# Effects of canopy cover and pH on polyketide synthase gene transcription in *Cladonia stygia* and the polyketides produced in natural conditions.

By

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## ABSTRACT

Polyketide synthase (PKS) genes encode large multidomain enzymes that synthesize polyketides (secondary metabolites) but little is known about their biosynthesis. The goals of this study were to identify PKS genes in the *Cladonia rangiferina* genome in order to target PKS genes in *Cladonia stygia,* and to compare PKS gene transcription and polyketide production in *Cladonia stygia* collected from two geologically different locations in Manitoba. The Antibiotic and Secondary Metabolite Analysis Shell (antiSMASH) program identified 41 secondary metabolite gene clusters consisting of 15 Type 1 PKS genes but only two Non-Reducing Type 1 PKS clusters. The significant difference in the concentration of the polyketides, atranorin and fumarprotocetraric acid, between the two locations corresponded with gene expression using the 21 Beta-Ketoacyl Synthase ($p=0.040$) and 26 Thioesterase domain ($p=0.081$), suggesting that these PKS domains may play a role in the production of fumarprotocetraric acid and atranorin, respectively, in the *C. stygia* thallus.

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# CHAPTER 1 Background

## 1.1 Introduction

Lichens are symbioses between mycobionts and photobionts. The mycobiont is generally an ascomycete and, in a small number of lichens, it can be a basidiomycete. The photobiont in the lichen can either be a cyanobacterium or green alga (Friedl and Büdel 2008). The name of the lichen species refers to the name of the fungal species present in the lichen (Friedl and Büdel 2008). The fungal partner in the lichen is considered to be ecologically obligate (Honegger 2008). Both the algal and the fungal partner must be growing together to produce the distinct lichen morphology that is observed in nature (Honegger 2008).

The type of photobiont found in the symbiosis is somewhat dependent on the type of habitat in which the lichen is found. Lichens present in mostly shaded environments have cyanobacteria as their photobiont, which do not require much light to be metabolically active but do require liquid water. Lichens found in open, less shaded environments, have green algae as their symbiotic partners which require more light than cyanobacteria and water vapour for photosynthesis (Friedl and Büdel 2008). The photobiont provides carbohydrates in the form of sugars to the fungal partner to be used in growth or production of carbon-based secondary metabolites (Honegger 2008). Different green algal partners produce different type of polyols such as ribitol in *Trebouxia*, erythritol in *Trentepohlia* and sorbitol in *Hyalococcus* (Palmqvist et al. 2008) and cyanobacterial photobionts produce glucose (Wastlhuber and Loos 1996), which may influence the type of secondary metabolite produced by the lichen. However, even though the major compounds have been used for many decades in chemotaxonomy, the range of

secondary metabolites produced by lichen fungi has had very little attention with the exception of two recent studies to identify genes (Abdel-Hameed et al. 2016a and b).

Lichen morphology is generally divided into three different forms. First, crustose lichens are directly attached by the entire thallus undersurface to the substrate or they grow within the substrate and the sexually-produced fruiting structures are visible on the substrate. Second, foliose lichens are lobe-like with an upper and lower surface of the thallus and have multiple attachment organs which are used in attachment of the thallus to the substrate or plants. The third type of growth form is fruticose, which are attached to the substrate via a single point of attachment, are pendant from trees or upright from soil or rock surface, and are circular with inner and outer surfaces (Büdel and Scheidegger 2008). Depending on the substrate, lichens may fall into different categories: epilithic lichens grow on the surface of rocks, endolithic lichens grow within rocks, epiphytic lichens grow on trees, rocks, or other structures, corticolous lichens grow on bark, and terricolous lichens are found on soil (Büdel and Scheidegger 2008). The growth form, the substrate and environmental conditions within the habitat may influence the photobiont and hence the type and quantity of secondary metabolites produced by the species. Lichens are found in a diversity of colors which are attributed to the accumulation of diverse secondary compounds present in the lichens (Shukla et al. 2014).

It has been well documented that the vast majority of lichenized ascomycetes have both sexual and asexual life cycles. Many of the lichen-forming ascomycetes reproduce sexually and disperse primarily via ascospores (Honegger et al. 2004), which results in genetic variation among populations. The other option is to disperse asexually via symbiotic propagules or via thallus fragmentation (McCarthy and Healey 1978, Fröberg et al. 2001), which results in genetically isolated populations. This has been documented in a study conducted by Zoller et al.

(1999) where it was observed that *Lobaria pulmonaria* produced abundant isidiate soredia with no apotheia and these populations were strongly fragmented and geographically isolated. Species of lichen-forming ascomycetes may have many fertile apothecia such as *Xanthoria parietina*, others may have fewer apothecia such as *Parmelia tiliacea,* in other species apothecia might be extremely rare such as in *Hypogymnia physodes,* and in other species ascomata might be completely lacking such as *Thammolia vermicularis*. The gametangia (before apothecia form) are composed of haploid somatic hyphae and dicaryotic ascogenous hyphae (Büdel and Scheidegger 2008). In lichens the mycobiont expresses the full sexual reproduction but the reproduction of the photobiont is reduced (Büdel and Scheidegger 2008). The reproductive structures found in ascomycete lichen fungi are collectively called ascomata, and include apothecia, perithecia, pseudothecia and cleistothecia (Henssen et al. 1981), the most common in lichens is apothecia. Lichens are also known to reproduce through various vegetative structures, which most commonly include conidiospores produced in pycnidia. Lichens produce a variety of vegetative propagules which disperse both symbionts together, such as soredia, isidia, lobules, schizidia, or thallus fragments. Certain mycobionts are only compatible with particular photobionts (Ward 1884; Werner 1931; Jahns et al. 1979; Schuster et al. 1985; Garty and Delarea 1988; Scheidegger 1995; Clayden 1998; Sanders 2002; Sanders and Lücking 2002). Studies have also found that the fungal-algal partnership might not always be fixed and the mycobiont might switch its algal partner depending on the partner which provides more benefit to the symbiosis (Peircey-Normore 2009; Wornik and Grube 2010). Some species of lichens such as species from the genus *Bryoria* and *Cladonia* have shown growth from thallus fragments Dibben (1971) showed that fragments from some *Cladonia* species may grow in phytotron experiments. In a study conducted by Sanders 2014 on *Cladonia puiggarii* it was observed that

there were both symbiotic and aposymbiotic modes of spore dispersal (Figure 1). It was found that numerous photobiont cells were observed within campylidia where the algal cells were encircled by the maturing macroconidia (Sanders 2014). The study conducted by Sanders (2014) also provided evidence that hyphae that emerged from *Calopodia* that had been dispersed aposymbiotiocally made contact with the unicellular green algae. This has also been docuemented in previous laboratory studies conducted on lichenization (Ahmadjian and Jacobs 1983; Joneson and Lutzoni 2009). The diverse fungal and algal cells are known to attach together in close physical associations which can be compared to biofilms in bacteria (Sanders 2001; 2005).

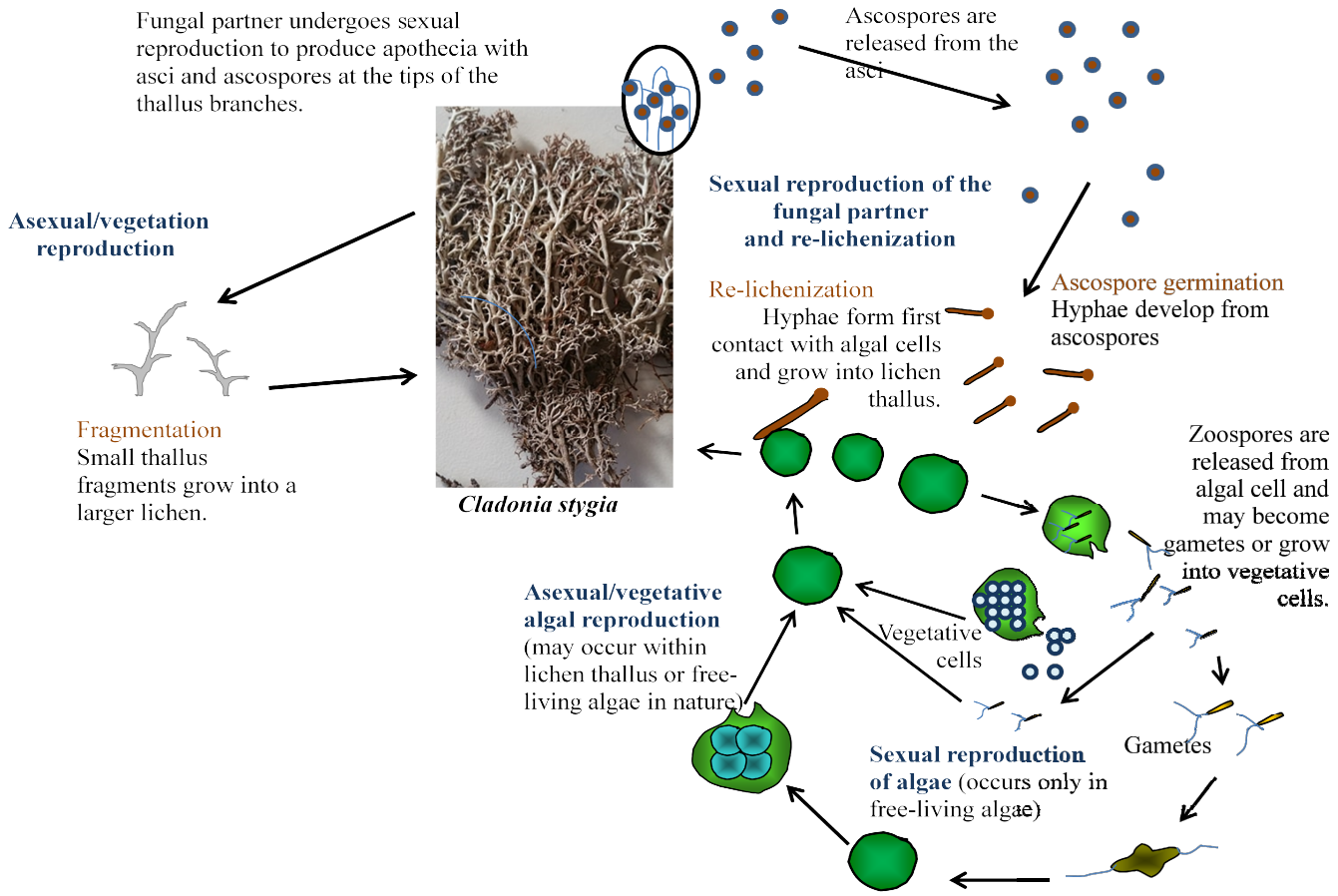



**Figure 1**: Skematic of lichen life cycle of *Cladonia stygia* created by Kamaldeep Chhoker. Figure represents both the asexual lifecyle and sexual life cycle of *C. stygia*.

The fungal partner in lichen associations produces secondary metabolites which aid lichens in adapting to the environment. Secondary metabolites produced by lichens were initially separated into four main groups: light-screening compounds, chemical weathering compounds, allelopathic compounds, and anti-herbivore defence compounds (Rundel 1978), but other groups of secondary metabolites have recently been described to have a variety of bioactive properties. The biological and pharmacological properties can be further subdivided into four main categories: antibiotic activity, antitumor and antimutagenic activity, antiviral activity, and enzyme inhibitory activities (Boustie and Grube 2005). Lichenized fungi are known to produce over 1,050 known secondary metabolites (Kirk et al. 2000; Molnár and Farkas 2010), which are mostly unique to lichen fungi (Rundel 1978) and some are very unique (Elix et al. 1984; Hauck and Huneck. 2007). Even though many secondary metabolites are unique to lichen fungi, some of the metabolites are common among large numbers of species and are consistently produced within species, which makes them a feature often used in chemotaxonomy (Frisvad et al. 2008; Molár and Farkas 2010).

Many fungal secondary metabolites are low molecular weight molecules and may be classified as Polyketides, Non-ribosomal peptides, Terpenes and Indole alkaloids (Keller 2005; Stocker-Wörgötter 2007). There are multiple theories regarding secondary metabolites: They might be a by-product of primary metabolism (Bu'Lock 1961; Keller et al. 2005), or they might have arisen from horizontal gene transfer where the genetic material is transferred between two different organisms, which then undergoes duplication and subsequent evolution (Kroken et al. 2003; Schmitt and Lumbsh 2008). Polyketides are small carbon-based molecules which are synthesized by large multidomain enzymes, polyketide synthases (PKS). The PKS are encoded by large genes which often have other genes used to tailor the polyketide. Few fungal

polyketides have been linked to the genes that encode the enzyme that produce the polyketides and none have been linked in lichen fungi with the possible exception of usnic acid (Abdel-Hameed et al. 2016). In *Penicilium nordicum*, expression of a PKS gene, *otapksPN,* was affected by pH which correlated with the production of ochratoxin A providing evidence that *otapksPN* is connected to the production of ochratoxin A (Geisen 2004). It has been well documented in previous studies that lichens and fungi contain multiple PKS genes even when only few secondary metabolites are produced (Grube and Blaha, 2003; Schmitt et al. 2005; Chooi et al. 2007) which makes it hard to determine which PKS genes might be co-related to the secondary metabolites present in those lichen species. This makes it crucial to try to identify the connection between the PKS genes and the secondary metabolite product in the lichens since the secondary metabolites produced by lichens have shown benifical pharmacological properties (Yilmaz et al. 2004; Melo et al. 2011; Backorova et al. 2012).

Two reindeer lichens, *Cladonia rangiferina* and *C. stygia*, produce both atranorin and fumarprotocetraric acid consistently (Brodo et al. 2001). Atranorin is thought to influence light penetration in the cortex of the lichen thallus and prevents the lichen from harmful UV rays (Rundel 1978; Nybakken and Julkunen-Tiitto 2006; Solhaug et al. 2010). Atranorin has also been shown to optimize the light for algal photosynthesis (Rundel 1978). Fumarprotocetraric acid is thought to play a role in protecting the lichen from hervivores and microbes (Rundel 1978; Reutimann and Scheidegger 1987; Yilmaz et al. 2004). Fumarprotocetraric acid has also been shown to act as a chelating agent in the lichen (Syers 1969; Ascaso and Galvan 1976). Both atranorin and fumarprotocetraric acid have also shown potential to be used as medicinal agents. Atranorin showed a significant decrease in the mitochondrial membrane potential (MMP) in cancer cell lines and usnic acid showed similar results, which was interpreted as being effective

against cancer (Backorova et al. 2012). Melo et al. (2011) found that atranorin concentrations of 1-100 µg/ml showed significant antioxidant effects depending on dose, and the concentration of 100 µg/ml also showed a high antioxidant effect. Both atranorin and fumarprotocetraric acid have also shown antimicrobial activity against a wide variety of microbes (Yilmaz et al. 2004). Despite the fact that lichen secondary metabolites provide many benefits to humans, the amount of research conducted on them is not significantly high. The lack of research on lichen polyketides is due to two main reasons: Firstly, lichens exhibit a very slow growth rate making them difficult to work with in the lab in terms of time to produce polyketides and avoidance of faster growing contaminants; and secondly, secondary metabolites produced in nature might not be the same as those that are produced in the lab because the growing conditions may be different (Miao et al. 2001). Since culture conditions have been optimized for *Cladonia rangiferina*/*stygia* (Athukorala et al. 2015; Elshobary et al. 2017), the species are widespread and easy to find, the genome has been sequenced (unpub. data), and they produce two major compounds atranorin and fumarprotocetraric acid, the species make good model species and provide the basis on which to build on the knowledge of the biosynthesis of their secondary metabolites.

### 1.2 Goals and Objectives

The goal of this thesis was to characterize the secondary metabolite genes in the genome of *C. rangiferina* and to use the information from the characterization to gain insights into polyketide biosynthesis in naturally occurring thalli of *Cladonia stygia* by examining correlations between gene transcription, polyketide quantity and two environmental variables. The specific objectives were:

1) to characterize more broadly the secondary metabolite genes in the genome of *C. rangiferina,*

2) to identify the non-reducing PKS genes, present in the genome of *Cladonia stygia* using the genome sequenced from *C. rangiferina*.

