvis 1997). As microscopes became more powerful in the 18th and 19th centuries and as knowledge of the distributions of species improved, additional characters such as the features of ascospores, mode of reproduction, biogeography and fine morphological characters were used to distinguish between lichen species (Letrouit-Galinou 1973, Hale and Culberson 1970). Fungal "chemotaxonomy" provides additional characters of the secondary products that are used in classification (Lumbsch 1998a, 1998b, Hawksworth 1976). Lichens that are morphologically identical but differ in the secondary compounds synthesized by the thallus are referred to as chemotypes or chemospecies. Identification of the commonly occurring chemospecies and characterization of the secondary metabolites, require the use of methods such as thin layer chromatography (TLC;

Culberson 1986, 1972), mass spectrometry, and high performance liquid chromatography (HPLC) (Nourish and Oliver 1976). Polyketide products can be separated and purified using TLC. The quantity and isolation of polyketides has been determined by HPLC. HPLC has been more widely used than TLC as an effective analytical tool for the separation and identification of lichen secondary metabolites (Feige et al. 1993). Over the last two decades, HPLC has become more popular among lichenologists and has often replaced the previously used TLC methods for natural product separation (Mietzsch et al. 1993, Elix et al. 1988).

1.3.3. Lichen morphology

The thallus of lichens can vary in size from less than one millimeter (mm) to over two meters (m). Some of the pendulous forms which hang from tree branches are longer than 2m (Nash 2008). However, calicioid lichens are very tiny, the thallus being visible only as a pale stain on wood (e. g. *Calicium trabinellum*) and the fruiting stalk ranges from 0.5-0.9mm tall (Brodo et al. 2001). Lichens are generally considered to be perennials and some species are reported to survive over 1000 years, which may be useful in dating rock surfaces (Beschel 1961). Many species have slow growth rates and grow from 0.005mm/year in a polar lichen (Green et al. 2011) to over 10mm/year in some foliose lichens such as members of the genus *Peltigera*. Hale (1973) reported the growth of crustose lichens to be up to 0.5-2mm per year, foliose lichens may grow up to 0.5-4mm per year and fruticose species may grow up to 1.5-5mm per year. Some lichens in the genera *Cladonia* and *Ramalina* are thought to grow faster than these reported rates.

Figure 1.0.   Illustrations of the thallus cross-section in fruticose lichen. A) Cross-section through the podetium in *Cladonia* spp. B) Thallus cross- section in *Ramalina* spp.

B

A

Outer cortex

Algal cells

Medulla

Inner layer

Inner hollow tube
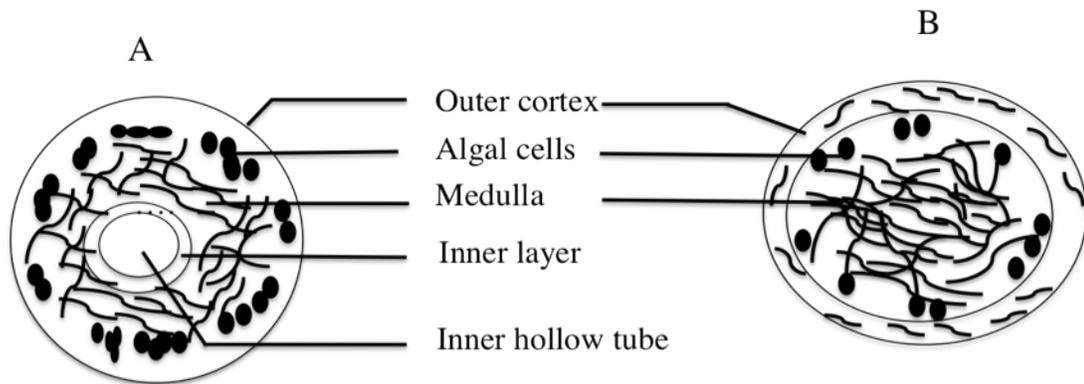
Thallus size may also vary where the squamules in some species of *Cladonia* (e. g. *C. cariosa)* can grow up to 30 mm in length and the podetia can grow up to 15 cm tall in *C. maxima* (Brodo et al. 2001). The pendent thallus of *Ramalina thrausta* and *R. menziesii* are reported to grow up to 100 cm long (Brodo et al. 2001).

Most lichens are terrestrial, and only a few species (e. g. *Peltigera hydrothyria)* are aquatic that occur submerged in fresh water or sub-aquatic (e. g. *Dermatocarpon luridum*) that are periodically inundated by fresh water. Species belonging to the *Verrucaria maura* group colonize the rocky shoreline around oceans and are considered marine lichens. Many terrestrial lichens occur commonly as epiphytes on trees and other plants (Seaward 2008, McCune et al. 2003). Some species of lichens are also found to colonize soil, rooftops, man-made materials, or as epilithic lichens on the surface of rocks, or endolithic within the rock substrate (Friedmann 1982). Some rapidly growing species are also found to colonize the leaf surface on tropical plants as epiphylls (Lucking and Bernecker-Lucking 2002). Regardless of the substratum, the appearance and habit of the lichen thallus is usually determined by the mycobiont (Büdel and Scheidegger 2008). Lichens are classified into three morphological groups (crustose, foliose and fruticose) on the basis of their growth forms, which may be variable within species. Crustose lichens are tightly attached to the substrate over the entire lower surface and cannot be removed without destruction of the thallus. Foliose lichens are leaf-like, flat and are only partially attached to the substrate with attachment organs such as rhizines or holdfasts. The foliose thallus is often divided into lobes with various degrees of branching and they have a distinct upper and lower surface. Fruticose lichens have strap-shaped, hair-like or shrubby thallus lobes with cylindrical branches having inner

and outer portions of the thallus. The thallus always stands out from the surface of the substrate and is usually attached by a single point. Some genera, such as *Cladonia*, develop a two-part thallus, which is differentiated into vertical and horizontal (squamulose) parts. If the vertical growth originates from primordia of the fruitbody and produces an apothecia-bearing stalk, it is called the podetium. If the vertical thallus develops from primordia of the horizontal thallus, it is called a pseudopodetium (Ahti 2000).

The thallus organization of crustose and foliose forms may be homoiomerous or heteromerous, where the majority of lichens have heteromerous thalli. Mycobionts and photobionts are evenly distributed in homoiomerous thallus organization such as in the jelly lichens. The heteromerous thallus contains highly organized layers of tissue and each layer has a specific function (Büdel and Scheidegger 2008). Fruticose thalli have outer, middle, and sometimes inner layers of tissue because of the cylindrical nature of the thallus extending upward (podetium; *Cladonia*) or outward (pendant or tufted; *Ramalina*) from the substratum, whereas foliose thalli have upper, middle and lower layers of tissue because of the flattened, leaf-like nature of the thallus against the substratum (Ahti 1966). The outer or upper layers may be comprised of a cortex with thick walled conglutinated fungal hyphae densely adhered to one another. This layer sometimes contains pigments or other secondary metabolites that have a number of hypothesized protective functions. The middle layer of tissue is comprised of the medulla, which is a layer of loosely woven fungal hyphae often with air spaces between the hyphal strands. Secondary metabolites that confer an external hydrophobic property, and a continuous or patchy layer of algal cells are present in the upper or outer layer of

the medulla. The lower or inner layer of tissue varies tremendously depending on the taxonomy and habitat of members of the genus. The podetium of the members of the genus *Cladonia* contains an inner hollow tube with a margin of conglutinated fungal hyphae similar to a cortex. This hollow tube is diagnostic of the genus and it provides a rigid upright podetial thallus to release fungal spores from the apothecia into the air stream for better dispersal. The inner layers of the primary squamulose thallus are comprised of medullary hyphae while the upper squamule sometimes contains an upper cortex and a photobiont layer and they are devoid of a lower cortex. Members of *Ramalina* contain an outer cortical layer, and loosely arranged medullary hyphae with no differentiated inner tissue (Bowler 1977).

1.3.4. Scientific progress on studying lichens

Studies on the variation in thallus morphology and secondary product biosynthesis with environmental change in lichen associations has lagged behind studies in non-lichenized fungi because of difficulties with isolation and culturing of lichen-forming fungi. Thallus morphology was suggested to exhibit a degree of plasticity depending on environmental conditions (Pintado et al. 1997) and growth form was shown to arise independent of species evolution (Stenroos and DePriest 1998). Secondary metabolite production may also be influenced by changes in culture conditions, which might be regarded as environmental changes (Stocker-Wörgötter 2008). Investigation of secondary product biosynthesis in lichen fungi has been hindered by problems associated with isolating and growing cultures of lichen fungi. Since the 1970's, a large portion of experimental lichenology was focused on the improvement and optimization of culture conditions of

lichen fungi. Growth conditions for some fungi were determined in early studies (Yamamoto et al. 1993, Hamada 1989, Ahmadjian 1961), and more recently culture conditions were optimized to examine polyketide production of cultured mycobionts (Fazio et al. 2009, Brunauer and Stocker-Wörgötter 2005, Cordeiro et al. 2004, Molina et al. 1997, Armaleo 1991). The improvement of culture techniques for lichen fungi have provided greater access to cultures of lichen symbionts and allowed a better understanding of thallus morphology in the genus *Cladonia* using scanning and transmission electron microscopy (Hammer 2001, Hammer 2000, Ahti 1980). Knowledge is also beginning to accumulate on phylogenetic histories including early studies on genetic variation (Grube and Blaha 2003, Stenroos and DePriest 1998, Grube et al. 1996, DePriest 1995); more recent studies on multi-gene phylogenies (de Paz et al. 2011, Crespo et al. 2010); on gene arrangement in entire genomes of lichen-forming fungi (*Cladonia grayi*, Armaleo et al. 2011, *Xanthoria parietina* [http://genome.jgi.doe.gov/Xanpa1/Xanpa1.home.html], and *Lobaria pulmonaria* [http://genome.jgi.doe.gov/pages/home.jsf?core=genome&query=Lobaria&searchType= Keyword]); on the genes involved in production of secondary metabolites from lichen fungi (Armaleo et al. 2011, Muggia et al. 2008, Opanowicz 2006, Schmidt et al. 2008, 2005); and on the effects of the environment on the expression of PKS genes (Brunauer et al. 2009, Chooi et al. 2008, Stocker-Wörgötter 2007).

1.3.5. Evolutionary studies of lichen fungi

At least five independent origins of lichenization were proposed by Gargas et al. (1995). However, Lutzoni et al. (2000) reported the origin of lichenization by multiple independent losses of the lichen symbiosis. The Ascomycotina lineages of non-lichen forming species are believed to have been derived from lichen-forming fungal ancestors (Lutzoni et al. 2001). Some non-lichenized fungi may have secondarily lost the ability to form a lichen association and as a result, lichenization has been viewed as a highly successful nutritional strategy (Wedin et al. 2004, Honegger 1998). The nutritional status of the fungus is thought to have improved after forming an association with algal cells (Honegger 2000). One of these lichen-forming fungal lineages diverged into a diversity of successful species in the Order Lecanorales, which include the families Cladoniaceae and Ramalinaceae.

1.3.6. Ramalinaceae

The genus *Ramalina* Ach. is a group of pendent or shrubby fruticose, epiphytic and saxicolous lichens with long, flattened and usually stiff thallus branches. Thalli can either be hollow or solid and compressed or terete (Joneson 2003). The genus *Ramalina* is represented by 46 species in North America (Esslinger 2011), which grow on branches, bark, twigs, tree trunks, moss, soil, and on siliceous rock. While some species within the genus grow on rocks or cliffs, other species prefer the bark of trees, and some of the generalists may be found on both rock and tree bark. The genus contains fruticose species that are attached to their substratum by a single or several holdfasts giving the thallus a tufted or sometimes pendant appearance. Members of the genus *Ramalina* are associated

with the green algal genus *Trebouxia*. Different species of *Trebouxia* may associate with

different members of the genus *Ramalina*. For example, *T. impressa* and *T. potteri*

associate with *R. sinensis* and *R. americana* (Francisco et al. 2012) while *T. decolorans*

associates with *R. menziesii* (Werth and Sork 2010).

The growth rate in *Ramalina* species can range between 1 to 5 mm per year but

R. menziesii is known to grow up to 90 mm per year (Hale 1973). The common method

of reproduction in the members of *Ramalina* is by ascospores, pycnospores, soredia or

isidia. Apothecia in *Ramalina* species may be laminal or submarginal which release

ascospores. There are eight spores in each ascus; spores are two-celled and colorless

(Brodo et al. 2001). The genus *Ramalina* has been well studied for secondary metabolism

and ecology (Stocker-Wörgötter et al. 2004, Elix and Wardlaw 1986, Bowler and Rundel

1978, Culberson et al. 1977). *Ramalina* species are found to produce β-orcinol depsides

and depsidones and orcinol depsides, which comprise the typical aromatic phenols. Usnic

acid is found in the cortex of all *Ramalina* species.

The heteromerous thallus in *Ramalina* (Figure 1B) consists of an outer thin layer called a

cortex present in many species, the middle and inner layers combined into the medulla

consisting of loosely woven fungal hyphae with many air spaces. A continuous layer of

algal cells is present in the outer part of the medulla.

Ramalina dilacerata (Hoffm.) Hoffm. is an epiphytic lichen with numerous apothecia and a shrubby thallus that produces usnic and divaricatic acids as the diagnostic compounds. The thallus in *R. dilacerata* is light green, corticolous and hollow (Joneson 2003). *Ramalina dilacerata* is sympatric to *R. pertusa* and can be found growing together corticolously on the same branch. These two species may be chemical races of the same species and can be separated by chemistry alone. *Ramalina pertusa* is a rare species found in Europe and has not been reported from North America. Unlike *R. dilacerata*, *R. pertusa* produce usnic and evernic acids. *Ramalina dilacerata* is readily distinguished from other North American species in the genus (Brodo et al. 2001); it has not been previously included in any phylogenetic study. The derived species are hypothesized to produce larger numbers of polyketides than the more primitive species (Stocker-Wörgötter 2004). Culberson et al. (1990) reported a high chemical and morphological variation in *Ramalina americana* complex which was later separated into *R. americana* and *R. culbersoniorum* (LaGreca 1999).

1.3.7. Cladoniaceae

The Cladoniaceae are a family of lichen-forming fungi that include more than 500 species. Many species are easily recognized because of their conspicuous colors. The apothecium is usually bright red or dark brown or may occasionally be pale brown or pale pink (Stenroos and DePriest 1998). Species of Cladoniaceae are found on all continents (Ahti 2000). Large thalli of the members of Cladoniaceae grow up to 15cm tall; exceptional thalli may grow up to 48cm in height (Ahti 2000). The genus *Cladonia* is a lichen forming species within the family Cladoniaceae (Miadlikowska et al. 2006, Ahti

2000) and its members are associated with *Asterochloris*, a coccoid green alga

(Miadlikowska et al. 2006, Rambold et al. 1998). Most other members of the Lecanorales

are associated with a closely related genus *Trebouxia* (Dahlkild et al. 2001, Helms et al.

2001). Approximately 168 species of *Cladonia* have been reported in North America

(Esslinger 2008) that are often mixed with mosses on wood, bark, rock, soil, and peat.

Members of the genus are mostly ground-dwelling on various substrata, but some species

prefer decaying wood or tree bases. All species have a primary crustose or squamulose

thallus in direct contact with the substratum and a vertical fruticose thallus (podetium)

often culminating in the sexually produced fruit body (apothecium) at its apex (Ahti

2000). The thallus in the genus *Cladonia* is referred to as cladoniform, which is a two-

part lichen thallus and is considered to be a composite of the crustose and fruticose

growth forms (Ahti 1982). The primary thallus is horizontal consisting of scaly

squamules or a crustose habit and the erect stalks (podetia) which develop from the

squamules or the crust. Podetia are the hollow, vertical thalli, and the squamules

constitute the horizontal crustose thalli (Ahti 1982a). Podetia grow from the horizontal

thallus, which may or may not persist for long periods but the podetium lasts for many

years (Ahti 1982b). The hollow podetia can be unbranched to highly branched, they may

form a cup-like structure, or be blunt or have a pointed tip. Podetia normally consist of

an outer thin layer called a cortex present in many species, the middle layer called

medulla consisting of loosely woven fungal hyphae and the inner layer of conglutinated

fungal hyphae in a stereome surrounding a hollow centre (Figure 1A). A continuous or

sometimes patchy layer of algal cells is present in the outer part of the medulla.

Sometimes the outer layer of the podetial thallus breaks due to the development of tiny squamules or soredia. Podetia growing from active meristematic tissue of the squamules ultimately give rise to the reproductive structures, the ascomata (Hammer 2000). A podetium is a lichenized portion and bears the hymenial discs and or conidiomata in a fruticose apothecium (Ahti 1982). Apothecia in the genus *Cladonia* may be red, light to dark brown and yellowish. In some species, apothecia are flat but otherwise they are generally convex and cap-like. Spores are one-celled, colorless and eight spores per ascus. Some species of *Cladonia* lack apothecia even though the podetium is well developed (Ahti 2000).

The genus *Cladonia* is comprised of seven taxonomic Sections (Ahti 2000) and also includes the former genus *Cladina* (Ahti and DePriest 2001). Section *Cladonia* consists of many of the pixie cup lichens and a number of species complexes including the *Cladonia chlorophaea* species complex. Polyphyly is reported in some of these complexes (Kotelko and Piercey-Normore 2010, Stenroos et al. 2002). Additional loci and a better understanding of the secondary metabolite production may help to resolve species concepts in lichen fungi. The difference in the chemical composition between lichen species is used as an important taxonomic character at various levels in lichen systematics (Culberson 1966). Morphogenetic plasticity of the phenotypes in many lichens may also be correlated with intraspecific chemical variation (Stocker-Wörgötter et al. 2004).

1.3.8. Fungal Secondary metabolites

The study of fungal secondary metabolism is extensive (Nielsen et al. 2009, Stocker-Wörgötter 2008, reviewed by Bennett and Ciegler 1983, Turner 1971). Polyketides constitute structurally diverse molecules produced by the successive condensation of small carboxylic acids, typically co-enzyme A activated malonate by a mechanism similar to fatty acid biosynthesis (Hopwood and Sherman 1990). Biosynthesis of polyketides takes place by sequential reactions catalyzed by PKS enzymes, and it occurs stepwise from carbon building blocks such as acetyl-CoA, propionyl-CoA, or butyryl CoA. The variety of polyketide structures produced from this pathway reflects the diversity of their biological activities. In non-lichenized fungi, PKS enzymes have been found to catalyze the synthesis of the precursors of fungal pigments like melanins, carcinogens such as aflatoxins, and some metabolic regulators like lovastatin (Vederas and Sorensen 2003).

Metabolic products in lichen-forming fungi are divided into two main groups: intracellular and extracellular products. Metabolic products that are deposited within the cell (intracellular) are referred to as primary metabolites, which are required for the survival, growth and development of an organism. Lichen primary metabolites include proteins, amino acids, carbohydrates, polyols, and vitamins. The intracellular products may be synthesized by both symbionts and can also occur in free-living fungi and algae (Hale 1983). Metabolic products that are deposited on the surface (extracellular) of hyphae and are not required for their survival are referred to as secondary metabolites. Primary metabolites are generally water-soluble and can be extracted with boiling water

(Fahselt 1994b). The majority of extracellular compounds synthesized by lichen-forming

fungi are secondary metabolites that are insoluble in water and can be extracted with

organic solvents only. Since secondary metabolism is not required for survival, its

products are considered to be dispensible and secondary metabolism is anabolic, whereas

primary metabolism is essential for survival with anabolic and catabolic activities to

maintain life (Bennett and Ciegler 1983). Secondary metabolites are chemically diverse

but are produced from a few key intermediates of primary metabolism, and are generally

categorized by these intermediates from which they are produced (Turner 1971). Bennett

and Ciegler (1983) summarize six categories (derived from Turner 1971) of secondary

metabolites derived from different primary intermediates. These six categories include

secondary metabolites that are synthesized: 1) without acetate, 2) from fatty acids, 3) as

polyketides, 4) as terpenes and steroids, 5) from the Tri-carboxylic acid (TCA) cycle, and

6) from amino acids. However, Turgeon and Bushley (2010) reported four main classes

of fungal secondary metabolites (polyketides, nonribosomal peptides, terpenoids, and

alkaloids).

Polyketides are a group of naturally occurring secondary metabolites produced by

a wide range of organisms – bacteria (prokaryotes), fungi, algae, higher plants and

animals. Many of these organisms live saprophytically in the soil where they are exposed

to harsh environmental conditions with other competing organisms. In these harsh

conditions secondary metabolites may provide selective advantage to those organisms

that produce them. Most lichen-forming fungal secondary metabolites are small aromatic

polyketides synthesized by the fungal partner in the symbiosis (Elix and Stocker-

Wörgötter 2008, Schmitt et al. 2007, Culberson and Armaleo 1992). About 1,050 lichen

substances have been identified (Stocker-Wörgötter 2008). Although fungal secondary

metabolites are extensive, they are generally produced by one of just a few major

pathways (Moore 1998). The three pathways in lichen-forming fungi are the mevalonic

acid pathway, the shikimic acid pathway, and most of the secondary metabolites in lichen

fungi are derived by acetyl-polymalonyl pathway (Huneck 2001, Culberson and Elix

1989). Major classes of secondary metabolites in lichen fungi are secondary aliphatic

acids, esters and related derivatives; polyketide derived aromatic compounds (acetyl-

polymalonyl pathway); terpenes and steroids (mevalonic acid pathway); and

terphenylquinones and pulvinic acid derivatives (shikimic acid pathway) (Elix and

Stocker-Wörgötter 2008).

1.3.9. Polyketide synthases

Polyketides are aromatic compounds that are produced by polyketide synthases,

which are encoded by polyketide synthase (PKS) genes. Polyketides are diverse in

structure and biological activity but they share a common biogenic origin. The protein

structure of polyketide synthases may differ (Nicholson et al. 2001, Bingle et al. 1999,

Cox et al. 1997, Hopwood 1997, Bedford et al. 1995). Type I and Type II PKS enzymes

are found in bacteria and fungi. Type III PKS enzymes are present in higher plants. Type

I PKS enzymes can be iterative or non-iterative. The bacterial Type I PKS enzymes are

non-iterative, and have separate modules for each methylmalonyl CoA addition. Fungal

PKS enzymes are limited to one module, with which they carry out repeated biosynthetic

reactions and are therefore called 'iterative PKS enzymes' or Type I PKS (Keller et al.

2005). Iterative PKS enzymes are a single protein complex (a single module) that

contains all the necessary domains and use their active sites repeatedly to produce a single polyketide. On the other hand, non-iterative PKS enzymes are known as modular PKS enzymes that consist of more than one module and the active sites located on different modules are used only once to produce a particular polyketide. Type III PKS enzymes lack the ACP moiety and use coenzyme A esters.

The fungal Type 1 PKS enzymes are structurally and mechanistically similar to fatty acid synthases. PKS enzymes are multidomain proteins that catalyse multiple carboxylic acid condensations (Keller et al. 2005). The fungal PKS enzymes consist of a linear succession of domains of ketosynthetase (KS), acyl transferase (AT), dehydratase (DH), enoyl reductase (ER), ketoreductase (KR), acyl carrier protein (ACP) and thioesterase (TE) (Graziani et al. 2004). The simplest fungal PKS enzymes include KS, AT and ACP domains, which are the minimal set of domains required for carboxylic acid condensation (Hopwood 1997). Some fungal PKS enzymes include KR, DH and ER domains, which catalyse the reduction of carbonyl groups after each cycle of condensation (Proctor et al. 2007). Fungal polyketides usually undergo modifications (reductions, oxygenations, esterifications, etc.) after they are formed by the activities of the PKS enzymes. This modification is catalysed by enzymes other than the PKS enzymes (Proctor et al. 2007). The genes encoding the PKS and modifying enzymes are often located adjacent to each other in gene clusters. The genes in a cluster are co-regulated with transcription of all the genes being repressed or activated simultaneously (Keller and Shwab 2008).

Figure 1.1. Illustration of the domain structure for a hypothetical polyketide synthase gene in Fungi. (Modified from Stocker-Wörgötter 2008). KS=Ketosynthase, AT=acyl transferase, ACP=acyl carrier protein, DH=dehydratase, ER=enoyl reductase, KR=ketoreductase, MT=methyl transferase.

Fungal polyketide synthases and fatty acid synthases (FAS) have similar ancestral KS, AT, KR, DH, ER and ACP domains (ACP domain is also known as the PP domain, which is the phosphopantetheine attachment site). The KS, AT and ACP/PP domains are three essential domains required for the minimal functioning of both PKSs and FASs. The three reducing domains KR, DH, and ER are found in all FASs but PKSs may either contain all (reducing PKS), some (partially-reducing PKS), or none (non-reducing PKS) of the reducing domains. The reducing domains that catalyze the step-wise reduction of a keto group to a hydroxyl group is keto-reductase (KR), dehydration of hydroxyl to an enoyl group is dehydratase (DH), and reduction of enoyl group to to alkyl group is catalysed by enoyl reductase (ER). Each successive chain elongation step is followed by a fixed sequence of ketoreduction, dehydration and enoylreduction in fatty acid biosynthesis (Turgeon and Bushley 2010, Elix and Stocker-Wörgötter 2008). As a result of the many possible modifications, polyketides are remarkably diverse in structure and complexity.

Based on the presence and absence of reducing domains in the enzyme, PKS genes may represent any one of three types:

i) Non-reducing PKS genes lack all the reducing domains and catalyze the synthesis of fully oxidized polyketide products such as depsides, depsidones, orsellinic acids, β-orcinol depsidones and dibenzofurans. Most of the common and well-known lichen polyketides are encoded by non-reducing PKS genes.

ii) Partially reducing PKS genes contain one or two reducing domains and catalyse the synthesis of partially reducing polyketide products commonly referred to as MSAS-type

(6-methyl salicylic acid type; Bingle et al.1999). Bourgeanic acid and anthrones are partially reduced polyketides (Stocker-Wörgötter 2008) but these are not very common in lichen fungi.

iii) Highly reducing PKS genes contain all the three reducing domains and catalyze the successive reduction of the keto group to the hydroxyl group, the hydroxyl to an enoyl, and the enoyl to an alkanoyl group resulting in the synthesis of highly reduced polyketide products such as lovastatin, which is synthesized by *Aspergillus terreus* and *A. nidulans* (Vederas and Sorensen 2003).

Figure 1.2. Illustration of the general domain architecture for three hypothetical polyketide synthase genes in Fungi. (A) Non-reducing, (B) Partially-reducing and (C) Highly reducing PKS gene complex. (Modified from Cox 2007). SAT= Starter unit, KS=ketosynthase, AT=acyltransferase, PT=product template, ACP=acyl carrier protein, CLC=Claisen cyclase, DH=dehydratase, KR=ketoreductase, ER=enoyl reductase, MT=Methyl transferase.
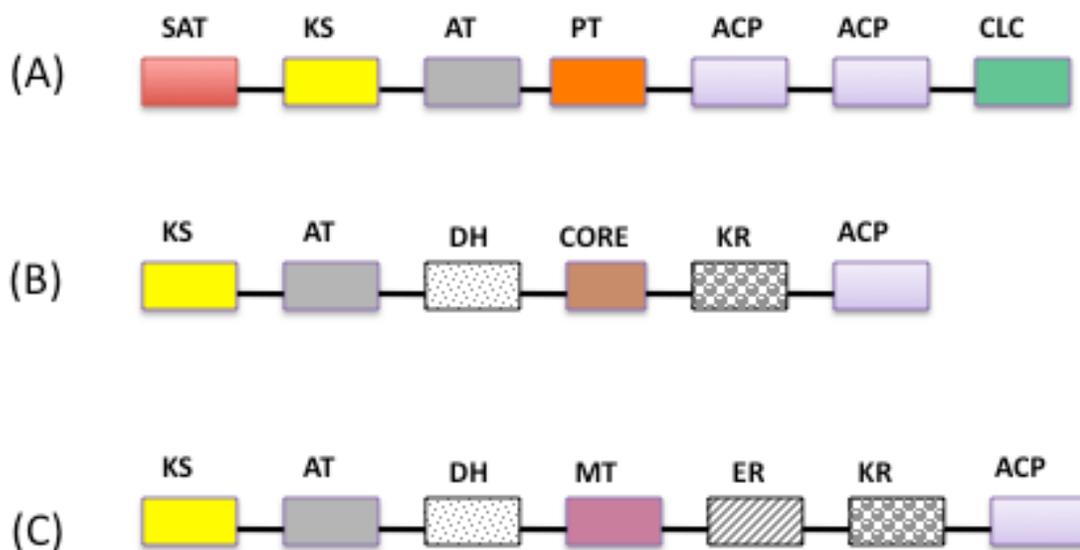
A single fungal genome contains more than one PKS gene and each species of fungus can produce more than one polyketide or polyketide family (Proctor et al. 2007). Each gene copy may encode for a particular functional polyketide product making the gene a paralog. Multiple paralogs of PKS genes have been detected in members of *Aspergillus* (Sanchez et al. 2008, Nierman et al. 2005), *Fusarium graminearum* (Hoffmeister and Keller 2007), *Gibberella moniliformis* (Schmidt et al. 2008) and the lichen family Parmeliaceae (Opanowicz et al. 2006) and the Cladoniaceae (Armaleo et al. 2011). Studies on PKS genes from different lichen groups suggest a high level of gene paralogy (Grube and Boustie 2005). Six paralogs of the KS domain of PKS genes have been detected so far in the Parmeliaceae and a higher number of paralogous PKS genes are expected to be present in the genomes of the Parmeliaceae because they are rich in diverse phenolic compounds (Opanowicz et al. 2006).

Paralogs may have arisen by gene duplication, mobile elements, gene fusion, or other mechanisms (reviewed by Long et al. 2003). Alternative explanations for multiple, apparently non-functional, genes include horizontal gene transfer from bacteria to fungi (Schmitt and Lumbsch 2009), horizontal gene transfer between different fungi (Khaldi et al. 2008), or as adaptation triggering gains and losses through evolution (Blanco et al. 2006). In many cases, the numbers of paralogs reported are high compared with the number of polyketides that are produced by any one fungal species. However, these numbers are expected to be even higher than currently reported because of recent knowledge of the numbers of paralogs present in a genome based on findings from genome sequencing projects in *Aspergillus* (Gilsenan et al. 2009), *Cladonia grayi*

(Armaleo et al. 2011), and more than 200 projects in progress or completed for other

ascomycetes (http://www.ncbi.nlm.nih.gov/genomes/leuks.cgi). It has been reported that

the number of genes responsible for producing secondary metabolites far exceeds the

number of known metabolites in an organism (Sanchez et al. 2008). For example in

Aspergillus nidulans as many as 27 polyketide synthase genes have been identified

whereas only seven secondary metabolites are known for this species and 13 paralogs are

reported for *C. grayi* when only two major polyketides are known to be produced by this

species (Armaleo et al. 2011). Genome sequencing has also revealed unique gene clusters

among various organisms; probably because an organism may have evolved to produce

different secondary metabolites to best suit its biological and ecological requirements

(Sanchez et al. 2008). Variation in the number of paralogs among individuals within a

single species (Opanowicz et al. 2006) may be explained because of the limitation of

primers available that may be specific to some paralogs and not others, where a larger

number of paralogs might be expected to be present in all genomes within a single

species. The numbers of gene paralogs and the effect of environmental conditions on

production of secondary metabolites is reviewed in Deduke et al. (2012).

1.3.10. Functional significance of polyketides

The lichen and its natural products have been used for centuries in traditional

medicines and are still of considerable interest as alternative treatments (Miao et al.

2001). Many of these natural products have bioactive properties (Huneck 1999, Huneck

and Yoshimura 1996) that are not required for primary metabolic functions and are

referred to as secondary metabolites (Keller et al. 2002), many of which are polyketides.

The finding of polyketides in forest soils, where they are exposed to harsh environmental conditions with other competing organisms, has led to the suggestion that those polyketides with antagonistic properties may structure the microbial communities in the soil (Kellner and Zak 2009). The polyketide-producing organisms that do not live in the soil, such as lichen-forming fungi, may benefit from these compounds as they allow lichens to survive in their ecological niche by reacting to environmental factors such as light or drought, or protecting the thallus from predators and parasites (Huneck 1999). Secondary metabolites have been hypothesized to play a role in herbivory defence, antibiotics, or as metal chelators for nutrient acquisition (Gauslaa 2005, Huneck 1999, Lawrey 1986). Recently it was hypothesized that polyketides play a role in protection against oxidative stress in fungi (Reverberi et al. 2010, Luo et al. 2009) and that some metabolites such as fumarprotocetraric acid, perlatolic, and thamnolic acids contribute to the ability of lichens to tolerate acidic solutions such as acid rain (Hauck et al. 2009, Hauck 2008). Regardless of the reason for secondary metabolite production (as a by-product, detoxification of primary metabolism, or leftover products after growth slows) they often elicit a function that is advantageous to survival of the lichen within its ecological niche. The advantage(s) may in part be understood by the location of the compounds within the thallus such as atranorin and usnic acid being produced more frequently by the cortical hyphae than the medullary hyphae and having a function related to photoprotection. These chemical characters are thought to be adaptive features because of their perceived ecological role.

Secondary metabolite producing organisms may benefit from these compounds as they allow them to survive in their ecological niche (Fox and Howlett 2008) and protect

them from predators and parasites. Some lichen-fungal secondary compounds have been thought to filter particular wavelengths of light for the photobiont or assist the fungal hyphae to penetrate the hard rocky substratum (Lawrey 1986, Rundel 1978). Polyketides are of great commercial interest as many of these compounds have pharmaceutical properties and they may be a novel source of antibiotics, anti-cancer agents, anti-tumor and cholesterol lowering drugs (Olsson et al. 2009).

1.3.11. Regulation of polyketide production

The development and reproduction of non-lichenized fungi and secondary metabolite production appear to be coordinated (Schwab and Keller 2008, reviewed in Bennett and Ciegler 1983,). Since development and sexual reproduction are thought to be similar between lichenized and non-lichenized fungi (Nash 2008) and the acetyl-polymalonyl pathways are thought to be present in both groups of fungi (Turgeon and Bushley 2010, Stocker-Wörgötter 2007), it may be assumed that reproduction and development is also coordinated with polyketide production in lichenized fungi. Morphogenesis of fruticose and foliose lichens is complex compared with that of the crustose lichens and the vegetative phase of many non-lichenized fungi (Honegger 2008). In most lichens, the thallus is comprised of differentiated "tissues" arranged in layers that often produce different metabolites. Thallus development in lichens has been examined using microscopy (Honegger 1993, 1990, Joneson and Lutzoni 2009) and recently a study has described a number of genes that correlate with symbiont recognition and early thallus development (Joneson et al. 2011). Observations of cultures of the lichen-forming fungus have suggested that thallus development may be linked with production of

secondary metabolites. For example, a major compound umbilicaric acid produced by *Umbilicaria mammulata* was produced by cultures of *U. mammulata* only after lobe-like structures were formed in dehydrating medium (Stocker-Wörgötter 2001). Similarly, cultures of *Cladonia crinita* produced its major substance, fumarprotocetraric acid and its satellite substances only after podetial structures were formed (Stocker-Wörgötter 2001). Species of *Ramalina* produced secondary metabolites only after layers of mycelia became differentiated (Stocker-Wörgötter 2001). As more research is conducted on development in lichens it is expected that links between development and production of secondary metabolites will become evident.

The production and regulation of secondary metabolites in non-lichenized fungi, mainly ascomycetes, has been reviewed with an emerging understanding of the biosynthesis and the pathways involved in regulation (Keller et al. 2005, Yu and Keller 2005). The regulation of fungal secondary metabolism to some extent is thought to depend upon the chromosomal organization of biosynthetic genes. A global transcription factor, which is encoded by genes that are unlinked to the polyketide biosynthetic gene clusters, is thought to control the production of secondary metabolites (Fox and Howlett 2008). Genes encoding global transcription factors regulate multiple physiological processes and are thought to respond to factors like pH, temperature, light, and nutrients in the surrounding environment. Fungal secondary metabolite production is also responsive to other environmental factors such as carbon and nitrogen sources (Shwab and Keller 2008). Microorganisms capable of growing over a wide range of pH levels have their gene expression under the control of the pH of their growth medium (Miguel and Herbert 2003). It is thought that the signals generated in response to environmental

conditions are relayed through Cys2His2Zinc-finger proteins including CreA for carbon, AreA for nitrogen and PacC for pH signaling. These proteins may have positive or negative effects on metabolite production. With regard to two *Cladonia* species, *C. pocillum* and *C. pyxidata*, it is believed that pH is the driving environmental factor that creates the diagnostic difference between the two morphotypes (Kotelko and Piercey-Normore 2010, Gilbert 1977). Members of the *Cladonia chlorophaea* species complex have been found to share virtually identical morphologies but different secondary metabolites (Culberson et al. 1988, Culberson 1986) that serve as key diagnostic features of their identification. *Cladonia grayi* and *C. merochlorophaea* grow at a lower pH than *C. chlorophaea sensu stricto* or other members of the species complex. Therefore, an effect of varying pH levels on phenotype has already been suggested to play a role in the phenotype in lichen-forming fungi.

The availability and type of carbon and nitrogen source affect production of polyketides in fungi (Shwab and Keller 2008, Keller et al. 2002). Sugars like glucose, sucrose or sorbitol as the sole carbon source supported high aflatoxin production along with higher fungal growth and sporulation. On the other hand, peptones and more complex sugars such as galactose, xylose, lactose and mannose do not support aflatoxin production. Studies on *Aspergillus* species have shown different effects of nitrogen sources used in the growth medium on aflatoxin and sterigmatocystin production (Keller et al. 2002). Keller et al. (1997) reported an increased amount of sterigmatocystin and aflatoxin production in an ammonia-based medium and a decreased amount in a nitrate-based medium. Little is known about the carbon and nitrogen requirements for many secondary metabolites and less is known about nutrient requirements in lichenized fungi.

Brunauer et al. (2007) reported high numbers of secondary compounds in the mycobiont of *Xanthoria elagans* subcultured in G-LB (modified Lilly & Barnett) medium as compared to the collected specimen.

A study on mycotoxin production and regulation of the genes responsible for mycotoxin production in species of *Aspergillus* showed that the gene, *veA,* regulates production of four biosynthetic genes in *A. flavus* (Duran et al. 2007). *veA* (velvet A) is a gene that is shown to be involved in both development and the synthesis of secondary metabolites in different species of *Aspergillus* (Fox and Howlett 2008). Deletion of *ve* A terminated *aflR* expression and aflatoxin production (Duran et al. 2007, Fox and Howlett 2008), and it has also been shown to regulate penicillin production in *A. nidulans* (Kato et al. 2003), suggesting that the regulatory mechanism may be conserved among species of *Aspergillus* (Duran et al. 2007). Another gene, *laeA,* has also been shown to regulate expression of biosynthetic gene clusters in species of *Aspergillus* (Fox and Howlett 2008, Keller et al. 2005, Bok and Keller 2004). *laeA*, a nuclear protein, is reported to be a master regulator of secondary metabolism in *Aspergillus* species. Its disruption resulted in lower levels of secondary metabolites in species of *Aspergillus* (Fox and Howlett 2008). *Ve*A is found to interact with laeA and VelB in *A. nidulans*. VeA and VelB interact in the cytoplasm and move to the nucleus where VeA acts as a bridge between laeA and VelB and triggers induction of genes involved in sterigmatocystin production (Fox and Howlett 2008). On the otherhand, it has also been shown that laeA negatively affects the regulation of veA (Kale et al. 2008).

The ability of the lichen to adapt to different light conditions depends on the stability of thylakoid membranes and protection offered by the cell from reactive oxygen

species (Berkelmans and van Oppen 2006). Therefore, the choice of algal partner may depend largely on the habitat conditions in which the developing lichen thallus is found (Piercey-Normore 2004). If the choice of alga depends on habitat conditions, and the particular starting unit for the polymalonyl-acetate pathway, then the polyketide production would also depend on the alga. For lichen thalli that are thought to contain multiple algae simultaneously (Hoyo et al. 2011, Piercey-Normore 2006), the predominant alga would provide the majority of starting carbohydrates, but a combination of carbohydrates may be available for different biosynthetic pathways.

It is expected that the regulation of polyketide synthesis in lichen fungi will depend on both the taxonomy and the environment to which it has adapted. Multiple gene paralogs should facilitate adaptation and different polyketides would be expected to be produced under different growing conditions. The genera *Cladonia* and *Ramalina* have species that can produce very few or many diverse polyketides. In this thesis I hope to use the accumulated knowledge of chemical diversity in these two groups to shed light on the environmental regulation of polyketides and to narrow the number of paralogs to more specific functional groups.

CHAPTER 2

Monophyly of some North American species of *Ramalina* and inferred polyketide synthase gene function

2.1. Abstract

Ramalina thrausta, *R. roesleri*, and *R. dilacerata* are three common North American species that have not been placed in a phylogeny and are sympatric in their distribution leading to uncertainty about monophyly. Species characters include secondary metabolites (polyketides) in *Ramalina*, which may be both hereditary and influenced by environmental conditions, but little is known about the function of polyketide synthase (PKS) genes. The main goal of this study was to examine the monophyly among some of the more common species in northern North America and secondarily to compare potential PKS gene function with the phylogeny. Nucleotide sequences of two genes, the Internal Transcribed Spacer 1 of ribosomal DNA (ITS1 rDNA) and the mitochondrial small subunit (mtSSU), were used to infer a phylogeny. Gene function was inferred from three PKS genes by dN/dS ratios. While seven species of *Ramalina* are highly supported

in monophyletic clades, two other species form clusters with low support, *R. americana* is paraphyletic, and *R. pollinaria* is polyphyletic. Three PKS genes were inferred to be functional but were not present in all samples. Functional PKS genes could enable adaptation to new habitats and facilitate species diversification.

2.2. Introduction

The genus *Ramalina* Ach. is a group of epiphytic and saxicolous pendant or shrubby fruticose lichen that has been well-studied for ecology and secondary metabolite composition (e.g. Stocker-Wörgötter et al. 2004, Elix and Wardlaw 1986, Bowler and Rundel 1978, Culberson et al. 1977), and more recently algal selection (Francisco et al. 2012, Casano et al. 2010, Werth and Sork 2010, Tschaikner et al. 2007). Isolated studies have examined phylogenies of selected species of *Ramalina* (Ohmura et al. 2008, Joneson et al. 2004, Stocker-Wörgötter et al. 2004, Joneson 2003, LaGreca and Lumbsch 2001, LaGreca 1999). *Ramalina farinacea* and *R. intermedia* have overlapping diagnostic characters and are also sympatric in northern North America, raising questions of species monophyly. European specimens of *R. farinacea* (L.) Ach. were shown to be monophyletic despite their chemical variability (Stocker-Wörgötter et al. 2004). The North American *R. farinacea* complex has at least four chemical races and no correlation with substrate preference (Bowler and Rundel 1978) suggesting that the chemotypes of *R. farinacea* do not warrant a "species-level" taxonomic change. The variation of morphological features in *R. intermedia* (Delise ex Nyl.) Nyl. may overlap with those of *R. farinacea* throughout their distribution in North America (Bowler and Rundel 1974). In fact, Howe (1914) described members of *R. intermedia* as "abraded, erose, generally

saxicolous states of [*R.*] *farinacea*" suggesting that *R. intermedia* may be a growth stage or ecotype of *R. farinacea*. *Ramalina farinacea* does not produce sekikaic acid, the diagnostic metabolite produced by *R. intermedia*. *Ramalina thrausta* (Ach.) Nyl. is a long slender pendant lichen similar in appearance to *Alectoria sarmentosa* (Ach.) Ach. and was previously classified in *Alectoria (*Motyka 1964), but *R. thrausta* has distinctive curled branch tips. In contrast, *R. dilacerata* (Hoffm.) Hoffm. has a short tufted inflated thallus with smooth hollow branches, no soredia, and usually bears numerous apothecia at the branch tips. In North America *R. dilacerata* can readily be distinguished from other species in the genus and is thought to be related to southern hemisphere species (Brodo et al. 2001). While *R. pollinaria* has a flat tufted thallus containing proliferations with irregular soralia that are often split at the lobe tips, R. *roesleri* has a small delicately tufted thallus with finely divided round branches terminating in granular soredia. The relationships among three (*R. dilacerata, R. roesleri*, and *R. thrausta*) North American species have not been previously examined.

Some species of *Ramalina* have been shown to be diverse in the types of secondary metabolites they produce (Stocker-Wörgötter et al. 2004, LaGreca 1999, Culberson et al. 1990) while others produce usnic acid alone (e.g. *R. sinensis* Jatta (Joneson et al. 2004, Joneson 2003), a northern chemotype of *R. americana* (LaGreca 1999). While environmental conditions are thought to influence production of some metabolites (Stocker-Wörgötter 2010, Stocker-Wörgötter 2001, Culberson et al. 1977,) suggesting phenotypic plasticity (Slepecky and Starmer 2009), it is also known that secondary metabolite production is hereditary. For example, several chemotypes of *Ramalina siliquosa* (Huds.) A.L.Sm. showed distinct ecological zonation along cliffs

based on different degrees of exposure to sea spray but chemical composition was maintained within zones suggesting a hereditary basis (Culberson and Culberson 1967, but see Culberson et al. 1993). However, the secondary metabolite composition of *R. farinacea* showed no correlation with substrata (Bowler and Rundel 1978). Stocker-Wörgötter et al. (2004) proposed the hypothesis that chemically-poor species containing usnic acid alone are considered to be basal to the more derived chemically-diverse species. This evolutionary pattern was limited to species collected from Europe and raises questions regarding the origin of the genes that produce secondary metabolites. Secondary metabolites produced by *Ramalina* species are polyketides, which are synthesized by the fungal partner in the lichen symbiosis (Culberson and Armaleo 1992, Elix and Stocker-Wörgötter 2008) but some fatty acids are thought to derive from the photobiont (Hanus et al. 2008). Polyketides are produced by an enzyme encoded by a large PKS multidomain gene. It is hypothesized that one gene is responsible for each polyketide (Armaleo et al. 2011) despite multiple paralogs with unknown functions reported for many fungi (Armaleo et al. 2011, Sanchez et al. 2008, Schmitt et al. 2008, Opanowicz et al. 2006). Alternatively, Brown et al. (2012) proposed that 3 or 4 orthologous PKS genes may be responsible for producing the same metabolite based on a phylogenomic analysis in *Fusarium* species. The influence of environmental and developmental changes (Fox and Howlett 2008) further complicates studies of PKS gene expression. With a growing number of PKS genes discovered in lichen-forming fungal genomes, questions of gene and secondary metabolite diversity can begin to be addressed.

The main goal of this study was to examine the phylogeny of some of the northern species of *Ramalina* in North America and secondarily to gain a better understanding of PKS gene function relative to secondary metabolite diversity. The objectives of the study were: 1) to examine the monophyly of nine North American species of *Ramalina*, and 2) to screen these nine species for presence of PKS gene paralogs and to infer function from PKS gene sequence alignments.

2.3. Materials and Methods

2.3.1. *Lichen material and Chromatography*

Fifty samples from nine North American species of *Ramalina* (*R. americana*, *R. dilacerata*, *R. farinacea*, *R. intermedia*, *R. menzisii*, *R. pollinaria*, *R. roesleri*, *R. sinensis* and *R. thrausta)* were collected for this study and 29 additional sequences from fourteen species (seven North American and seven European) were retrieved from NCBI GenBank to contribute to the analyses (Table 2.1). North American species were chosen from NCBI to broaden the representation in North America. The European species were chosen to place the North American species in a perspective of an already published phylogeny of European species (Stocker-Wörgötter et al. 2004). Specimens are deposited in the University of Manitoba herbarium or the University of British Columbia herbarium.

Thin Layer Chromatography (TLC) was performed on all samples collected in this study to confirm the major secondary metabolites present in each thallus. A TLC protocol was modified from Culberson (1972) and Orange et al. (2001) using solvent A (toluene 180mL: dioxane 45mL: glacial acetic acid 5mL), and spot characteristics

outlined in Orange et al. (2001) were consulted for compound identification. For some

samples a longer incubation time (2 hours) in hot acetone was used and an increased

amount of extract was spotted onto the plate to detect trace amounts of some compounds.

HPLC was performed for some samples of *R. farinacea* because it is more variable than

other species and the compounds were more easily identified in HPLC. Lichen thalli (3-4

small branches of the *Ramalina* samples) were placed in tubes and extracted in methanol

for 4 hours; the extracts were then transferred to HPLC vials and an aliquot of 20 μL

from every sample was injected. The secondary compounds were analysed by HPLC

using a Merck-Hitachi system with two pumps, a DAD (photodiode array detector; 190-

900 nm wavelength, covering UV and visible spectra) connected to a computer system.

Retention time indexes (R_I) were calculated from Benzoic acid and solorinic acid controls

(Elix and Stocker-Wörgötter 2008, Feige et al. 1993). Standard solutions of benzoic acid

and E-1-(9-anthryl)-2-phenylethene (gift from J. Elix) were used, producing peaks at the

beginning and end of the run. Two solvent systems were used: (1) 1% aqueous

orthophosphoric acid and methanol in the ratio 7:3 and (2) methanol. The run started with

100% of solvent 1 and was raised to 58% solvent 2 within 15 min, then to 100% solvent

2 after a further 15 min, followed by isocratic elution in 100% solvent 2 for a further 10

min. The spectra were identified by means of a spectrum library (comparison with

reference substances), and chemical data listed in Huneck and Yoshimura (1996).

Table 2.1. List of species, collector and collection numbers, location and date of collection, and GenBank accession numbers for the four genes of the specimens used in this study. SPF (Sandilands Provincial Forest), WPP (Whiteshell Provincial Park), WNP (Wapusk National Park), SWPP (Spruce Woods Provincial Park), and HIPP (Hecla Island Provincial Park). Md indicates that the specimen was used in the phylogenetic analysis with "missing data" coded for one gene. "na" indicates that no sequence was obtained and it is "not applicable" for this analysis.

Species	Collector and collection number	Location and date of collection	Accession for ITS1 rDNA	Accession for mtSSU	Accession for wA 1	Accession for wA 2
Cliostomum griffithi	unknown	unknown	AF282076	GU138667	na	na
Ramalina americana	Normore 4352	Canada, Manitoba, SPF, N49° 17' 21", W95° 45' 22"; 2005. **TLC:** usnic acid	JQ003081	JQ003105	na	JQ003167
R. americana	Normore 4358	Canada, Manitoba, SPF, N49° 17' 21", W95° 45' 22"; 2005. **TLC:** usnic acid	JQ003100	Md	na	na
R. americana	Normore 5531	Canada, Manitoba, HIPP; N51° 10' 52.2", W96° 40' 9.3"; 2006. **TLC:** usnic acid	JQ003083	JQ003104	na	JQ003168
R. americana	Normore 6895	Canada, New Brunswick; N46° 46' 41.6", W66° 18' 1.1"; 2006. **TLC:** usnic acid	JQ003082	JQ003103	na	na
R. americana	LaGreca 495	USA, North Carolina; (LaGreca, 1999)	AF109236	Md	na	na
R. americana	LaGreca 507	USA, New York; (LaGreca, 1999)	Af109237	Md	na	na
R. americana	LaGreca 506	USA, New York; (LaGreca, 1999)	AF109238	Md	na	na
R. americana	LaGreca 497	USA, North Carolina; (LaGreca, 1999)	AF109235	Md	na	na
R. culbersoniorum	LaGreca 500	USA, North Carolina; (LaGreca, 1999)	AF109231	Md	na	na
R. culbersonoirum	LaGreca 493	USA, North Carolina; (LaGreca, 1999)	AF109232	Md	na	na
R. culbersoniorum	LaGreca 510	USA, North Carolina; (LaGreca, 1999)	AF109233	Md	na	na
R. dilacerata	Normore 8786	Canada, Manitoba, SPF; 2009. **TLC:** usnic acid and	JQ003068	JQ003110	JQ003150	na

Species	Voucher	Locality/TLC				
R. dilacerata	Normore 724	Canada, Nova Scotia; N45° 26' 45.1", W63° 55' 14.5"; 2004. **TLC:** usnic acid and divaricatic acid	JQ003066	JQ003106	JQ003148	JQ003169
R. dilacerata	Normore 2867	Canada, Manitoba; N50° 43' 41.1", W96° 8' 12.7"; 2003 **TLC:** usnic acid and divaricatic acid	JQ003067	JQ003107	JQ003149	JQ003170
R. dilacerata	Normore 9647	Canada, Manitoba, WPP; 2010. **TLC:** usnic acid and divaricatic acid	JQ003069	JQ003109	JQ003151	na
R. dilacerata	Normore 9643	Canada, Manitoba, SPF; N49° 25' 27.8", W96° 15' 41.7"; 2009. **TLC:** usnic acid and divaricatic acid	JQ003070	JQ003108	na	na
R. farinacea	Stocker-Worgotter S.n.	Italy (Stocker-Worgotter et al. 2004)	AY462052	Md	na	na
R. farinacea	Pruegger SN024.16/1	Slovenia (Stocker-Worgotter et al. 2004)	AY462053	Md	na	na
R. farinacea	Pruegger SN038.10/1	Slovenia (Stocker-Worgotter et al. 2004)	AY462046	Md	na	na
R. farinacea	Pruegger SN003.3/1	Slovenia (Stocker-Worgotter et al. 2004)	AY462048	Md	na	na
R. farinacea	EDNA09-01609	Kelly et al. (2011)	FR799283	Md	na	na
R. farinacea	Normore 9839	Canada, Manitoba, WNP; N57° 35' 0.2", W92° 42' 23.7"; 2010. **TLC**: usnic acid, noristictic acid and variolaric acid.	JQ003075	JQ003112	na	na
R. farinacea	Normore 6340	Canada, Manitoba, WNP; N58° 6' 56.3", W92° 53' 17.5"; 2006. **TLC**: usnic acid, salazinic acid and protocetraric (tr.) acid	JQ003074	JQ003114	na	na
R. farinacea	Normore 6501	Canada, Manitoba, WNP; N58° 3' 31.1", W92° 51' 30.9"; 2006. **TLC**: usnic acid, noristictic acid and variolaric acid.	JQ003073	JQ003115	na	na
R. farinacea	Normore 6503	Canada, Manitoba, WNP; N58° 7' 48.7", W92° 57' 54.9"; 2006. **TLC**: usnic acid and salazinic acid	na	na	JQ003152	na
R. farinacea	Normore 6463	Canada, Manitoba, WNP; N58^0 7' 48.7", W92^0 57' 54.9"; 2006. **TLC**: usnic acid and salazinic acid	JQ0030772	Md	na	na

Species	Collection	Locality / TLC				
R. farinacea	Normore 6512	Canada, Manitoba; WNP; N58^0 3' 26.2', W92^0 51' 29.7''; 2006. **TLC:** usnic acid, salazinic acid	JQ003071	Md	na	na
R. fastigiata	Groner 1479	Switzerland (Groner and LaGreca 1997)	U84583	Md	na	na
R. fraxinea	LaGreca 577	Denmark (LaGreca and Lumbsch 2001)	AF249907	Md	na	na
R. fraxinea	Pruegger SN066.34/1	Slovenia (Stocker-Worgotter et al. 2004)	AY462054	Md	na	na
R. intermedia	Goward 9-635	Canada, British Columbia; N48° 43.23', W123° 22.2'; 2009. **TLC:** usnic acid, sekikaic acid, 4'-O-demethylsekikaic, and homosekikaic acids (tr.)	JQ003079	JQ003121	na	na
R. intermedia	Garton 22116	Canada, Ontario; N49° 20' W88° 07'; 1983. **TLC:** usnic, sekikaic acid, 4'-O-demethylsekikaic, and homosekikaic acids (tr.)	Md	JQ003123	JQ003153	JQ003171
R. intermedia	Normore 5512	Canada, Manitoba, HIPP; N51° 12' 0.4'', W96° 37' 2.3''; 2006. **TLC:** usnic acid, sekikaic acid, 4'-O-demethylsekikaic, and homosekikaic acids	JQ003076	JQ003119	na	na
R. intermedia	Normore 3618	Canada, Manitoba; N58° 58', W101° 11'; 2004. **TLC:** usnic, sekikaic, 4'-O-demethylsekikaic, and homosekikaic acids (tr.)	Md	JQ003117	na	na
R. intermedia	Normore 4614	Canada, Manitoba; N53° 21', W101° 03'; 2005. **TLC:** usnic, sekikaic, 4'-O-demethylsekikaic, and homosekikaic acids (tr.)	JQ003077	JQ003120	JQ003154	na
R. intermedia	Normore 7269	Canada, Ontario; N49° 00' 33.5''; W88° 9' 36.4''; 2006. **TLC:** usnic, sekikaic, 4'-O-demethylsekikaic, and homosekikaic acids (tr.)	JQ003078	JQ003116	na	na
R. intermedia	Normore 4626	Canada, Manitoba, On Hwy 60, 75 Km East of Hwy 10; 2005. **TLC:** usnic, sekikaic, 4'-O-demethylsekikaic, and homosekikaic acids	Md	JQ003118	na	na
R. intermedia	Goward 7-82	Canada, British Columbia; N53° 53.5', W122° 49'; 2007. **TLC:** usnic, sekikaic acids (tr) and unknown (yellow-green after H+; Rf=4 in Sol. A)	JQ003080	JQ003122	na	na
R. intermedia	LaGreca 508	USA, New York; (LaGreca, 1999)	AF109239	Md	na	na