3) to correlate environmental factors such as canopy cover and pH with the quantity of atranorin and fumarprotocetraric acid produced by *Cladonia stygia* in its natural environment.

4) to compare quantity of polyketides with non-reducing PKS gene transcription in *C. stygia* in its natural environment.

# Chapter 2 Literature Review

## 2.1 Genomes sequenced from fungi

In recent years the number of fungal genomes that have been sequenced have drastically increased. In 2013, the Joint Genome Institute (JGI) added 63 new genomes from fungi. Examples include genomes of two basidiomycete fungi, *Malassezia globosa* and *Malassezia restricta,* which were sequenced in 2012 (Martinez et al. 2012; Sharma. 2014). Some fungi which affect human health have also been recently sequenced such as *Aspergillus fumigates* Af293, which has been known to cause Aspergillosis (Denning et al. 2002; Pain et al. 2004); *Candida albicans* Sc5314 which can cause severe systemic disease (Jones et al., 2014); *Trichophyton rubrum* CBS118892, which can cause human fungal infections (Martinez et al. 2012); *Arthroderma benhamiae* which infects keratinized tissue (Burmester et al. 2011); and *Trichophyton vernicosum,* which causes inflammation in the scalp, nails and skin (Burmester et al. 2011). An international team of researchers collaborating with the Joint Genome Institute of the Department of Energy (JGI) have been adding more sequences of fungi to the 1000 Fungal Genomes Project. As of 2018, the Fungal Genomics Resource at JGI contains more than 800 genomes out of which many have been published. The complete list can be accessed on https://genome.jgi.doe.gov/fungi/fungi.info.html.

To date, 9 different lichen-forming fungal genomes have been sequenced and are present in the JGI database (https://genome.jgi.doe.gov/lichens/lichens.info.html). *Xanthoria parietina* was the first lichen forming fungus to be sequenced in January 2011. *X. parietina* has a genome assembly size of 31.90 (Mbp) which contains 11,065 genes and contains 302 contigs. Other lichen species that have been sequenced are *Cladonia grayi*, *Cladonia rangiferina* (unpub. data),

*Endocarpon pusillum* (Wang et al. 2014), *Lobaria pulmonaria*, *Sclerophora sanguine*, *Symbiochloris reticulata*, *Trypethelim eluteriae* and *Usnea florida*. Previous studies have identified multiple polyketide synthases in both lichenized and non-lichenized fungi and the genes involved (Fujii et al. 1998; Armaleo et al. 2011). A study conducted on *Cladonia macilenta* isolated and characterized the non-reducing polyketide synthase gene *CmaPKS1* which was non-reducing type 1 PKS based on the domains present: β-ketoacylsynthase (KS), acyltransferase acyl carrier protein (ACP) and Thioesterase (TE) domain (Jeong et al. 2015). Another study by Jung et al (2012) isolated the PKS1 domain named CmPKS1 which contained five domains: β-ketoacylsynthase (KS), acyltransferase (AT), dehydratase (DH), β-ketoreductase (KR) and the phosphopantetheine attaching site (PP). Previous studies over the years have determined the type of PKS and the domain architecture of the specific PKS genes in various lichen species (Chooi et al. 2008; Brunauer et al. 2009; Gagunashvili et al. 2009; Valarmathi et al. 2009; Armaleo et al. 2011; Wang et al. 2011; Yu et al. 2013).

The most common pipeline used to characterize fungal secondary metabolite genes is antiSMASH (antibiotics & Secondary Metabolite Analysis Shell). The antiSMASH pipeline works by identifying the regions at the gene cluster level and then aligning them with the nearest relative in the database which contains the gene clusters that have been previously identified (Medema et al. 2011). antiSMASH has had increasing activity by researchers since 2011 and reliably identifying gene clusters of secondary metabolite compounds in bacteria, plants and fungi (Medema et al. 2001; Weber et al. 2015; Blin et al. 2017; Blin et al. 2017). antiSMASH is able to detect all known classes of secondary metabolite biosynthetic gene clusters, predict the non-ribosomal peptide synthetases (NRPS)/ Polyketide Synthase (PKS) product chemical structure and also provide detailed annotation of NRPS/PKS clusters. antiSMASH has shown

much more reliability and has been shown to identify clusters which had been not identified via other previous softwares (Medema et al. 2011; Weber et al. 2015). The newest version, antiSMASH 4.0, has introduced a more user friendly interface and uses more pipelines to better predict the gene clusters than previous versions (Blin et al. 2017).

### 2.2 Secondary metabolites in fungi

Secondary metabolites are compounds which are not essential for survival, but may be useful in some stressful conditions, facilitating survival under those conditions. The fact that they are not essential for survival and may vary in quantity in different environmental conditions, distinguishes them from compounds produced via primary metabolism (Keller et al. 2005; Hoffmeister and Keller 2007 Sanchez et al. 2008). To date, more than 1,050 different lichen secondary metabolites have been identified in the 18,500 lichens species that are presently known (Stocker-Wörgötter 2010; Molnár and Farkas 2010). One theory to explain the origin of secondary metabolites is that they may arise from by-products of primary metabolism (Keller et al. 2005) or detoxification of by-products. Other theories that explain the evolution and the production of secondary metabolites were described in Bu'Lock (1961) and more recent reviews suggesting that they might have arisen from horizontal gene transfer (Kroken et al 2003; Schmitt and Lumbsh 2008). This has been backed by studies that compared the pathway of penicillin in both fungi and prokaryotes which showed that some portions of the pathway might have been acquired through horizontal transfer from prokaryotes (Weigel et al. 1988; Penalva et al. 1990). It has been thought that the precursors of secondary metabolism are supplied by the metabolic pathways of primary metabolism (Drew and Demain 1977). There are different ways that this can be achieved. Primary metabolite end products are sometimes used as precursors in secondary metabolism biosynthesis. There has been evidence to show that both secondary pathways and

primary pathways share intermediates which make up branched sequences meaning that the intermediate can go into either primary metabolism or secondary metabolism depending on the enzyme present (Drew and Demain 1977). Secondary metabolism has also been shown to be affected by the carbon source (Elshobary et al. 2016), nitrogen content and the ATP energy source (Drew and Demain 1977). Studies have shown evidence that polyketide synthase genes have been transferred from different organisms via horizontal gene transfer (Kroken et al. 2003; Jenke-Kodama et al. 2005; Schmitt and Lumbsch 2008). In a study conducted by Schmitt and Lumbsch (2008) using bacterial and fungal PKS genes they observed that fungal 6-Methylsalicylic acid (MSAS)-type clade was more closely related to bacterial than fungal PKSs. They observed that the fungal 6-MSAS-type PKS gene might have been horizontally transferred from an ancestor who included bacterial iterative PKSs and a small group of bacterial modular PKSs. In bacterial modular PKS every module consisting of KS, AT, ACP and reduction or dehydration domain, carry out either initiation or one round of chain reaction. As the number of modules increases the length of the polyketide chain length also increases as well (Donadio and Staver 1991; Khosla 1997; Hill. 2006). In contrast the bacterial iterative PKS, the activity is encoded by a separate enzyme that can be used several times during the biosynthesis of the aromatic product (Hutchinson. 1997 and Kennedy et al. 1999). The iterative PKS is also the most common type found in fungi. In both fungi and bacteria a single organism can contain multiple PKS genes which is thought to have been caused by gene duplication over the evolutionary process (Kroken et al. 2003 and Jenke-Kodama et al. 2005).

The production of secondary metabolites in lichens is thought to begin when the mycelial growth slows down and there is an accumulation of carbohydrates (Culberson and Armaleo 1992; Stocker-Wörgötter 2008), and nutrients may become limiting (Moore 1998). The

accumulated carbohydrates that are not used in primary metabolism may then be used in secondary metabolism. Solhaug and Gauslaa (2004) showed that the production of parietin in *Xanthoria parietina,* increased when the carbohydrate provided by the photobiont also increased due to increased photosynthesis. Since secondary metabolite production may occur under stressful conditions (Stocker-Wörgötter 2007), it may not be beneficial for the lichen to produce secondary metabolites which do not provide any benefit to the lichen. Therefore, the quantity of secondary metabolite within a species may change depending on the environmental conditions in which individual thalli are growing (Comerio et al. 1998; Feng and Leonard 1998; Marin et al. 1999).

Recent studies have shown that lichen secondary metabolites can act as antibiotic agents, and have other biological activities such as antifungal, anticancer, and antioxidant activities (Boustie et al. 2005; Stocker-Wörgötter 2008; Boustie et al. 2011 and Shrestha et al. 2013). Abo-Khatwa et al. (1996) observed that usnic acid acted against Gram-positive bacteria due to usnic acid having the properties which helped it uncouple the oxidative phosphorylation in these bacteria. It has been observed that usnic acid helps the inhibition of bacterial biofilm formation on different polymers. Francolini et al. (2004) looked at the effects of usnic acid on biofilm formation by *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The study found that there was no significant biofilm growth on the (+)- usnic acid-loaded polymer when compared to the control with no (+)- usnic acid on the polymer by *S. aureus*. *P. aeruginosa* formed biofilm on both the control and (+)- usnic acid treated polymer but the morphology of both the biofilms were different from the control, which indicated that usnic acid was in fact affecting the morphology of the biofilms (Fancolini et al. 2004). In a study conducted by Mayer et al. (2005), usnic acid showed decreased proliferation of human lung cancer cells and human breast cancer

cells without causing any DNA damage. Some of these secondary metabolites produced by either fungi or bacteria play an important role in human medicine such as erythromycin which acts as an antibiotic, lovastatin which has been used to lower cholesterol (Cortes et al. 1990; Donadio et al. 1991; Kao et al. 1994), rapamycin which can act as an immunosuppressant (Schwecke et al. 1995; Kennedy et al. 1999)., and brefeldin A which acts as an antiviral agent (Yamamoto et al. 1985; Kennedy et al.  Hutchinson et al. 2000). Some lichen compounds have also shown antioxidant activity by scavenging for free oxygen radicals such as sphaerophorin and pannarin, which have shown inhibition of superoxide anion formation (Hidalgo et al. 1994; Odabasoglu et al. 2004; Russo et al. 2008; Karakus et al. 2009). In addition to these benefits, lichen secondary metabolites have the potential to be used in the development of new drugs (Huneck. 1999; Shukla et al. 2010; Stojanovic et al. 2012; Zambare and Christopher 2012).

The type and amount of secondary metabolite produced may be dependent on the species as well as the habitat of the lichen. For example, since some lichen species are found in the Antarctic and arctic exposed to high levels of ultraviolet (UV) light, they may produce secondary metabolites such as usnic acid, melanin or atranorin which protect the lichen from harmful UV rays (Rundel 1978; Bell and Wheeler 1986). Secondary metabolites such as fumarprotocetraric acid and squamatic acid can help the lichen in chelating minerals from the rocks on which they are found (Ascaso and Galvan 1976). Some secondary metabolites provide protective roles to the lichen and fungi from different pathogens (Rundel 1978; Khosla et al. 1999). Both atranorin and fumarprotocetraric acid are also thought to have anti-herbivore and anti-microbial activities (Asplund et al. 2010; Lawrey 1984). Secondary metabolites like barbatic acid have allelopathic properties against higher plants by impairing the function of their photosystem II (Hager et al. 2007). Other broad classes of secondary metabolites produced by lichens are anthraquinones

such as parietin which act as a light filter to provide the optimal intensity of light reaching the algal layer (Rundel 1978). A study conducted on *Xanthoria parietina* observed that the concentration of parietin was strongly related with a crude light gradient (Hill and Woolhouse, 1966). Pulvinic acid derivatives are another broad group of compounds that are derived from the shikimic acid pathway and are present in 25 lichen genera (Rundel 1978). A study conducted on (+)-enantiomer of vulpinic acid which is a pulvinic acid derivative showed that for LD50 level for ingestions by *Spodoptera litoralis* larvae was 90.8 and 111.0 μmol $g^{-1}$ dry wt, respectively and showed a strong mortality rate (Emmerich et al. 1993). Lichens are also known to produce terpenes and steroids via the mevalonic acid pathway which provides them with various benefits (Rundel 1978), such as carotenoids acting as light harvesting pigments (Czeczuga, 1985; Czeczuga et al. 1988). A diterpene furanoid isolated from lichen have been shown to have inhibitory effect against the protein tyrosine phosphatase 1B (PTP1B) activity (Yinglan et al. 2012). Zeorin which is common in red-fruited *Cladonia* species has shown significant cytotoxic activity against P-388 cancer cells (Wong et al. 1986; Brinker et al. 2007).

### 2.3 Induction and structure of lichen polyketide synthases

The photobiont or its sugar has been shown to influence production of secondary metabolites by the fungal partner (Stocker-Wörgötter 2007; Elshobary et al. 2016). Photosynthesis of the algal partner results in production of carbon through sugars, which may be used by the fungal partner in biosynthesis of the secondary metabolite (Honegger 2008; Stocker-Wörgötter 2008). A study conducted on *Cladonia rangiferina* examined the effect of algal carbohydrates on fungal polyketide synthesis. Atranorin was not present in any of the extracts containing glucose, ribitol or sorbitol; but fumarprotocetraric acid was present in algal cultures that contained ribitol (Elshobary et al. 2016). Fumarprotocetraric acid was only found in cultures

that grew on agar containing 1% and 5% ribitol but none in the culture amended with 10% ribitol (Elshobary et al. 2016). This study showed that both the type of carbohydrate and the concentration of carbohydrate can affect the synthesis of polyketides in lichens. This phenomenon has been observed in previous studies where it was observed that *Xanthoria elegans* reacted strongly to the addition of mannitol and ribitol (Brunauer et al. 2007). Mannitol also helped produce an unknown substance when compared to cultured media without mannitol (Brunauer et al. 2007). The production of lichen secondary metabolites may also be dependent on the environmental or growing conditions. It is known that stable culture conditions of fungi will form fat droplets on the surface of mycelia due to the biosynthesis of fatty acids (Stocker-Wörgötter 2007). When the conditions change from optimal to stressful the fatty acid pathway is thought to be switched to the acetyl-polymalonyl pathway which produces polyketides (Stocker-Wörgötter 2007). These environmental stresses such as cold-warm temperature treatments, high light exposure, moisture content, pH levels and simulated day-night cycles may affect secondary metabolite production (Espeso et al. 1993; Tilburn et al. 1995; Keller et al 1997; Stocker-Wörgötter 2007).