Species	Collection	Locality				
R. leptocarpha	Kroken s.n.	USA, California, Marin Co.: Point Reyes National Seashore; 1994	AF249908	Md	na	na
R. menziesii	Goward 9-344	Canada, British Columbia; N51° 48', W120° 1.5'; 2009. **TLC**: usnic acid	JQ003093	JQ003125	na	na
R. menziesii	Riddell s.n. 1	USA, California; N34° 42.560, W120° 03.426; 2008. **TLC**: usnic acid	JQ003095	JQ003124	JQ003156	JQ003174
R. menziesii	Bjork18095	Canada, British Columbia, 48° 23.5', 123° 39.5'; 2009. **TLC**: usnic acid	JQ003094	JQ003126	na	na
R. menziesii	Withey S.n.	USA, California; (LaGreca and Lumbsch 2001)	AF249909	Md	na	na
R. menziesii	Isolate 2	LaGreca (submitted 2000; unpublished).	AF249910	Md	na	na
R. montagnei	LaGreca 515	USA, Florida; (LaGreca and Lumbsch 2001)	AF249911	Md	na	na
R. paludosa	LaGreca 512	USA, North Carolina; (LaGreca 1999)	AF109240	Md	na	na
R. panizzei	Groner 1480	Switzerland (Groner and LaGreca 1997)	U84585	Md	na	na
R. pollinaria	LG 41227 R501	Belgium (Serusiaux et al. 2010)	GU827324	Md	na	na
R. pollinaria	Tuerk 40707	Brunauer (submitted 2000; unpublished)	EF432560	Md	na	na
R. pollinaria	Hur H06019	Korea (Han et al. unpublished; submitted 2007)	EU034670	Md	na	na
R. pollinaria	Normore 3748	Canada, Manitoba, HIPP; N51° 6' 31"; W96° 39' 34.7"; 2004. **TLC**: usnic acid and evernic acid	JQ003096	JQ003127	JQ003155	JQ003173
R. pollinaria	Riewe 582	Canada, North West Territories, N67° 20'; W126° 25'; 1976. **TLC**: usnic acid and evernic acid	JQ003099	JQ003128	na	JQ003172
R. pollinaria	Bjork 18610	Canada, British Columbia; 55° 55.544'N 118°, 2009. **TLC**: usnic acid and evernic acid	JQ003098	JQ003130	na	na
R. pollinaria	Goward 7-81	Canada, British Columbia; 53° 53.500'N 122°, 2007. **TLC**: usnic acid and evernic acid	JQ003097	JQ003129	na	na
R. roesleri	Normore 9838	Canada, Manitoba, WNP; N57° 35' 0.2", W92° 42' 27.3"; 2010. **TLC**: usnic acid and sekikaic acid	JQ003089	JQ003132	JQ003160	na
R. roesleri	Normore 7065	Canada, Newfoundland; N50° 06' 49.5", W57° 39' 51.3"; 2006. **TLC**: usnic acid and sekikaic acid	JQ003091	JQ003133	na	na

Species	Collection	Locality				
R. roesleri	Normore 5191	Canada, New Brunswick; 2005. **TLC**: usnic acid and sekikaic acid	Md	JQ003134	JQ003157	na
R. roesleri	Normore 7252	Canada, Ontario; N48° 55' 9.6"; W87° 46' 8.7", 2006. **TLC**: usnic acid and sekikaic acid	JQ003092	JQ003137	na	na
R. roesleri	Normore 6500	Canada, Manitoba, WNP; N58° 3' 31.1"; W92° 51' 30.9"; 2006. **TLC**: usnic acid and sekikaic acid	JQ003090	JQ003131	JQ003158	JQ003175
R. roesleri	Normore 7080	Canada, Newfoundland; N50° 23' 32.8", W57° 31' 1.1"; 2006. **TLC**: usnic acid and sekikaic acid	JQ003102	JQ003135	na	na
R. roesleri	Normore 7090	Canada, Newfoundland; N50° 34' 32.9", W57° 15' 11.4"; 2006. **TLC**: usnic acid and sekikaic acid	Md	JQ003136	JQ003159	na
R. siliquosa	Culberson 13087	U. K., Wales; (Groner and LaGreca 1997)	U84586	Md	na	na
R. sinensis	Normore 5491	Canada, Manitoba, WPP; N50° 08' 43.8", W95° 48' 24.3"; 2006. **TLC**: usnic acid	JQ003088	JQ003140	JQ003164	JQ003178
R. sinensis	Normore 7319	Canada, Manitoba, SPF; N49° 32' 45.9", W95° 34' 30.5"; 25 March 2007. **TLC**: usnic acid	JQ003086	JQ003139	JQ003163	na
R. sinensis	Normore 7334	Canada, Manitoba, SPF; N49° 16' 20.7", W95° 12' 41.6"; 2007. **TLC**: usnic acid	JQ003085	JQ003142	JQ003165	na
R. sinensis	Normore 5250	Canada, Manitoba, SWPP; 2006. **TLC**: usnic acid	JQ003101	JQ003141	JQ003162	JQ003177
R. sinensis	Normore 4514	Canada, Manitoba, SPF; N49° 26' 50.8", W95° 25' 21.8"; 2005. **TLC**: usnic acid	JQ003084	JQ003138	JQ003161	JQ003176
R. sinensis	Normore 9477	Canada, Manitoba, SWPP; N49° 39' 39.1", W99° 16' 6.5"; 2009. **TLC**: usnic acid	JQ003087	Md	na	na
R. sinensis	Hur CH050098	Hur (submitted 2006, unpublished)	DQ383646	Md	na	na
R. sinensis	St. Clair S.n.	USA, Utah (LaGreca 1999)	AF109241	Md	na	na
R. sinensis	Benito Tan #95-1977	China, Qinghai Province; 2001 3930 m elevation; Farlow Herbarium Barcode number 00377003	AF249905	Md	na	na

		TLC: Usnic acid (tr.), probably variolaric acid (++) (LaGreca pers. Comm.)				
R. thrausta	McCarthy 44	Canada, Newfoundland; 2008. **TLC**: usnic acid and stenosporic acid	JQ003064	JQ003143	JQ003166	na
R. thrausta	Bjork 21704	Canada, British Columbia, N51° 27.800', W120° 8.100'; 2010. **TLC**: usnic acid and stenosporic acid	JQ003062	JQ003144	na	na
R. thrausta	Goward 7-270	Canada, British Columbia, N55° 18.508'; W122° 40.315'; 2007. **TLC**: usnic acid and stenosporic acid	JQ003063	JQ003146	na	na
R. thrausta	Goward 6-288	Canada, British Columbia; N55°22', W127° 41'; 2006. **TLC**: usnic acid and stenosporic acid	JQ003065	JQ003147	na	na

2.3.2. DNA extraction and amplification

Total DNA was extracted for two reasons, one for sequencing the genes for the phylogeny and the other was to screen samples for presence of PKS genes by PCR. DNA was extracted from 2 cm thallus branch following a CTAB DNA extraction protocol modified from Grube et al. (1995). The Internal Transcribed spacer 1 (ITS1) of the nuclear ribosomal DNA (rDNA) was amplified using the primer pair ITS1F and ITS2 (Table 2.2) and a touch down PCR cycle. The cycle consists of initial denaturing at 94°C for 5 min, then 30 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 1min, 58°C for 1 min, 56°C for 1 min, 54°C for 1 min and 52°C for 1 min and extension at 72°C for 1 min. The gene for the mitochondrial small subunit (mtSSU) was amplified with mssu1 and mssu2R primers (Zoller et al. 1999) with initial denaturing at 94°C for 5 min, then 30 cycles of denaturing at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1 min.

The amplification of a 300bp region of the wA1-type PKS gene was done using RsinF2 and RsinR (Table 2.2) designed from Schmitt et al. (2008). These primers were designed from fungal wA-type PKS genes and a BLAST search always resulted in significant matches with fungal PKS genes. Amplification was done with initial denaturing at 94°C for 5 min, then denaturing at 94°C for 45 sec, annealing at 56°C for 45 sec, extension at 72°C for 50 sec for 36 cycles and final extension for 10 minutes. Part of the ketosynthase domain of a wA2-type PKS gene complex using the primer pairs PKS1F and PKS2R (designed from Bingle et al. 1999) and newly designed nested primers, RamPKS-F and RamPKS-R (Table 2.2) was amplified using the same cycle used for the ITS region. Partial MSAS-type genes were amplified by RFarBERTc and RFarLC3c

primers designed from Schmitt et al. (2008) using the same cycle as in RsinF2 and

RsinR. All amplifications were performed in a thermal cycler (Biometra T-Gradient;

Tampa, FL, USA) in 20 μL reaction volumes with 1X buffer (200mM Tris-HCl, 500mM

KCl) with 1.25 units GO Taq (Go Taq $^®$ Hot Start polymerase, Promega), 3.125 mM

MgCl$_2$ 1.25 mM of each dNTP, 1.0 M of each primer, and between 40 to 60ng of DNA.

Table 2.2. List of primers showing primer names, target gene, primer sequences (5'to 3'), source

of primer, and the length (bp) of the amplified product.

Primer name	Gene	Primer sequence (5'-3')	Source of primers	Product size (bp)
RSinF2	PKS wA 1	CTGTCTTACACCACAACCGCC	this lab (from Schmidt et al. 2008)	300
RSinR	PKS wA 1	ACCATCAACGAGTGGGAGAAA	this lab (from Schmidt et al. 2008)	300
RFarBERTc	PKS MSAS	GTCTTGAGCTGTCCACTCAT	this lab (from Schmidt et al. 2008)	1050
RFarLC3c	PKS MSAS	CTGCAAGGCGAGTCTAAGAT	this lab (from Schmidt et al. 2008)	1050
RamPKS-F	PKS wA 2 (nested)	CAAGGACGTGCATGCGAGCT	this lab (from *Ramalina)*	300
RamPKS-R	PKS wA 2 (nested)	CGGTCAGACGAGCGACGACT	this lab (from *Ramalina)*	300
PKS1F	PKS wA 2	TACGAAGCCCTAGAAATGGC	this lab (from Bingle et al. 1999)	450
PKS2R	PKS wA 2	ACGTTTGGCAGTTTCCTGTC	this lab (from Bingle et al. 1999*)*	450
ITS1F	ITS1 rDNA	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns (1993)	300
ITS2	ITS1 rDNA	GCTGCGTTCTTCATCGATGC	White et al. (1990)	300
mrssu1	mt SSU	AGCAGTGAGGAATATTGGTC	Zoller et al. (1999)	400
mrssu2R	mt SSU	CCTTCGTCCTTCAACGTCAG	Zoller et al. (1999)	400

2.3.3. DNA sequencing and alignment

Six replicates of 50μL reaction volumes of PCR product were pooled for DNA

sequencing. The pooled 300μL PCR product was precipitated by adding 2.5 volumes of

100% ethanol and 0.2 volumes of 5M NaCl and centrifuged at 13000 rpm for 10 min.

The DNA pellet was dissolved in 20μL sterile distilled water, and gel purified by

excising the band from 1% agarose gel and purified using the Wizard ® SV Gel and PCR

Clean-Up System (Promega, Madison, WI, USA) following the manufacturer's

instructions. Cycle sequencing reaction volumes were 20μL, containing about 40-60ng of

purified DNA, BigDye V3.1 (Applied Biosystem, Foster City, CA, USA) and the same

PCR primers were used for sequencing. Post reaction clean up followed the

manufacturer's instructions for the ethylene diamine tetraacetic acid (EDTA) and ethanol

precipitation. The dried product was dissolved in 20μL formamide, denatured at 95ºC for

5 minutes, placed on ice, and loaded into a 96-well plate for sequencing on a 3130

Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The PCR product of

MSAS-type gene was sequenced from two samples (*R. intermedia*, N4614 and *R.

dilacerata,* N8786) to confirm the MSAS-type gene. Species of *Ramalina* (Table 2.1)

were then screened for the presence or absence of the PKS gene using PCR. The PKS

genes were scored by the presence or absence of the PCR product for each primer pair. A

positive control, the mitochondrial small subunit, was included with each amplification to

reduce the likelihood of false results. The other PKS primers for sequencing wA1 and

wA2 regions, as well as the ITS rDNA and mtSSU primers, were the same as the PCR

primers (Table 2.2). Nucleotide sequences were compiled in the program Sequencher 4.6

(Gene Codes Corporation, Ann Arbor, MI, U.S.A.) and manually aligned in Se-Al v 1.0

(Rambaut 2001). Two sets of alignments were produced that correspond with combined ITS1 and mtSSU for the phylogenetic analyses. Two additional PKS alignments were used for functional analyses of the PKS genes.

2.3.4. Data analysis

Four nucleotide sequence alignments were analysed; two alignments for the phylogenetic analysis (ITS1 and mtSSU) and two alignments for the PKS functional analysis (wA1 and wA2 type PKS genes). The fungal ITS1 rDNA phylogeny was inferred from 46 collected samples and 29 accessioned DNA sequences retrieved from NCBI GenBank. The mtSSU phylogeny was inferred from 42 collected samples and three samples from GenBank. The ITS and mtSSU sequences were first analyzed separately (not shown) and then the alignments were combined. One wA type PKS gene (wA1) was sequenced from 20 samples the other wA type gene (wA2) was sequenced from 12 samples. In addition, the MSAS gene region was sequenced from two samples to confirm that the PCR fragments reported for 46 samples (Figure 1) represent the MSAS gene region. All nucleotide sequences generated in this study have been deposited in NCBI GenBank and accession numbers are indicated in Table 2.1. Aligned sequences of the combined ITS rDNA and mtSSU alignment were subjected to phylogenetic analyses using PAUP* 4.0b10 (Swofford 2003) and MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003, Huelsenbeck et al. 2001). Phylogenetic determinations were based on Maximum Parsimony (MP) and Bayesian analyses. The option for the MP analyses was tree bisection and reconnection (TBR) branch swapping. Heuristic searches were conducted using 1000 random addition replicates and bootstrap searches of 500 resamplings

(Felsenstein 1985). Bootstrap was done using the MP option in PAUP and values greater than 70 are reported in the phylogenies. For the combined ITS and mtSSU Bayesian analysis a six parameter hLRTs (Hierarchial Likelihood Ratio Tests) model was applied with a gamma shaped parameter and proportion of invariable sites uniformly distributed. This model was the best model estimated with Modeltest 3.7 (Posada and Crandall 1998). Bayesian analyses were performed using 5,000,000 generations for all analyses. Both runs converged on similar likelihood values and were terminated when the standard deviation of split frequencies fell below 0.01. The number of burnin trees discarded for each analysis included 1250 burnin trees. Posterior probability values greater than 90 are reported on the phylogenies. The outgroup taxon, *Cliostomum griffithi*, was chosen so the tree in this study will have the same reference as in Stocker-Wörgötter et al. (2004).

Gene function was inferred by the ratio of synonymous (silent) and non-synonymous (amino acid altering) substitutions in the Synonymous Non-synonymous Analysis Program (SNAP) (HIV database website www.hiv.lanl.gov) and Korber (2000) using a set of codon-aligned nucleotide sequences. The open reading frames of all three gene regions were determined by the ORF finder program in NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The number of synonymous substitutions per synonymous site (ds) and the number of non-synonymous substitutions per nonsynonymous site (dn) is thought to reflect the type and degree of selection that acts on the gene region (Hughes and Nei, 1989). If (ds/dn) ratio is greater than 1.0 across lineages, the amino acids are considered to be conserved by purifying selection. If the ds/dn ratio is less than 1.0 the selection is positive to promote non-silent changes and the

emergence of new phenotypes. If there is no selection on the gene region, the ds and dn are expected to be the same.

2.4. Results

All species in this study contained usnic acid (Table 2.1). *Ramalina dilacerata* produced divaricatic acid in addition to usnic acid. The chemical variability in *R. farinacea* was examined by HPLC and two specimens contained usnic acid, norstictic acid and variolaric acid; three contained usnic acid and salazinic acid; and one contained usnic acid, salazinic acid and protocetraric acid. Seven samples of *R. intermedia* produced usnic, sekikaic and homosekikaic acids. One sample of *R. intermedia* produced an unknown compound (yellow-green after H+; Rf=4 in Sol. A). All samples of *R. intermedia* examined also contained 4'-O-demethylsekikaic acid. *Ramalina pollinaria*, produced usnic and evernic acids. *Ramalina roesleri* produced usnic and sekikaic acids. *Ramalina thrausta* produced usnic and stenosporic acid. Three species, *R. sinensis, R. americana*, and *R. menziesii,* produced usnic acid alone.

Among the 67 samples that represented 12 North American species of *Ramalina*, 43 samples were used in the ITS1 rDNA analysis and 42 samples were used in the mtSSU DNA analysis (Table 2.1). The ITS1 rDNA alignment contains 23 nucleotides of the nuclear 18S gene, then 192 nucleotides of the ITS1, and 50 nucleotides of the 5.8S gene. All sequences from each of *R. menziesii* and *R. sinensis* (and *R. leptocarpha* (AF249908, *R. pollinaria* (582), and *R. montagnei* (AF240011)) include 30 nucleotides of an insertion that were not found in any other sample. The data were analyzed with the insertion and by removing the insertion the tree topology changed by the placement of *R.*

sinensis closer to *R. menziesii* and remaining in a basal position. *Ramalina sinensis* was still monophyletic with bootstrap support of 81%. The alignment based on the mitochondrial SSU gene has 363 total characters. The separate analyses were consistent with one another except there was low resolution in the mtSSU phylogeny as compared to the ITS phylogeny; *R. menziesii* was basal to *R. sinenesis* in mtSSU tree but *R. sinensis* was basal in ITS tree; and *R. americana* was not included in the J clade in mtSSU phylogeny. The combined ITS1 rDNA and mtSSU phylogeny consists of 75 ingroup samples and one sample assigned as an outgroup. The outgroup was the same species (*Cliostomum griffithi*) for both ITS and mtSSU but different individuals. The combined MP analysis contains 628 total characters and 99 parsimoniously informative characters and a tree length of 332 steps. The CI and RI of the phylogenetic tree are 0.6566 and 0.8470, respectively.

The two-gene phylogeny contains ten clades (A to J) with high support for one or both of bootstrap and posterior probability. Although the backbone is not strongly supported, seven of the clades can be argued to represent monophyletic species: *R. thrausta* (Clade A), *R. dilacerata* (Clade B), *R. culbersoniorum* (Clade C), *R. intermedia* (Clade F), *R. fraxinea* (Clade H), *R. menziesii* (Clade I), and *R. sinensis* (Clade J) (Figure 2.1). Two internal nodes with strong support were clade D (*R. dilacerata*, *R. culbersoniorum*, and *R. roesleri*) and clade E. Clade E is comprised of 8 species. Members of *R. intermedia* (Clade F) have 81% bootstrap support but a low posterior probability value. *Ramalina farinacea* and *R. roesleri* each have less than 50% support but fall into separate clades and may be considered to be monophyletic but with low support. Members of *R. americana* have less than 50% support, and are grouped together

with one sample each of *R. panizzei* and *R. siliquosa*. *Ramalina pollinaria* is paraphyletic with four samples within clade E and three samples outside clade E. Seven species are basal to clade E (*R. fraxinea*, *R. menziesii*, *R. montagnei*, *R. paludosa*, *R. pollinaria* in part, *R. leptocarpha*, and *R. sinensis*). Three of these basal species produced highly supported monophyletic clades (*R. fraxinea* (Clade H), *R. menziesii* (Clade I), and *R. sinensis* (Clade J).

Figure 2.1. Phylogeny of North American species of Ramalina based on the combined

ITS1 rDNA and mtSSU nucleotide sequence alignment. This is one of 485 most

parsimonious trees, which is consistent with the Bayesian tree. The assigned outgroup is

Cliostomum griffithii. Numbers above branches represent maximum parsimony bootstrap

support, numbers below the line represent the bootstrap support for the ITS rDNA

analysis, thick lines show the posterior probabilities greater than 90 from the Bayesian

analysis. Clades discussed in the text are indicated by letters from A to J. Collection

numbers and GenBank accession numbers follow the species epithet. The table at the

right shows the polyketide gene amplified product as present (+) or absent (-) for each of

three gene paralogs tested (wa1, wA2, and MSAS), or unknown (U) for the GenBank

accessions, and the number (no.) of polyketides detected by TLC in this study,

corresponding to each sample in the tree.

Two wA type PKS partial genes were sequenced. One wA type (wA 1) is 300 bp long and forms part of the acyltransferase domain. Blast (blastx) searches with the nucleotide sequence of *R. sinensis* (5250) produced the AT region in *C. grayi* isolate PKS 15 putative polyketide synthase gene as the most similar significant result with maximum identity of 60% in 315bp and 2×10^{-26} as the E-value. A second wA type partial gene (wA 2) is about 450 bp long. Blast (blastn) searches with the nucleotide sequence produced the KS domain of *C. grayi* isolate PKS 15 putative polyketide synthase gene as the most similar significant result with maximum identity of 92% in 405bp and 1×10^{-159} as the E-value. It is not clear if these represent two different domains of the same gene or different PKS genes. The MSAS-type of partial PKS gene was sequenced in two samples and other samples were screened for the presence or absence of the gene. A blast (blastx) search with the nucleotide sequence from *R. dilacerata* (8786) gave 6-methylsalicylic acid synthase (*Aspergillus terreus* XP_001215453) as the most similar result with maximum identity of 67% in 957 nucleotides and 1×10^{-159} as the E-value.

Members of nine species of *Ramalina* in the phylogenetic tree (Figure 2.1) were screened for the presence of each of the three PKS gene regions by PCR. The wA-types and MSAS-type PKS gene regions were amplified from 43 samples of nine species of *Ramalina*. The wA1 and wA2 genes show variation with respect to their presence or absence within species. The wA1-type gene was absent in 10 of the 43 samples tested, the wA2-type gene was absent in 14 of the 43 samples tested, and the MSAS region was absent in 6 of the 43 samples tested. The three gene paralogs, in total, are absent in 20 of 84 (23.8%) recorded in the derived species and in 10 of 45 (22.2%) recorded occurrences for the basal species, suggesting the function of the paralogs are not different between

derived or basal species. This may be explained by a true absence of the gene, or presence of mutations at the primer site where the genes may be present but no products were amplified. The unequal distribution of the two wA-type genes among samples suggest they are not parts of the same gene, but represent two different PKS genes. In support of the theory, that species which produce larger numbers of secondary metabolites (see inserted Table in Figure 2.1) are more derived than those that produce only one or a few metabolites, the derived clade E contains samples with two or more metabolites represented and no samples with a single metabolite produced. The more basal species have a single metabolite except the *R. pollinaria* samples and one *R. americana* sample. However, the similar proportion of functional PKS gene paralogs between derived and basal species does not support the hypothesis, but these tests were made on a small portion of the PKS gene.

Gene function for each of the three PKS gene regions was inferred by using open reading frames (ORF) with a single stop codon. The presence of an ORF indicated that the gene could potentially be expressed. The three possible ORFs were examined for each of the gene regions to determine the longest frame. ORF 1 for wA1 was 294 nucleotides, ORF 2 was 297 nucleotides, and ORF 3 was 257 nucleotides long. ORF 1 for wA2 was 186 nucleotides, ORF 2 was 183 nucleotides, and ORF 3 was 183 nucleotides long. ORF 1 for the MSAS type was 319 nucleotides, ORF 2 was 318 nucleotides, and ORF 3 was 317 nucleotides long. Therefore the chosen ORFs for each region was ORF 2 for wA1, ORF 1 for wA2, and ORF 1 for MSAS region to be analysed for non-synonymous to synonymous ratios. The SNAP analysis showed an average ds/dn of 5.4860 for wA1, 2.0126 for wA2, and 1.1234 for the MSAS type gene region.

2.5. Discussion

2.5.1. Monophyly in North American species of Ramalina

The phylogenetic tree is supported by other phylogenies (Stocker-Wörgötter et al. 2004, LaGreca and Lumbsch 2001) and shows seven highly supported monophyletic species, one polyphyletic species (*R. pollinaria*), two monophyletic species with low support (*R. roesleri, R. farinacea*) and one paraphyletic species (*R. americana*) based on two genes from the nuclear and the mitochondrial genomes. The polyphyly of *R. pollinaria* could not be explained in this study but additional species such as *R. sekika* and *R. yasudae* may provide better resolution where the size of soredia was thought to be a distinguishing feature (Ohmura et al. 2008). In addition, Clade G comprises two southern USA coastal plain species, which is supported by unpublished analyses (LaGreca pers. Comm.).

One of the seven monophyletic species, *R. thrausta*, is strongly supported and placed within clade E. *Ramalina thrausta*, contains the spores and cortical features typical of other *Ramalina* species (Bowler 1977) such as *R. leptocarpha* and *R. menziesii*, but phylogenetically it is clearly distinct from these species (Figure 2.1). Goward (1999) reported that *R. thrausta* contains usnic and stenosporic acids, and later Elix and Tonsberg (2005) included perlatolic acid, in addition to the other two compounds. Only usnic and stenosporic acids are detectable by TLC, explaining why only two compounds are reported in this study (Table 2.1). *Ramalina thrausta* is morphologically similar to *Alectoria sarmentosa* (Brodo and Hawksworth 1977) and was a member of the genus

Alectoria before the spore features were observed (Motyka 1964). This is the first report of the phylogenetic placement of *R. thrausta* within the genus *Ramalina*.

The close relationship between *R. dilacerata* and southern hemisphere species (Brodo et al. 2001) may explain the strong support for North American specimens of *R. dilacerata* in this study. Unexpected results were the placement of *R. culbersoniorum* in clade D. *Ramalina culbersoniorum* is a distinct phylogenetic species and chemotype from *R. americana* (Figure 2.1; Stocker-Wörgötter et al. 2004, LaGreca 1999) despite the morphological similarity. Both species were once members of the larger chemically heterogeneous *R. americana* until they were separated by LaGreca (1999). However, the cluster of *R. americana* has low support in Stocker-Wörgötter et al. (2004) and in this study (Figure 2.1). *Ramalina sinensis* formed a highly supported clade regardless of the geographic location of collections from Canada, China, and USA. Nucleotide sequences representing all *R. sinensis* specimens in this study are conspecific despite the large geographic distance and size variation. Though geographic distance may reflect relatedness to some degree since three samples from China fell outside the Manitoba clade in Francisco et al. (2012). The Manitoba samples were collected within 400km of each other and ranged in size from 1 to 9cm at the widest place of the thallus, suggesting that thallus size variation did not influence genetic relatedness. The high degree of morphological variation of *R. sinensis* within the prairie regions (Wylie 1977) is not reflected in the phylogeny in this study (Figure 2.1).

The monophyly of widely distributed members of *R. farinacea* was supported only with low levels of bootstrap and posterior probability values in our two-locus phylogeny. We included ITS sequences of four chemically diverse individuals from

Stocker-Wörgötter et al. (2004), and they form a clade with low support in this study. The phenotypic and ecological differences among the chemical variants within *R. farinacea* were not considered to be distinct enough by Bowler and Rundel (1978) to separate the species from other closely related species. A morphologically similar species, *Ramalina intermedia*, forms a group supported by 81% bootstrap in the ITS rDNA analysis separating them from the unresolved individuals of *R. pollinaria*. Further morphological study of *R. pollinaria* with additional genes may resolve the polyphyly presented in this study. Bowler and Rundel (1974) further reported that *R. roesleri* can be misidentified as *R. intermedia* even though *R. intermedia* falls outside clade D containing *R. roesleri*. Joneson (2003) also had difficulties to distinguish closely related species but found the ITS region to be the best region so far examined to distinguish among morphologically similar species.

2.5.2. *PKS gene function in* Ramalina

This study reports that species of *Ramalina* contain a MSAS-type gene in addition to two wA-type PKS genes. The wA-type of PKS is also known as non-reducing PKS in which the enzyme lacks all three reducing domains and produces compounds with no chemical reduction in structure. The MSAS-type of PKS gene contains any of the reducing domains and catalyses the synthesis of polyketides with various degrees of chemical reduction in structure. The presence of the MSAS type gene was unexpected because partially reducing polyketides such as 6-methylsalicylic acids are generally not reported for species of *Ramalina*. One exception is bourgeanic acid, a MSAS-type polyketide which is occasionally produced by *Ramalina bourgeana* and *R. evernioides*

(Stocker-Wörgötter 2008). If the bourgeanic acid synthase gene is present in *Ramalina bourgeana,* the gene may also be present in closely related species. The MSAS-type PKS gene in *Aspergillus* has been shown to have high homology with those of bacterial genomes (Sanchez et al. 2008, Nip and Chu 1977). The presence of MSAS-type PKS genes has been reported for other lichenized fungi (Kim et al. 2012, Schmitt et al. 2008). The wA1- and wA2-type PKS genes are represented by different domains in this study, suggesting that these regions may belong to a single gene or two different wA type genes. Most polyketides produced by *Ramalina* are encoded by wA-type PKS genes.

If a PKS gene is not functional, then it would not be responsible for production of the secondary metabolites. Since none of the genes were present in 100% of the samples, but usnic acid was produced by all samples, we conclude that none of these three gene paralogs are responsible for production of usnic acid. However, we cannot rule out an absence of gene function for any of the three paralogs studied so we inferred function from the coding regions that were amplified and sequenced. The three PKS genes reported are likely to be functional based on evidence from the ds/dn ratio being greater than 1, indicating that the gene regions are under purifying selective constraints (Muggia et al 2008). Further evidence to support function of these genes is the significant match between each of the three gene regions and other PKS genes through BLAST analysis. The E-scores were more significant than those considered by Brown et al. (2012) who considered an E-score of 1×10^{-7} to be a significant match for a functional PKS gene in *Fusarium* spp. Unusual PKS products have been reported for fungi growing under conditions different from those found in their optimal environments (Stocker-Wörgötter

2001, Culberson et al. 1977) suggesting that non-expressed genes may be present and triggered under specific environmental conditions (Fox and Howlett 2008).