Lichen fungi produce polyketide synthase (PKS) enzymes that catalyze the biosynthesis of polyketides (Udwary et al. 2002; Kroken et al. 2003; Schumann et al. 2006), which are derived from carboxylic acids and are formed as a result of condensation steps in the biosynthetic pathway (Elix 1996). Polyketides have an extremely large diversity in their structures due to polyketides having different numbers of subunits, type of subunits and degrees of chemical reduction (O'Hagan. 1995; Liou and Khosla. 2003; Amnuaykanjanasin et al. 2009). Unique PKS genes which are present in lichenized fungi are the reason for the production of unique polyketides (Duke et al. 2003). There are two types of PKSs found in fungi known as

Type 1 and Type 2 (Stocker-Wörgötter 2008). Type 1 PKSs are produced by genes that encode large multifunctional proteins which are iterative and contain the necessary information to produce a particular polyketide (Stocker-Wörgötter 2008; Keller et al. 2005). Both fatty acid synthases (FASs) and polyketide synthases (PKSs) have similar domains including the ketoacylsynthase (KS), acyltransferase (AT), ketoreductase (KS), dehydratase (DH), enoylreductase (ER) and acyl carrier protein (ACP) domains (Stocker-Wörgötter 2008). The main difference between FASs and PKSs is that the full reduction of the β-carbon is an optional event in PKSs, which is why the simplest functional PKS core domains include only KS, AT and ACP, while reducing domains such as KR, DH and ER are not present in all lichen fungi (Keller et al. 2005). Depending on the domains present, the Type 1 PKS can be further subdivided into three main classes: non-reducing (NR), partially reducing (PR) and highly reduced (HR) (Bingle et al. 1999; Kroken et al. 2003; Cox. 2007; Elix and Stocker-Wörgötter. 2008; Stocker-Wörgötter 2008). The NR-PKSs have the keto synthase (KS) domain that elongates the polyketide backbone by catalyzing repeated decarboxylative condensation, an acyltransferase (AT) domain that selects the extension unit, an acylcarrier protein (ACP) domain which has a phosphopantetheinyl arm to act as a tether for the growing polyketide and the completed polyketides, a methyl transferase (ME) domain which sometimes introduces a methyl group to the polyketide chain, the Thioesterase/Claisen cyclise (TE/CYC) domain that cyclises and releases the products (Bingle et al. 1999; Hopwood 1997; Kim et al. 2012; Li et al. 2010; Schmitt et al. 2008). Previous studies have shown that a variety of starter units such as acetyl-CoA, ethyl-CoA, propionyl-CoA and butyryl-CoA or their equivalents can be utilized by the PKSs. Chain elongation occurs through several cycles of condensation and modification reactions (Chooi and Tang 2012). In addition to acetyl-coenzyme A and malonyl-coenzyme A,

the PKSs can also use other chemical substrates to assemble the carbon chain which help them produce a wide variety secondary metabolites that are often biologically active (Rawlings 1999 and Hutchinson et al. 2000). The three core domains (KS, AT and ACP) are primarily found in iterative type I PKSs (Miao et al. 2001; Shen 2003; Wang et al. 2012). Type 1 PKSs fall under two different classes: modular and iterative. Modular type 1 PKSs are generally associated with bacteria while the type 1 iterative class is associated with fungi (Lin et al. 2012). In bacteria, there are specific domains or modules that correspond to only one biosynthetic step. The ways in which these domains are arranged in the genome indicate the molecular structure of the final product (Staunton and Weissman 2001). Fungal type 1 PKSs differ from this arrangement because some domains are iterative and are used multiple times in the course of chemical condensation (Lin et al. 2012). Different secondary metabolites are produced using different pathways such as the shikimic acid pathway (SA), mevalonic acid pathway and acetyl-polymalonyl pathway (APM) (Edwards et al 2003).

### 2.4 *Cladonia stygia* as a species to study polyketides

*Cladonia stygia* (Figure 2) is a highly branched reindeer lichen in the family *Cladoniaceae* and genus *Cladonia.* The genus has approximately 459 species worldwide (Athukorala et al. 2016; Pino-Bodas et al. 2016) with seven taxonomic sections (Ahti 2000). One of the sections contains the reindeer lichens with highly branched thalli. The family, *Cladoniaceae,* falls within the class Lecanoromycetes, which is generally known as the most diverse and among the largest groups of lichen-forming fungi (Stenroos et al. 2002).

**Figure 2**: Specimen of *Cladonia stygia* (collection number: Normore 7680) showing a highly branched thallus with gray coloration and a blackened base. Picture taken by Kamaldeep Chhoker.

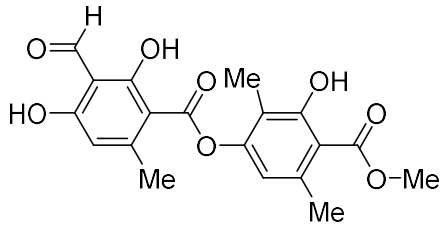
Previous studies have shown that there are about 60 secondary compounds found in the family *Cladoniaceae* out of which 30 are significant for taxonomy in the genus *Cladonia* and the remaining are minor compounds which vary in their occurrence (Huneck and Yoshimura, 1996). Species of *Cladonia,* including *Cladonia stygia,* have a mutualistic relationship with a green algal photobiont in the genus *Asterochloris* (Chlorophyta). C*ladonia stygia* is closely related to *C. rangiferina* (Stenroos et al. 2002; Athukorala et al. 2016) and they have similar morphological features. While *C. stygia* from *C. rangiferina* have been separated based on pink pycnidial jelly and a black stereome (Ahti and Hyvonen 1985; Ruoss and Ahti 1989), the names have been used interchangeably in the literature. It is unknown whether these features are a result of the different habitats they inhabit (bogs for *C. stygia* and drier sites for *C. rangiferina*) or if they have a

genetic basis even though they were shown to be conspecific by Athukorala et al. (2016). *C. rangiferina* has had its genome sequenced (unpub.) and it would be expected that both species would have very similar genes because of their morphological and phylogenetic similarity. Both species, *C. stygia* and *C. rangiferina,* are widespread in Europe, Asia, Arctic and boreal regions of North America (Ruoss and Ahti 1989). They are most common at the northern boreal timberline zone (Ahti 1964). *Cladonia stygia* is known to produce two main secondary metabolites, a depside known as atranorin and a depsidone known as fumarprotocetraric acid (Fontaniella et al. 2000), which provide different benefits to the lichen. Both of these compounds are known to arise from the acetyl-polymalonyl pathway (Edwards et al. 2003).

## 2.5 Polyketides produced by *Cladonia stygia*

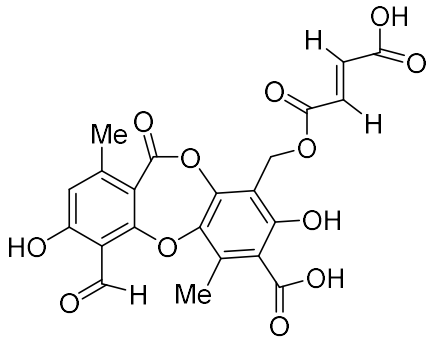
Atranorin is a depside consisting of two phenolic rings joined by an ester linkage which undergoes a condensation step to remove the water and join the molecules together (Figure 3a) (Rundel 1978; Schmitt et al. 2008).

A




Atranorin

B




Fumarprotocetraric acid

**Figure 3**: Schematic representing structure of (a) atranorin and (b) Fumarprotocetraric acid. Structures were created by Kamaldeep Chhoker and are based on those present in a study by Edwards et al. 2003.

Atranorin is found in the outer layers of the thallus around the photobiont where it helps to protect the photobiont from harmful UV rays (Nybakken and Julkunen-Tiitto 2006). Atranorin has the ability to screen light which is why it is located in the cortex of the thallus (Rundel 1978). A study conducted by Solhaug et al. (2010) examined reflectance by *Physcia aipolia* which contains atranorin. The study showed that reflectance values were different between the hydrated control thalli when compared to the hydrated thalli deficient of atranorin, suggesting that atranorin helps the lichen by reflecting the harmful UV rays. The hydrated thalli containing atranorin might be able to reflect more light than thalli deficient of atranorin due to atranorin having hydrophobic properties. In this same study, it was hypothesized that atranorin may have increased the reflectance by preventing water from entering the spaces present in the cortex (Solhaug et al. 2010). Begore and Fahselt (2001) observed that the amount of atranorin increased when lichens were subjected to UV-A (high or low) and higher visible light when compared to a control treatment. Atranorin is thought to change the wavelength of the light to either make the light less harmful or to optimize it for algal photosynthesis (Rundel 1978). Atranorin has also been shown to have antiherbivore properties. Nimis and Skert (2004) studied the damage caused by herbivores where grazed species of lichens produced low levels of atranorin. It was observed that the damage caused to species producing atranorin was between low to medium range compared to some other species that lacked atranorin which showed higher damage caused by the hervibores (Nimis and skert 2004).

Another polyketide produced by *Cladonia stygia* is fumarprotocetraric acid, which is a depsidone (Figure 3b). Depsidones are bicyclic phenolic compounds containing a linkage between two monocyclic units that have both ester and ether bonds (Fontaniella et al 2000). Previous studies have proposed that depsidones are produced by depsides through

dehydrogenative coupling due to the similarities in structures between depsides and depsidones (Erdtman and Wachmeister 1957; Culberson 1964). The evidence for this was provided by a study conducted in 1964, where an oxidative coupling reaction was utilized in the synthesis of a naturally occurring depsidone (Culberson 1964). Vicente et al. (1984) proposed that atranorin a depsides might also be a probable precursor to fumarprotocetraric acid. In a study conducted on *Cladonia sandstedei* it was observed that the production of fumarprotocetraric acid was inversely related to atranorin accumulation (Vicente et al. 1984). Elix et al. (1987) devised a chemical process which has been used for the synthesis of many depsidones. In the first step, two individual polyketides are synthesized from acetate/malonate and cyclised to orcinol or β-orcinol rings, which are then linked by esterification to form a depside. The depside is then changed to a depsidone in four steps called hydroxylation, acyl migration, smiles rearrangement, and esterification (Elix et al. 1987; Armaleo et al 2011). The study conducted by Fontanielle et al. (2000) was performed on alginate-immobilized cells of *Cladonia verticillaris* (Figure 4A). Fumarprotocetraric acid was only produced when the cells were supplemented with FMN and acetate which implied that fumaric acid is added through a reducing flavin-dependent coupling reaction which used the pool of succinyl-CoA from the fungal partner (Fontanielle et al. 2000).

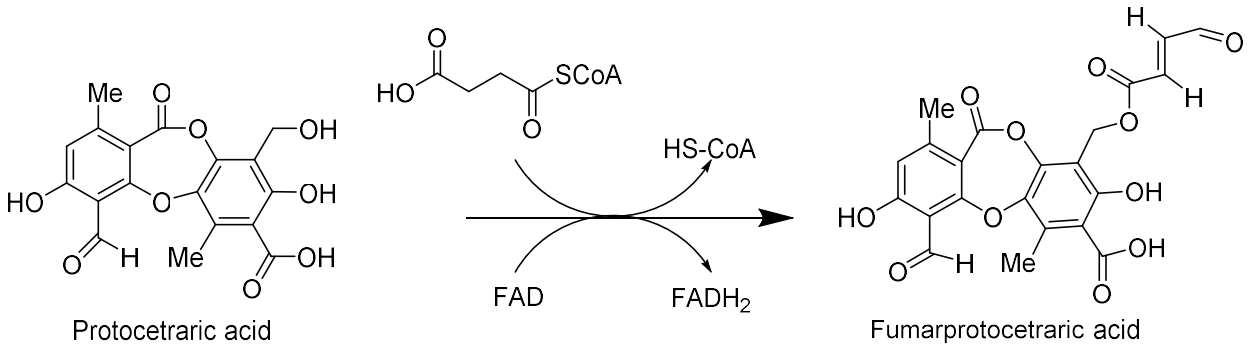



**Figure 4A**: Schematic representing the synthesis of fumarprotocetraric acid using protocetraric acid as a precursor. Fumarprotocetraric acid is synthesized by the acetyl-polymalonyl pathway (Fontanielle et al. 2000; Edwards et al 2003). Schematic created by Kamaldeep Chhoker.

Fumarprotocetraric acid has been hypothesized to have both anti-herbivore and anti-microbial properties. In an early study conducted by Liano (1948) it was observed that reindeer did not feed on *C. arbuscula* or *C. rangiferina* which contained fumarprotocetraric acid. However, Rundel (1948) showed that reindeer did indeed feed on *C rangiferina* but did not feed on *C. arbuscular,* which had a higher concentration of fumarprotocetraric acid than that found in *C. rangiferina.* This study suggested that the concentration of a particular secondary metabolite can have a larger effect then just having the compound present or absent. Fumarprotocetraric acid has also been shown to have allelopathic properties. In a study conducted on a vascular plant, *Lactuca sativa,* it was observed that the seedling germination and the size of the leaf area was reduced when the germinated seedlings were transferred to petri plates containing water solutions of the different organic extracts (fumarprotocetraric acid and protocetraric acid) compared to the control where fumarprotocetraric acid or protocetraric acid were absent (Tigre et al. 2012). Fumarprotocetraric acid was also shown to have antimicrobial activity on a wide variety of microbes (Yilmaz et al. 2004). When compared with usnic acid and atranorin it was observed that usnic acid had the highest antimicrobial activity followed by fumarprotocetraric acid and then atranorin with the least activity. There is also evidence that fumarprotocetraric acid

can act as a chelating agent where Syers (1969) examined the chelating ability of fumarprotocetraric acid and *Parmelia conspersa* growing on granite. Based on his results it was hypothesized that the chelating ability of *P. conspersa* might be affected by the presence of fumarprotocetraric acid in the thallus. Fumarprotocetraric acid contains side groups that can function as electron donors which can aid in chelation (Syers 1969).

Neelakantan et al. (1962) examined the synthesis of atranorin using trifluoracetic anhydride as a condensing agent to condense both haematommic acid and methyl β-oricnol carboxylate to synthesise atranorin (Figure 4B). Another study conducted on *Himantormia lugubris*, a genus of lichenized fungi found in Antarctica, showed that Methyl β-orcinol carboxylate is a precursor to the production of atranorin (Mateos et al. 1991). Garcia-Junceda et al. (1987) observed that atranorin accumulated in the thalli of *Pseudevernia furfuracea* while it was floating on distilled water under both dark and light conditions. There was a large increase in the concentration of atranorin when the thallus was placed in light by production of (3-(3,4-dichlorophenyl)-1,1-dimethylurea) (DCMU). Based on their study, two pathways for initiating the biosynthesis of lichen phenols have been suggested: 1) stored carbohydrates are mobilized to produce acetate, and 2) catabolism of mannitol (or glucose) to act as photoassimilates (Garcia-Junceda et al. 1987). Both of these pathways are thought to produce compounds that have been derived from the orcinol and β-orcinol series, where the presence of a CH3 group on the third carbon on β-orcinol differentiates the molecules (Armaleo et al. 2011) The β-orcinol series is produced by esterification of two orsellinic acids that are catalyzed by several orsellinate depside hydrolases (Vicente et al. 2003). Atranorin has been shown to be produced from acetate even when it is a sole source of carbon but the production is increased when oxygen is supplied. The use of oxygen in the synthesis of atranorin was supported by the increase in atranorin content

extracted from field-collected samples of *Evernia prunastri* when the media was supplemented by NAD+ (Vicente et al. 2013).

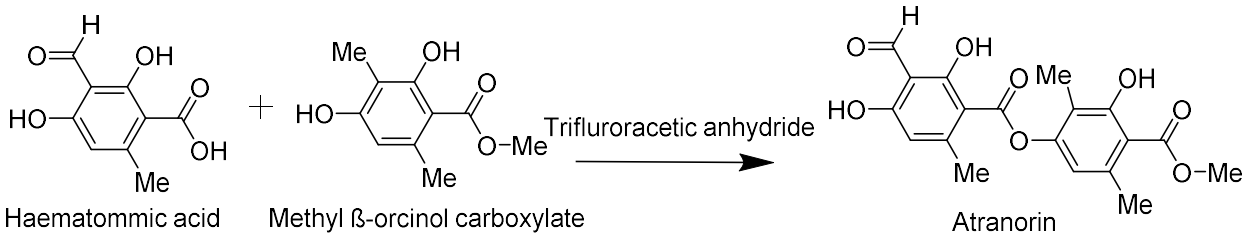



**Figure 4B:** Schematic representing the synthesis of atranorin from condensation of haematommic acid and methyl β-orcinol carboxylate using trifluoracetic anhydride as a condensing agent (Neelakantan et al. 1962). Schematic created by Kamaldeep Chhoker.

## 2.6 Effect of pH levels on secondary metabolite production

There have been previous studies conducted on fungal PKSs that show the effect of pH on PKS gene transcription. A study conducted by Brzonkalik et al. (2005) examined how production of alternariol (AOH) and alternariol monomethylether (AME), a mycotoxin produced by species of the genus *Alternaria,* are affected by changes in pH levels. The pH ranged from 3.5 to 7.5 in different shaking flasks containing *Alternaria*. It was observed that the highest mycotoxin concentrations were present at acidic pH levels. As the pH increased from 5.5 to 7.5 the mycotoxin concentration decreased with the increasing pH level (Brzonkalik et al. 2012). Bizukojc et al. (2012) examined the effect of different pH levels on the biosysnthesis of mevinolinic acid and other secondary metabolites by *Aspergillus terreus*. It was observed that mevinolinic acid concentration increased with increasing pH where a pH of 3.5 produced the lowest and both 6.5 and 7.5 pH levels produced higher concentrations. Monacolin L was found to be present in higher amounts at pH levels below 5.5 and it was present in very low amounts compared to mevinolinic acid at a pH level of 9.5. Another metabolite, (+)-Geodin, was only found to be present at an initial pH of 6.5 (Bizukojc et al. 2012).