Seven species, the outgroup, and several European conspecifics from Stocker-Wörgötter et al. (2004) were included in the analysis (see Table 2.1) to ensure the same phylogenetic backbone as in Stocker-Wörgötter et al. (2004) to compare the placement of North American species. The distribution of the secondary metabolites mapped onto the phylogenetic tree inconclusively supports the hypothesis proposed by Stocker-Wörgötter et al. (2004). Some of the PKS gene paralogs are consistently produced within each species (Figure 2.1) but the large knowledge gap in linkage between the genes and the polyketide product prevents any conclusion from being made. Early gene duplication and subsequent purifying selection of PKS genes was proposed by Muggia et al. (2008) and accumulation of mutations was proposed for *R. americana* (LaGreca 1999). If speciation is occurring within Clade E, gene duplication might account for the slightly larger number of polyketides produced by members of this clade. Whereas longer evolutionary times represented by longer branches in the more basal species might explain an accumulation of mutations and a reduction in the number of polyketide products. An explanation for the hypothesis may be that the presence of large numbers of PKS paralogs known to occur in fungi (Armaleo et al. 2011, Sanchez et al. 2008, Schmitt et al. 2008, Opanowicz et al. 2006) and the morphological plasticity apparent in fungi (Slepecky and Starmer 2009) might account for a chemical diversification of species as new niches are colonized and species adapt to environmental changes in habitats.

In conclusion, this study shows the placement of three species not previously included in any phylogeny (*R. dilacerata*, *R. roesleri*, and *R. thrausta*) and confirms that

R. thrausta belongs in the genus *Ramalina*. We report monophyly for seven species and two species (*R. americana* and *R. farinacea*) that were previously shown to be monophyletic are not monophyletic in this study reflecting a lack of resolution by the ITS rDNA. The large size of the genus and recent speciation of its members would require using a larger number of taxa and genes than used in this study to reconstruct a more resolved evolutionary hypothesis. The polyphyletic finding for *R. pollinaria* is also new and requires further examination. This study also supports the theory that chemically diverse species of *Ramalina* are more derived that those containing a single polyketide. If the PKS genes are present but latent in the basal species of *R. sinensis* and *R. menziesii*, these species may have the ability to produce a number of unreported polyketides. The genus *Ramalina* has undergone diversification revealing chemical diversity in the derived species. Diversification of gene function with species diversification would be beneficial as species shift to new microhabitats and adapt to new challenges imposed by a changing environment.

CHAPTER 3

Effect of aposymbiotic conditions on colony growth and secondary metabolite production in the lichen-forming fungus, *Ramalina dilacerata*

3.1. Abstract

The production of secondary metabolites by aposymbiotic lichen-forming fungi in culture is thought to be influenced by environmental conditions. Environmental conditions may be controlled by culturing in defined conditions to provide a better understanding of the role of the large number of polyketide synthase (PKS) gene paralogs detected in the genomes of many fungi. The objectives of this study were to examine the effects of different culture conditions (media composition and pH level) on the colony growth, the numbers of secondary products, and the expression of two PKS genes by the lichen-forming fungus, *Ramalina dilacerata*. Four types of growth media at four different pH levels were prepared to culture spore isolates of *Ramalina dilacerata*. Colony

diameter and texture were recorded. The number of secondary compounds were determined by TLC and HPLC. Expression of two PKS genes (wA1 and MSAS) were compared with expression of an internal control (mitochondrial small subunit gene). The results showed that media containing yeast extracts produced the largest colony diameters and the fewest number of secondary metabolites. Colony growth rates also varied with different media conditions, and a significant negative relationship occurred between colony diameter and number of secondary metabolites. Expression of the wA-type (non-reducing) PKS gene was significantly higher at pH 6.5 on the glucose malt agar than any other media, and expression of the MSAS-type (partially-reducing) PKS gene was significantly higher at pH 8.5 on malt agar. Gene expression was correlated with the pH level and media conditions that produced the larger numbers of secondary products. It is speculated that slower growth from the absence of a nitrogen source and a pH level higher than that found in its natural environment may be the conditions needed to trigger PKS gene expression.

3.2. Introduction

Lichen forming fungi (ascomycetes) synthesize a variety of secondary metabolites in the symbiotic lichen association (Stocker-Wörgötter 2007). To a lesser extent lichen-forming fungi also produce secondary metabolites that consist primarily of phenolic polyketides under aposymbiotic growing conditions (Stocker-Wörgötter 2007). The production of secondary metabolites by the fungus in culture is influenced by growing conditions (Hager et al. 2008, Zocher and Stocker-Wörgötter 2005, Stocker-Wörgötter 2002, Hamada 1996). When grown on different nutrient media *Parmotrema reticulatum*

(Taylor) M. Choisy did not produce atranorin and salazinic acid, but both metabolites were detected in the voucher specimen (Fazio et al. 2009). The effect of varying temperature conditions on secondary metabolite profiles was examined in *Heterodea muelleri* (Hampe) Nyl., where the mycobiont in culture did not produce any detectable secondary compounds (Hager et al. 2008). However, other studies demonstrated that the diagnostic secondary metabolites could be detected aposymbiotically (Verma et al. 2012, Behera et al. 2006, Cordeiro et al. 2004, Hamada and Ueno 1990). It has been reported that the availability and type of carbon and nitrogen sources may affect the production of secondary metabolites by non-lichenized fungi (Keller et al. 2002) and aposymbiotic lichenized fungi (Verma et al. 2012, Behera et al. 2006). Sugars such as glucose, sucrose, or sorbitol when used as the sole carbon source have been found to support high aflatoxin production along with higher fungal growth and sporulation in *Aspergillus* species. On the other hand, peptones and more complex sugars such as galactose, xylose, lactose and mannitol did not support aflatoxin production (Keller et al. 2002). Different effects of the nitrogen source have also been shown for aflatoxin and sterigmatocystin production (Keller et al. 2002), where an increased amount of sterigmatocystin and aflatoxin production in ammonia-based media and a decreased amount in nitrate-based media was reported for *Apergillus* species. The theory that slower mycelial growth resulting from inadequate nutrients is linked with production of secondary metabolites (Bu'Lock 1961) has not been experimentally tested with lichen fungi. An understanding of the conditions responsible for production of secondary metabolites may also provide insight into the manipulation of biosynthetic pathways for further experimentation and discovery of new polyketides. Growth conditions for some fungi were determined in early studies (Hamada

1989, Ahmadjian 1961), and more recently to examine polyketide production of cultured

mycobionts (Fazio et al. 2009, Brunauer and Stocker-Wörgötter 2005, Cordeiro et al.

2004, Molina et al. 1997, Armaleo 1991) but no conditions have been reported for

culturing *Ramalina dilacerata* (Hoffm.) Hoffm. Knowledge of optimal growing

conditions for a particular species must be determined before a comparison can be made

with less optimal growing conditions (Stocker-Wörgötter 2007).

Polyketides may help an organism survive in its ecological niche if they are

produced in response to environmental stresses such as high light levels or drought, or to

protect the organism from predators and parasites (Huneck 1999). Secondary metabolites

have also been hypothesized to play a role in herbivory defence, to serve as antibiotics, or

as metal chelators for nutrient acquisition (Gauslaa 2005, Huneck 1999, Lawrey 1986).

Polyketide biosynthesis is catalyzed by polyketide synthase (PKS) enzymes that are large

multifunctional enzymes with a linear succession of individual catalytic domains in many

fungi. The minimal requirement for polyketide assembly is a PKS gene that contains a

ketosynthase (KS), an acyl transferase (AT), and an acyl carrier protein (ACP) domain

(Hopwood 1997). More complex reducing PKS have dehydratase (DH), enoyl reductase

(ER), ketoreductase (KR), and thioesterase (TE) domains in addition to the KS, AT, and

ACP domains (Graziani et al. 2004). Fungal PKS enzymes can be classified as either

MSAS-type or wA-type PKS (Bingle et al. 1999) depending on the presence or absence

of reducing domains, respectively. The wA-type of PKS, also known as non-reducing

PKS and produces compounds, typically aromatic phenols, that lack a chemical reduction

during the assembly of their carbon skeleton. The MSAS-type of PKS gene, exemplified

by 6-methylsalicylic acid synthase, also contain at least one KR and DH reducing

domains in addition to KS, AT and ACP domains. The MSAS-type enzyme produces polyketides with varying degrees of chemical reduction in structure depending on the occurrence of reducing domains. The known polyketides produced by most *Ramalina* species represent chemical structures consistent with the occurrence of a wA-type non-reducing PKS. However, *Ramalina bourgeana* Mont. Ex Nyl. and *R. evernioides* (syn. *R. lacera* (With.) J. R. Laundon) occasionally produce partially-reduced polyketides such as bourgeanic acid (Stocker-Wörgötter 2008). More typically *R. dilacerata* produces two polyketides, usnic acid and divaricatic acid (Brodo et al. 2001) that have chemical structures consistent with wA-type polyketide synthases. The polyketides, usnic acid and divaricatic acid, can be detected by chemical analysis in the lichen, *R. dilacerata*, but the complete nucleotide sequence of the genes responsible for their biosynthesis is unknown. The large number of PKS gene paralogs that have been detected in other ascomycetes (Armaleo et at. 2011, Gilsenan et al. 2009) suggests that there may be potential for a large number of gene products to be present. However, little is known about the function of the paralogs or the conditions required for expression of the genes involved in production of the polyketide secondary metabolites in lichen-forming ascomycetes.

Ramalina dilacerata is an epiphytic lichen with numerous apothecia and a shrubby thallus that produces both usnic acid and divaricatic acid. It has been shown to be monophyletic and a derived species in the genus (Timsina et al. 2012) and is readily distinguished from other North American species in the genus (Brodo et al. 2001). As a derived species, it is hypothesized to produce larger numbers of polyketides than the more primitive species (Stocker-Wörgötter 2004), and three polyketide synthase gene paralogs have been detected in the species (Timsina et al. 2012). Therefore, *R. dilacerata*

would be suitable for aposymbiotic culture because of numerous apothecia, the absence of taxonomic issues in North America, and it may have the potential to produce a large number of polyketides. In addition *R. dilacerata* grows on a wide range of tree species suggesting that it can adapt to a wide range of conditions, making this species likely to be more amenable to culturing aposymbiotically. While variability is present in spore morphology (Howe 1914), it might be expected that other features such as growth and secondary metabolite production would also show variability. Experimental studies on the PKS gene expression and variability of spore growth and polyketide production from spore cultures within and between different apothecia is the first step to improve our understanding of the adaptability of this species and provide insight into how other species may adapt to changing environments.

The goal of the present study was to investigate the effect of environmental conditions on the cultured mycobiont, *R. dilacerata*. The specific objectives of the study were to examine the influence of different media and pH levels on colony growth and morphology, secondary metabolite production, and PKS gene expression; compare the growth and morphology of *R. dilacerata* between spores within and between apothecia; and to test whether the rate of colony growth influences production of numbers of secondary metabolites.

3.3. Materials and Methods

3.3.1. *Lichen material and mycobiont isolation*

The lichen-forming fungus used in this study was *Ramalina dilacerata* and the thalli were collected from Manitoba, Canada (Normore 9643, Sandilands Provincial Forest; and Normore 9647, Whiteshell Provincial Park). Both samples are deposited in the University of Manitoba herbarium.

Isolation of the mycobiont followed a procedure described by Yoshimura et al. (2002) with some modifications. Apothecia were removed from a thallus branch, washed under running tap water for 90 minutes, transferred to a 50 mL beaker and washed for 30 minutes with sterile distilled water using a magnetic stir bar. The wash was repeated in sterile distilled water by adding 150 μL of Tween 80. The water was changed and the wash was repeated three times. Finally apothecia were transferred to a sterile beaker containing sterilized distilled water and stirred using a magnetic stir bar for 15 minutes covered with aluminum foil. Apothecia were then cut on a glass slide using a razor blade inside a laminar flow hood. Vaseline was placed on the underside of the top cover of a Petri plate. One apothecium each was placed on each spot of vaseline using sterile forceps with the hymenium layer facing toward the medium so spores would be released directly onto the medium. To ensure that the petri-plate remained moist, which enhanced spore release, three to four triangular pieces of sterile wet blotting paper were placed between the apothecia on the petri plate cover. Culture plates were then sealed with parafilm, labeled, and incubated at 20 ^{0}C. Malt-extract agar (1.5% malt and 1.4% agar) was the medium used for spore discharge.

3.3.2. *Generation of single spore mycobiont cultures*

The experiments consisted of five types of media and four pH levels and comparisons were made at about 100 days of growth. Replicates of at least three plates were made for each experiment and incubated at 20 ^{0}C. The pH level of the medium was adjusted using concentrated HCl or NaOH before autoclaving. Four pH levels (pH 3.5, pH 4.5, pH 6.5 and pH 8.5) were prepared for each type of five media compositions; malt agar (MA; 15 g agar and 14 g malt extract in 1 L distilled water); glucose agar (GA; 15 g agar and 10 g Glucose in 1 L distilled water); malt-glucose agar (MGA; 15 g agar, 14 g malt extract, and 10 g glucose in 1 L distilled water); malt-glucose-yeast agar (MGYA; 15 g agar, 5 g malt extract, 5 g yeast extract, 10 g of glucose in 1 L distilled water); and malt-yeast agar (MYA; 15 g agar, 14 g malt extract, and 14 g yeast extract). All chemicals were obtained from Sigma unless otherwise indicated. There was no growth in the glucose media at pH 3.5, pH 4.5, and pH 8.5. In addition, the media with glucose (MGA and MGYA) failed to solidify at pH 3.5. Therefore these five experiments were excluded from the analysis resulting in 15 experiments used in the study (see Table 3.1).

Seven days after preparing the petri plates, but before germination, the spores from each apothecium were transferred to freshly prepared media using a dissecting microscope. A spore suspension was spread over the new media to obtain single spore colonies by adding two to three drops of sterile distilled water to resuspend the spores. The colony growth was monitored over time on five different media compositions (GA, MA, MGA, MYA, and MGYA) at pH 6.5 and incubated at 20 ^{0}C in the dark for a total of 180 days. Replicates of 20 measurements of colony diameter were made for each medium at each time period (30, 70, 100, 140, 160, and 180 days). Two perpendicular colony

diameters were measured to the nearest mm and the average was recorded as a single measurement for each colony.

Initially spore rains of 15 apothecia were set up for sample Normore 9643 and 24 apothecia for sample Normore 9647. These numbers were reduced to 11 apothecia for Normore 9643 and 15 apothecia for Normore 9647 because some of the apothecia failed to release spores. The resulting combinations of experiments and replicates (with the exception of five glucose treatments above) resulted in at least 1170 plates for both samples. Measurements were recorded for approximately six colonies per plate for all experiments.

Table 3.1. Number of secondary metabolites detected from two samples Normore 9643 and Normore 9647 by TLC when spore colonies were grown with different media compositions and pH levels. Glucose agar [GA], malt [MA], malt-yeast [MYA], malt-glucose [MGA], malt-glucose-yeast [MGYA].

Growth medium (sample)	pH 3.5	pH 4.5	pH 6.5	pH 8.5	Growth rates (μm /day ± SD in the linear phase)
GA (9643)	No growth	No growth	6	No growth	6.07 ± 1.46
MA (9643)	3	4	6	7	67.85 ± 5.21
MYA (9643)	4	6	7	5	53.29 ± 3.71
MGA (9643)	Media not solid	5	6	6	55.07 ± 14.17
MGYA (9643)	Media not solid	5	6	5	80.29 ± 5.30
GA (9647)	No growth	No growth	6	No growth	6.07 ± 1.46
MA (9647)	3	5	6	7	67.85 ± 5.21
MYA (9647)	4	6	7	6	53.29 ± 3.71
MGA (9647)	Media not solid	4	6	7	55.07 ± 14.17
MGYA (9647)	Media not solid	4	6	5	80.29 ± 5.30

3.3.3. *DNA extraction and sequencing*

To confirm that the cultured mycobiont was *R. dilacerata*, two fresh colonies were placed in 1.5 mL micro-centrifuge tubes and total DNA was extracted following the cetylmethylammonium bromide (CTAB) extraction protocol modified from Grube et al. (1995). The Internal Transcribed spacer 1 (ITS1) of the nuclear ribosomal DNA (rDNA) was amplified using the fungal primers ITS1F and ITS2 (Table 3.2). A touchdown PCR cycle consisted of initial denaturing at 94 ºC for 5 minutes, then 30 cycles of denaturing at 94 ºC for 1 minute, annealing at 60 ºC for 1 minute, then 58 ºC, 56 ºC, 54 ºC and 52 ºC each for 1 minute and extension at 72 ºC for 1 minute. The mitochondrial small subunit gene (mtSSU) was amplified with mrSSU1 and mrSSU2R primers (Table 3.2) using a PCR cycle of initial denaturing at 94 ºC for 5 minutes, then 30 cycles of denaturing at 94 ºC for 1 minute, annealing at 54 ºC for 1 minute, and extension at 72 ºC for 1 minute. Amplifications were performed in a thermal cycler (Biometra T-Gradient; Tampa, FL, USA) in 20 μL reaction volumes with 1X buffer (200 mM Tris-HCl, 500 mM KCl), 1.25 units GO Taq (Go Taq ® Hot Start polymerase, Promega), 3.125 mM $MgCl_2$ 1.25 mM of each dNTP, 1.0 M of each primer, and 40 to 60 ng of DNA. The dried and purified product was dissolved in 20μL formamide, denatured at 95 ^{0}C for 5 minutes, placed on ice, and loaded into a 96-well plate for sequencing on a 3130 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The same PCR primers as above were used for the sequencing reactions.

Table 3.2. List of primers showing primer names, target gene, primer sequences (5'to 3'),

source of primer, and the length (bp) of the amplified product.

Primer name	Gene	Primer sequence (5'-3')	Source of primers	Product size
RSinF2	PKSwA1	CTGTCTTACACCACAACCGCC	Timsina et al. (2012)	300
RSinR	PKSwA1	ACCATCAACGAGTGGGAGAAA	Timsina et al. (2012)	300
PKSwA1F	PKSwA1	CAGTGTGAGCGAGAGATTGG	this lab	143
PKSwA1R	PKSwA1	CCATTGAGCTCGTACAGCTG	this lab	143
RFarBERTc	PKS MSAS	GTCTTGAGCTGTCCACTCAT	Timsina et al. (2012)	1050
RFarLC3c	PKS MSAS	CTGCAAGGCGAGTCTAAGAT	Timsina et al. (2012)	1050
MSAS-F	MSAS	TGCTCCTCCCTCAAGATG	this lab	134
MSAS-R	MSAS	CTACGTCTACTCCGCTTG	this lab	134
ITS1F	ITS1 rDNA	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns (1993)	300
ITS2	ITS1 rDNA	GCTGCGTTCTTCATCGATGC	White et al. (1990)	300
mrSSU1	mtSSU	AGCAGTGAGGAATATTGGTC	Zoller et al. (1999)	400
mrSSU2R	mtSSU	CCTTCGTCCTTCAACGTCAG	Zoller et al. (1999)	400

3.3.4. *RNA isolation and cDNA synthesis*

Total RNA was extracted from 100-120 mg of cultured mycobiont colonies under RNase-free conditions using a TRIZOL® Reagent method following the manufacturer's instructions (Invitrogen). RNA quality and quantity were determined with a NANODROP 2000c spectrophotometer (Thermo Scientific). Prior to cDNA synthesis, 1.0 µg of total RNA was treated with DNase I, RNase-free following the manufacturer's instructions (Invitrogen). The DNased RNA samples were amplified by wA1 specific primers RSinF2 and RSinR (Table 3.2) to confirm that any residual DNA has been removed. The cDNA was then synthesized from 1.0 µg of DNased RNA using Maxima ® First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas Life Scientific). The 5X Reaction Mix contain both oligo $(dT)_{18}$ and random hexamer primers.

Amplification of a fragment of the AT domain of a non-reducing polyketide synthase gene (wA1) was achieved using a primer pair PKSwA1F and PKSwA1R and the amplification of the partially reducing PKS gene (MSAS-type) was done using MSAS-F and MSAS-R (Table 3.2). Both wA1 and MSAS type PKS genes were amplified using an initial denaturing at 94°C for 4 minutes, then 35 cycles of denaturing at 94°C for 10 seconds, annealing at 63°C (wA1) and 57°C (MSAS) for 10 seconds, and extension at 72°C for 10 seconds. The internal control gene, mitochondrial small sub unit ribosomal cDNA (mtSSU), was amplified using mrSSU1 and mrSSU2R (Table 3.2) using a PCR cycle of initial denaturing at 94 °C for 5 minutes, then 25 cycles of denaturing at 94 °C for 1 minute, annealing at 54 °C for 30 seconds, and extension at 72 °C for 1 minute. Amplifications were performed in a thermal cycler (Biometra T-

Gradient; Tampa, FL, USA) in 20 μL reaction volumes with 1X buffer (200 mM Tris-HCl, 500 mM KCl), 1.25 units GO Taq (Go Taq ® Hot Start polymerase, Promega), 3.125 mM MgCl$_2$ 0.6 mM of each dNTP, and 0.5 M of each primer. Following the separation of the bands on agarose gels, the PCR products were purified by the Wizard ® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) using the manufacturer's instructions. Purified PCR products were sequenced as described above.

3.3.5. *Quantitative PCR (qPCR)*

Quantitative PCR for the non-reducing and partially reducing PKS genes was accomplished with PKS wA1 and MSAS primers (described above) on a MiniOpticon™ Real-Time PCR System (BIO-RAD), using the reagents in SsoFast™ EvaGreen® Supermix (BIO-RAD). The Real Time -PCR cycle was initial denaturing at 95 ℃ for 4 minutes, then 40 cycles of denaturing at 95 ℃ for 10 seconds, annealing at 63 ℃ (wA1) and 57 ℃ (MSAS) for 10 seconds, and extension at 72 ℃ for 10 seconds. Messenger RNA expression levels were measured in triplicate (for each treatment) samples of cDNA reverse transcribed from 50 ng of total RNA. Messenger RNA expression for wA1 and MSAS type PKS gene was analyzed using the mrSSU gene as the internal control gene to normalize the expression. The internal control gene was equally expressed in all treatments. The total RNA was isolated from fungi exposed to a pairwise combination of four different pH levels (3.5, 4.5, 6.5, and 8.5) and four different media (MA, MYA, MGA, MGYA) conditions resulting in 16 media and pH conditions (except the five conditions indicated in section "*Generation of single spore mycobiont cultures*"). Expression of wA1-type and MSAS-type PKS genes was analysed.

3.3.6. *Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC)*

Thin Layer Chromatography (TLC) was performed on acetone extracts from the natural lichen thallus and from the cultured mycobiont. Samples of the cultured mycobiont representing all four media compositions and four pH levels were conducted in replicates of three to determine the secondary metabolites present in a subset of the cultured colonies (122 samples). Secondary metabolites were extracted from colonies by three extractions with acetone for a total of 20 minutes followed by spotting the crude acetone extract on silica coated glass plates (Fisher Scientific, Ottawa, Ontario, Canada). A TLC protocol was modified from Orange et al. (2001) and Culberson (1972) using toluene (180 mL): dioxane (45 mL): glacial acetic acid (5 mL) as eluting solvent followed by spraying with 10% sulfuric acid and color development in an 80 ^{0}C oven for 10 minutes. An R_f standard consisted of known samples of usnic acid (Chromadex) and divaricatic acid (from *Evernia mesomorpha* Nyl.) were applied to each TLC plate, and spot characteristics as described in Orange et al. (2001) were consulted to confirm compound identification. The unknown compounds were assigned numbers and the plate characteristics were recorded to remain consistent between samples.

For HPLC analysis, a Waters HPLC Separations Module 2695, combined with a PDA Detector Model 2996 was used. The column was a mBondapak Waters C_{18} (3.9 X 300 mm) column with particle diameter of 15–20 μm, with 125 Å pores. The flow rate was 1 mL/min and the eluent was monitored continuously at 210–600 nm and HPLC traces were displayed at 220 nm and 254 nm. The gradient was held at 20% acetonitrile

in 0.075% aqueous trifluoracetic acid for 10 min then a linear gradient to 80%

acetonitrile and held at that composition for 20 minutes followed by a linear gradient

back to 20% acetonitrile for 10 minutes and held there for an additional 10 minutes. The

total run time was 60 minutes. The number of peaks within each sample was compared

with those from the media alone, and were counted for each trace.

3.3.7. *Data analyses*

The independent variables examined were pH level and type of medium. The dependent

variables were the colony growth and number of secondary metabolites produced. One-

way ANOVA was used to compare means between apothecia within each treatment. The

Bonferroni test was applied to this analysis as implemented in IBM SPSS Statistics (ver.

19). Results of this test showed low levels of variation between apothecia and provided

justification for combining data from each apothecium for subsequent tests between

treatments. To test whether there were differences in colony growth between different pH

levels and media composition, one-way ANOVA was used for each treatment and

Tukey's tests were applied to determine pairwise significance. To test whether an

interaction occurred between the independent variables (pH level and media composition)

to predict the dependent variables (colony diameter and number of secondary

metabolites), a two-way ANOVA (Sokal and Rohlf 1981) was performed in IBM SPSS

Statistics (ver. 19). Finally, a linear regression was performed to test whether the number

of secondary metabolites produced can be explained by colony growth between pH level

but within each media composition. JMP (ver 8.0.x) was used to analyze most of the

comparisons and IBM SPSS Statistics (ver. 19) was used where indicated.

3.4. Results

Both ITS rDNA and mtSSU genes were sequenced from the two colonies that were isolated from field collected specimens of *R. dilacerata* (Normore 9643 and Normore 9647). The nucleotide sequences were 100% identical with the nucleotide sequence obtained from the previously collected thallus of *R. dilacerata* (GenBank accession numbers: JQ003070, JQ003108). The blast (BLASTn) searches with each of the ITS rDNA and mtSSU sequences matched several species of *Ramalina* as the most significant results with 100% identity confirming that the cultures are *R. dilacerata*.

The diameter of more than 4500 spore colonies (as described in section "*Generation of single spore mycobiont cultures*") from different apothecia showed small amounts of size differences within the same pH and growth medium. When variation in colony diameter for colonies representing each apothecium was examined using the Bonferroni test, 6 out of 90 apothecial comparisons showed significant differences (p=0.001) for Normore 9643 and none of the apothecial comparisons were significantly different from one another (p=0.001) for Normore 9647. Since there were less than 10% significant differences, the data from all apothecia on both specimens were combined, and differences in colony diameter were compared among pH levels and media conditions.

3.4.1. *Changes in colony diameter over time*

Diameter of the fungal colonies was measured over time beginning at 30 days after inoculation. An increasing linear phase was observed between 30 days and 100 days suggesting a radial increase in growth over this time period. The growth then started to plateau in a deceleration phase. This pattern of growth was observed in four types of media except the medium with glucose alone (Figure 3.1). The diameter of the colonies in the media containing glucose and another nutrient source were comparable to those in the media without glucose and other nutrients. Colonies continued to grow radially as measured by their diameter until about 140 days and then most of the growth was by vertical hyphae after 140 days, which was not measured in this study. During the 140 days of growth, the rate was the highest at 100 days in all media compositions tested (Figure 3.1). When compared at 100 days, the growth rates were slowest on glucose ($6.07 \pm 1.46\ \mu$m/day), faster on malt-extract ($67.85 \pm 5.21\ \mu$m/day), and fastest on malt-yeast-glucose extract ($80.29 \pm 5.30\ \mu$m/day) (Table 3.1). Subsequent comparisons between treatments were made at 100 days of growth. All measurements were compared using standard deviations and sample sizes were between 3 and 5 colonies.

Colony features, including color and texture, changed as the colony diameter increased in size over time (Figure 3.2). The color of the colonies was pale pink to light brown before 30 days of age, and they turned dark brown, and appeared dry and rough in older colonies at 90 days of age and older. The media surrounding the colonies also turned brown with age in these older colonies.