# CHAPTER 3 Methods

## 3.1 Experimental Design

Two locations were selected for field collection of specimens in July 2016, which were on Athapap Road and Sherridan Road in Manitoba. These locations differed in the composition of the ground and surrounding vegetation and underlying geological make-up of the bedrock (Doering 2014). Athapap was comprised of limestone bedrock which made the underlying substrate basic and Sherridan had granite bedrock which made the substrate acidic. Both these locations had different types of trees and the canopy cover varied due to the number and density of trees present (Table 1). Three smaller sites were selected from the Athapap area and four sites were selected from Sherridan, which were at least 1 km apart. Three quadrats were randomly placed within each of the sites taking into account that each quadrat had *Cladonia stygia* present. The quadrats were 0.5 m by 0.5 m. In total, there were 9 quadrats from 3 sites in Athapap, and 12 quadrats from 4 sites in Sherridan, which resulted in a total of 21 quadrats in two locations.

**Table 1:** Study locations in Manitoba (Athapap and Sherridan) during July 2016. Each site is represented by three pH values (1 value per quadrat) and three canopy cover percent values (1 value per quadrat). (See Appendix D for full fieldwork spreadsheet).

| Location | Habitat | Latitude and longitude | pH | Canopy cover |
|---|---|---|---|---|
| Athapap Site 1: 12.6 Km east from Hwy 10, parallel to Athapapuskow Lake. | Limestone Table, *Abies balsamea, Aralia nudicaulis*, *Betula papyrifera, Linnaea borealis*, *Picea glauca*, *Populus tremuloides, Rosa arkansana*. | N54°30'40.2" W101° 32'43.3" | 5.5-6.0<br>5.5-6.0<br>5.5-6.0 | 25%<br>25%<br>40% |
| Athapap Site 2: 11.2 Km east from Hwy 10, parallel to Athapapuskow Lake. | Limestone Table, *Abies balsamea*, *Alnus glutinosa*, *Aralia nudicaulis*, *Betula papyrifera*, *Cornus canadensis, Empetrum nigrum*, *Fragaria virginiana*, *Linnaea borealis, Rhododendron tomentosum*, *Rosa arkansana*, *Picea glauca*, *Populus tremuloides*, *Vaccinium* spp. | 54°31'03.6"N 101°31'38.5"W | 5.5-6.0<br>5.5-6.0<br>5.5-6.0 | 23%<br>25%<br>18% |
| Athapap Site 3: 9.7 Km east from Hwy 10, parallel to Athapapuskow Lake. | Limestone Table, *Abies balsamea*, *Betula papyrifera*, *Empetrum nigrum*, *Fragaria virginiana*, *Kalmia* sp, *Linnaea borealis, Picea glauca*, *Pinus banksiana*, *Populus tremuloides*, *Rosa arkansana* | N54°31'26.5" W101°30'31.5" | 5.5-6.0<br>5.5-6.0<br>5.5-6.0 | 80%<br>72%<br>90% |
| Sherridan Site 1: Near Naeosap Lake, 28 Km from Hwy 10. | Granodiorite rock Mat forming lichens and mosses, east facing granite rock ridge, *Picea glauca Pinus banksiana*. | N54°50'38.4", W101°28'45.6" | 5.0-5.5<br>5.0-5.5<br>5.0-5.5 | 50%<br>60%<br>45% |
| Sherridan Site 2: 21-22 Km from Hwy 10. | Sandy Plain between granite ridges; *Gaultheria*, *Pinus banksiana*, *Vaccinium* spp. | N54°47'50.3", W101°27'44.3" | 5.0-5.5<br>5.0-5.5<br>5.0-5.5 | 80%<br>80%<br>70% |
| Sherridan Site 3: 18-19 Km from Hwy 10. | Granite rock table, *Betula papyrifera*, *Linnaea borealis*, *Picea glauca Pinus banksiana*, *Vaccinium* spp. | N54°46'24.9", W101°28'40.4" | 5.0-5.5<br>5.0-5.5<br>5.0-5.5 | 90%<br>0%<br>0% |
| Sherridan Site 4: 16-17 Km from Hwy 10. | Granite rock table, *Picea glauca*, *Pinus banksiana*, *Rhododendron tomentosum*, *Vaccinium* spp. | N54°45'46.5" W101° 30'10.1" | 5.0-5.5<br>5.0-5.5<br>5.0-5.5 | 0%<br>0%<br>0% |

### 3.2 Variables measured

The variables that were recorded include soil pH, percent ground cover of each species in the quadrats, and canopy cover.

The pH measurements were conducted using a pH strip (Sigma-Aldrich, Oakville, ON, Canada) with increments of pH of 0.5 and ranged from pH 5 to pH 14. Sterilized distilled water was used to moisten the soil before the pH strip was placed in the soil. The pH of the soil was measured by placing the pH strip directly on moist soil approximately 5 cm below the soil surface and holding it in the soil for about 5 minutes until there was no more color change. The color on the strip was compared to the standard color chart based on different pH values provided by the manufacturer.

Canopy cover was also recorded from each quadrat by three people who estimated the proportion of canopy cover directly above each of the quadrats. If there were no canopy branches directly above the quadrat, the canopy cover was assigned a value of 0, and if the view was completely covered by canopy the canopy was assigned a value of 100%. If the values from three individuals ranged between ± 10%, the average of the three values were obtained to get the final value per quadrat.

The thallus samples were collected by preparing 90 labelled Eppendorf tubes containing 1 mL RNAlater (Fisher Scientific, Nepean, ON, Canada) before going into the field. Four different thallus samples of *Cladonia stygia* were collected from each quadrat. The top 2 cm portion of the branching thallus was broken off with clean forceps and placed directly within each tube of RNALater. Four additional thallus samples of *C. stygia* were collected and dried as specimen vouchers.

Representative specimens of all lichen species in the quadrat were collected for confirmation of identification. The percent cover of each species present in the quadrat was recorded using a 0.5 m by 0.5 m quadrat divided into 2.5 cm grid and the cover was estimated for each species. All specimen vouchers were deposited in the Memorial University of NL, Sir Wilfred Grenfell Campus Herbarium (SWGC). To obtain of frequency of occurrence of lichen and bryophyte present in each location. The total number of times that particular lichen or bryophyte was found in Athapap was divided by the total number of quadrats in Athapap and the total number of times that particular lichen or bryophyte was found in Sherridan was divided by the total number of quadrats in Sherridan.

### 3.3 Thin Layer Chromatography

Thin layer chromatography (TLC) was used to determine the major secondary metabolites of all species that needed confirmation through their secondary products. The procedure followed that of Orange et al. (2001). Briefly, 5 mg of thallus sample was obtained from each specimen, gently crushed, and placed in an eppendorf tube. About 250 µL of acetone (depending on the size of the sample) was added to the samples to cover the lightly crushed lichen thallus. A larger amount of acetone can dilute the secondary metabolites making them difficult to be observed on the TLC plate. After adding the acetone to the tubes with thallus samples, the samples were incubated on the bench top for 30 minutes to provide enough time for secondary metabolites to dissolve in the acetone.

The TLC aluminum plate coated with a layer of silica powder (Fisher Scientific, Nepean, ON, Canada), was prepared by marking spots with a blunt pencil equidistant across the line of origin. By using a 5µL capillary tube, the acetone from each incubated thallus sample was dissolved onto each pencil spot 25 times. The complete TLC plate was submersed into a TLC

tank containing solvent A [10% toluene, dioxane and acetic acid (180:45.5 ml)]. When the TLC plate was immersed in the solvent solution, the solvent diffused up the silica coating on the plate carrying the secondary compounds along with it. The TLC plate was submersed in the solution for approximately 8 minutes until the solvent had reached the top of the plate. The TLC plate was then removed from the solution and left outside the tank to dry in the fume hood. After the TLC plate had dried the plate was observed under an UV lamp at both short (254 nm) and long (365 nm) wavelengths, and any compound present was marked with a blunt pencil. Other characters were also recorded such as quenching or fluorescence. The TLC plate was then removed and placed in the fume hood to be developed with 10% sulphuric acid using a brush to coat the TLC plate, and left to dry for about 30 minutes. The dried TLC plate was then placed in the oven to be baked for 6-8 minutes at 80ºC, followed by cooling inside the fumehood, and then the plates were scanned to preserve electronic images.

Rf classes were assigned based on the Rf classes and color characteristics of the known compounds (atranorin and fumarprotocetraric acid) present in the control *Cladonia rangiferina* (collection number: Normore 1547). The characteristics of the compounds present on the TLC plate were compared with a standard spreadsheet (Culberson, unpub) as well as Orange et al. (2001) which contained information about the compound color, florescence, and quenching, under visible light, short wave, long wave and the Rf class. See appendix Figure A1 for sample image.

### 3.4 High Performance Liquid Chromatography

A preliminary test was first conducted on extra thalli of *Cladonia stygia* to optimize the method. Then the 9 samples from Athapap and 12 samples from Sherridan were tested for the quantification of the two major compounds in each sample (atranorin and fumarprotocetraric

acid) by High Performance Liquid Chromatography (HPLC) analysis. The samples were ground using a ceramic mortar and pestle until a fine powder was obtained. Empty scintillation vials were weighed and then the powder was added to them by scraping with a metal spatula to a final weight of 0.080g for each of the 21 samples. Two mL of acetone were then added to the samples, which were then left in acetone for 30 minutes on the bench. For each sample, one capillary tube was also prepared by adding a small amount of cotton to act as filter. The acetone powder mixture was then transferred to the smaller vials through the capillary tube. The cotton acted as a filter and only allowed acetone containing the secondary compounds to go through and preventing the thallus debris from passing through. The vials were then placed in the fume hood for two days to let the acetone evaporate leaving the crystals behind.

Once the acetone had evaporated, the vials were reweighed. The weight of the empty vials was subtracted from the weight of vials containing the dried mass of crystals to obtain the weight of the crystalline product. The samples were then re-dissolved in acetone. The amount of acetone varied depending on the weight of dry residue present in the vials resulting in a final concentration of 2000µg/mL. The samples were then sonified for 60 minutes to clean the vials from any residue present and properly dissolve the crystals in the acetone.

To use the samples in HPLC, 50µg/mL samples were prepared from the starting concentration of 2000 µg/mL for each vial using the equation C1 (50 µg) x V1 (1 mL) = C2 (2000 µg) x V2 (?). A Waters HPLC Separations Module 2695 combined with a PDA Detector Model 2996 was used for HPLC analysis. The column was a µBondapak Waters c18 (3.9 x 300mm), which had 125 Å pores with a column particle diameter of 15-20µm. The flow rate was steady at 1 mL/minute. The gradient was held at 20 % methanol to 80 % water in 0.075 % aqueous trifluoracetic acid for 10 minutes then the percent of methanol was linearly increased up

to 80 % and held at that composition for 20 minutes followed by a linear gradient back to 20 % methanol for 10 minutes and held there for 10 minutes. Standards for atranorin and fumarprotocetraric acid (ChromaDex) were used as references. The standards were diluted to different concentrations ranging from 500 µg/mL to 5 µg/mL and ran on HPLC which were then used to calculate a standard curve equation for both of the metabolites. The cycle contained two Methanol Hydroxide (MeOH) wash steps in the beginning, two at the end and one MeOH wash step between every sample. Each sample took approximately 2 hours to run.

### 3.5 RNA Extraction

Samples for RNA extractions were obtained from the field-collected samples stored in RNALater (Fisher Scientific, Nepean, ON, Canada). Sterilized forceps were used to break the branched thallus which was then transferred to a tube containing Trizol (Invitrogen, Burlington, ON) and the manufacturer's instructions were followed. The branched thallus was placed in the Eppendorf tubes, which were homogenized using 1ml of Trizol (Invitrogen, Burlington, ON). After the addition of the Trizol, blue sterilized pestles were used to grind the sample until it was completely homogenized. The tubes were then left in the fume hood to incubate for 5 minutes. Phase separation involved adding 0.2 mL chloroform (Sigma-Aldrich, Oakville, ON, Canada) to the tubes containing the homogenized thallus sample. After the chloroform was added, the tubes were shaken vigorously for about 15 seconds with a 3 minute incubation period at RT on the bench. The samples were then centrifuged for 15 minutes at 10,000 RPM at 4°C, and the tubes were carefully removed to avoid mixing of the three layers. The aqueous layer on top was carefully removed in 0.2 mL increments by using a pipette and placed in a sterile tube for further isolation of the RNA. Precipitation of the nucleic acid was performed by adding 0.5 mL of 100% isopropanol (Sigma) which was added to the tubes containing the aqueous phases. The tubes

were then mixed by inverting them several times and incubated at RT on the bench for 10 minutes. The tubes were then centrifuged at 10,000 RPM for 10 minutes at 4°C and the supernatant was gently removed from each tube. One mL of 75% cold ethanol (4°C; Sigma) was added to each tube to wash proteins and salts from the pellet. Once the cold ethanol had been added, the samples were finger vortexed and then centrifuged at 7,800 RPM for 5 minutes at 4°C. The supernatant was then discarded. The tubes were then inverted on the bench to allow the RNA pellet in the tube to air dry. After drying, 35 µl of RNAase free water was added to the samples in the tubes. The tubes with samples were then finger vortexed to dissolve the pellet in the water. The samples were then placed in a water bath at 58°C for 15 minutes to further dissolve the pellets. The samples were then placed in -20°C freezer for storage.

### 3.6 Removal of DNA from the RNA extraction

The RNA obtained was subjected to DNase 1 (Invitrogen, Burlington, ON, Canada) following the manufacturer's instructions to remove any DNA present in the RNA extraction. The final concentration of RNA was brought to 1 µg/mL. After adding the appropriate volumes from each sample, 1 µL of 10x DNAse 1 reaction mix (Invitrogen, Burlington, ON, Canada) was added to the vials. This was followed by the addition of 1 µL DNase 1 (Amp Grade, 1 U/µL; Invitrogen, Burlington, ON, Canada). The total volume of the sample was then brought to 10 µL by adding DEPC-treated water (Sigma). The samples were then incubated for 15 minutes at room temperature and the DNase I was then inactivated by adding 1 µL of 25 mM Ethylenediaminetetraacetic acid (EDTA) and heated for 10 minutes at 65ºC. The DNased RNA was then ready to be used to prepare cDNA. To ensure DNA was absent from the RNA, a PCR was conducted on random samples to ensure that the DNase procedure had worked.

### 3.7 cDNA Synthesis

Each DNA-free RNA sample was prepared by adding 4 µL of 5x Reaction Mix (Fisher Scientific, Nepean, ON, Canada), 2 µL of Maxima Enzyme Mix (Fisher Scientific), 2 µL of 1 µg/mL RNA template (Fisher Scientific) and 12 µL of nuclease free water (Fisher Scientific). For the reverse transcriptase control, no enzyme mix was added and 1 µL of each RNA template was added followed by the addition of nuclease free water to bring the total volume to 20 µL. For the negative control, there was no RNA template and nuclease free water was 14 µL. The samples were mixed and centrifuged followed by an incubation for 10 minutes at 25ºC and then at 50ºC for 15 minutes. The reaction was then terminated by heating at 85ºC for 5 minutes. The cDNA was then stored in a freezer at -20ºC.