Figure 3.1. Radial growth (mm) over time (days) for colonies of *Ramalina dilacerata* isolated from apothecia of Normore 9643, on five types of solid medium (glucose [GA], solid circles; malt [MA], solid diamonds; malt-yeast [MYA], solid squares; malt-glucose [MGA], open circles; and malt-glucose-yeast [MGYA], open triangles).
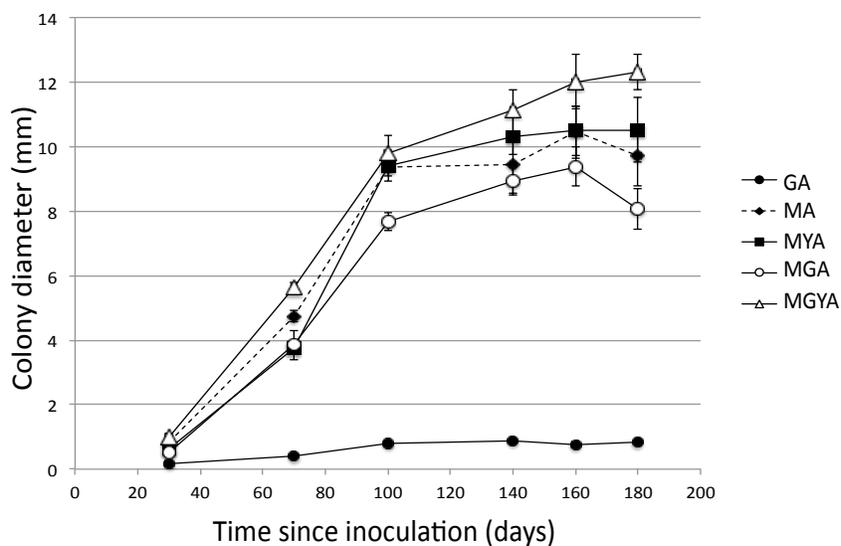
Figure 3.2. Colonies of *R. dilacerata* in five different media at pH 6.5 showing variation in colour and texture at 25-30 days of growth (a to e) and at 90-100 days of growth (f to j). Photos a and f are grown in MA (malt), b and g are grown in MGA (malt-glucose), c and h are grown in MYA (malt-yeast), d and I are grown in MGYA (malt-glucose-yeast), and e and j are grown in GA (glucose).
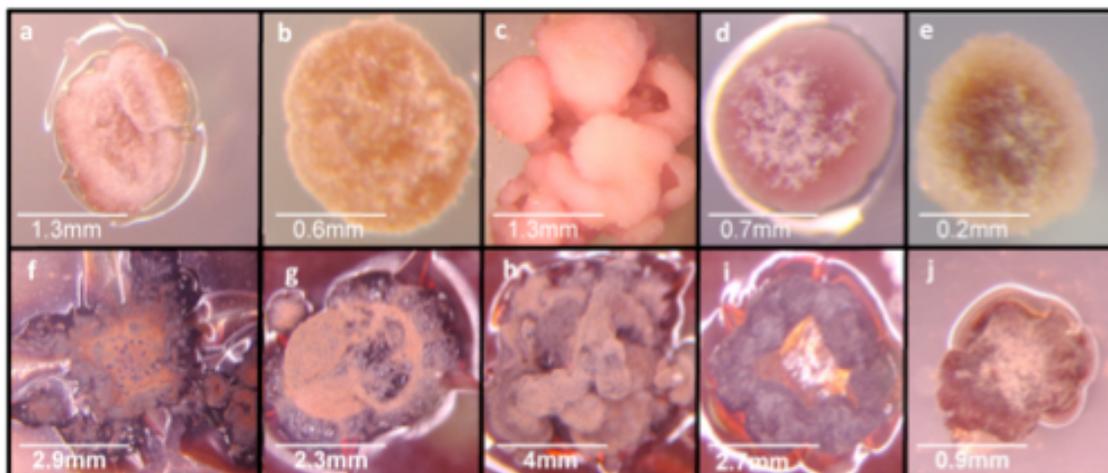
3.4.2. *Effect of media composition and pH level on colony diameter*

Young colonies (less than 30 days) growing on a glucose-containing medium (GA, MGA, MGYA) had a brown color and those on non-glucose media (MA and MYA) had a pink color. Older colonies (90-100 days) were all dark red in color. Colony growth comparisons were made during the linear phase after 100 days.

The effect of growth medium composition and pH level on colony diameter of *R. dilacerata* was examined (Figure 3.3). A comparison among media types (within each pH level) showed that colony diameter was significantly higher in MA than MYA at pH 3.5. At pH 4.5 the colony diameter was significantly higher when grown in MGYA and lowest in MYA. Also at pH 4.5 the colony diameter in MGA was significantly lower than those grown in MGYA, and significantly higher than those grown in MYA, but there was no significant difference from those in MA. At pH 6.5, colony diameter was significantly higher in MGYA than any other media, and extremely low in the GA (Figure 3.3, noted in caption). This was the only pH tested using agar with glucose alone. At pH 8.5, colony diameter was significantly lower in MYA, and similar in the other three types of agar. There was a significant interaction between pH level and media suggesting that both variables influence growth together to produce a synergistic interaction. Based on these comparisons, *R. dilacerata* produced the largest colonies at pH 4.5 and pH 6.5 in MGYA. The smallest colonies (with the exception of glucose alone) were in MYA at all pH levels tested.

Figure 3.3. Effect of growth medium and pH on colony diameter of *R. dilacerata* showing mean and standard deviation for growth medium within each pH level. Different lower case letters within each cluster represent significant differences at p=0.05. Average colony diameter in glucose agar at pH 6.5 was 0.8 mm, which is not shown in the figure. MA (malt), MYA (malt-yeast), MGA (malt-glucose), MGYA (malt-glucose-yeast).
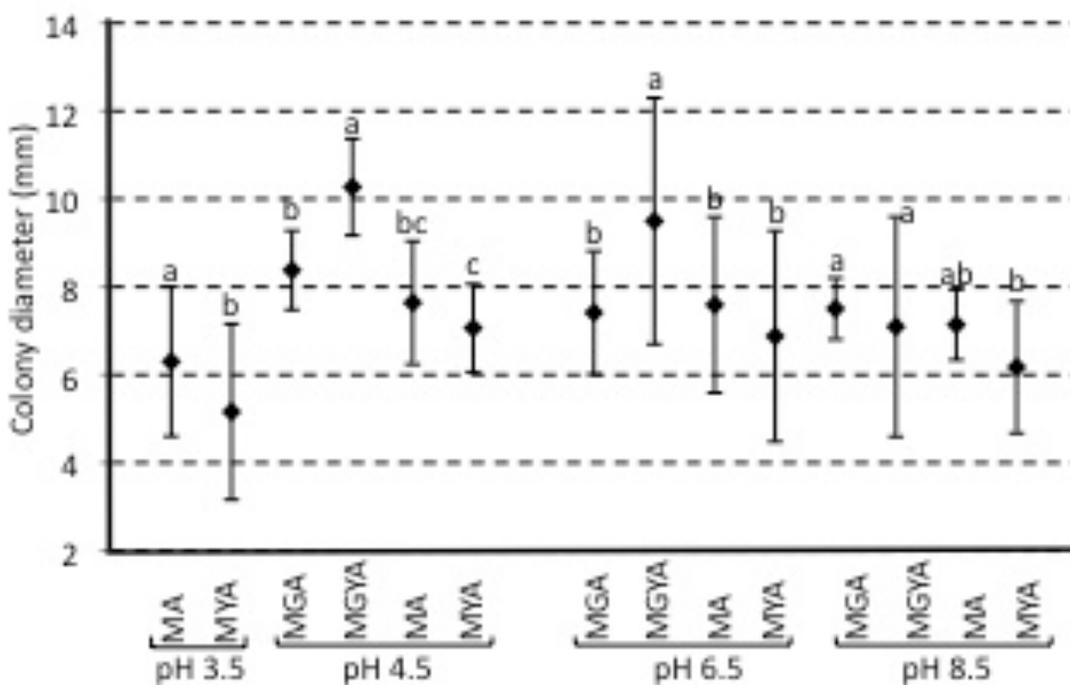
3.4.3. Effect of media and pH on number of secondary metabolites

There were a larger number of secondary metabolites detected in the acetone extract from the cultured mycobiont when compared to the acetone extract of the natural lichen thallus (results not shown). Only two metabolites, usnic acid and divaricatic acid, were detected in the acetone extract of the voucher specimens but as many as seven metabolites were detected from the 100-day old cultured aposymbiotic mycobiont (Table 3.1). The lowest numbers of metabolites were detected at pH 3.5 for both media compositions (MA and MYA) tested and both lichen samples. At pH 4.5, the number of metabolites was highest in MYA. Similarly, at pH 6.5 the number of metabolites was also highest in MYA. At pH 8.5, the number of metabolites was highest in MA, next in MGA and lowest in MYA.

While the TLC results were consistent with the HPLC results (Figure 3.4), only two unknown samples could be collected in sufficient quantities for structural determination. The crude residue left behind after evaporation of the acetone extract from the pH 6.5 on MYA experiment was subjected to analysis by ^{1}H NMR in $CDCl_3$. The only peaks observed in the ^{1}H NMR were consistent with the presence of a triacylglyceride (TAG) and any other signals were below the detection limit of the ^{1}H NMR. However this result is consistent with the HPLC and TLC data, as the expected phenolic secondary metabolites would have a higher extinction coefficient than the TGA's detected by NMR, reflecting the higher sensitivity of HPLC than TLC for the detection of the phenolic secondary metabolites.

Figure 3.4. HPLC traces (A) and TLC spots (B) showing number of secondary metabolites produced by *R. dilacerata* in Malt-agar medium at pH 8.5. N=2. There are 7 spots on the TLC plate. We did not record the spot between 1 & 3. The spot between 1 & 3 is very weak and may correspond to the first peak (16.383min) in HPLC.
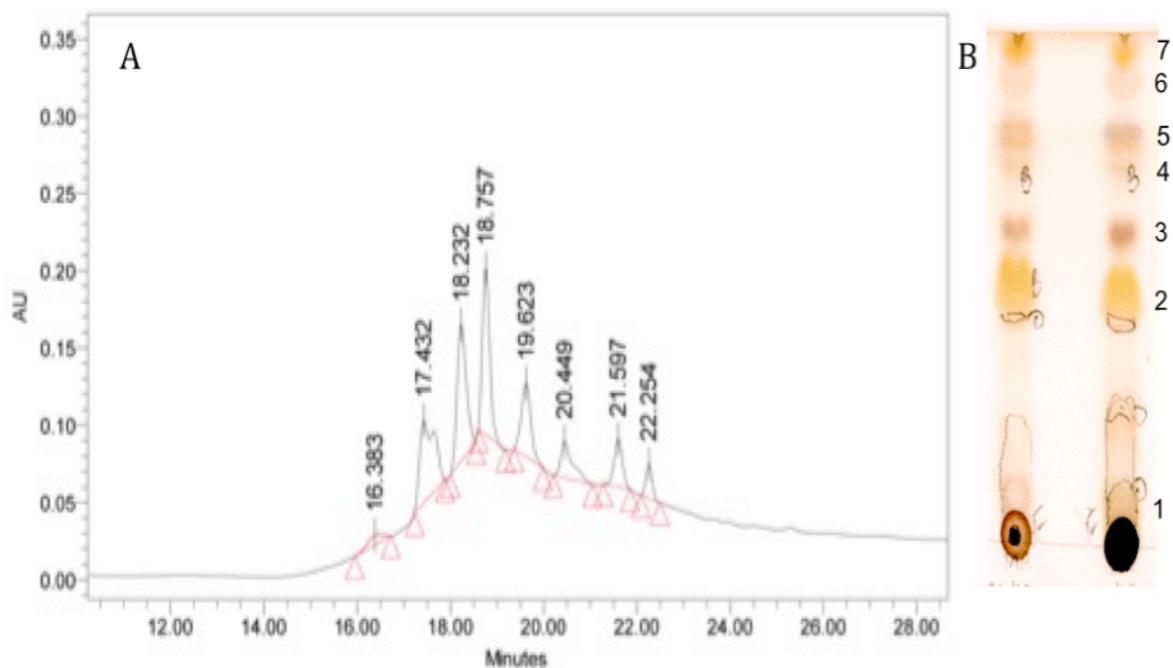
3.4.4. Effect of colony growth on secondary metabolite production

A subset of 26 samples were selected to correlate colony diameter with number of secondary metabolites produced as determined by TLC and HPLC analysis. It was observed that the mean colony diameter was not significantly different between media composition and pH level for the subset of samples selected. Therefore the colony diameter data were combined into a single dataset to test for a relationship between colony diameter and number of secondary metabolites within each type of media composition. A reduction in the number of secondary metabolites can be explained by an increase in colony diameter when they are grown on MYA (p=0.019; Figure 3.5). There was no significant relationship between colony diameter and number of secondary metabolites produced for the other three growth media tested.

3.4.5. Realtive mRNA expression of wA1 and MSAS type PKS genes

The primer pair (PKSwA1F and PKSwA1R) for the wA1-type PKS gene produced a sequence 143 bp long which showed 100% identity to the nucleotide sequence obtained from a previously sequenced thallus of *R. dilacerata* (Normore 9647; JQ003151). Similarly, the primer pair (MSAS-F and MSAS-R) for the MSAS-type PKS gene produced an amplified sequence of 134 bp in length which displayed 100% identity to *R. dilacerata* (Normore 8786; Acc. no). The primer pair (mrSSU1 and mrSSU2R) for the mitochondrial small subunit ribosomal gene produced an amplified sequence of 400 bp long, which was identical with the mtSSU of the previously sequenced gene from the thallus of *R. dilacerata* (Normore 9647; JQ003109). The comparison with all known

sequences (BLASTn) using the nucleotide sequence of the wA1-type PKS gene produced

a putative non-reducing polyketide synthase in *Ramalina* species as the most similar

significant result with maximum identity (JQ003150, JQ003108). The same comparison

(BLASTn) for the MSAS type PKS gene sequence produced 6-methylsalicylic acid

synthase (*Aspergillus terreus* Thom; XP_001215453) as the most similar significant

result.

Figure 3. 5. Relationship between number of secondary metabolites and mycobiont

colony diameter (mm) for all pH levels on MYA (malt-yeast).

The chart shows the equation $y = -0.2994x + 7.5683$ and $R^2 = 0.13944$. The x-axis is labeled "Colony diameter (mm)" and the y-axis is labeled "No. secondary metabolites".
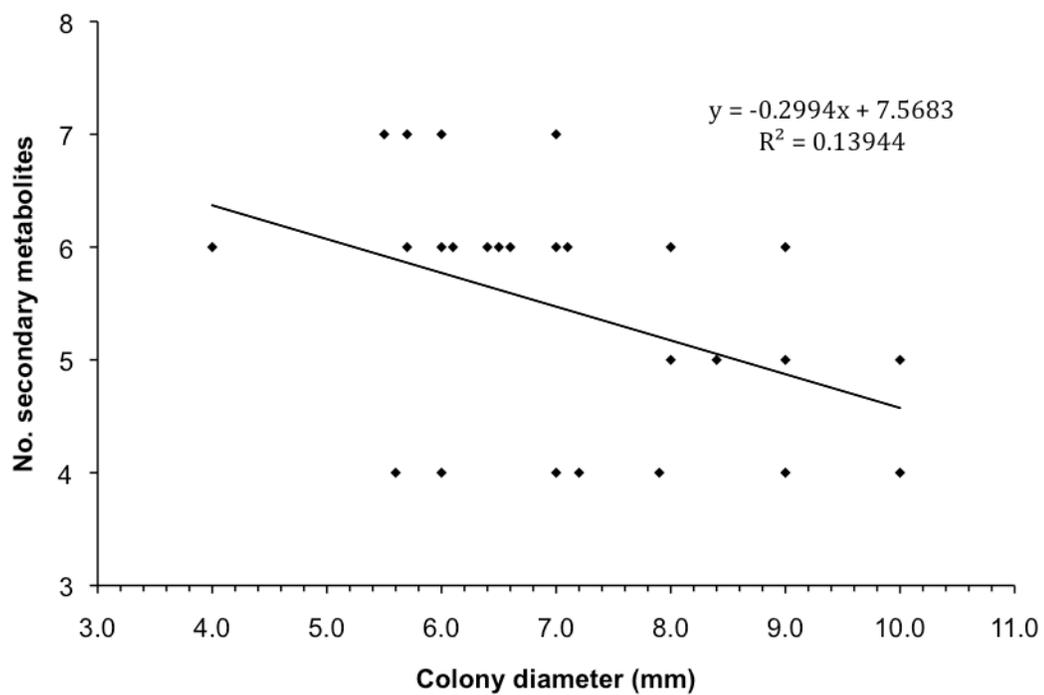
The expression level of the wA1-type PKS gene was compared relative to that of an internal standard (mtSSU), which was equally expressed among treatments (N=3). Gene expression of the wA1-type PKS gene was present in only two media (MA and MGA; Figure 3.6A). In MGA the expression level was higher at pH 6.5 (P= <0.0001) whereas no expression was detected at pH 4.5. In MA, the expression level was higher at pH 8.5 and no expression was detected at the other three pH levels. The expression of the wA1-type gene was not detected in the other growing conditions (Figure 3.6A). The expression level of the MSAS-type gene was significantly higher at pH 8.5 in MA than at pH 6.5 in MGA (P=0.0001), but there was no expression at the other pH values or the other media (Figure 3.6B). When comparisons were made between four media compositions at the same pH level for wA1 (pH 4.5), no significant difference in mRNA expression was detected (Figure 3.6C). However, at pH 6.5 the expression level of the wA1-type gene in MGA was significantly higher (P=<0.0008) when compared to expression levels in the other media compositions at pH 6.5 (Figure 3.6D). A rehydrated thallus of *R. dilacerata* that had been stored in a desiccated state at room temperature for several months was tested for wA1-type gene expression as a comparison. The wA1-type expression level in the rehydrated thallus was significantly higher (P=0.0007) than the expression level in the cultured mycobiont at pH 4.5 (Figure 3.6C) but lower than that of the cultured mycobiont at pH 6.5 (Figure 3.6D). These pH levels and media conditions also showed the largest number and relative amounts of secondary metabolites (Table 3.1).

Figure 3. 6. Real Time PCR analysis of A) the wA1-type PKS gene and B) the MSAS-type PKS gene, C) the wA-type PKS gene at pH 4.5, and D) the wA-type PKS gene at pH 6.5. Real time analyses are relative to expression of the internal standard, mtSSU gene. Expression levels were compared between pH levels (3.5, 4.5, 6.5, and 8.5) within each of four media {MA (malt), MGA (malt-glucose), MGYA (malt-glucose-yeast), MYA (malt-yeast)} and the rehydrated thallus (T). Expression levels on the y-axis represent expression of the PKS genes relative to the internal control. Vertical bars represent standard deviation of three replicates.
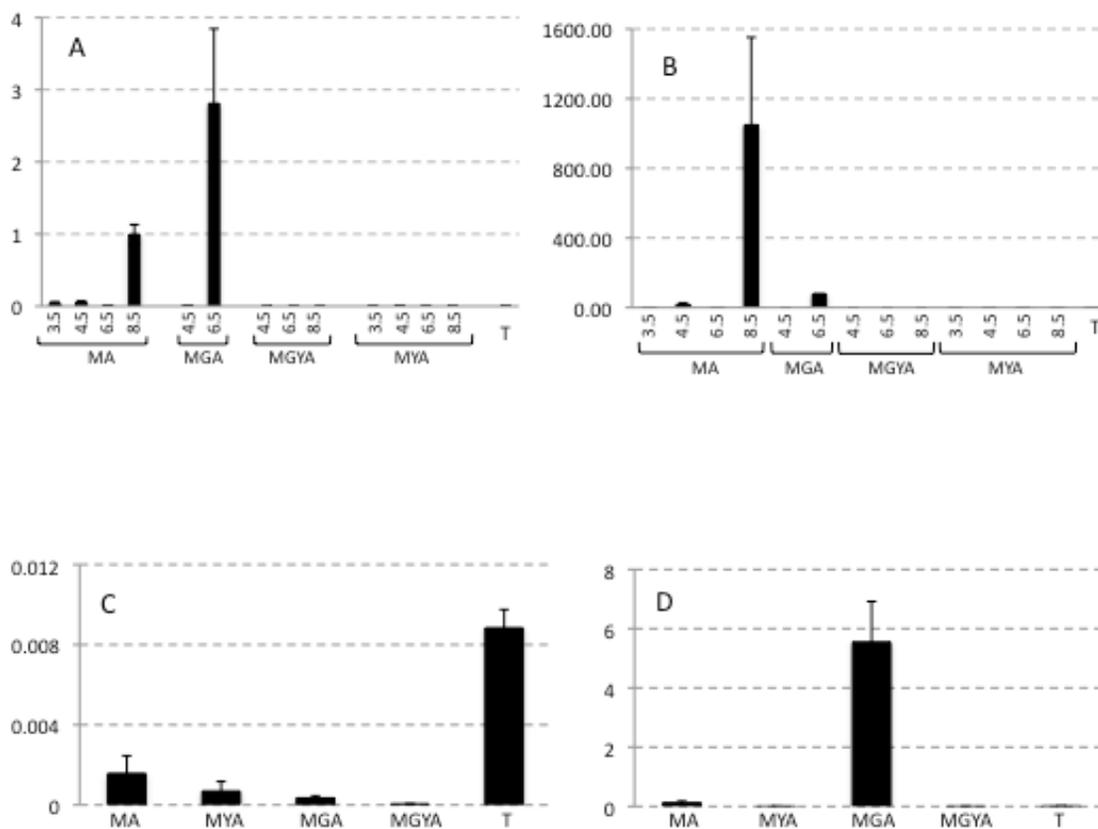
3.5. Discussion

3.5.1. Growth of R. dilacerata

The standard growth curve for colonies of *R. dilacerata* reflects the logarithmic growth as described for other non-lichen fungi (Meletiadis et al. 2001; Trinci 1969), and for lichen-forming fungi, *R. siliquosa* (Hamada 1989) and *Acarospora fuscata* (Ahmadjian 1961). As the radial growth began to slow down, growth continued to be observed as an increase in vertical colony size but that was not measured in this study to avoid destructive sampling so colonies can be used for polyketide detection. Vertical growth has also been observed in other lichen fungi in culture (Fazio et al. 2009, Culberson and Armaleo 1992). Variability in growth of colonies that were isolated from different apothecia may reflect slightly different microconditions in the media. Variability in growth of *Entomosporium mespili* from conidiospores was also reported (Baudoin 1986). In the natural habitat, the wide range of tree species that can serve as substrate for *R. dilacerata* (Goward and Ahti 1992, Gowan and Brodo 1988, Bird et al. 1980, Ahti 1964) suggests this species can tolerate a breadth of conditions including a range of bark pH levels and nutrient availability.

This study shows that the nutrient composition and pH level of the growth media influence radial colony growth and colony morphology of *R. dilacerata*. The differences in colony color and texture of *R. dilacerata* sub-cultured on five different media (GA, MA, MYA, MGA, MGYA) may be partially explained by the carbohydrate and nitrogen sources. Although Fazio et al. (2009) reported no morphological difference between colonies grown on Lilly and Barnett (LB), and two types of malt-yeast media, a color

difference was apparent in this study between the glucose-containing media and the non-glucose media (Figure 3.2). Similar color changes were reported for *R. siliquosa* (Huds.) A.L.Sm. (Hamada 1989) and *R. fastigiata* (Pers.) Ach. (Ahmadjian 1961) and for the plant pathogenic biotroph, *E. mespili* (DC.) Sacc. (Baudoin 1986a, 1986b). The form of carbohydrate may also influence the growth of the mycobiont. Maltose is better than glucose for growth of *Aspergillus niger* (Xu et al. 1989) and dextrose, yeast extract and sodium thioglycolate produced the best growth for most of 12 mycobionts tested (Ahmadjian 1961). The yeast extract, which provides a nitrogen source, would be expected to promote growth. However, the addition of glucose to a medium that already contains other nutrients may not impede fungal growth. In sufficient quantities with other nutrients, glucose may promote growth but the influence of glucose may be specific to the fungal species. For example, Guo et al. (2009) reported the optimal concentration of glucose in their study to be 40 g/L, whereas Diedhiou et al. (2004) reported growth of Thelephoroid fungal species at 0.1 g/L. Fungal growth using other levels of glucose were measured in *Scleroderma dictyosporum*, which required between 1.0 g/L and 10 g/L to sustain growth, and *Aspergillus nidulans*, which required a glucose concentration of 1.2 g/L (Trinci 1969). The glucose concentration in this study was 10 g/L, falling within the range reported, but glucose must be supplemented with other nutrients to be effective for growth as shown by negligible growth in the medium containing glucose alone (Figure 3.1).

The optimal pH level for the largest colony growth of *R. dilacerata* is pH 4.5 in MGYA, and pH 6.5 in MGYA (Figure 3.3). The optimal pH level for culture growth of non-lichenized fungi is reported to be between pH 4.0 and 7.0 (Guo et al. 2009, Joe et al.

2004, Lee et al. 2004, Nakamura et al. 2004, Xu et al. 2003). Growth and polyketide production for *R. siliquosa* occurred at pH 6.5 (Hamada 1989) and the range of pH levels for many mycobionts was pH 4.5 to pH 6.5 (Ahmadjian 1961). This may be explained if more nutrients are soluble at lower pH levels than at higher levels making them more available to plants and fungi. The findings in this study and those from the literature suggest that the lower pH levels between 4.5 and 6.5 are optimal for growth of *R. dilacerata* and a pH level outside this range may prevent many nutrients from being available.

3.5.2. Stress induces the production of secondary metabolites

The optimal pH level for growth of *R. dilacerata* (pH 4.5 to 6.5) is lower than the pH level, which produces the largest number of compounds (pH 8.5; Table 3.2). One of the triggers for production of secondary compounds is stress (Fox and Howlett 2008). During maximum growth most available nutrients are used to build tissue, but when one or more nutrients become depleted and the medium begins to dehydrate, mycelial growth begins to slow down. The accumulated carbohydrates may be shifted to other pathways to produce secondary metabolites that are not essential for growth. Production of secondary metabolites in cultured mycobionts has been linked to dehydration (Stocker-Wörgötter 2001, Culberson and Armaleo 1992).

Secondary metabolism may include the use of malonate to produce polyketides and fatty acids and these molecules may undergo further chemical transformations to produce additional secondary metabolites (Moore 2003, p. 115). Although usnic and divaricatic acids were not detected from the aposymbiotic mycobiont, other products

were detected that included fatty acids based on ^{1}H NMR analysis. It is not unusual for polyketides (usnic and divaricatic acids) to be absent from the cultured mycobiont (Molina et al. 2003). The numbers of byproducts from sub-optimal growing conditions may be an indication of the shifting of metabolism either as a byproduct or in response to stressful conditions such as elevated pH, which may be linked to pH-dependent nutrient availability and tolerance (Paul et al. 2009).

Environmental conditions, reflected by the culturing conditions in this study, influenced the production of secondary metabolites in *R. dilacerata*. is the results are supported by the findings of Brunauer et al. (2007) where a higher number of secondary metabolites were produced by the cultured mycobiont when compared to the lichen, *Xanthoria elegans*. In contrast, Solhaug and Gauslaa (2004) tested the effect of different carbohydrates on the activation of polyketide production in naturally grown lichens. They observed increased levels of polyketide production when carbohydrates were supplied by photobionts rather than artificially. The findings of this study are supported by other studies where environmental conditions were suggested to affect levels of secondary metabolism in fungi (Shwab and Keller 2008, Stocker-Wörgötter 2007, Penalva and Arst 2002).

3.5.3. PKS gene expression

The increase in expression levels of the wA1-type PKS gene in the maltose media when supplemented with glucose (MGA, pH 6.5; Figure 3.6A) may be explained if the unavailable nutrients at the high pH level prevent carbon from being incorporated into growth allowing for an accumulation of carbon. The carbon accumulated would be

diverted into the acetate polymalonyl pathway. However, when the MG medium was supplemented with yeast (MGYA)**,** a nitrogen source, the wA1 PKS gene expression was downregulated, which may be explained if the presence of nitrogen diverted the carbon into a different metabolic pathway leading to better growth and development. This result was consistent with the finding that colony diameter was highest when *R*. *dilacerata* was grown in MGY agar. Fazio et al. (2009) indicated that lichenized fungi may switch between polyketide and fatty acid production and the production of fatty acids and acylglycerides increased when growth conditions are unfavorable for the synthesis of lichen secondary metabolites. The mycobiont of *Parmotrema reticulata* Taylor when cultured on solid media (MYA and malt-yeast-sucrose) produced triacylglycerides but not lichen secondary metabolites (Bertoni et al. 2000). Bertoni et al. (2000) reported similar results with the mycobiont of *P*. *eciliatum* (Nyl.) Hale and *Flavoparmelia exornata* (Zahlbr.) Hale cultured on malt-yeast-sucrose. Certain environmental factors are essential for the induction and suppression of the acetate polymalonyl pathways (Stocker-Wörgötter 2007). Since the wA1-type PKS genes are common in lichen fungi (Armaleo et al. 2011, Sanchez et al. 2008, Schmitt et al. 2008, Opanowicz et al. 2006), it would be expected that growth conditions would influence the expression of the wA1 type gene in *R*. *dilacerata*.

Expression of the MSAS-type gene was detected at pH 8.5 in MA (Figure 3.6B), which are slightly different set of conditions than those described for the wA1-type PKS gene expression. The MSAS-type genes have been reported to occur in lichen-fungi (Schmidt et al. 2008) and bourgeanic acid, a MSAS-type polyketide is known to be produced by some members of the genus *Ramalina* (Stocker-Wörgötter 2008) but it has

not been reported in *R. dilacerata*. These results indicate that gene expression may be

influenced by both nutrient source and pH level of the media. The pH level of the growth

medium for *Aspergillus nidulans* was found to regulate *pal* and *pacC* gene expression

(Caddick et al. 1986). These genes encode transcription factors, which may be involved

in the regulation of the expression of genes such as PKS gene clusters (Fox and Howlett

2008). The level of PKS gene expression in many fungal PKS clusters may be very low

and often individual metabolites cannot be detected under cultured conditions (Brakhage

et al. 2008). While the wA1-type gene was found to be upregulated in only two sets of

conditions (Figure 3.6A), neither usnic nor divaricatic acids could be detected in the

cultured colonies. The absence of usnic and divaricatic acids may be explained if the

wA1-type gene paralog that was amplified in this study was not responsible for encoding

the usnic and divaricatic acid polyketide synthases. Large numbers of paralogs with

unknown functions have been reported for fungi (Armaleo et at. 2011, Gilsenan et al.