### 3.8 PCR on cDNA

Two types of PCR reactions were performed. First, PCR was performed on cDNA to optimize the primer reactions. (See appendix C1). Ten µL reactions were prepared, which included 5 µL SYBER Green Real-Time PCR Master Mix (Fisher Scientific, Nepean, ON, Canada), 1 µL of 10 µM forward primer (Sigma), 1 µL of 10 µM Reverse primer (Sigma) and 3 µL of 1:10 diluted cDNA (= 1µg RNA) was added to each sample. For the negative control, 3 µL of Ultra Pure Distilled Water (Invitrogen, Burlington, ON, Canada) was added instead of the cDNA. The PCR vials were then added to the MJ Mini Personal Thermal cycler (BIO-RAD, Mississauga, ON, Canada) with an initial 5 min at 95ºC, and then 34 cycles of 95ºC for 15 seconds, 62ºC for 15 seconds and 72ºC for 15 seconds, and ending with 72ºC for 2 minutes followed by 25ºC for infinite hold. The samples were then run on the 1% agarose (Fisher) gel in 1X Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA).

The second type of PCR reaction was conducted to determine the optimal cycle number for qPCR. After the PCR reaction was optimized, the reaction volume was increased to 15 µL of SYBER, 3 µL of 10 µM forward primer and 3 µL of 10 µM reverse primer and 8 µL of 1:10 diluted cDNA. The negative control consisted of 5 µL SYBER, 1 µL of 10 µM forward primer, 1 µL of µM reverse primer and 3 µL Ultra Pure Distilled Water. Six µL of the reaction mix was removed from the PCR tubes containing the cDNA and reaction mixes at cycle numbers 32, 34, 36, 38, 40 (to observe the quantitative expression increase at each cycle). The samples were run on a 1% agarose gel in 1X TAE buffer. This PCR was conducted to identify the increasing transcription level of the genes represented by the cDNA product to determine the optimal cycle number for semi-quantative PCR (see Appendix C2).

### 3.9 Electrophoresis

Electrophoresis was conducted by adding 2.5 µL of loading dye (bromophenol blue; Invitrogen, Burlington, ON, Canada) to 8 µL of each sample to the wells of the 1.7% agarose gel. Two µL of 100 bp DNA ladder (Invitrogen, Burlington, ON, Canada) was also mixed with loading dye and added to the gel. The gel electrophoresis was run for approximately 30 minutes at 115 volts or until the dye reached 3/4 distance of the gel. The gel was then removed and observed under 254 nm UV light and the image was taken by Quantity One-4.6.9 software (BIO-RAD).

### 3.10 Semi-quantitative PCR

After obtaining the images, the best 3 cycle number for semi-quantitative PCR was determined based on where the transcription could be seen increasing as the cycle numbers increased before saturation of transcription had occurred. For most samples, cycle numbers 30,

32, or 34 were chosen but for the positive control, cycle number 20, 22 and 24 were chosen. Semi-quantitative PCR was performed using 14 thallus samples; 7 samples from Athapap and 6 samples from Sherridan. For each sample, a master mix was made consisting of 10 µL of SYBER, 2 µL of 10 µM forward primer, 2 µL of 10 µM reverse primer (Table 2) and 8 µL of 1:10 diluted cDNA (= 1µg RNA) cDNA. The tubes were then mixed properly and placed in the MJ Mini personal thermal cycler. The PCR cycle was an initial 5 min at 95ºC, and then 34 cycles of 95ºC for 15 seconds, 62ºC for 15 seconds and 72ºC for 15seconds, and then 72ºC for 2 minutes and ending with 25ºC for infinite hold. Seven µL aliquots were removed from the PCR tubes at cycle numbers 32, 34 and 36 for target genes and cycle numbers 20, 22, 24 for the control gene (MrSSU) to quantify the change in the gene transcription. The control gene was being expressed much higher than the others, which is why it was removed at earlier cycles to better estimate the change in transcription levels. The samples were then run on a 1.7% agarose gel in 1X TAE buffer. Seven µL of sample and 2.5 µL of loading dye were mixed and loaded into the 1.7% agarose gel. The gel was then run for an hour at 100 Volts and was then removed from the electrophoresis chamber and placed in a solution consisting of 150ml 1X TAE buffer and 2.5 µL of 0.5 µg/ml ethidium bromide (Fisher Scientific, Nepean, ON, Canada). The gel was incubated in the solution for 35 minutes while being constantly shaken at a medium pace. After 35 minutes the gel was removed and then incubated in 150 mL sterile distilled water for 15 minutes while being shaken. After 15 minutes the gel was removed and an image was taken using Quantity One-4.6.9 software (See Appendix C3).

**Table 2:** Primers designed from sequences of the Beta-ketoacyl synthase and Thioesterase domains. The table also includes the primers for the mitochondrial small subunit (MrSSU) gene used as the reference gene.

| Oligo Name | Sequence 5' to 3' (include modification codes if applicable) | Annealing Temperaure |
|---|---|---|
| MrSSU1 | AGCAGTGAGGAATATTGGTG | 62° C |
| MrSSU2R | CCTTCGTCCTTCAACGTCAG | |
| CrPKS1-KS771F | TGCCTTTCAAGCGATGGACT | 62° C |
| CrPKS1-KS930R | GGCTCTGATCATACCCGTCG | |
| CrPKS10-KS1806F | TTGGCGTTACGATTGGGTGA | 62° C |
| CrPKS10-KS2369R | TGCTGGCGTCATGGATCTT | |
| CrPKS10-TE770F | TCCGCTGTTCCGACTAGAGA | 62° C |
| CrPKS10-TE1328R | CAGAAACATCGACCCTCGCT | |
| CrPKKS20-KS 5538F | GTATGTACTCCAGGACGCCG | 62° C |
| CrPKS20-KS5703R | GAGCGAGTGTTCTGACTGCT | |
| CrPKS21-KS1485F | AGTGCCGTCTTTGTTTTCGC | 62° C |
| CrPKS21-KS1660R | TGGGCGAGGACCAATAAACC | |
| CrPKS23-KS270F | TCTTGGCAGCGTCGTAAGTT | 62° C |
| CrPKS23-KS428R | CGCTGATCATCGAGCCTCTT | |
| CrPKS26-KSF | TCTCACGGCAAGCATGGAAT | 62° C |
| CrPKS26-KSR | AGTGCGGAGATGGGTCTACT | |
| CrPKS26-TE1613F | GCGCTATCGTTTGCGTTCAT | 62° C |
| CrPKS26-TE1771R | CAGGGCGATCGACGACATAA | |

* Multiple primer pairs were also designed for Cluster 14 but all of them failed to amplify any of the bands; hence, that cluster was removed from the experiment.

### 3.11 Genes chosen for this study and primer design

The internet program, antiSMASH, was used to determine the secondary metabolite clusters in *Cladonia rangiferina* genome (accessed at https://antismash.secondarymetabolites.org/#!/start). The fasta file of the *C. rangiferina* genome (unpub.) was uploaded to the antiSMASH online software. The software took multiple hours to identify all the secondary metabolite clusters in the *C. rangiferina* genome. Eight target genes representing the two non-reducing clusters and four partially reducing clusters were used for this study. A portion of the Mitochondrial Small Subunit was used as a control and as a reference gene. To design the primers, the clusters obtained from antiSMASH were downloaded which provided the sequences of domains present in the clusters. Genome Complier (TWIST BIOSCIENCE) software was used to view the clusters and to obtain the specific sequences of the Beta-Ketoacyl Synthase domains and the Thioesterase domains from the target genes. The sequences were then used to design the primers on PrimerQuest Tool (Integrated DNA Technologies) accessed at https://www.idtdna.com/Primerquest/Home/Index.

To prepare samples for sequencing, the PCR was conducted using a larger reaction mixture of 60 uL (36 µL SYBER, 7 µL of 10 µM Forward primer, 7µl of µM Reverse Primer and 10µl cDNA mix (88 ng/µL). The amplified PCR product was then run on a 1% agarose gel in a 1X Tris-Borate (TBE) buffer and the bands were excised from the gel and cleaned using the Wizard SV-Gel and PCR Clean-up kit (Promega, Madison, Wisconsin, USA) following the manufacturer's directions. The cleaned PCR product was then resuspended in 36 µL of nuclease free water provided by the manufacturer in the kit. The samples were first cycle-sequenced using Big Dye v.3.1 (Applied Biosystems, Foster City, CA) and then were cleaned using ethylenediaminetetraacetic acid (EDTA), following the manufacturer's instructions. The product

was then resuspended in Hi-Di formamide and sequenced using a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequences were then cleaned using Chromas Pro v2 (Technelysium Pty Ltd, South Brisbane, Australia). The cleaned sequences were submitted to the NCBI database for a BLAST search and the results were obtained. The remaining sequences were sent to Genomics Services DNA Sequencing Services (Memorial University of NL, St. John's, Newfoundland, Canada) for sequencing using Applied Biosystems Big Dye chemistry on an ABI 3730 DNA Analyzer.

### 3.12 Amplification of Purified cDNA

To make the serial dilution of the target cDNA concentrations, samples were obtained using the similar steps used during the samples being prepared for sequencing using Wizard SV-Gel and PCR Clean-up kit protocol. The concentration of the purified product was calculated using a NanoDrop 2000/2000c Spectrophotometer (Fisher Scientific) which was then used to make serial dilutions. The serial dilutions ranged from 1 nanogram (ng 1.0E9) to 1 zeptogram (zg 1.0E-21). Known cDNA dilutions were then run through PCR along with one sample containing the cDNA from the target gene. The PCR reaction volume was 10 µL containing 5 µL SYBER, 1 µL of 10 µM Forward primer, 1 µL of 10 µM Reverser primer and 3 µL of either of the dilution of known concentration or the 1:10 diluted cDNA (= 1µg RNA) cDNA. The PCR cycle was an initial 5 min at 95ºC, then 30 cycles of 95ºC for 15 seconds, 62ºC for 15 seconds and 72ºC for 15seconds, and a final extension of 72ºC for 2 minutes, ending with 25ºC for infinite hold. After the PCR was done, the samples were run on 1% agarose gel. The image was then observed under Image Lab 6.0 software. (See appendix C5)

### 3.13 General overview of semi-quantitative PCR

1. The cycle range where the signal strength was increasing visibly on the gel to observe the "log-phase" of the temeplate before it reached saturation was determined.
2. The cycle ranges chosen were 20-24 for the MrSSU control and 30-34 for the target genes.
3. The band density (Volume INT*$mm^2$) values obtained for the control MrSSU were then used to standardize the data by dividing the band density (Volume INT*$mm^2$) values of the target genes by the band desnity values of the control MrSSU gene.
4. Dilution of the purified cDNA PCR product was obtained for each of the target genes to obtain the expression strength of the target gene. The dilution series for each of the target genes was then made and the reactions were subjected to PCR with one sample containing the target gene. The values obtained were then used to calculate an approximate value of expression of the target gene. This provided an approximate measure of which target gene is expressed more than others.

### 3.14 Statistical analysis

The unpaired t-test was used to analyse the data for the quantity of secondary metabolites obtained from two locations with different canopy covers and pH values. The difference in atranorin concentration and fumarprotocetraric acid concentration produced by *Cladonia stygia* in Athapap and Sherridan was tested using HPLC concentrations, which were calculated using the area obtained under the curve at 254 nm wavelength. The value for area was inserted into the standard curve equation obtained using the dilutions of known atranorin and fumarprotocetraric acid concentrations based on the standard atranorin and fumarprotocetraric acid concentrations

obtained from the HPLC with help of M. Zraik (University of Manitoba) (Table 3). For each sample, the equation (in section 3.6) was used to obtain the final concentration. Once the final concentrations were obtained they were used in an unpaired t-test using the QuickCalcs t-test calculator (https://www.graphpad.com/quickcalcs/ttest1.cfm; accessed on December 10 2016). The unpaired t-test determined whether there was a significant difference in the secondary metabolite concentrations between the two locations. The same procedure was followed for fumarprotocetraric acid by using the standard curve equation obtained in the same manner. If the two locations experiencing differences in pH and canopy cover had an effect on secondary metabolite production we would expect to see a difference in concentration of atranorin and fumarprotocetraric acid in the two locations. The unpaired t-test was also performed between sites in the same location but yielded no significant results. Data obtained from HPLC and semi-quantitative PCR were organized in Excel spreadsheets.

**Table 3**: Regression line equations obtained using the serial dilutions of known standard concentration values of atranorin and fumarprotocetraric acid in HPLC. The $R^2$ value indicates the significance of the regression line at p=0.05 (See Appendix B2 for graphs).

| Sample | Regression equation | $R^2$ | p-value |
|---|---|---|---|
| Atranorin | Y=64664x - 450974 | 0.993 | <0.00001 |
| Fumarprotocetraric acid | Y=12597x + 39057 | 0.991 | 0.00099 |

For semi-quantitative PCR, ~500 bp portion of the Mitochondrial Small Subunit (MrSSU) region was used as a control gene and to standardize the data (Weihrauch 2006; Blaesse et al. 2010). From the gel image, Quantity One-4.6.9 software was used to calculate the intensity of the bands which provided a measure of relative quantity for each band. An empty square was placed at a random spot on the gel which provided the background intensity which was then subtracted from the intensity of each band. The intensity values of bands were used in an unpaired t-test to compare the two locations, Athapap and Sherridan, using https://www.graphpad.com/quickcalcs/ttest1.cfm (Accessed on December 24 2017) to ensure that the control did not show any significant difference in gene expression between locations. The same procedure was followed to obtain the band intensity for all the target genes. Once the data for all the target genes were obtained, the intensity of the control gene was used to obtain a ratio of the intensity of bands for each target gene to the intensity of the control gene. The ratios were then used in an unpaired t-test to check if the target gene showed any significant difference in the expression between two locations.

To determine how much the control and target genes were being expressed relative to each other in the *Cladonia stygia* sample. Purified product obtained by excising the template of the target gene was first obtained for each target gene which was then diluted by adding DNase/RNase-free distilled water (Fisher). The intensity of each band of the dilution and cDNA was observed from the agarose gel using Image Lab 6.0 software (BIO-RAD) due to image being read only by this software and not by Quantity One-4.6.9 software due to different machines. This did not affect the results since both the software process the images the same way and provide the same information regarding band intensity. Known dilutions were prepared for each target gene which were then used to obtain the intensity of the band and used in a standard curve

equation (Table 4). This was done for all the target genes to obtain the relative expression for all the target genes. Depending on how much that target gene was being expressed relative to the other target genes the intensity of the bands would be higher or lower while keeping the PCR conditions similar.

**Table 4:** Logarithmic Regression line equations obtained by preparing known concentration dilutions of the control (MrSSU) and the target genes. (See Appendix C6 for graphs).

| Target Gene | Logarithmic Regression Line Equation | $R^2$ |
|---|---|---|
| MrSSU (Mitochondrial Small Subunit) | y = 81517ln(x) + 3E+06 | 0.940 |
| 23 Beta-Ketoacyl Synthase | y = 80418ln(x) + 3E+06 | 0.897 |
| 26 Beta-Ketoacyl Synthase | y = 98853ln(x) + 5E+06 | 0.794 |
| 21 Beta-Ketoacyl Synthase | y = 56246ln(x) + 2E+06 | 0.896 |
| 20 Beta-Ketoacyl Synthase | y = 86505ln(x) + 4E+06 | 0.808 |
| 26 Thioesterase | y = 81478ln(x) + 3E+06 | 0.980 |
| 1 Beta-Ketoacyl Synthase | y = 69946ln(x) + 3E+06 | 0.944 |
| 10 Beta-Ketoacyl Synthase | y = 76571ln(x) + 3E+06 | 0.997 |
| 10 Thioesterase | y = 54114ln(x) + 2E+06 | 0.936 |

To confirm the target genes, DNA sequencing was performed using the same procedures described above. Once the sequences were obtained they were submitted to the BLAST program in NCBI GenBank for comparison with known genes in the database on NCBI database to check the identity of the gene and to obtain the E-value and the Indentity percent value of the significant alignment. Phylogenetic tree using the target sequences and the significant alignments obtained through the NCBI Blast program were created using MEGA7. The sequences were first aligned using ClustalW by keeping the standard parameters. Maximum Likelihood tree with Bootstrap method (Bootstrap set to 100) was then constructed using the aligned sequences. All the parameters were kept same except Gaps/Missing Data treatment was changed to "Use all sites".