2009). It may also be explained if colony growth had not progressed to the stage required

for production of these two polyketides and were producing intermediate metabolites. If

limiting nutrients promoted the production of aerial hyphae, the micro-environment, with

desiccating conditions, would trigger polyketide biosynthesis (Culberson and Armaleo

1992). In this study the media composition and pH levels needed for slower growing

colonies may have provided the conditions necessary for the initiation of some polyketide

biosynthesis along with an increase in the production of triglycerides. However, the

desiccating conditions may not have been favorable, or progressed far enough, for the

production of the polyketide products observed in the native thallus.

3.5.4. Relationship of optimal culture conditions to habitat

The habitat of *R. dilacerata* includes open, well-lit habitats on a wide range of

trees such as *Alnus, Quercus, Populus, Betula, Picea,* and *Salix* (Goward and Ahti 1992,

Gowan and Brodo 1988, Bird et al. 1980, Ahti 1964). Depending on the habitat, *R.*

dilacerata may tolerate different pH levels of the substrata. For example, the bark pH

level of *Quercus* is pH 4.5 to pH 6.0 (Spier et al. 2010) while the bark pH level of *Picea*

and *Pinus* is pH 3 to pH 4.5 which changes depending on height within the tree canopy

(Marmor et al. 2010). Meanwhile the bark pH level of *Alnus, Betula, Prunus, Populus,*

and *Salix* is pH 4.5 to pH 6.0 (Werth 2001). The pH level of the optimal growing

conditions for the mycobiont is consistent with the bark pH level of its habitat. Similarly,

the use of a complex nitrogenous medium for best growth also reflects its habitat

described by McCune and Geiser (2009) as eutrophic and requiring moderate to high

nitrogen.

In summary, this study showed that pH level and media composition can

influence the production of secondary products and expression of two types of PKS genes

in a cultured lichen fungus. Further study is needed to determine the identity of the

natural products and the genes specific for the products. It is difficult to use culture

conditions of one symbiont to draw conclusions of the habitat of the lichenized symbiont;

however, the pH levels and media composition used in this study are similar with those of

the natural habitat of the lichen, suggesting a comparison can be made. The negative

relationship between colony diameter and the number of secondary metabolites produced

is consistent with the theory that cell growth and secondary metabolism are competing

processes (Bu'Lock 1961) where secondary metabolites are produced only after one or more nutrients become limiting to mycelial growth. Although optimal growth occurs at the lower pH levels (4.5 and 6.5) and in the more complex medium (MGYA), the larger numbers of compounds are produced at the higher pH levels (6.5 and 8.5) and in three malt-containing media. High pH levels may cause nutrients to be insoluble resulting in the accumulation of carbon compounds that can be utilized by secondary metabolism. Although progress is being made on the characterization and expression of PKS genes in fungi, little is known about PKS gene expression in lichen-fungi. This is the first study, to our knowledge, to focus on gene expression in the cultured mycobiont belonging to the genus *Ramalina*. An understanding of the growth conditions that influences expression of secondary metabolite genes will provide insight into the biosynthesis of related secondary metabolites.

CHAPTER 4

Exploring the function of PKS paralogs in the *Cladonia chlorophaea* species complex

4.1. Abstract

Lichen-forming fungi produce a broad diversity of polyketides, which are synthesized by multidomain polyketide synthases (PKS). Multiple genes that encode PKS have been detected in fungal genomes, and phylogenetic hypotheses have been proposed, but the function of many gene paralogs is still unknown. In this study, 56 members of a closely related species complex, the *Cladonia chlorophaea* complex, were screened with 12 PKS primer sets for detecting PKS paralogs known to be present in one member of the complex, *C. grayi*. A PKS gene phylogeny was constructed from the deduced amino acid sequences and gene function was inferred using the synonymous and non-synonymous substitution ratio. A species tree was also inferred from the ITS rDNA. Both reducing and non-reducing PKS genes were detected in members of *C. chlorophaea* complex, even

though all polyketides detected from these species were not reduced polyketides. The gene phylogeny indicated three independent origins of non-reducing paralogs in the *C. chlorophaea* complex, and there was evidence for purifying and positive selection. This research provided insight into the evolution of the PKS genes in the *C. chlorophaea* group, inferred functional groups of genes, and identified potential genes that produce the non-reduced polyketides present in the *C. chlorophaea* species complex.

4.2. Introduction

Members of the genus *Cladonia* are lichen-forming fungal species within the family Cladoniaceae (Miadlikowska et al. 2006, Ahti 2000). The genus *Cladonia* is comprised of seven taxonomic Sections (Ahti 2000) and three sections in the former genus *Cladina* (Ahti and DePriest 2001), which was proposed for further revision based on phylogenetic analyses (Stenroos et al. 2002, DePriest 2000). *Cladonia* Section *Cladonia* consists of many of the cup-forming lichens with brown apothecia and a number of species complexes including the *Cladonia chlorophaea* species complex (Ahti 1966). There are 13 chemical variants known in the species complex producing 14 secondary products (Culberson and Kristinsson 1969). Genetic variability was reported for *C. chlorophaea* (DePriest 1993) and the *C. chlorophaea* species complex was shown to be polymorphic (Kotelko and Piercey-Normore 2010, Beiggi and Piercey-Normore 2007, DePriest 1994) but they may be restricted to different habitats (Oksanen 1987, Hennings 1983, Wetherbee 1969).

Fungal polyketide synthesis is catalysed by iterative Type I Polyketide Synthase (PKS), a multidomain protein (Keller et al. 2005) with a linear succession of

ketosynthetase (KS), acyl transferase (AT), dehydratase (DH), enoyl reductase (ER),

ketoreductase (KR), acyl carrier protein (ACP) and thioesterase (TE) domains (Graziani

et al. 2004). The KS, AT, and ACP domains comprise the simplest fungal PKSs, which

are required for carboxylic acid condensation (Hopwood 1997) but they lack any

chemical reduction in their chemical structure (Bingle et al. 1999). The fungal PKSs that

include KR, DH and ER domains, produce polyketides with varying degrees of chemical

reduction in structure. The known polyketides produced by most *Cladonia* species

represent non-reduced chemical structures and most PKS genes for specific polyketides

have not yet been identified.

Polyketide synthase (PKS) genes are known to have large numbers of paralogs in

fungi (Armaleo et al. 2011, Sanchez et al. 2008, Schmidt et al. 2008, Hoffmeister and

Keller 2007, Opanowicz et al. 2006, Nierman et al. 2005). Gene paralogs are thought to

arise through gene duplication events, gene decay, mobile elements, gene fusion, or other

mechanisms (reviewed by Long et al. 2003). Alternative explanations for multiple genes

include horizontal gene transfer from bacteria to fungi (Schmitt and Lumbsch 2009), or

between different fungi (Khaldi et al. 2008), which may have resulted in gains and losses

of genes (xenologs/paralogs) through evolution (Blanco et al. 2006, Koonin 2005).

Current phylogenomic analyses based on PKS gene evolution suggest that a single fungal

genome may contain more than one PKS gene paralog (Schmitt and Lumbsch 2009,

Kroken et al. 2003) but produce even fewer polyketides (Sanchez et al. 2008). In lichen-

forming fungi, six paralogs of the KS domain of PKS genes have been detected in

members of the lichen family Parmeliaceae (Opanowicz et al. 2006) and 12 PKS paralogs

in the Cladoniaceae (Armaleo et al. 2011). Non-lichenized fungi also show high levels of

gene parology such as *Aspergillus nidulans*, which contains 27 PKS paralogs but produces only seven gene products (Sanchez et al. 2008).

The production of fumarprotocetraric acid in the *C. chlorophaea* complex (Culberson and Kristinssen 1969) has been shown to vary with geographic location (Culberson et al. 1977). The presence of additional polyketides such as grayanic and merochlorophaeic, acids define the chemical species in the complex (Orange 1992, Culberson et al. 1985). Other secondary metabolites are sometimes present in small amounts. If a single gene is responsible for production of one or related polyketides then one PKS paralog should be conserved among allies of the species complex. If several gene paralogs are responsible then these genes should also be equally conserved among individuals of close allies. Most studies that report paralogs show their occurrence in one or a few individuals of the same species. Few studies have screened for their occurrence across a broad range of individuals in the same and closely related species.

The general goal of this study was to explore the distribution and infer function of the 12 PKS paralogs from *C. grayi* among closely related members of the *Cladonia chlorophaea* complex, specifically *C. pyxidata*, *C. pocillum*, and *C. chlorophaea* s.s. The specific objectives of this study were: i) to screen *C. chlorophaea* allies for the presence of PKS paralogs, ii) to estimate a PKS gene phylogeny in a closely related group of species, and ii) to investigate PKS gene function among the species.

4.3. Materials and Methods

4.3.1. Lichen material and Chromatography

Thin Layer Chromatography (TLC) was performed on all samples collected in this study (Table 4.1) to confirm the major secondary metabolites present in each thallus. The TLC protocol was modified from Orange et al. (2001) and Culberson (1972) using solvent A (toluene 180mL: dioxane 45mL: glacial acetic acid 5mL). Spot characteristics of secondary metabolites outlined in Orange et al. (2001) and Huovinen and Ahti (1982) were consulted for polyketide identification. Positive controls were taken from herbarium specimens that are known to contain large quantities of the metabolite such as merochlorophaeic acid in *C. wainioi* (Normore 7209), grayanic acid in *C. grayi* (Wallace 298e), fumarprotocetraric acid in *C. verruculosa* (Brodo 26541), sekikaic acid in *Cliostomum toensbergii* (Brodo 17414), and homosekikaic acid in *C. rei* (Brodo 29600B) and *Ramalina intermedia* (Normore 5512).

Table 4.1. List of species, collector and collection numbers, location and date of

collection, and NCBI GenBank accession numbers for the nucleotide sequences (ITS1

rDNA and PKS) from the specimens used in this study.

Species	Collector and collection number	Location and date of collection	Accession for ITS	Accession for PKS
Aspergillus clavatus	Strain = "NRRL 1"	USA, submitted by Nierman, W.C,		XP001273762
Aspergillus clavatus	Strain = "NRRL 1"	USA, submitted by Nierman, W.C.		XM001268489
Aspergillus flavus	Strain = "NRRL3357"	USA, submitted by Nierman, W.C.		XP002381496
Aspergillus niger	Strain = "CBS 513.88"			XM001402371
Aspergillus terreus	Strain = NIH2624	USA, submitted by Nierman, W.C.		XP001210231
Bipolaris maydis	Strain="C4; ATCC 48331"	USA, submitted by Kroken, S. and Turgeon, B.G.		AAR90276
Botryotinia fuckeliana	Isolate T4	Genoscope, C. E.A. France		CCD56082
Cladonia chlorophaea	Kotelko 916	Canada, Yukon, Whitehorse windmills, 2005.		
Cladonia chlorophaea	Yahr 5307	U.K., Scotland, S. Aberdeenshire, Creg Choinnich wood, 2006.		
Cladonia chlorophaea	Yahr 5335	U.K., England, Northumbria, Hepburn Wood, 2007.		
Cladonia chlorophaea	Yahr 5336	U.K., England, Northumbria, Hepburn Wood, 2007.		1DA-KF483879 5DA-KF483907 11DA-KF483934 13DA-KF483949
Cladonia	Yahr 5342	U.K., Scotland, Mid	KF378715	1DA-KF483880

chlorophaea		Perthshire, Enochdhu, 2007.		2DA-KF483892 5DA-KF483908 7DA-KF483918
Cladonia chlorophaea	Yahr 5346	U.K., Scotland, S. Aberdeenshire, Creg Choinnich wood; 2007.	KF378718	
Cladonia chlorophaea	Yahr 5347	U.K., Scotland, S. Aberdeenshire, Creg Choinnich wood; 2007.		
Cladonia chlorophaea	Yahr 5361	U.K., England, S Lancashire, Chapeltown.	KF378717	1DA-KF483881 2DA-KF483891 11DA-KF483935 13DA-KF483948
Cladonia chlorophaea	Kelly	U.K., Richmond, Surrey, Kew, Royal Botanic Gardens	FR799156	
Cladonia coccifera	Normore 10134	Canada, Manitoba, Wapusk National Park, Noochewaywum Creek, 2010.	KF378721	
Cladonia coccifera	Normore 6592	Canada, Ontario, east of Borups Corners, 2006.	KF378722	
Cladonia coccifera	Strain CL93	Czech Republic (Steinova et al., 2011), unpublished	HE611161	
Cladonia ecmocyna	Burgaz, isolate 6MACRO	Spain, (Pino-Bodas et al., 2011)	JN811398	
Cladonia ecmocyna	Rui and Timdal, isolate 1ECMO	Norway, (Pino-Bodas et al., 2011)	JN811399	
Cladonia ecmocyna	Hansen 747 (H)	Greenland, (Stenroos et al., 2002)	AF455199	
Cladonia fimbriata	Normore 9932	Canada, Manitoba, Wapusk National Park, vegetated beach ridge, 2010.	KF378724	
Cladonia fimbriata	Normore 5600	Canada, Manitoba, north of Grand Rapids, 2006.	KF378725	
Cladonia fimbriata	Yahr 5328	U.K., England, Northumbria, Hepburn Wood, 2007.	KF378723	2DA-KF483895 7DA-KF483926 13DA-KF483957 MSAS-DA-KF483974

Cladonia fimbriata	Dolnik 1060	Germany (Dolnik et al., 2010)	GU188406	
Cladonia gracilis	Normore 7988	Canada, Manitoba (Fontaine et al., 2008)	FJ536358	
Cladonia gracilis	Ahti, isolate 23GRAC	Russia (Pino-Bodas et al., 2011)	JN811389	
Cladonia gracilis	Talbot and Scholfield, isolate 28GRAC	USA (Pino-Bodas et al., 2011)	JN811396	
Cladonia grayi	Culberson 19971	USA, North Carolina (Duke University cryptogamic herbarium)		1DA-KF483877 2DA-KF483888 3DA-KF483905 5DA-KF483916 7DA-KF483927 10DA-KF483932 11DA-KF483939 12DA-KF483945 13DA-KF483947 14DA-KF483966 MSAS DA-KF483975
Cladonia grayi	Normore 10130	Canada, Manitoba, Wapusk National Park, Noochewaywum Creek, 2010.	KF378727	
Cladonia grayi	Normore 9644	Canada, Manitoba, Sandilands Provincial Forest, Hwy 210, 2009.	KF378716	1DA-KF483883 3DA-KF483898 5DA-KF483910 7DA-KF483920 10DA-KF483930 11DA-KF483937 12DA-KF483942 13DA-KF483951 14DA-KF483960 MSAS-DA-KF483969
Cladonia grayi	Normore 7209	Canada, Ontario, west of Spanish, 2006.	KF378726	
Cladonia grayi	Normore 971	Canada (Beiggi and Normore, 2007)	DQ530201	
Cladonia grayi		Armaleo et al. (2011)		GU930713
Cladonia magyarica	Sohrabi 4553	Iran, East Azerbaijan, 2005	FJ756727	

Cladonia magyarica	MACB: 98243	Hungary (Pino-Bodas et al., 2013)	KC526136	
Cladonia magyarica	Nadeina (H)	Ukraine (Pino-Bodas et al., 2013)	KC526137	
Cladonia maxima	Normore 7934	Canada (Fontaine et al., 2010)	FJ536367	
Cladonia maxima	Normore 7861	Canada (Fontaine et al., 2010)	FJ536370	
Cladonia maxima	Normore 7935	Canada (Fontaine et al., 2010)	FJ536371	
Cladonia merochlorop haea	Normore 8787	Canada, Manitoba, Sandilands Provincial Forest, 2009.		1DA-KF483878 3DA-KF483896 5DA-KF483906 7DA-KF483917 10DA-KF483928 11DA-KF483933 12DA-KF483940 13DA-KF483946 14DA-KF483958 MSAS-DA-KF483967 16DA-KF483976
Cladonia merochlorop haea	Normore 8792	Canada, Manitoba, Sandilands Provincial Forest, 2009.		1DA-KF483882 3DA-KF483897 5DA-KF483909 7DA-KF483919 10DA-KF483929 11DA-KF483936 12DA-KF483941 13DA-KF483950 14DA-KF483959 MSAS-DA-KF483968
Cladonia merochlorop haea	Normore 9914	Canada, Manitoba, Wapusk National Park, vegetated beach ridge, 2010.	KF378720	
Cladonia merochlorop haea	Normore 10138	Canada, Manitoba, Wapusk National Park, Noochewaywum Creek, 2010.	KF378719	
Cladonia merochlorop haea	Normore 6824	Canada, Quebec, northwest of Le Domaine, 2006.		

Cladonia merochlorophaea	Stenroos 5168	Finland (Stenroos et al., 2002).	AF455227	
Cladonia pocillum	Kotelko 962	Canada, Yukon, Destruction Bay, 2005.	KF378729	
Cladonia pocillum	Kotelko 869	Canada, British Columbia, north of Buckinghorse River, 2005.	KF378728	2DA-KF483893 3DA-KF483899 5DA-KF483911 7DA-KF483921 12DA-KF483943 13DA-KF483952 14DA-KF483961 MSAS- KF483970
Cladonia pocillum	Kotelko 945	Canada, Yukon, Takini burn, 2005.		
Cladonia pocillum	Kotelko 951	Canada, Yukon, Aishihik Lake Road, 2005.		
Cladonia pocillum	Kotelko 966	Canada, Yukon, Congdon Creek Campground, 2005.		
Cladonia pocillum	Kotelko 974	Canada, Yukon, east of Aishihik Lake Rd., 2005.		
Cladonia pocillum	Normore 5556	Canada, Manitoba, Long Point, 2006.		
Cladonia pocillum	Normore 6081	Canada, Manitoba, north of The Pas, 2006.		
Cladonia pocillum	Normore 6085	Canada, Manitoba, south of The Pas, 2006.		
Cladonia pocillum	Normore 9061	Canada, Manitoba, Wapusk National Park, open sandy beach ridge, 2009.		1DA-KF483884 2DA-KF483894 3DA-KF483900 5DA-KF483913 7DA-KF483922 10DA-KF483931 11DA-KF483938 13DA-KF483953 14DA-KF483962 MSAS-DA-KF483971
Cladonia pocillum	Normore 9460	Canada, Manitoba, Hwy 391 outside Leaf Rapids, 2009.		1DA-KF483885 3DA-KF483901 5DA-KF483912 7DA-KF483923 13DA-KF483954 14DA-KF483963 MSAS-KF483972

16DA-KF483977

Cladonia pocillum	Normore 10200	Canada, Manitoba, Wapusk National Park, south of Owl River, 2010.		
Cladonia pocillum	Normore 6102	Canada, Manitoba, Mossy Portage Road, 2006.		
Cladonia pocillum	Normore 6112	Canada, Manitoba, Highway 6 south of Highway 60, 2006.		
Cladonia pocillum	Normore 6118	Canada, Manitoba, Long Point, 2006.		
Cladonia pocillum	Kotelko 870	Canada, British Columbia, north of Buckinghorse River, 2005.	FJ756728	
Cladonia pocillum	Kotelko 946	Canada, Yukon, Takhini burn, 2005.	FJ756729	
Cladonia pyxidata	Kotelko 726	Canada, British Columbia, 2005		1DA-KF483886 2DA-KF483889 3DA-KF483902 5DA-KF483914 7DA-KF483924 12DA-KF483944 13DA-KF483955 14DA-KF483964
Cladonia pyxidata	Kotelko 905	Canada, Yukon, west of Johnsons Crossing, 2005.		
Cladonia pyxidata	Kotelko 938	Canada, Yukon, Fox Lake, 2005.		
Cladonia pyxidata	Kotelko 950	Canada, Yukon, Aishihik Lake Road, 2005.		
Cladonia pyxidata	Kotelko 999	Canada, Yukon, Mount. Goldenside, 2005.		
Cladonia pyxidata	Normore 9458	Canada, Manitoba, Hwy 391 outside Leaf Rapids, 2009.		1DA-KF483887 2DA-KF483890 3DA-KF483903 5DA-KF483915 7DA-KF483925 13DA-KF483956 14DA-KF483965 MSAS-DA-KF483973

Cladonia pyxidata	Normore 9817	Canada, Manitoba, Wapusk National Park, Rupert Creek, 2010.		
Cladonia pyxidata	Normore 9996	Canada, Manitoba, Wapusk National Park, forested beach ridge, 2010.		
Cladonia pyxidata	Normore 6086	Canada, Manitoba, south of West Ray, 2006.		
Cladonia pyxidata	Normore 6115	Canada, Manitoba, Long Point, 2006.		
Cladonia pyxidata	Normore 6576	Canada, Ontario, east of Kenora, 2006.		
Cladonia pyxidata	Yahr 4952	U.K., Scotland, Mid Perthshire, Carie forest, 2006.		
Cladonia pyxidata	Yahr 4953	U.K., Scotland, E Perthshire, Blair Atholl.		
Cladonia pyxidata	Yahr 5309b	U.K., Scotland, Midlothian, Traprain Law, 2006.		
Cladonia pyxidata	Yahr 5340	U.K., Scotland, Mid Perthshire, Enochdhu, 2007.	KF378730	3DA-KF483904
Cladonia pyxidata	Yahr 5343	U.K., Scotland, E Perthshire, Blair Atholl, 2007.		
Cladonia pyxidata	P Lusy s.n	U.K., England, NW Yorkshire, Wensleydale, 2007.		
Cladonia pyxidata	Normore 6578	Canada, Ontario, east of Kenora, 2006.	FJ756757	
Cladonia pyxidata	Normore 5750	Canada, Manitoba, Hwy 391, 2006.	FJ756758	
Cladonia rei	Vondrak 7024	Czech Republic (Pino-Bodas et al. 2010)	FN868587	
Cladonia rei	Vondrak 7006	Czech Republic (Pino-Bodas et al. 2010)	FN868588	
Cladonia rei	Aptroot 68588	Netherlands (Pino-Bodas et al. 2010)	FN868590	

Gibberella moniliformis	Strain="ATCC 38932; FGSC 7600"	USA, submitted by Kroken, S. and Turgeon, B. G.	AY495595
Sordaria macrospora	Strain="k-hell"	Germany, submitted by Nowrousian, M.	XP003350625
Umbilicaria torrefacta	Strain = "gbM25"	Austria, submitted by Brunauer, G.	ACJ24814
Zymoseptoria tritici	Strain = "IPO323"	Goodwin et al. (2011)	XP003849645

4.3.2. DNA extraction and amplification

Total DNA was extracted from 1-2 podetia following a CTAB DNA extraction protocol modified from Grube et al. (1995). The ITS1 of the nuclear ribosomal DNA (rDNA) was amplified using the primer pair ITS1F (Gardes and Bruns 1993) and ITS2 (White et al. 1990) and a PCR cycle consisting of initial denaturing at 94°C for 5 min, then 30 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 45 sec for 2 cycles, then 58°C for 45 sec for 2 cycles, 56°C for 45 sec for 2 cycles, 54°C for 45 sec for 2 cycles and 52°C for 45 sec for the remaining 22 cycles, and extension at 72°C f or 1 min. The amplification of PKS paralogs was done using a series of primers designed in this lab (Table 4.2). These primers were designed from fungal PKS sequences (kindly provided from D. Armaleo, Duke University) that are represented by scaffolds of Nuclear Genome Assembly V1.0 from the Joint Genome Insititute (JGI; http://genome.jgi-psf.org/Clagr2/Clagr2.home.html). Amplification of the PKS genes was done with initial denaturing at 94°C for 5 min, then 33 cycles of denaturing at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1.5 min. All amplifications were performed in a thermal cycler (Biometra T-Gradient; Tampa, FL, USA) in 20 μL reaction volumes with 1X buffer (200 mM Tris-HCl, 500 mM KCl) with 1.25 units GO Taq (Go Taq [®] Hot Start polymerase, Promega), 3.125 mM $MgCl_2$ 1.25 mM of each dNTP, 1.0 M of each primer, and about 50 ng of DNA.

Table 4.2. List of primers showing primer names, target gene, primer sequences (5'to 3'),
source of primer, and the length (base pairs) of the amplified product.

Primer name	Source	Primer sequence (5'-3')	Product size (bp)
ITS-1F	Gardes and Bruns (1993)	CTTGGTCATTTAGAGGAAGTA	300
ITS2	White et al (1990)	GCTGCGTTCTTCATCGATGC	300
PKS1F	This lab (from Bingle et al.1999)	TACGAAGCCCTAGAAATGGC	450
PKS2R	This lab (from Bingle et al.1999)	ACGTTTGGCAGTTTCCTGTC	450
PKS-1-DA-F	This study	TGCCTTTCAAGCGATGGACT	600
PKS-1-DA-R	This study	CAGGAGAATGCGGAATCGTT	600
PKS-2-DA-F	This study	ATAGCCACTCAGGGACAGAT	650
PKS-2-DA-R	This study	TGTGTTTCGCATCAGGCACT	650
PKS-3-DA-F	This study	GGTGAGCTATGAAGCGCT	700
PKS-3-DA-R	This study	GGCATCGTAATACCAGCAGT	700
PKS-5-DA-F	This study	CATCGTCCAACACTGAGTCT	700
PKS-5-DA-R	This study	GCCAGCATTCTTGTAGGTCT	700
PKS-7-DA-F	This study	AAGCCCTTGAGAATGCT	500
PKS-7-DA-R	This study	AGAGTCTCCATCTCGGAT	500
PKS-10-DA-F	This study	AAGTCACGTACGAAGCCGT	750
PKS-10-DA-R	This study	TACGCCGTATCAGCCAGAT	750
PKS-11-DA-F	This study	ATGCTTGGAAGGAGGTCT	750
PKS-11-DA-R	This study	AGGCTTCCCGAATAAGGT	750
PKS-12-DA-F	This study	ACGAGGCATTTGAGAACGGT	800
PKS-12-DA-R	This study	GAACCTAGTCTCACTGGTGT	800
PKS-13-DA-F	This study	GCAGCTGAAACTGATCCT	700
PKS-13-DA-R	This study	GTGCATCTCGACATAGCT	700
PKS-14-DA-F	This study	GATCGCAGAGACCAAAGT	1250
PKS-14-DA-R	This study	TGCGTGATAGACACTGCT	1250
PKS-16-DA-F	This study	CGATGTGGAGAAGATCCTT	1400
PKS-16-DA-R	This study	CCAGCATGTGGATGCGTTAT	1400
PKS-ORS-FDA-F	This study	ATGGATCCGCAGCAAAGACT	700
PKS-ORS-DA-R	This study	GACCTCCACCAGCTTTCAAT	700

4.3.3. Extracting DNA sequences from NCBI and JGI databases

Fifty-six samples (42 from North America and 14 from Europe) that include representatives from the seven broadly defined *Cladonia chlorophaea* complex species (*C. chlorophaea, C. fimbriata, C. grayi, C. magyrica, C. merochlorophaea, C. pocillum,* and *C. pyxidata;* Huovinen and Ahti 1982) in addition to *C. coccifera*, as a reference species, were used in this study to screen for the presence of PKS gene paralogs. BLASTn using each of the 12 paralogous sequences as queries, allowed for the recovery of twelve PKS gene sequences from eight fungal genera from NCBI GenBank. This data base search contributed to the PKS gene phylogeny data set which containined 91 nucleotide sequences from representatives of the *Cladonia chlorophaea* species complex and 11 *C. grayi* sequences from the GenBank and JGI databases.

Nucleotide sequences of the Internal Transcribed Spacer of ribosomal DNA (ITS rDNA) representing 11 species of *Cladonia* were used to construct a phylogeny to examine species evolution. Specimens are deposited with the cryptogams in the University of Manitoba Herbarium (WIN).

4.3.4. DNA sequencing and alignment

Four to six replicates of 50 μL reaction volumes of PCR product were pooled for DNA sequencing. The pooled 200-300 μL PCR product was precipitated by adding 2.5 volumes of 100% ethanol and 0.2 volumes of 5M NaCl and centrifuged at 13000 rpm for 10 min. The DNA pellet was dissolved in 20 μL sterile distilled water, and gel purified by excising the band from 1% agarose gel and purified using the Wizard ® SV Gel and

PCR Clean-Up System (Promega, Madison, WI, USA) following the manufacturer's

instructions. Cycle sequencing reaction volumes were 20 μL, which contained about 50

ng of purified DNA. BigDye V3.1 (Applied Biosystem, Foster City, CA, USA) and the

same PCR primers were used for sequencing. Post reaction clean up followed the

manufacturer's instructions for the ethylene diamine tetraacetic acid (EDTA) and ethanol

precipitation method. The dried product was dissolved in 20 μL formamide, denatured at

95ºC for 5 min., placed on ice, and loaded into a 96-well plate for sequencing on a 3130

Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

4.3.5. Data analysis

The fungal ITS1 rDNA phylogeny was inferred from 17 collected samples and 22

accessioned DNA sequences retrieved from NCBI GenBank. The PKS phylogeny was

inferred using the amino acid sequences from 113 sequences (91 collected samples, 11

Cladonia grayi sequences and 11 samples from NCBI GenBank). All nucleotide

sequences generated in this study have been deposited in NCBI GenBank. ITS sequences

were aligned using the program Se-Al v2.0a11 (Rambaut 2001) and were manually

adjusted. Amino acid sequences for the PKS paralogs were aligned using ClustalX 2.1

(Thompson et al. 1997).

Aligned sequences of both ITS1 rDNA and PKS alignments were subjected to

phylogenetic analyses using Maximum Parsimony (MP) in PAUP* 4.0b10 (Swofford

2003) and Bayesian analysis in MrBayes v3.2.1 x64 (Ronquist et al. 2011). The option

for the MP analyses was tree bisection and reconnection (TBR) branch swapping,

heuristic searches using 1000 random addition replicates, and bootstrap searches of 500

resamplings (Felsenstein 1985) using the MP option in PAUP. Bootstrap values greater than 70 are reported in the phylogenies. For Bayesian analysis of the ITS sequences, a six parameter hLRTs (Hierarchial Likelihood Ratio Tests) model was applied with a gamma shaped parameter and proportion of invariable sites uniformly distributed. This model was the best model estimated with Modeltest 3.7 (Posada and Crandall 1998). Bayesian analyses were performed using 7,500,000 generations for the PKS gene alignment and 1,000,000 for ITS1 rDNA alignment. Both runs converged on similar likelihood values and were terminated when the standard deviation of split frequencies fell below 0.01. Every 500th tree was sampled, and the first 25% of trees were discarded as burn-in for both PKS and ITS runs. Posterior probability values greater than 90 are reported on the phylogenies.

Possible gene function was inferred using Codon-based Test of Purifying Selection (Z-test of Selection) for analysis averaging over all sequence pairs as implemented in MEGA5 (Tamura et al. 2011). The variance of the difference was computed using the bootstrap method (500 replicates). The analyses in part are based on estimating the rates of Synonymous and Nonsynonymous substitutions (i.e., dN/dS) using the Nei-Gojobori method (Nei and Gojobori 1986).

To estimate the number of alleles for each PKS paralog, the aligned nucleotide (alleles) sequences were exported as Clustal W and saved as Pretty files. All the sequences in the paralog were compared to the same standard sequence (*C. merochlorophaea*, Normore 8787). The bases in the pretty file alignment, that were different from those in the standard sequence, were recorded and the number of alleles

were reported by manually recording the differences. Single changes were discarded and only two or more base changes were recorded when determining alleles.

The polyketide synthase gene region (domain) for each PKS paralog was confirmed by BLASTp searches in NCBI GenBank and JGI databases. The amino acid sequence of one sample from each paralog was used as the query and the three most similar sequences with e-score and maximum identity were recorded.

4.3.6. Scoring PKS paralogs

The presence and absence of each PKS paralog was screened using 14 sets of PKS specific primers (Table 4.2) to determine the presence or absence of PKS paralogs in 56 samples belonging to 8 different species of *Cladonia*. The presence or absence of PCR bands in the amplified PCR product was recorded by gel electrophoresis. ITS1 rDNA and the mitochondrial small subunit (mtSSU) of rDNA primers were used as positive controls to ensure the DNA was present even when no PKS fragments were amplified.

Principle Components Analysis was performed using frequency of each paralog in each of four species with JMP (ver 8.0.x).

4.4. Results

4.4.1. Polyketides

Fumarprotocetraric acid was detected in all individuals of all species examined, except for two samples of *C. coccifera* (Normore 6592 and Normore 10,134). All three samples of *C. fimbriata*, 17 samples of *C. pocillum* and nine samples of *C. pyxidata*

contained only fumarprotocetraric acid. All other specimens contained at least one additional major metabolite and some specimens contained trace amounts of other metabolites (Table 4.3). Polyketides are not listed for those species whose sequences were obtained from GenBank.

Table 4.3. Comparison of secondary products among members of *Cladonia chlorophaea* complex used in this study and findings from literature sources.

Species	Secondary products (from literature sources)	Secondary products (from this study)
C. chlorophaea	Fumarprotocetraric acid (Archer 1983, Hennings 1983, Culberson and Kristinsson 1969).	Fumarprotocetraric acid (N10201, N9813, N9862, N10022).
	Fumarprotocetraric acid, protocetraric acid and Cph-2 (Huovinen et al. 1990).	Fumarprotocetraric acid and sekikaic acid (tr) (Y5307).
		Fumarprotocetraric acid, sekikaic acid and merochlorophaeic acid (Y5335, Y5336, Y5342).
		Fumarprotocetraric acid, protocetraric acid and unknown at Rf=3 (Y5346).
		Fumarprotocetraric acid and protocetraric acid (Y5347).
		Fumarprotocetraric acid and protocetraric acid + atranorin (Y 5361).
C. mero-chlorophaea	Fumarprotocetraric acid, merochlorophaeic acid, 4'-O-methlycryptochlorophaeic acid, homosekikaic acid (tr), and cryptochlorophaeic acid (tr) (Archer 1983).	Fumarprotocetraric acid and merochlorophaeic acid (N9914, N10138).
	Fumarprotocetraric acid, merochlorophaeic acid, 4'-O-methlycryptochlorophaeic acid, cryptochlorophaeic acid (tr), paludosic acid (tr), lower homologue of merochlorophaeic acid (tr) (Hennings 1983).	Fumarprotocetraric acid, merochlorophaeic acid, and homosekikic acid (tr) (N6824).
		Fumarprotocetraric acid, merochlorophaeic acid (tr), and sekikaic acid (N8787).
	Sekikaic acid, homosekikaic acid (Hennings 1983).	Fumarprotocetraric acid, merochlorophaeic acid and 4'-O-methlycryptochlorophaeic acid (N8792).
	Merochlorophaeic acid, 4'-O-methlycryptochlorophaeic acid,	

	sekikaic acid, homosekikaic acid and 5 unknowns (Huovinen et al. 1990).	
C. mero-chlorophaea var. novo-chlorophaea	Fumarprotocetraric acid, 4'-O-methylnorsekikaic (tr); 4'-O-methylnorhomo-sekikaic (tr), unknown (tr) (Hennings 1983)	
C. pyxidata	Fumarprotocetraric acid protocetraric acid and Cph-2 (Huovinen et al. 1990).	Fumarprotocetraric acid and protocetraric acid (K726, K879, K999, N9996, Y4952, Y5309b).
		Fumarprotocetraric acid (K905, N6115, N6578, N9458, N9817, Y4953, Y5340, Y5343, s.n).
C. pocillum	Fumarprotocetraric acid, protocetraric acid and Cph-2 (Huovinen et al. 1990).	Fumarprotocetraric acid (K869, K938, K945, K946, K950, K951, K962, K966, K974, N5556, N6085, N6086, N6112, N6118, N9061, N9460, N10200).
		Fumarprotocetraric acid and atranorin (K870).
C. grayi	Fumarprotocetraric acid, grayanic acid, cryptochlorophaeic acid, and 5 unknown products (Huovinen et al. 1990).	Fumarprotocetraric acid and grayanic acids (N7209, N9644, N10130).
	Fumarprotocetraric acid and grayanic acid (Bowler 1972; Culberson and Kristinsson 1969; Holien and Tonsberg 1985).	
C. fimbriata	Fumarprotocetraric acid (Hennings 1983).	Fumarprotocetraric acid (N5600, N9932, Y5328).
C. magyarica	Fumarprotocetraric acid, atranorin, protocetraric acid, and Cph-2 (Huovinen et al. 1990).	Atranorin and fumarprotocetraric acid (S4553).
C. coccifera	Zeorin, usnic, porphyrilic and conporphyrilic acids (Ahti and Stenroos, 1986)	Usnic acid and zeorin (N6592, N10134).

The results from the NCBI BLASTp analyses showed that the KS domains of
PKS genes from other fungi are the most significant matches (Table 4.4) for each of the
12 PKS gene paralogs in this study. The 1-DA, 2-DA, 13-DA, 14-DA and 16-DA
paralogs generated the most significant matches with *C. grayi* putative polyketide
synthase genes (see Table 4.4). Paralog 3-DA generated the best match with the PKS
genes of *Sordaria macrospora*, 5-DA generated the best match with *Aspergillus flavus*, 7-
DA generated the best match with *Zymoseptoria tritici*, 10-DA generated the best match
with *Aspergillus clavatus*, 11-DA generated the best match with *Gibberella moniliformis*,
12-DA generated the best match with *Aspergillus clavatus*, and ORS-DA generated the
best match with *Aspergillus niger* MSAS type polyketide synthase genes. BLASTp
searches for all paralogs using the JGI database of *C. grayi* identified the PKS gene for *C.
grayi* with an e-score of 0.0 and 100% identity except for paralog 12-DA, which resulted
in no match.

Table 4.4. Results from BLAST searches of amino acid sequences for each of

the 12 PKS paralogs.

Paralog	BLASTp match	GenBank Accession number	E value	Domain	Max identity
1 DA	*Cladonia grayi*	ADX36084	1.00E-96	KS	91%
	Bipolaris maydis	AAR90276	2.00E-97	KS	79%
	Botryotinia fuckeliana	CCD56082	4.00E-100	Not available	80%
2DA	*Cladonia grayi*	ADM79462	2.00E-119	Not available	87%
	Botryotinia fuckeliana	CCD56082	2.00E-36	KS	43%
	Cochliobolus heterostrophus	AAR90276	3.00E-35	Not available	37%
3 DA	*Sordaria macrospora*	XP_003350625	5.00E-58	KS	54%
	Glomerella graminicola	EFQ28216	5.00E-58	KS	54%
	Macrophomina phaseolina	EKG17457	4.00E-61	KS	53%
	Colletotrichum gloeosporioides	ELA26923	1.00E-57	Not available	54%
5 DA	*Aspergillus flavus*	XP_002381496	3.00E-85	KS	66%
	Glarea lozoyensis	EHL00839	7.00E-89	KS	69%
	Aspergillus niger	XP_001394029	4.00E-85	KS	67%
	Aspergillus oryzae	XP_001824383	5.00E-84	KS	66%
7 DA	*Zymoseptoria tritici*	XP_003849645	2.00E-25	KS	64%
	Peyronellaea zeae-maydis	AAR85531	1.00E-17	KS	59%
	Talaromyces marneffei	XP_002146288	1.00E-15	KS	60%
10 DA	*Aspergillus clavatus*	XP_001273762	1.00E-41	KS	41%
	Aspergillus fumigatus	XP_748462	2.00E-43	KS	42%
	Neosartorya fischeri	XP_001258783	1.00E-41	KS	41%

	Colletotrichum higginsianum	CCF44055	8.00E-45	KS	38%
11 DA	*Gibberella moniliformis*	AY495595	1.00E-87	KS	73%
	Verticillium albo-atrum	XM003007480	2.00E-87	Not available	78%
	Peltigera membranacea	HM180411	1.00E-73	Not available	67%
12 DA	*Aspergillus clavatus*	XM001268489	1.00E-58	Not available	56%
	Verticillium albo-atrum	XM003007480	4.00E-57	KS	60%
	Pyrenophors teres	XM003302099	6.00E-54	Not available	59%
13 DA	*Cladonia grayi*	ADX36085	7.00E-151	FabB	100%
	Umbilicaria torrefacta	ACJ24814	5.00E-133	KS	81%
	Lecanora muralis	ACJ24817	3.00E-126	KS	78%
	Pertusaria amara	AAY00077	2.00E-123	KS	74%
ORS DA	*Aspergillus niger*	XM_001402371	1.00E-08	Not available	37%
	Pertusaria pustulata	EF192113	2.00E-05	Not available	42%
	Pertusaria subfallens	EF192114	8.00E-114	KS	83%
14 DA	*Cladonia grayi*	ADX36086	2.00E-179	KS	96%
	Aspergillus terreus	XP_001210231	3.00E-146	KS	89%
	Ascochyta rabiei	ACS74449	1.00E-132	KS	73%
16 DA	*Cladonia grayi*	ADM79459	3.00E-39		83%
	Evernia prunastri	EF212820	1.00E-17	KS	65%
	Coccotrema cucurbitula	AY918716	3.00E-13	Not available	44%

4.4.2. PKS paralog screening

Fifty-two individuals representing eight species of *Cladonia* exhibited variable numbers of the 14 possible PKS paralogs (including 15-DA which was not sequenced and PKS1F/2R which was designed from Bingle et al. 1999; see Kotelko and Piercey-Normore 2010) based on PCR screening (Figure 4.1). The mtSSU and ITS1 rDNA were used as internal controls for this screening and produced bands in all 52 samples tested. All paralogs were amplified from four species but not all individuals (*C. chlorophaea*, *C. merochlorophaea*, *C. pocillum* and *C. pyxidata*). The paralog 15-DA was present in a single individual of *C. pyxidata*. PCR products derived from the primer sets PKS1F/PKS2R, PKS 1-DA and 3-DA were obtained in all samples of *C. pocillum* screened; and PKS1F/PKS2R, PKS 3-DA 5-DA, and 7-DA produced PCR products in all samples of *C. pyxidata* tested. Four additional species (*C. grayi*, *C. coccifera*, *C. fimbriata* and *C. magyarica*), represented by six individuals also produced PCR products (Figure 4.1). Based on the PCR screening *Cladonia coccifera* contained 5 paralogs, *C. fimbriata* contained 7 paralogs, *C. magyarica* contained 5 paralogs, and 3 individuals of *C. grayi* contained 7, 8, and 13 paralogs, respectively.

Figure 4.1. Percentage of PKS paralogs present by PCR screening for each of eight species of *Cladonia chlorophaea* complex. The y-axis represents percent of occurrence and the x-axis represents the 14 PKS paralogs tested. The number of samples tested for each species is presented in the legend as "n".
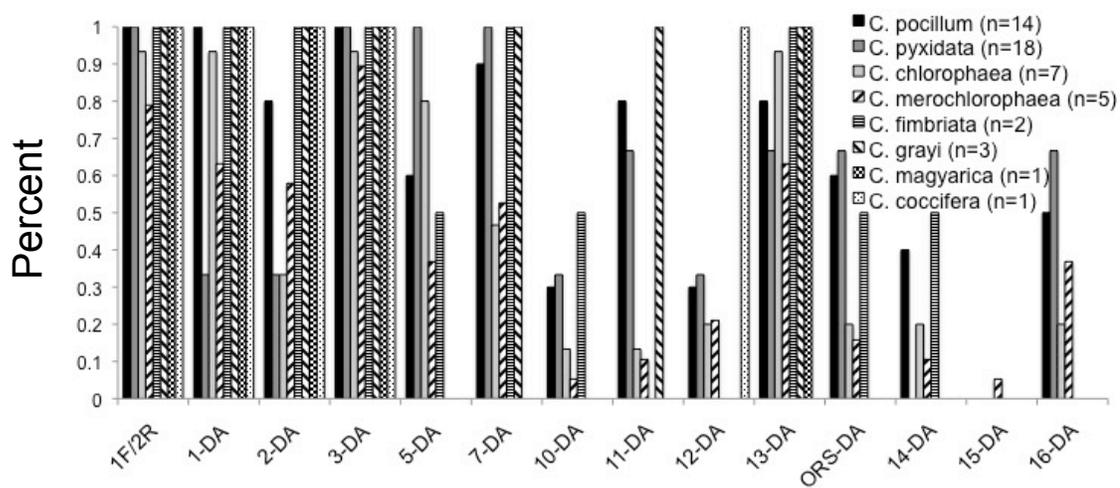
Figure 4.2. PCA (Principle Component Analysis) using the presence

and absence of the 14 PKS paralogs from PCR screening. Component 1 is the

horizontal axis and component 2 is the vertical axis.
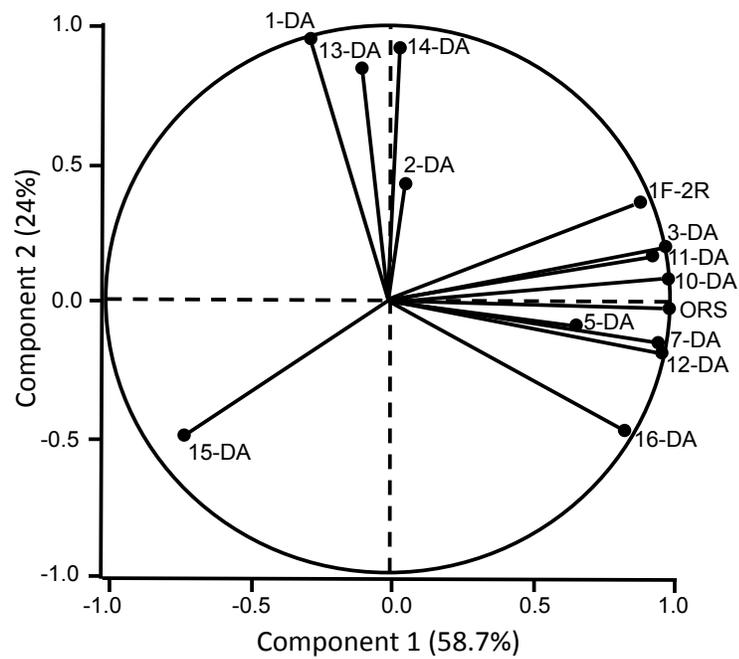
A Principle Components Analysis (PCA) showed that the paralogs were clusterd into two main groups, one cluster (PKS1F/2R, 3-DA, 5-DA, 7-DA, 10-DA, 11-DA, 12-DA, 16-DA and ORS-DA) around the positive values of the horizontal axis and the other cluster (1-DA, 2-DA, 13-DA, and 14-DA) around the positive values of the vertical axis. One paralog (15-DA) formed an outlying paralog in the negative direction of both axes. The horizontal axis explained 58.7% of the variation in the data obtained from the presence/absence of the paralogs, and the vertical axis explained 24% of the variation, resulting in 82.7% of the variation being explained by these first axes (Figure 4.2).

4.4.3. PKS gene phylogeny

The PKS gene phylogeny was inferred from the amino acid sequences belonging to 12 PKS paralogs (15-DA and 1F/2R were excluded from the analysis; 15-DA was present in a single sample and 1F/2R was not included in the 12 paralogs from Armaleo et al. (2011). The PKS alignment for the MP tree contains 478 total characters with 270 parsimoniously informative characters and the length of the tree is 2979 steps. The Consistency and Retention Indices of the phylogenetic tree are 0.7150 and 0.9281, respectively. All 12 paralogs formed highly supported monophyletic clades with 100% bootstrap support except sub-clades 13-DA and 14-DA, which have a bootstrap support of 95% and 98%, respectively (Figure 4.3). Six major clades (A to E) are indicated in the PKS gene tree representing an evolutionary history, where clade A has a high bootstrap support of 98% and contains two smaller clades; clade B with 1-DA, 13-DA and 14-DA which is supported by 88% bootstrap; and clade C with 3-DA, 5-DA, 10-DA, and 7-DA which is supported by 81% bootstrap. Clade D is sister in position to these clades A, B, and C, and contains 2-DA, while clade E contains 11-DA and 12-DA and clade F

contains members of 16-DA. The ORS-DA sequence was assigned as an outgroup. Clade

B corresponds with component 2 of the PCA but 2-DA lies outside the clade and is closer

to the origin in frequency of occurrence among individuals. Clade C corresponds with

component 1 of the PCA plot, which also contains the paralogs in clade E and ORS-DA.

Putative reducing and non-reducing lineages are indicated on the tree and were deduced

from the BLASTp results. Non-reducing lineages include all of clades B and D and the

clade with 16-DA. Reducing lineages includes all members of clades C and E and the

outgroup ORS-DA. Clade A contains both reducing and non-reducing members.

Figure 4.3. Phylogenetic tree showing the genealogy of PKS gene paralogs from the

Cladonia chlorophaea complex based on the amino acid sequence alignment deduced

from the DNA sequence of the KS domain. This is one of 422 most parsimonious trees,

which is consistent with the Bayesian tree (not shown). Major clades are indicated by

brackets and circled letters (A to F). The assigned outgroup is ORS-PKS. Numbers above

branches represent node support values greater then 70 % based on maximum parsimony

analysis and thickened lines indicate that the posterior probabilities are greater than 90 %

based on Bayesian analysis. The boxed words (reducing and non-reducing) represent

clades that contain a known gene structure from NCBI GenBank. Indicated are the gain

of the ME domain early within the evolution of the PKS genes and the eventual loss of

the ME domain in the potential ancestor of the PKS-3DA, 5DA, and 10DA paralogs.

PKS-1DA
(KS-AT-PP- (PP)
- ME- (CYC)

Non-reducing (B)

PKS-13DA*
(KS-AT-PP-(PP)
-ME-(CYC)

PKS-14DA*
(KS-AT-PP-(PP) -
ME-(CYC)

(A)

PKS-3DA*
(KS-AT-DH-ER-
KR-PPb)

loss of ME domain

PKS-5DA*
(KS-AT-DH-ER-KR-PP
-AA -PP-TE

Reducing (C)

PKS-10DA*
(KS)-AT-DH-ER-KR-PPb

PKS-7DA
(KS-AT-DH-MT-ER-KR-PPb)

Non-reducing (D)

PKS-2DA
(KS-AT-PP- (PP)-ME-(CYC)

Gain of MET domain

PKS-11DA*
(KS-AT-DH-(ME)-ER-KR-PP)

Reducing (E)

PKS-12DA
(KS-AT-DH-MT-ER-KR)

Non-reducing (F)

PKS-16DA** (KS-AT-PPb-PPb-TE)

Reducing

PKS-ORS-DA** (KS-AT-KR-PPb)

10 changes
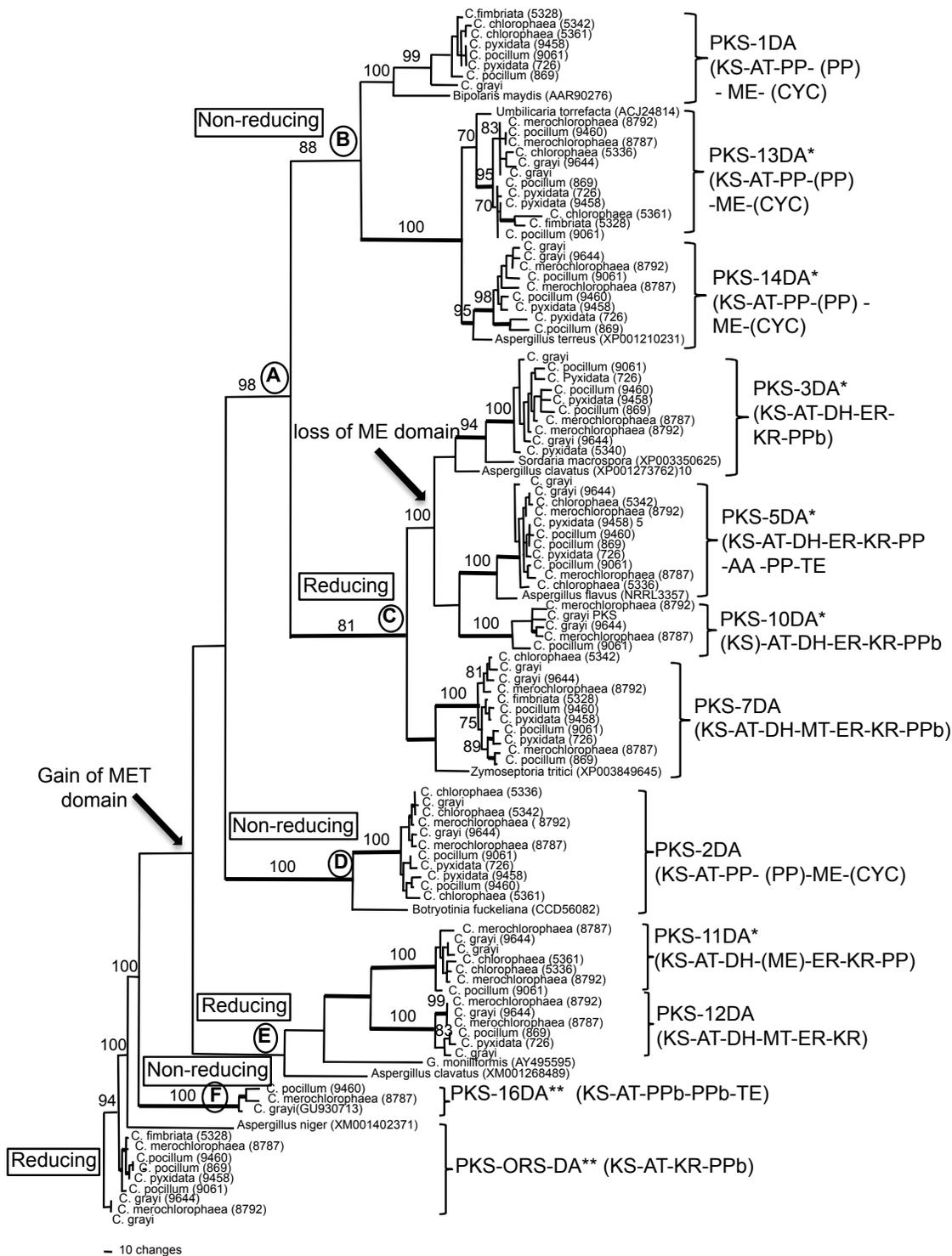
4.4.4. ITS1 rDNA phylogeny

Fungal ITS rDNA was sequenced for 16 samples from 7 species (*C. chlorophaea, C. coccifera, C. fimbriata, C. grayi, C. merochlorophaea, C. pocillum,* and *C. pyxidata*). Twenty-three sequences representing 12 species (*C. chlorophaea, C. coccifera, C. ecmocyna, C. fimbriata, C. gracilis, C. grayi, C. magyarica, C. maxima, C. merochlorophaea, C. pocillum, C. pyxidata* and *C. rei*) were recovered from GenBank. *Cladonia coccifera* was selected as an outgroup because it is morphologically distinct from the *C. chlorophaea* group and it falls within Section *Cocciferae*. The ITS1 rDNA alignment contains 560 characters with 91 parsimoniously informative characters and the length of the most parsimonious tree is 266 steps. The Consistency and Retention indices of the phylogenetic tree are 0.7556 and 0.8438, respectively. The ITS1 rDNA phylogeny contains two major clades A and B (Figure 4.4). Three of the eight species in clade A form monophyletic sub-clades (*C. ecmocyna, C. maxima,* and *C. magyarica*). Sub-clade (i), with three representatives of *C. ecmocyna,* and sub-clade (ii) with three representatives of *C. maxima,* is supported by 98% and 97% posterior probability values, respectively, but the bootstrap support is less than 70%. *C. ecmocyna, C. maxima* and *C. gracilis* are grouped together in subclade (iv), in which the three representatives of *C. gracilis* are unresolved but the subclade is supported with a bootstrap support of 79% and a posterior probability value of 90. Both the posterior probability value and boostrap support for sub-clade (iii) containing two representatives of *C. magyriaca* is 100%. Other representatives of Clade A include *C. chlorophaea, C. merochlorophaea, C. grayi,* and *C. pyxidata,* which form a polyphyletic group of species within the clade A. Clade B is comprised of 4 species (*C. fimbriata, C. rei, C. pocillum* and *C. pyxidata*).

Representatives of each of *C. fimbriata* and *C. rei* form monophyletic sub-clades (v and vi, respectively) where subclade (v) has high posterior probability value and boostrap support of 100% and 97% respectively. The posterior probability value for representatives of sub-clade *C. rei* (vi) is 100 with bootstrap support of 80%. *Cladonia pocillum* is paraphyletic within clade B but *C. pyxidata* is polyphyletic and members assigned to *C. pyxidata* are present in both clades A and B.