# CHAPTER 4 Results

## 4.1 antiSMASH results

### 4.1.1 Characterization of secondary metabolite genes from *Cladonia rangiferina*

In total, there were 32 clusters of nine types of secondary metabolite genes and nine clusters of secondary metabolite genes with unknown identity in the *C. rangiferina* genome identified by the AntiSMASH software (Table 5). There were a total of 15 Type 1 PKS clusters and 3 Type 3 PKS clusters. There were also 6 non-ribosomal peptide synthetase (NRPS) clusters, and 1 NRPS-Type 1 PKS hybrid cluster. Other types of secondary metabolites that were present in low numbers were: 1 Indole cluster, 1 Siderophore cluster, 1 Terpene cluster and 1 Terpene-Type 1 PKS hybrid. Nine of the 41 clusters could not be identified by the program.

**Table 5:** Secondary metabolites, the cluster numbers within each type, and the total number of clusters of each type, which were identified by antiSMASH using a *Cladonia rangiferina* genome (accessed on 17 June 2017).

| Secondary metabolite | Cluster Number | Total no. of clusters |
|---|---|---|
| Type 1 PKS | 1, 2, 3, 5, 6, 10, 14, 17, 20, 21, 23, 26, 29, 31, 36 | 15 |
| Type 3 PKS | 11, 13, 39 | 3 |
| Non-ribosomal peptide synthetase (NRPS) | 25, 27, 35, 38, 40, 41 | 6 |
| Terpene-Type 1 PKS hybrid | 37 | 1 |
| Indole | 4,15 | 2 |
| Siderophore | 22 | 1 |
| Terpene | 18, 19, 24 | 3 |
| NRPS-Type 1 PKS hybrid | 34 | 1 |
| Other (Unidentified) | 7, 8, 9, 12, 16, 28, 30, 32, 33 | 9 |

The Type 1 PKS clusters consisted of two non-reducing clusters, 7 partially-reducing clusters, and 6 highly reducing clusters (Table 6). The Type 1 PKS clusters consisted of the three core domains: Acyltransferase (AT), β -ketoacyl synthase (KS), acyl carrier protein (ACP) and the Thioesterase (TE) domain. The 7 partially reducing Type 1 PKS domains contained the three core domains in addition to β-keto reductase (KR) and/or dehydratase (DH) domains. Of the 7 partially reducing Type 1 PKS clusters, only the clusters 1, 10 and 14 contained the dehydratase (DH) domain but lacked the β-keto reductase (KR) domain. The 6 highly reducing Type 1 PKS clusters contained the three core domains in addition to β-keto reductase (KR), dehydratase (DH) and enoyl reductase (ER) domains.

**Table 6**: Type 1 PKS clusters showing the degree of molecular reduction based on the domains present in the cluster. The findings are based on the absence or presence of reducing domains identified by antiSMASH (accessed on 17 June 2017).

| Subdivision type | Cluster Number |
|---|---|
| Non-reducing Type 1 PKS (AT, KS, ACP and TE/CYC) | 21, 26 |
| Partially reducing Type 1 PKS (KR and DH sometimes) | 1, 5, 10, 14, 20, 23, 31 (KR domain absent from clusters 1, 10 and 14) |
| Highly reduced Type 1 PKS (KR, DH and ER) | 2, 3, 6, 17, 29, 36 |

#### 4.1.2 Illustrations of the Non-reducing, partially reducing and highly reduced clusters.

The two Type 1 PKS non-reducing clusters (clusters 21 and 26) found by antiSMASH (Fig. 5) consist of the three core domains: domains β-ketoacyl synthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) in addition to a Thioesterase (TE) domain. Both of these clusters also consist of an o-methyltransferase domain further downstream. There is also an argininosuccinate synthase domain present in cluster 26.

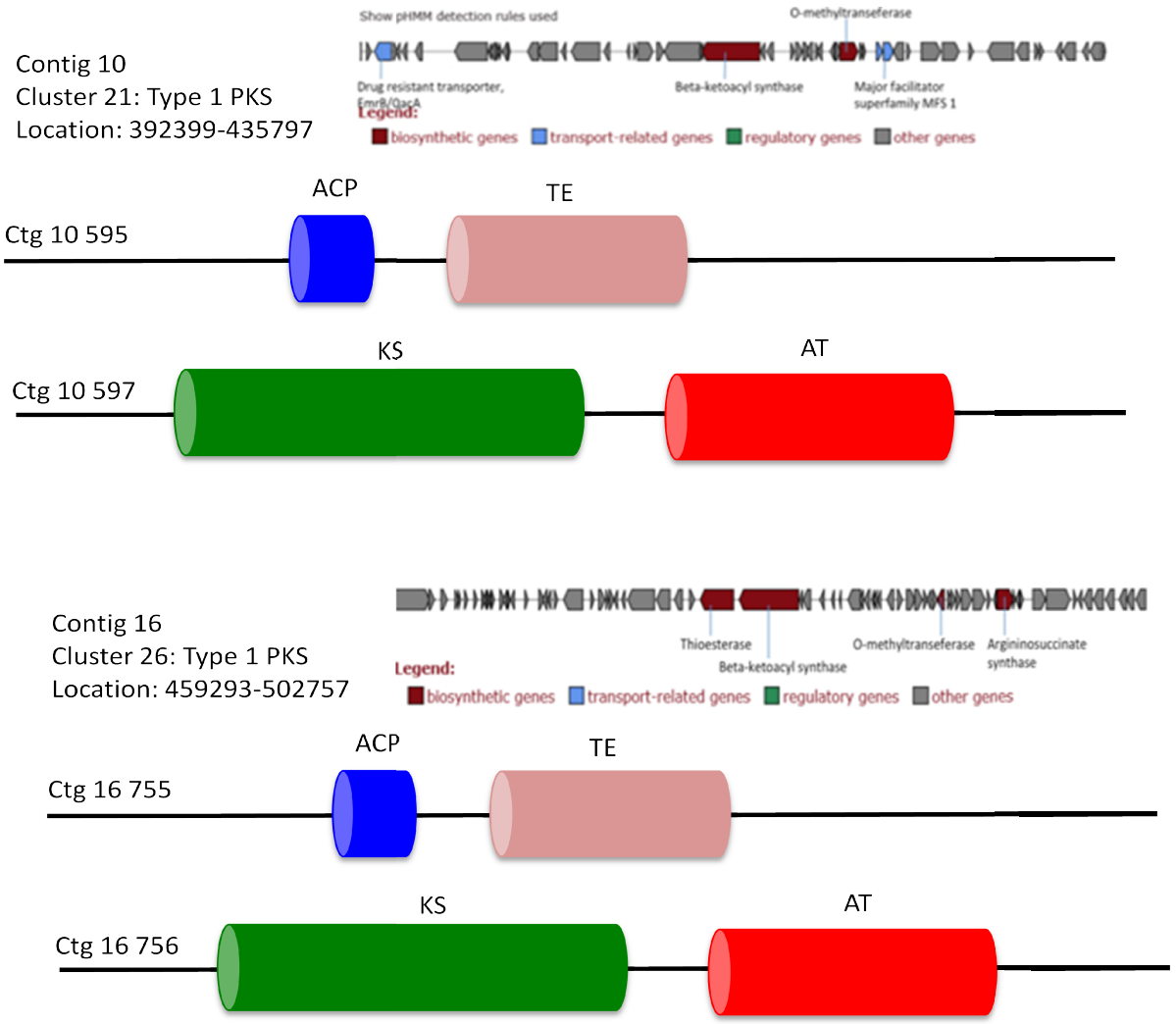



**Figure 5:** Illustration of the Type 1PKS non-reducing clusters 21 and 26 showing the putative gene functional domains, relative size and location of the domains, and direction of transcription. The image was obtained from antiSMASH results using the *Cladonia rangiferina* whole genome (accessed on 17 June 2017).

Two of the Type 1 PKS partially reducing clusters (clusters 1 and clusters 10) found by antiSMASH (Fig. 6) consist of the three core domains: domains β-ketoacyl synthase (KS), acyltransferase (AT) and acyl carrier protein (ACP). Both of these clusters have an additional dehydratase (DH) domain making these clusters partially reducing. Cluster 1 also has an o-Methyl transferase domain whereas cluster 10 has an additional Thioesterase (TE) domain. There are two cytochrome P450, FAD linked oxidase domains and an aldo/keto reductase family oxidoreductase domain present in cluster 1.

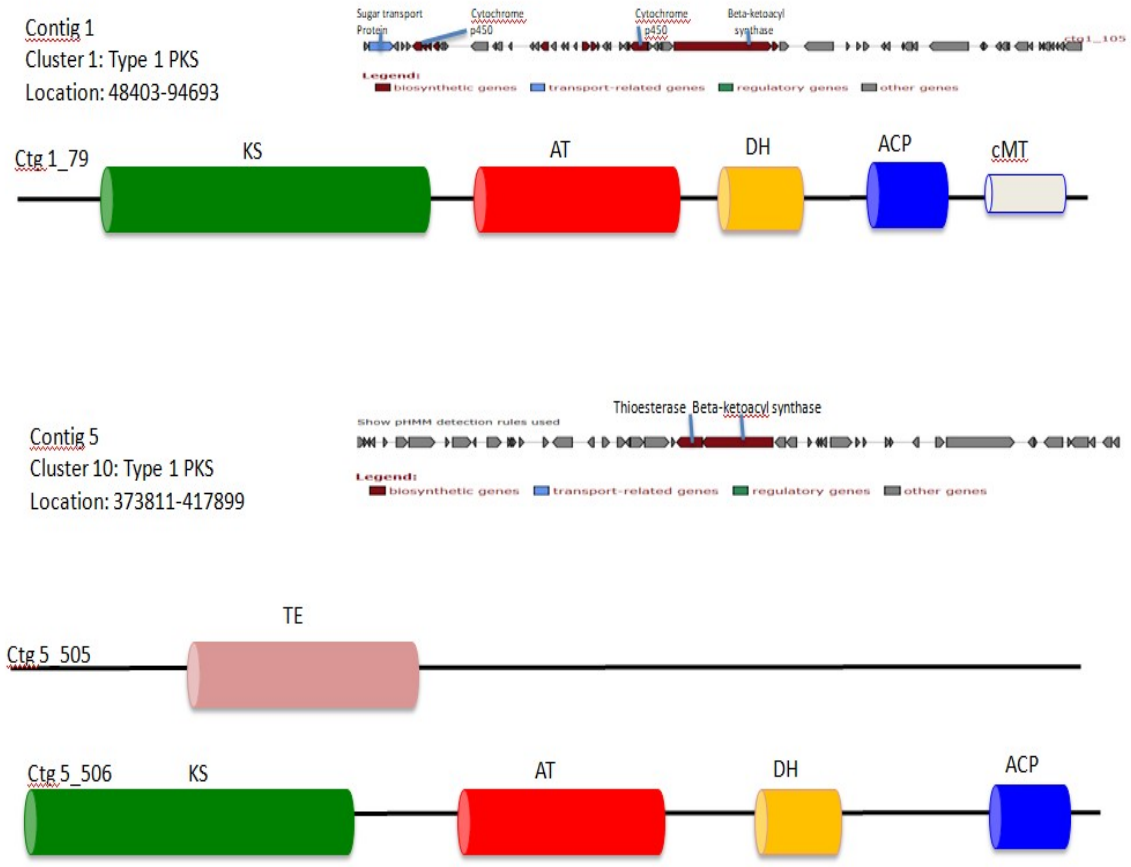



**Figure 6:** Illustration of the Type 1 PKS partially reducing clusters 1 and 10 showing the putative gene function, relative size and location of the domains, and direction of transcription. The image was obtained from antiSMASH results using the *Cladonia rangiferina* whole genome (accessed on 17 June 2017).

One of the type 1 PKS highly reducing cluster (cluster 17) found by antiSMASH (Fig. 7). Consist of the three core domains: domains β-ketoacyl synthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) in addition to the dehydratase (DH) and an Enoyl-reductase domain making this cluster highly reduceding. This cluster also contains an o-mehyltransferase domain, two cytochrome p450 domains, a drug resistant transporter, EmrB/QacA domain, and one short-chain dehydrogenase/reductase SDR domains.

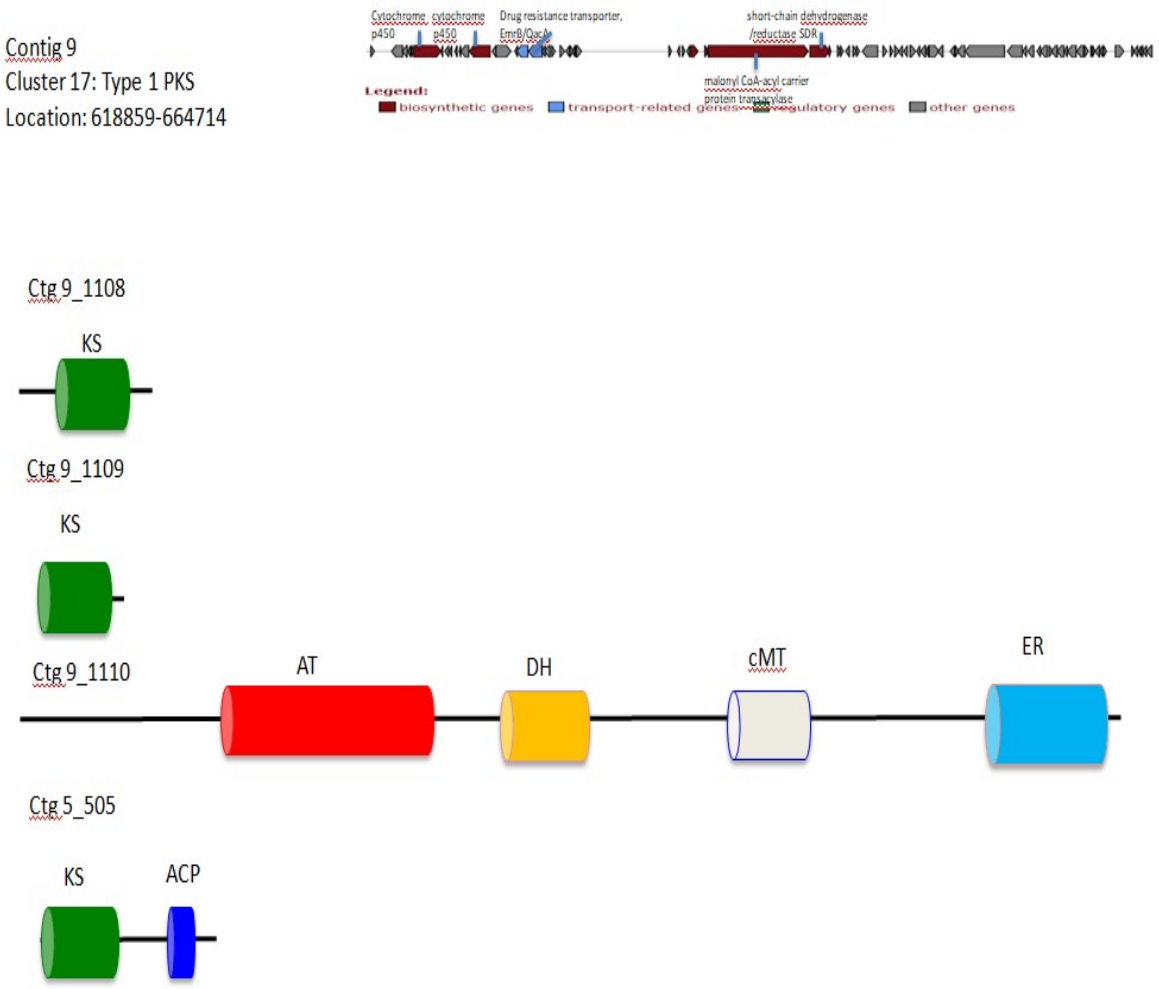



**Figure 7:** Illustration of the Type 1 PKS highly reduced cluster 17 showing the putative gene function, relative size and location of the domains, and direction of transcription. The image was obtained from antiSMASH results using the *Cladonia rangiferina* whole genome (accessed on 17 June 2017).

**4.2 Field work Data**

The soil pH and canopy cover are shown in Table 1. All pH values in Athapap ranged between 5.5-6.0 and all values in Sherridan were between 5.0-5.5. In Athapap, site 2 had the lowest canopy cover with values ranging between 18-25% and site 3 had the highest canopy cover with values ranging between 70-90%. In Sherridan, site 4 and the lowest canopy cover with 0% cover in all three of the quadrats followed by site 3 which had a canopy cover of 90% in quadrat 1 but 0% in both quadrat 2 and quadrat 3. The highest canopy cover in Sherridan was recorded for site 2 with cover of more than 70% for all three quadrats.

The highest frequency of occurrence was 100% for *Cladonia stygia* in both Athapap and Sherridan because the quadrats were selected for the presence of *C. stygia* (Fig. 8). *Cladonia stygia* was followed by *C. arbuscula* with 100% occurrence in Sherridan and 88% occurrence in Athapap. Five of the 18 lichen species (*C. arbuscula, C. crispata, C, phyllophora, C. pyxidata* and *C. stygia*) were present in both locations. *Cladonia chlorophaea* and *C. multiformis* were present only in Athapap whereas 11 lichen species (*Cetraria ericetorum*, *Cetraria islandica*, *Cladonia cornuta*, *C. deformis*, *C. gracilis* ssp. *gracilis, C. gracilis* ssp. *turbinata, C. stellaris, C. uncialis, Diploschistes scruposus, Peltigera leucophlebia, Vulpicida pinastri*) were present only in Sherridan.

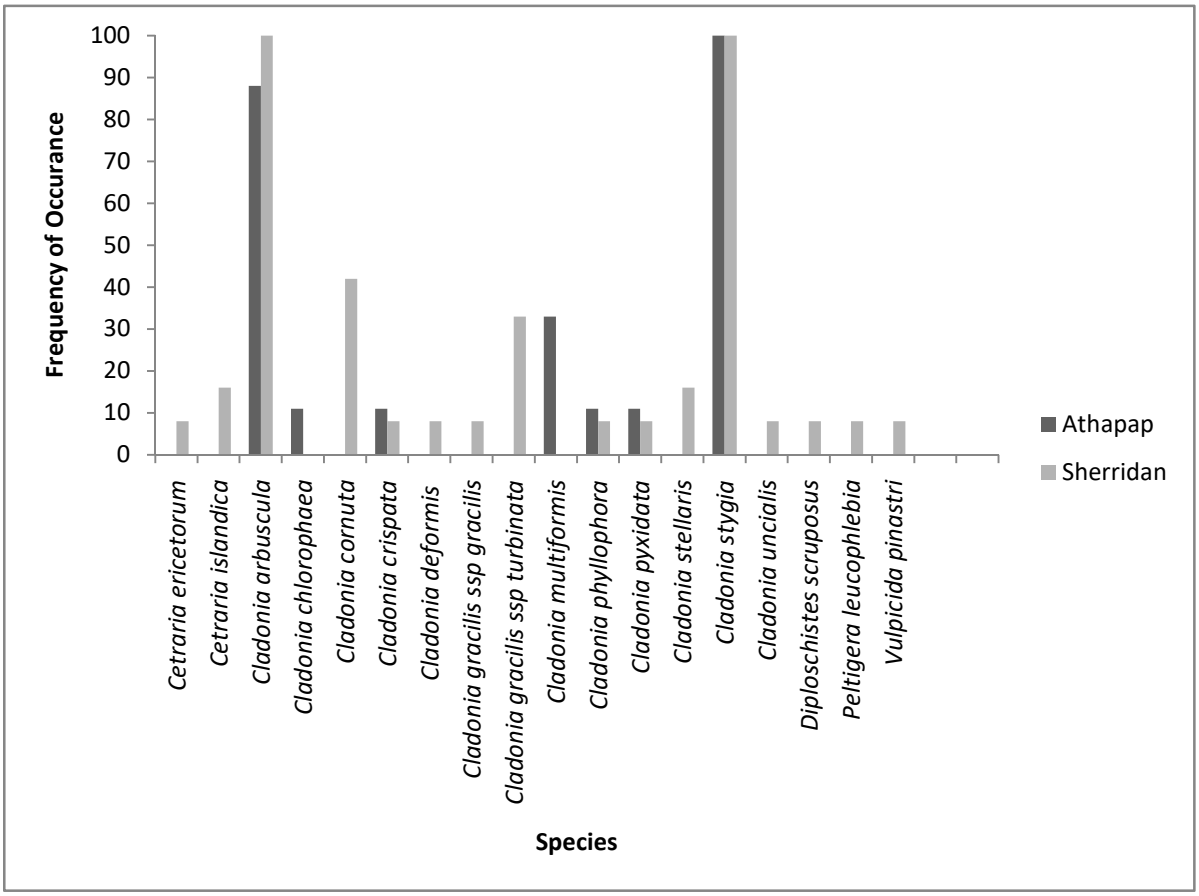



**Figure 8:** Lichen distribution based on frequency of occurrence of species present in two different locations (Athapap and Sherridan) in Manitoba.

The highest bryophyte frequency of occurrence for Athapap was *Pleurozium schreberi* at 100% followed by *Dicranum majus* with 33% occurrence (Fig. 9). For Sherridan, the highest frequency of occurrence was vascular plants with 58%, followed by *Pleurozium schreberi* with 42%, and *Dicranum polysetum* with a frequency of occurrence of 33%. Five of the 13 bryophyte species were present in both Athapap and Sherridan (*Dicranum majus, D. polysetum, Hylocomium splendens, Pleurozium schreberi* and *Polytrichum juniperinum*). Five bryophyte species (*Brachythecium campestre, Dicranella* sp., *Leptodictyum trichopodium, Thuidium abietinum* and *Thuidium delicatulum*) were present only in Athapap whereas three bryophyte species (*Pohlia nutans, Pohlia* sp*., Ptilidium pulcherrimum*) were present only in Sherridan.

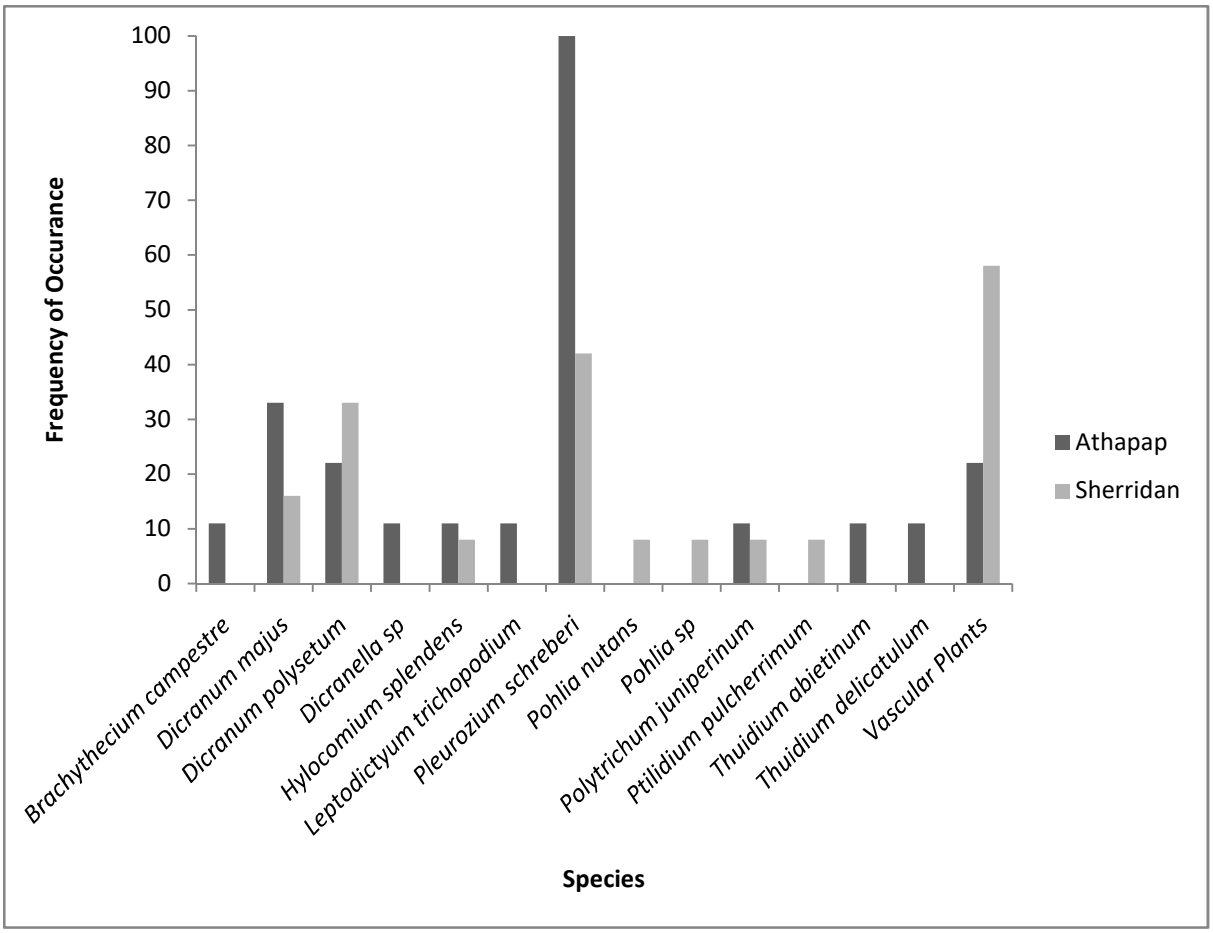



**Figure 9:** Bryophyte distribution based on frequency of occurrence of species present in two different locations (Athapap and Sherridan) in Manitoba.

The lowest percent ground cover for all the lichen species in both Athapap and Sherridan for all of the lichens was 0% except *Cladonia stygia* with a lowest percent cover of 30% in Athapap and 25% in Sherridan and *Cladonia arbuscula* with a lowest percent cover of 1% in Sherridan Table 7). The highest lichen cover in Athapap was for *Cladonia stygia* which was 75% at its highest and 30% at its lowest, followed by *Cladonia arbuscula* at 30% at its highest and 0% at its lowest. The highest percent cover in Sherridan was *Cladonia stygia* with 75% at its highest and 25% at its lowest, followed by *Cladonia arbuscula* with 31% at its highest and 1% at its lowest.

**Table 7:** The minimum and maximum percent cover of lichen species found in each location. n represents the total number of times each species was found out of 21 total quadrats.

| Species | Athapap (n=3) Minimum - Maximum | Sherridan (n=4) Minimum – Maximum |
|---|---|---|
| *Cetraria ericetorum* *n=1* | 0 - 0 | 0 - 1 |
| *Cetraria islandica* *n=2* | 0 - 0 | 0 - 2 |
| *Cladonia arbuscula* *n=20* | 0 - 30 | 1 - 31 |
| *Cladonia chlorophaea* *n=1* | 0 - 1 | 0 - 0 |
| *Cladonia cornuta* *n=5* | 0 - 0 | 0 - 3 |
| *Cladonia crispata* *n=2* | 0 - 1 | 0 - 3 |
| *Cladonia deformis* *n=1* | 0 - 0 | 0 - 1 |
| *Cladonia gracilis* ssp *gracilis* *n=1* | 0 - 0 | 0 - 1 |
| *Cladonia gracilis* ssp *turbinata* *n=4* | 0 - 0 | 0 - 4 |
| *Cladonia multiformis* *n=3* | 0 - 5 | 0 - 0 |
| *Cladonia phyllophora* *n=2* | 0 - 1 | 0 - 4 |
| *Cladonia pyxidata* *n=2* | 0 - 4 | 0 - 1 |
| *Cladonia stellaris* *n=2* | 0 - 0 | 0 - 7 |
| *Cladonia stygia* *n=21* | 30 - 75 | 25 - 75 |
| *Cladonia uncialis* *n=1* | 0 - 0 | 0 - 2 |
| *Diploschistes scruposus* *n=1* | 0 - 0 | 0 - 1 |
| *Peltigera leucophlebia* *n=1* | 0 - 0 | 0 - 1 |
| *Vulpicida pinastri* *n=1* | 0 - 0 | 0 - 2 |

The lowest percent cover for all the bryophyte species in both Athapap and Sherridan for all of the bryophytes was 0% except *Pleurozium schreberi* which had the lowest percent cover of 2% in Athapap (Table 8). The highest percent cover for Athapap was for *Pleurizium schreberi* with a cover of 45% at its highest and 2% it its lowest followed by *Dicranum polysetum* with the second highest cover of 15% at its highest and 0% at its lowest. The highest percent cover in Sherridan was also for *Pleurizium schreberi* with a cover of 40% at its highest and 0% at its lowest followed by *Polytrichum juniperinum* as the second highest with a cover of 30% at its highest and 0% at its lowest.

**Table 8:** The minimum and maximum percent cover of bryophyte species found in each location. n represents the total number of times each species was found out of 21 total quadrats.

| Species | Athapap (n=3) Minimum - Maximum | Sherridan (n=4) Minimum – Maximum |
|---|---|---|
| *Brachythecium campestre* *n=1* | 0 - 5 | 0 - 0 |
| *Dicranum majus* *n=5* | 0 - 1 | 0 - 6 |
| *Dicranum polysetum* *n=6* | 0 - 15 | 0 - 7 |
| *Dicranella* sp. n=1 | 0 - 6 | 0 - 0 |
| *Hylocomium splendens* *n=2* | 0 - 4 | 0 - 17 |
| *Leptodictyum trichopodium* *n=1* | 0 - 2 | 0 - 0 |
| *Pleurozium schreberi* *n=14* | 2 - 45 | 0 - 40 |
| *Pholea nutans* *n=1* | 0 - 0 | 0 - 5 |
| *Pohlia* sp. n=1 | 0 - 0 | 0 - 7 |
| *Polytrichum juniperinum* *n=2* | 0 - 1 | 0 - 30 |
| *Ptilidium pulcherrimum* *n=1* | 0 - 0 | 0 - 5 |
| *Thuidium abietinum* *n=1* | 0 - 4 | 0 - 0 |
| *Thuidium delicatulum* *n=1* | 0 - 5 | 0 - 0 |
| Vascular Plants n=9 | 0 - 2 | 0 - 1 |

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### 4.3 HPLC analysis

The atranorin concentration from the nine quadrats from Athapap had an average concentration of 18.22 µg/mL ± 6.22 µg/mL and 12 quadrats from Sherridan had an average concentration of 60.67 µg/mL ± 11.16 µg/mL (Figure 10). The fumarprotocetraric acid concentration from the nine quadrats from Athapap had an average concentration of 137.86 µg/mL ± 31.40 µg/mL (concentration of polyketide/ mL) and 12 quadrats from Sherridan had an average concentration of 219.80 µg/mL ± 31.34 µg/mL (concentration of polyketide/ mL) (Figure 10). To determine the difference in concentration of each polyketide between locations, the p-value was obtained using the unpaired t-test which was $p = 0.0070$ for atranorin and $p = 0.086$ for Sherridan.