Figure 4.4. Phylogeny of the ITS1 rDNA in the *Cladonia chlorophaea* complex based on nucleotide sequence alignment. This is one of 390 most parsimonious trees, which is consistent with the Bayesian tree. The assigned outgroup is *C. coccifera*. Numbers above branches represent bootstrap support greater than 60% based on maximum parsimony and thickened lines indicate posterior probabilities greater than 90 % based on Bayesian analysis. Clades discussed in the text are indicated by circled letters (A & B) and sub-clades are indicated by brackets (Roman numerals i to vi).
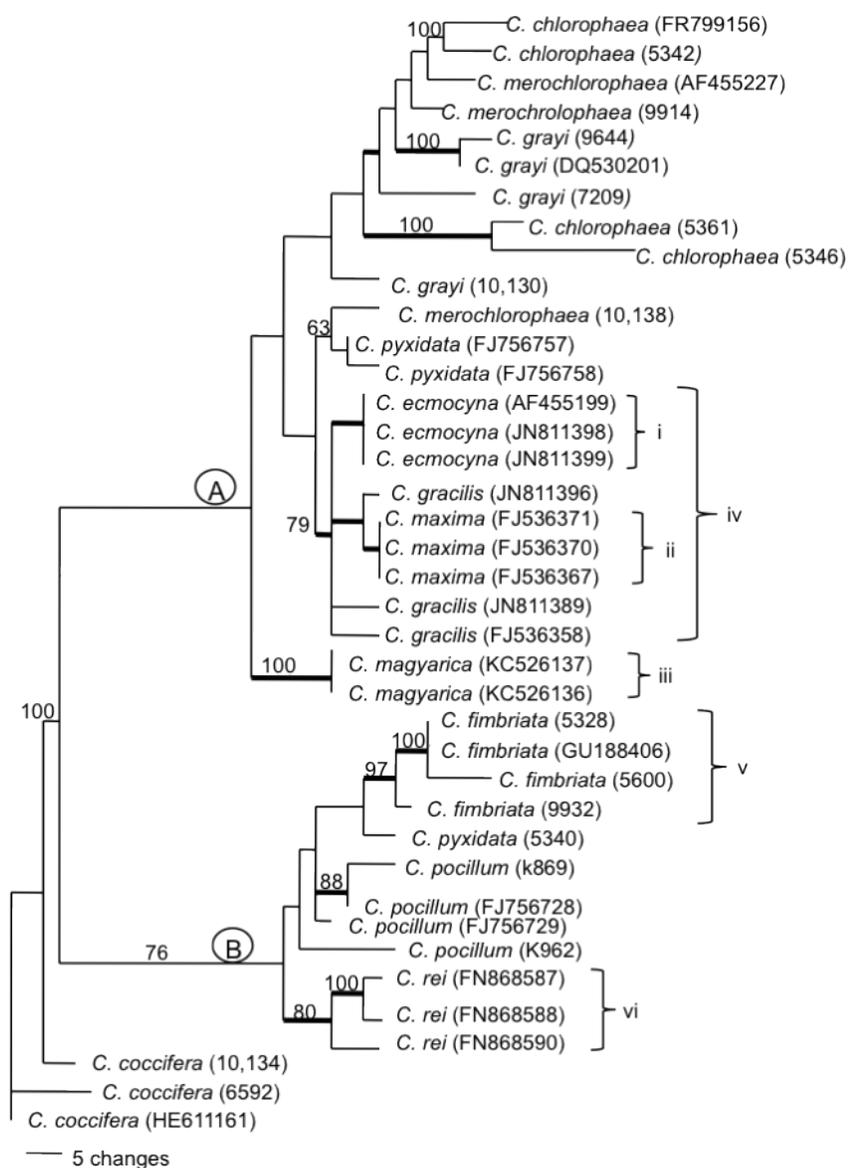
4.4.5. Alleles

The number of alleles deduced based on nucleotide substitutions noted within the DNA sequences range from 3 alleles in 16-DA to 11 alleles in 7-DA.

4.4.6. dN/dS estimations

Z-test of Selection in *MEGA* 5 for purifying selection (dN < dS) gave the probability values of 0.0 for 3-DA, 5-DA, 10-DA, 11-DA, 13-DA and 14-DA; 1.0 for 12-DA and ORS-DA; 0.336 for 1-DA; and 0.056 for 7-DA (see Table 4.5), with values of p < 0.005 being viewed as significant. The Z-test of Selection for positive selection (dN >dS) gave the probability values of 1.0 for all paralogs except 12-DA (0.269) and ORS-DA (0.001) (Table 4.5).

Table 4.5. Results from dN and dS comparisons inferring selection acting on PKS

paralogs used in this study (* paralog under purifying selection, ** paralog under positive

selection). Probability is the p-value where p<0.05 is significant and the statistic obtained

from MEGA 5 where test statistic (dS - dN) is shown in the Statistics column.

PKS paralogs	Purifying selection (dN<dS)		Positive selection (dN>dS)	
	Probability	**Statistics**	**Probability**	**Statistics**
DA-1 (8 sequences)	0.336	0.423	1.000	-0.405
DA-2 (11 sequences)	0.349	0.389	1.000	-0.423
DA-3 (10 sequences)	0.000*	4.234	1.000	-4.263
DA-5 (12 sequences)	0.000*	6.557	1.000	-6.529
DA-7 (11 sequences)	0.056	1.598	1.000	-1.619
DA-10 (5 sequences)	0.000*	3.694	1.000	-3.508
DA-11 (7 sequences)	0.000*	4.365	1.000	-4.787
DA-12 (6 sequences)	1.000	-0.569	0.269	0.617
DA-13 (12 sequences)	0.000*	5.231	1.000	-5.084
DA-14 (9 sequences)	0.000*	3.609	1.000	-3.543
DA-16 (3 sequences)	1.000	-2.816	0.001**	3.057
DA-ORS (9sequences)	1.000	-3.162	0.001**	3.258

4.5. Discussion

4.5.1. Genes responsible for polyketide production in the C. chlorophaea *group*

One to three secondary metabolites were detected in all the samples examined in this study but the literature indicates that a small proportion of individuals in any one of the species may have no compounds produced or they may produce from one to six polyketides as for *C. merochlorophaea* (Table 4.3). Even with these numbers of polyketides reported, the numbers of gene paralogs encoding PKS enzymes is much higher than the number of polyketides produced (Figure 4.1), which is consistent with the findings in other studies. For example, Sanchez et al. (2008) reported the presence of 27 *PKS* genes whereas only seven secondary metabolites are known from *Aspergillus nidulans*. Hoffmeister and Keller (2007) reported 15 *PKS* genes and four secondary metabolites in *Fusarium graminearum*. Similarly, Armaleo et al. (2011) reported 12 *PKS* genes and two major polyketides in *Cladonia grayi*. Recent knowledge about the number of paralogs present from genome sequencing projects in *Aspergillus* have predicted the number of *PKS* genes to be even higher than those previously reported (Gilsenan et al. 2009).

The genome of *C. grayi* (Armaleo et al. 2011), and its allies in this study, contains at least 12 PKS paralogs, some of which contain reducing domains (Figure 4.3). Reduced polyketides are not known to be present in the *C. chlorophaea* complex even though the genes may be present. Since we know that the metabolites produced by this group are produced by non-reducing PKS, we can eliminate seven genes which promote the production of polyketides with reducing domains, leaving five genes that might be

responsible for polyketide production in the *C. chlorophaea* group. The species *C. chlorophaea sensu stricto* and *C. merochlorphaea*, which are thought to be more closely related to *C. grayi* (Culberson et al. 1988), also had a larger number of detected paralogs than *C. pyxidata* and *C. pocillum*, which are not as closely related to *C. grayi*. There might be a relationship between the number and types of paralogs and the taxonomic relatedness among members of *Chlorophaea* group.

Armaleo et al. (2011) hypothesized that PKS 16-DA is involved in the production of grayanic acid, a non-reduced orcinol depsidone. The production of ß-orcinol depsides and depsidones by *C. chlorophaea, C. pocillum* and *C. pyxidata* such as fumarprotocetraric acid and atranorin, would require the ME domain for methylation of the third carbon in the polyketide. However, the orcinol products such as protocetraric, merochlorophaeic, sekikaic, homosekikaic, and 4-*o*-methylcryptochlorophaeic acids (Huovinen and Ahti 1982) would not require the ME domain. While the presence or absence of the ME domain may explain the production of the ß-orcinol products, domain skipping has also been suggested to occur during protein synthesis (Beck et al. 2002). If the ME domain is absent or not expressed, the polyketide would not be methylated, resulting in an orcinol product (Armaleo et al. 2011). All specimens produce at least one polyketide (fumarprotocetraric acid), but no single paralog could be detected in all members of the species group. Explanations for these observations are that more than one PKS may be required for production of a single product such as for citrinin and lovastatin (Abe et al. 2002, Kennedy et al. 1999) or additional paralogs are present in the genomes that have not yet been detected. The link between the gene responsible for the production of a certain polyketide is still unknown, but the numbers of possible genes involved in

polyketide synthesis for *chlorophaea* have been reduced from 12 to 5 genes allowing

further research to focus on these five genes.

4.5.2. *Function inferred from dN/dS ratios among the PKS paralogs*

This study suggests that purifying selection may occur on 3-DA, 5-DA, 10-DA,

11-DA, 13-DA, and 14- DA (Table 4.5) and positive selection may occur on 16-DA and

ORS-DA. Both purifying and positive selection was reported for fungal genes involved in

plant pathology (Gladieux et al. 2013). Selection pressure on orthologs is approximately

two times higher in bacteria and three times higher in mammals (Kondrashow et al.

2002). After duplication events (Long et al. 2003), one gene copy such as the ortholog

may be sufficient to perform the respective function while selection pressure on a paralog

would be relaxed allowing for accumulation of potential beneficial and deleterious

mutations. Another scenario is that soon after duplication, both the duplicated genes may

undergo a period of relaxed selection, where both genes might accumulate enough

mutations that impair the ancestral function (Kondrashow et al. 2002) resulting in

pseudogenes. In this study, PKS 3-DA, 5-DA, 10-DA, 11-DA, 13-DA, and 14-DA are

likely to be functional (Table 4.5) because the difference between synonymous and non-

synonymous substitutions is higher than 1.0 suggesting significant purifying (i.e. no

change at the amino acid sequence level) selection (Nei and Gojobori 1986) where high

selection for a gene product is preferred under certain environmental conditions. If the

dS/dN ratio is less than 1.0, the gene is assumed to be unusually variable (i.e. changes at

the amino acid level) and is thought to have evolved under positive selection where

selection acts on recent changes for a new or better function. Since members of *Cladonia*

chlorophaea sensu lato are widely distributed (Ahti 2000), and grow on a wide variety of substrates (Esslinger 2008), PKS gene duplication may be a general mechanism, followed by drift, purifying and positive selection on different paralogs, which would allow for subspecialization and enable them to adapt to variable conditions of environmental stress (Kondrashow et al. 2002).

4.5.3. *Three origins of non-reducing PKS in the* C. chlorophaea *group*

The *PKS* gene phylogeny with high bootstrap and posterior probability support shows that at least 12 paralogs are distributed among members of this small group of *Cladonia* species. It further suggests an evolutionary hypothesis that the reducing and non-reducing PKS genes roughly correspond with the three separate groupings in Figure 4.2. The PKS - PCA clusters represent the non-reducing PKS genes (except 16-DA) and the reducing PKS genes suggesting related gene function within each clade. The reducing paralogs are clustered along the horizontal axis in the PCA plot with the exception of 15-DA, which may be an artifact of having a single occurrence in the data, and 16-DA being separated from the non-reducing members corresponding with its basal position in the tree. Clustering methods are commonly used for gene expression data (Eisen et al. 1998) based on the idea that co-expression is related to co-regulation (Ross et al. 2000). This study shows the presence of genes rather than expression profiles, providing insight into the possible function and evolution of these genes.

Since 16-DA is suggested to be responsible for the production of grayanic acid (Armaleo et al. 2011), its basal position (Figure 4.3) may indicate a more distant relationship from the other orcinol polyketides. Since members of this species complex

are named according to the polyketide that is produced (Huovinen and Ahti 1982), the separation of 16-DA from clade A may also reflect the hypothesized reproductive isolation of *C. grayi* detected by Culberson et al. (1988). However, *C. grayi* could not be separated from the *C. chlorophaea* complex by the ITS rDNA (Figure 4.4). The ITS rDNA tree supports conspecificity within the *C. chlorophaea* species complex (Figure 4.4), but the suggestion that specific lineages within the complex separate from others has also been made (Kotelko and Piercey-Normore 2010) with subclades having high support (Figure 4.4). For example, *C. magyarica* forms a highly supported clade A (iii) and *C. fimbriata* also forms a highly supported clade B (v), both considered members of the *C. chlorophaea* group. Similarly, some members of the same species form a dicotomy with high support, but there is no instance in the tree with high support for a dichotomy of two different species. While the ITS rDNA provides sufficient variation to resolve species in some "non-chlorophaea" species (clade B; Figure 4.4), the same gene (ITS rDNA) cannot resolve the "chlorophaea" group.

The domain structure of the PKS gene varies according to the non-lichenized taxon associated with each clade by BLAST searching (Table 4.4; Figure 4.3). However, Kroken et al. (2003) suggested that KS-AT-DH-ME-ER-KR-PP is the ancestral domain structure, which would suggest that the non-reducing gene structures are derived from the loss of the reducing domains. While seven of the paralogs in this study appear to represent reducing PKS based on BLAST comparisons, five paralogs (1-DA, 2-DA, 13-DA, 14-DA, and 16-DA) represent non-reducing PKSs, which are more commonly represented in lichen-forming fungi than the reducing PKSs. The position of three paralogs 1-DA, 13-DA, and 14-DA are consistent with the non-reducing clade III, while

one paralog 11-DA was consistent with the reducing PKS clade I of Kroken et al. (2003). The common occurrence of non-reducing PKSs in lichen fungi may be explained by the slower growth of lichen-fungi and lower metabolic rates than non-lichenized fungi. The lower metabolic rates may result in fewer oxygen free radicals and less opportunity for molecular reduction and less selection pressure on the reducing function of the enzyme. The gene rearrangement with the ME domain downstream from the PP-binding domain may be the intermediate stage in evolution of gene structure before the loss of the reducing domains (Kroken et al. 2003). The absence of reduced polyketides in lichen-fungi suggests that 3-DA, 5-DA, 7-DA and 10-DA are not functional or are silenced because the conditions for their expression may not have been encountered.

In conclusion, it is becoming commonly known that a genome may contain many PKS genes but the organism produces fewer polyketides than there are genes that encode PKS. The PKS genes are part of a gene family that includes many paralogs that may be redundant in function and members might be either pseudogenes or evolving new functions associatied with the production of secondary metabolites. This study focused on a species complex in an attempt to reduce the breadth of taxonomic relationships that may obscure functional analyses, to gain insight into gene distribution and the inferred function of PKS genes based on knowledge of a working model species, *C. grayi*. There were three main findings in this study: 1) Five (1-DA, 2-DA, 13-DA, 14-DA and 16-DA) of the 12 PKS paralogs detected in *Cladonia chlorophaea* complex encode putative non-reducing PKS enzymes and four of these are hypothesized to be under selection (2-DA, 13-DA, 14-DA, and 16-DA). Since no reduced polyketide has been detected in these species so far, and if 16-DA is responsible for grayanic acid (Armaleo et al. 2011), then

1-DA, 2-DA, 13-DA and 14-DA must be responsible for polyketides detected in the *C. chlorophaea* group. 2) The non-reducing clades arise three times independently of the reducing clades in the PKS gene phylogeny suggesting that there may be more than one origin of non-reducing paralogs in the *C. chlorophaea* complex. This interpretation would be consistent with the multiple origins of lichenization suggested by Lutzoni et al. (2001) and Gargas et al. (1995), and suggests that the *C. chlorophaea* complex may be equipped to readily adapt to changing environmental conditions. 3) The results of both the PCA analysis and the PKS gene phylogeny clustered the 12 paralogs into two clusters or five clades, respectively, while one paralog was assigned as an outgroup. The phylogenetic data allows one to propose a possible model for the evolution of the various PKS paralogs placing the 16-DA at the node from which two lines of PKS paralogs can be derived which split into additional lineages (Figure 4.5). We hypothesize that the function of the PKS paralogs within each of the clades are related. While one explanation is that some paralogs may be responsible for catalyzing the synthesis of intermediates or precursors in the biosynthetic pathway, another explanation may be that multiple genes produce a single product. The expansion of the PKS gene family and the possible redundancy in gene function might be advantageous for lichen fungi providing them with the genetic plasticity needed to adapt to harsh conditions, such as substrates with limited nutrients, exposure to temperature/light extremes and the potential of being consumed by grazers.

Figure 4.5. Proposed evolutionary model for PKS gene paralogs. The symbols * and **
indicate that in some paralogs purifying and positive selection can be detected
respectively. PR=partially reducing, NR=non-reducing and R=reducing PKS paralog.
The model shows that reducing PKS paralogs have been derived from non-reducing PKS
ancestors. The variety of PKS paralogs adds to the genomic plasticity in lichen fungi
probably necessary to survive in harsh environments.
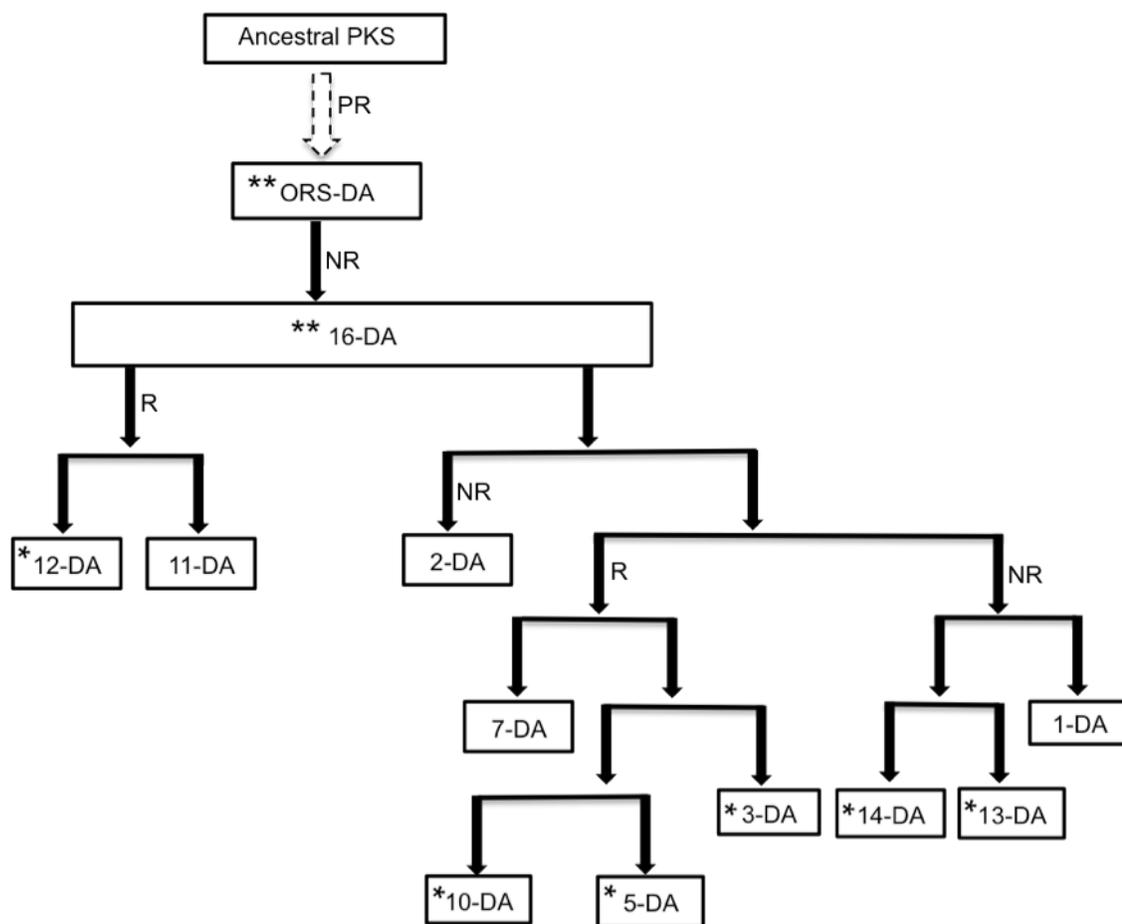
CHAPTER 5

Final Discussion and Conclusion

Nine northern North American species of *Ramalina* were investigated for their evolutionary relationships and compared with several species from Europe (Chapter 2). The *Ramalina* phylogeny based on the ITS1 rDNA and mtSSU rDNA showed that seven of these species are monophyletic and the evolutionary position for members of the other two species was unresolved. The seven species (*R. dilacerata*, *R. farinacea*, *R. intermedia*, *R. menziesii*, *R. roesleri*, *R sinensis* and *R. thrausta*) form monophyletic clades with strong bootstrap and posterior probability support. While this phylogenetic tree is consistent with other phylogenies from earlier studies (Stocker-Wörgötter et al. 2004, LaGreca and Lumbsch 2001), it shows the placement of three species that were not previously included in any phylogeny (*R. dilacerata*, *R. roesleri*, and *R. thrausta*) and confirms that *R. thrausta* belongs in the genus *Ramalina*, which is morphologically different from other species in the genus. Two species (*R. americana* and *R. farinacea*) that were previously shown to be monophyletic (Stocker-Wörgötter et al. 2004) were not resolved or had low support as clades in this study. This study also supported the hypothesis that more ancestral species in the genus produced fewer polyketides than the more derived species (Stocker-Wörgötter et al. 2004).

The *Ramalina* phylogeny provided the opportunity to examine three PKS paralogs (two wA-type and one MSAS-type genes) that were detected in species of *Ramalina* using three PKS specific primer pairs and to map the presence of the paralogs onto the

phylogenetic tree topology. Both non-reducing and partially reducing (6-MSAS) types of PKSs were discovered in members of the genus *Ramalina,* eventhough the MSAS-type of PKS genes are rare in lichens and *Ramalina* generally does not produce partially reduced polyketides. This was an interesting finding and led to the follow-up study (chapter3). While only three paralogs were examined in this study, a large number of paralogous PKS genes are expected to be present in the genomes of *Ramalina* because they are rich in diverse phenolic compounds (LaGreca 1999, Culberson et al. 1990). All three PKS paralogs detected in this study are likely to be functional in species of *Ramalina* based on the dN/dS ratio, indicating that the gene regions are under purifying selective constraints (Muggia et al. 2008). Purifying selection suggests high selection for a gene because of higher preference for the gene product under certain environmental conditions. Some species of *Ramalina* have been shown to be diverse in the types of secondary metabolites they produce (Stocker-Wörgötter et al. 2004, Culberson et al. 1990, LaGreca 1999). Chemical diversity may help an organism survive better in its ecological niche (Huneck 1999). Species that are chemically rich would exhibit greater plasticity and they would be better equipped to adapt to changing environments with stress from abiotic and biotic sources. Fungi are well known for their ability to switch growth forms in response to environmental stress (Rayner and Coates 1987) and they may also have a similar ability for other phenotypic changes such as polyketide production.

The polyketides produced by *Ramalina dilacerata* include usnic and divaricatic acids. *Ramalina dilacerata,* one of the derived species studied in Chapter 2, forms a strong monophyletic clade (Figure 2.1), and grows on a wide range of tree species suggesting adaptability to varied substrates. It also produces large numbers of apothecia

in its natural habitat, thus making it amenable to culturing aposymbiotically. Since *R. dilacerata* is distinct from other North American species, it has a solid taxonomy to avoid potential problems with related species in further studies, and it was chosen to examine the effects of environmental conditions on the fungus and polyketide production. A follow-up study (from chapter 2) was done to examine the function of MSAS-type gene in both a natural lichen thallus and the cultured mycobiont of *R. dilacerata*. A standard growth pattern consisting of a lag, exponential and stationary phases, was observed in the cultured mycobiont of *R. dilacerata*. Further experiments used 100 day-old colonies because it was the growth phase that allowed for a large colony diameter during active growth. During this time period, growth media containing yeast extracts and pH 4.5 to 6.5 produced mycobiont colonies with the largest diameter, indicating that these are the best growing conditions of those conditions examined for the *R. dilacerata* mycobiont. However, the number of secondary metabolites detected by TLC and HPLC was higher in colonies with smaller diameters that were grown under less optimal conditions. Under these less optimal growing conditions, the mycobiont may be under stress and may have produced a large number of compounds presumably to overcome the stressful condition or as by-products from primary metabolism where the absence of certain nutrients prevented maximum growth. One of the triggers for production of secondary compounds is stress (Fox and Howlett 2008). Earlier studies by Culberson and Armaleo (1992) and Stocker-Wörgötter (2001) have linked the production of secondary metabolites in cultured mycobionts to dehydration. The theory that cell growth and secondary metabolism are competing processes (Bu'Lock 1961) where secondary metabolites are produced only after one or more nutrients become limiting to mycelial growth, is

consistent with the findings in this study. High pH levels and the non-nitrogen medium may have been less favorable conditions for growth and induced the expression of wA-type and MSAS-type PKS genes in the cultured mycobionts of *R. dilacerata*. This explanation supports the theory that slower growth promotes the production of polyketides and an imbalance in the carbon nutrient balance hypothesis (Bryant et al. 1983) for polyketide production.

The expression of the wA-type gene in maltose media supplemented with glucose at pH 6.5 and the expression of MSAS-type PKS gene in maltose media without glucose and at pH 8.5, suggests that gene expression may be influenced by both nutrient source and pH level of the media. Environmental factors have been suggested to be essential for the induction and suppression of the acetate polymalonyl pathways (Stocker-Wörgötter 2007). These results show that high pH levels and non-nitrogen medium (sub-optimal conditions for colony growth) induced the expression of both wA-type and MSAS-type PKS genes. The number of secondary metabolites also correlated with PKS gene expression. Since the wA1-type PKS genes are common in lichen fungi (Armaleo et al. 2011, Sanchez et al. 2008, Schmitt et al. 2008, Opanowicz et al. 2006), it would be expected that growth conditions would influence the expression of the wA1 type gene in *R. dilacerata*.

While three PKS genes were studied in *R. dilacerata*, 14 PKS paralogs were detected in the *Cladonia chlorophaea* complex and six polyketides are reported to be produced by members of this complex (Huovinen and Ahti 1982). Hence, the numbers of gene paralogs encoding PKS are much higher than the number of polyketides reported for the *C. chlorophaea* species complex. The finding of a larger numbers of PKS genes than

polyketides is consistant with earlier studies (Armaleo et al. 2011, Sanchez et al. 2008, Hoffmeister and Keller 2007). The genome sequencing projects in *Aspergillus* have predicted the number of PKS genes to be even higher than those reported (Gilsenan et al. 2009). The presence of large numbers of PKS paralogs known to occur in fungi (Armaleo et al. 2011, Sanchez et al. 2008, Schmitt et al. 2008, Opanowicz et al. 2006) and the morphological plasticity apparent in fungi (Slepecky and Starmer 2009) might account for a chemical diversification of species as new niches are colonized and species adapt to habitats. Recently duplicated genes are thought to play a role in adaptation to environmental conditions (Kondrashow et al. 2002). This would also suggest that gene duplication may occur in response to stressful conditions (Blanco et al. 2006) as a mechanism to facilitate adaptation. Multiple duplications of genes is reported to be one of the responses to selection pressure (Brown et al. 1998). Gene duplication and higher number of gene paralogs may enable an organism to synthesize a larger number of and/or more variable secondary metabolites, which would increase the potential for adaptation to environmental changes. However, a better understanding may result from genome comparisons, PKS transformation and expression under different conditions.

The presence of five clades of non-reducing and seven clades of reducing PKS paralogs in the *Cladonia chlorophaea* complex, is unusual since no reduced polyketide has so far been detected from these species. This might suggest that the reducing paralogs are either non-functional or not expressed in the present environmental conditions. It is thought that certain environmental factors may be essential for the induction and suppression of the acetate polymalonyl pathways responsible for the synthesis of fungal polyketides (Stocker-Wörgötter 2007), and the levels of secondary metabolism in fungi is

suggested to be affected by environmental conditions (Shwab and Keller 2008, Stocker-Wörgötter 2007, Penalva and Arst 2002). The non-reducing clades in the PKS gene phylogeny show three independent evolutionary origins suggesting the possibility of multiple origins of non-reducing paralogs. This is consistent with the idea of multiple origins of lichenization suggested by Lutzoni et al. (2001) and Gargas et al. (1995) and of growth form by Stenroos and DePriest (1998). The explanation for multiple origins of a feature is based on the idea that fungi exhibit plasticity in their capacity to adapt when environmental conditions change. This can be better explained in future by further studies on parallel PKS gene phylogenies in other groups of organisms.

Fungal plasticity reflected by multiple PKS gene paralogs is also supported by the consistency between clustering of the PKS paralogs in the Principle Component Analysis based on frequency of occurrence within four members of the *C. chlorophaea* complex, and the PKS gene phylogeny. These results suggest that more than one paralog may be responsible for encoding one gene product or a chemically related group of products. Synthesis of secondary metabolites such as citrinin and lovastatin is reported to be catalysed by more than one PKS enzyme (Abe et al. 2002, Kennedy et al. 1999). The clusters of paralogs roughly correspond with reducing and non-reducing genes. If the gene clustering reflects function, then the taxonomic status of species may be linked with gene function. Even though the *C. chlorophaea* complex of species do not form monophyletic clades in the ITS rDNA species tree, the paralog frequencies are different between the *C. chlorophaea* — *C. merochlorophaea* pair of species and the *C. pyxidata* — *C. pocillum* pair of species, suggesting functional relationships that correspond with taxonomic relationships.

In conclusion, a better understanding of the growth conditions that influence expression of polyketide synthase genes has provided insight into the biosynthesis of related secondary metabolites and the manipulation of biosynthetic pathways for further experimentation and discovery of polyketides. These natural products with novel structures and functions can be identified using techniques like NMR. This also provides a platform for the heterologous expression of the gene of interest in a surrogate host and the functional characterization of fungal biosynthetic genes. While this thesis provided insight into the growing conditions required to produce larger numbers of polyketides in *Ramalina dilacerata*, it also revealed that PKS genes in the *Cladonia chlorophaea* group may provide the lichen with the adaptive capacity to survive under variable conditions with multiple evolutionary origins, and having silent genes that may be ready for induction when certain conditions arise. The discovery of both reducing and non-reducing PKSs in the *Cladonia chlorophaea* complex is very interesting because all the polyketides reported from *Chlorophaea* complex are not reduced. In addition, since only some of these PKSs are found to be under selection pressure, it raises a question of whether this has anything to do with the fact that reduced polyketides are so rare in these lichen fungi. Further study is needed to address these questions. Since fungal polyketides have been found to have a wide range of biological and ecological activities, knowledge of their ecological function can be valuable for conservation management and policy makers as well as being harnessed for the pharmaceutical roles of these natural products.

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