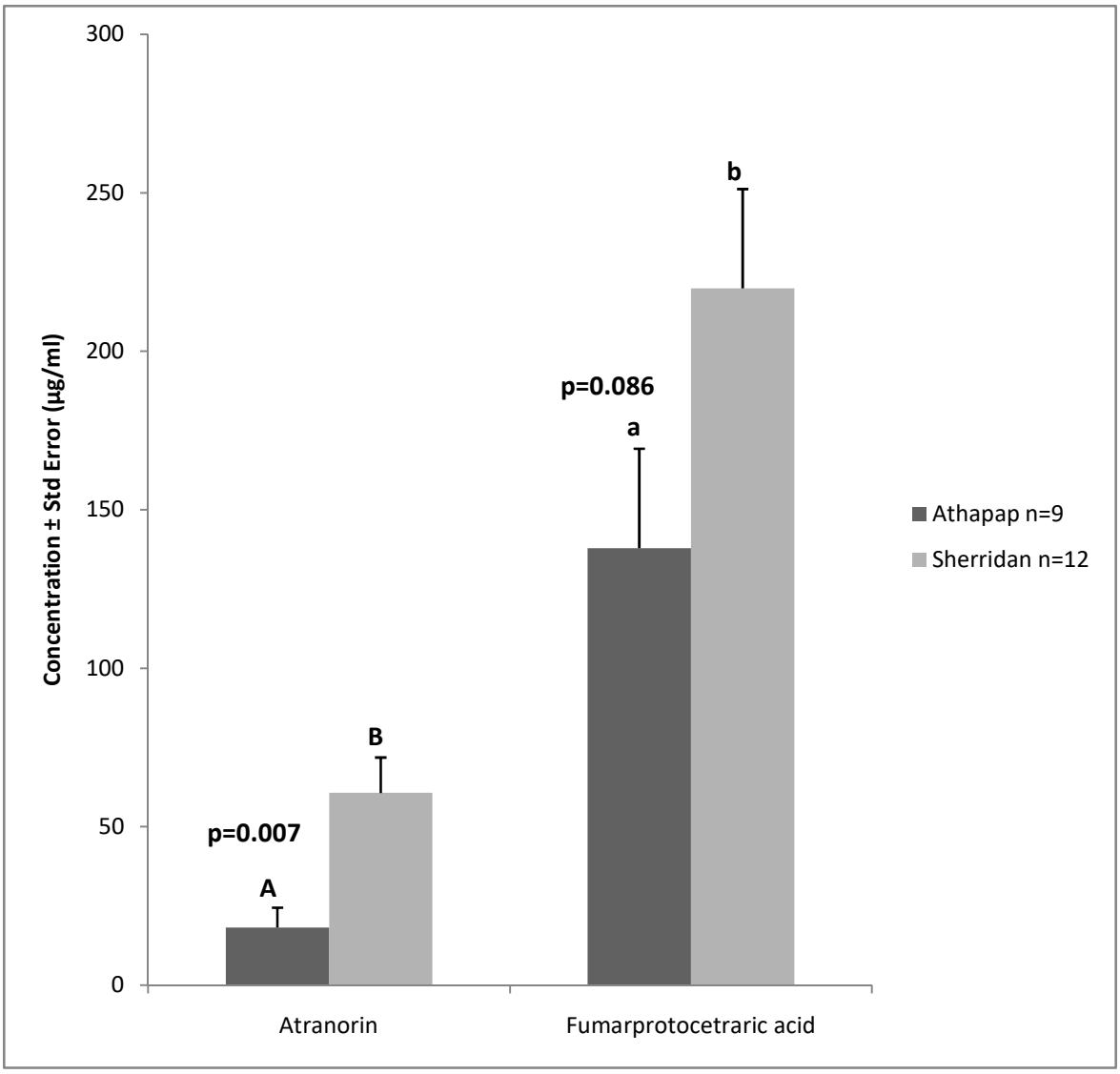


**Figure 10:** Average concentration (µg/mL) of atranorin and fumarprotocetraric acid present in *Cladonia stygia* samples based on HPLC analyses using specimens of *Cladonia stygia* (0.080g) from different sites and quadrats in two locations (Athapap and Sherridan). Error bars show the standard error of mean.

### 4.4 Semi-quantitative PCR

The semi-quantitative PCR using the mitochondrial small subunit control gene performed on 14 samples of *C. stygia* collected from Athapap and Sherridan showed that the ideal number of cycles was between 20 and 24 cycles (Figure 11). The MrSSU was used as a reference control. Seven µL aliquots of the reaction mixture were removed at cycle numbers 20, 22 and 24, which is represented by each of the three lanes between the vertical lines. The final data were based on band intensity at cycle number 22, which is the second band after each vertical line (See appendix C4 for Intensity values).

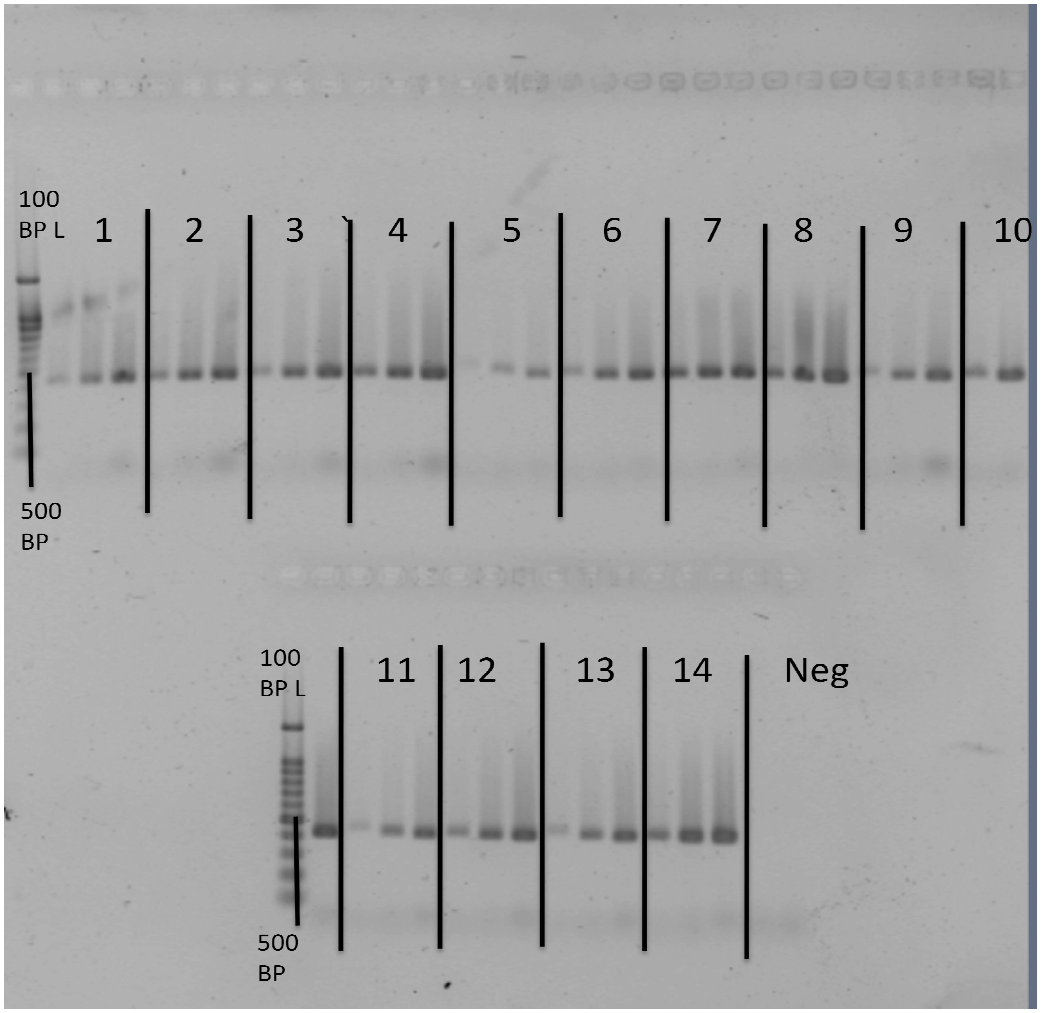


**Figure 11:** Agarose gel image showing bands obtained from semi-quantitative PCR of 14 samples (numbered) using the mitochondrial small subunit gene of the mitochondrial ribosomal DNA (MrSSU) as the control gene. The vertical bars separate samples. The three bands between vertical lines indicate the expression change with increasing cycle number. Neg represents the negative PCR control. The far left lane shows the DNA marker (100 bp ladder), with the 500 bp band marked for reference. The far right lane in the bottom tier is the PCR negative control. Sample numbers one through eight represents samples from Athapap and sample numbers nine through fourteen represent samples from Sherridan.

### 4.5 Target gene expression

The difference in gene expression between two locations is shown for all eight genes examined (Figure 12). Cluster 21 Beta-Ketoacyl Synthase showed a significant difference between the two locations ($p = 0.0408$) and Cluster 26 Thioesterase domain was almost significantly different ($p = 0.0817$). However, the other six genes examined showed non-significant differences between locations.

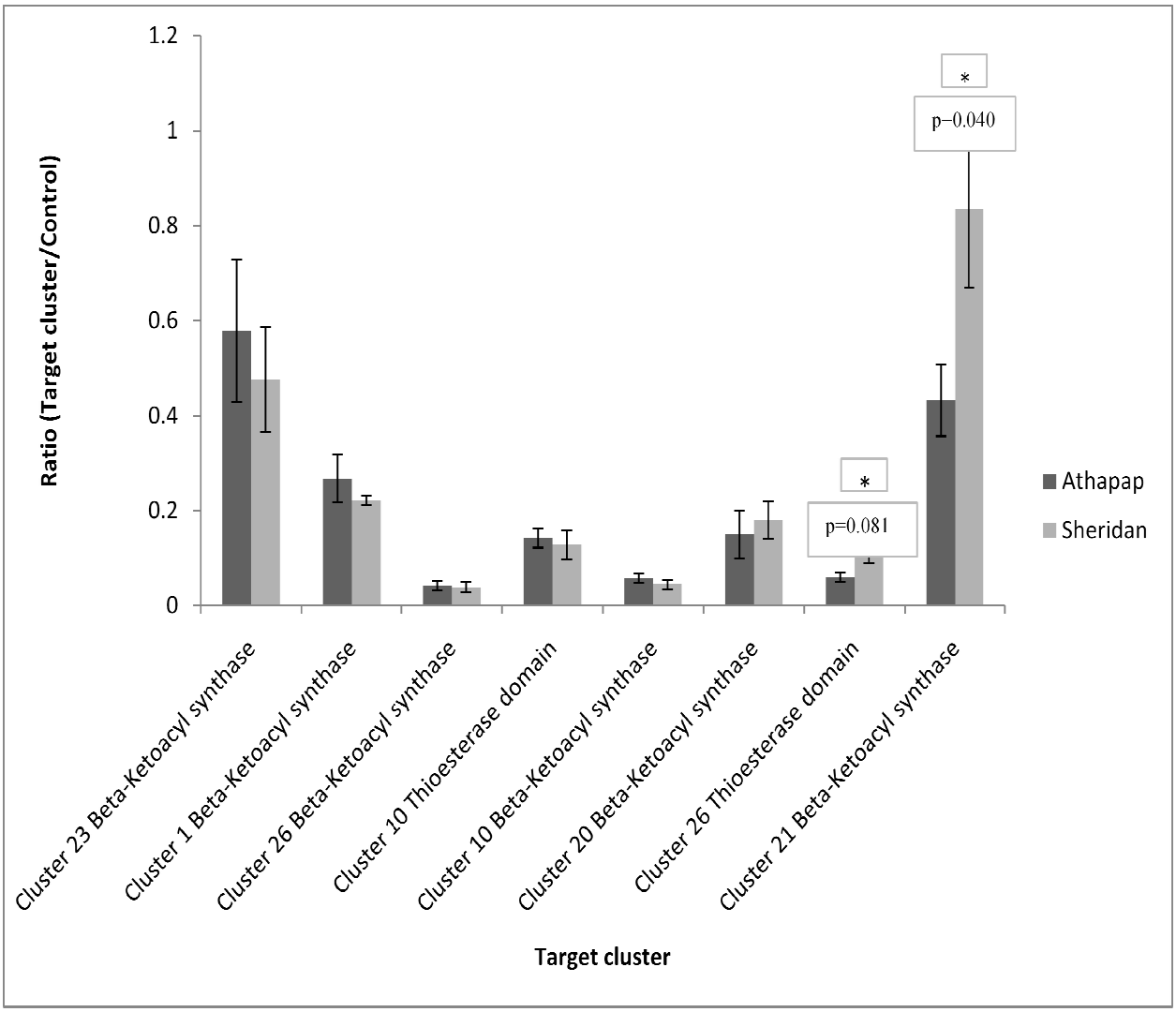


**Figure 12:** Bar graph representing the ratio of Intensity (Volume INT x $mm^2$) obtained by dividing the Intensity (Volume INT x $mm^2$) of target genes by Intensity (Volume INT x $mm^2$) of MrSSU (Mitochondrial small subunit) control for 7 samples from Athapap and 6 samples from Sherridan were compared. The unpaired t-test was performed to determine significant difference the expression of two locations with a p-value of 0.05. The vertical bars show the standard error of the mean. Ratio obtained using band Intensity of target cluster divided by the band Intensity of the control.

### 4.6 Sequence identify and Phylogenetic analysis

The best match between each of the sequenced cDNA PCR product and GenBank genes are shown in Table 9. The results show that e-values of the Mithochondrial small subunit and 10 Beta-Ketoacyl Synthase genes are greater than e-50 which is considered significant matches. However, the four genes (1 Beta-Ketoacyl Synthase, 23 Beta-Ketoacyl Synthase, 20 Beta-Ketoacyl Synthase and 10 Thioesterase), which are from lichen fungi, show e-values which are close to the e-50. The gene (21 Beta-Ketoacyl Synthase and 26 Thioesterase) are from non-lichenized fungi and show poor matches. The percent identity values of the Beta-Ketoacyl synthase domains, which contain all the three core domains of Ketoacyl Snthase (KS), Acyl Transferase (AT) and Acyl Carrier Protein (ACP), and are all greater than 70%. For cluster 26, the Thioesterase domain was sequenced.

**Table 9:** Results of the BLAST searches of the target genes in the NCBI GenBank database showing the gene of the best match, the e-value, and the percent identity with the amplified and sequenced cDNA of *Cladonia stygia* samples.

| Target gene | Significant alignment | E-value | Indent |
|---|---|---|---|
| Mitochondrial small subunit | *Cladonia stygia* AFTOL-ID 4940 small subunit ribosomal RNA gene, partial sequence | 1e-137 | 95% |
| | *Cladonia rangiferina* voucher NY:Lendemer 46392 mitochondiron, complete genome | 1e-136 | 95% |
| 1 Beta-Ketoacyl Synthase | *Cladonia pocillum* strain Normore 9061 polyketide synthase (PKS-2DA) gene | 3e-34 | 91% |
| 23 Beta-Ketoacyl Synthase | *Cladonia macilenta* voucher Hur CH050136 type I polyketide synthase (PKS1) | 1e-34 | 90% |
| 20 Beta-Ketoacyl Synthase | *Cladonia grayi* putative non-reducing PKS (PKS2) gene, partial cds | 1e-28 | 85% |
| 26 Thioesterase | *Colletotrichum graminicola* M1.001 beta-ketoacyl synthase domain-containing protein partial mRNA | 6.1 | 80% |
| 26 Beta-Ketoacyl Synthase | *Cladonia macilenta* voucher Hur CH050136 type 1 polyketide synthase (PKS1) mRNA complete | 3e-10 | 72% |
| 21 Beta-Ketoacyl Synthase | *Fusarium babinda* strain NRRL 25539 putative polyketide synthase (Fba_258) mRNA, complete cds | 7e-04 | 78% |
| 10 Beta-Ketoacyl Synthase | *Cladonia uncialis* subsp *uncialis* contig 2 genomic sequence | 4e-150 | 85% |
| 10 Thioesterase | *Cladonia uncialis* subsp *uncialis* contig 2 genomic sequence | 3e-43 | 76% |

Target gene 26 Thioesterase was most closely related to *Colletotrichum graminicola* M1.001 beta-ketoacyl synthase domain-containing protein partial mRNA with a strong bootstrap support of 98 (Figure 13). 10 Thioesterase was most closely related to *Cladonia uncialis* subsp. *uncialis* contig 2 genomic sequence.

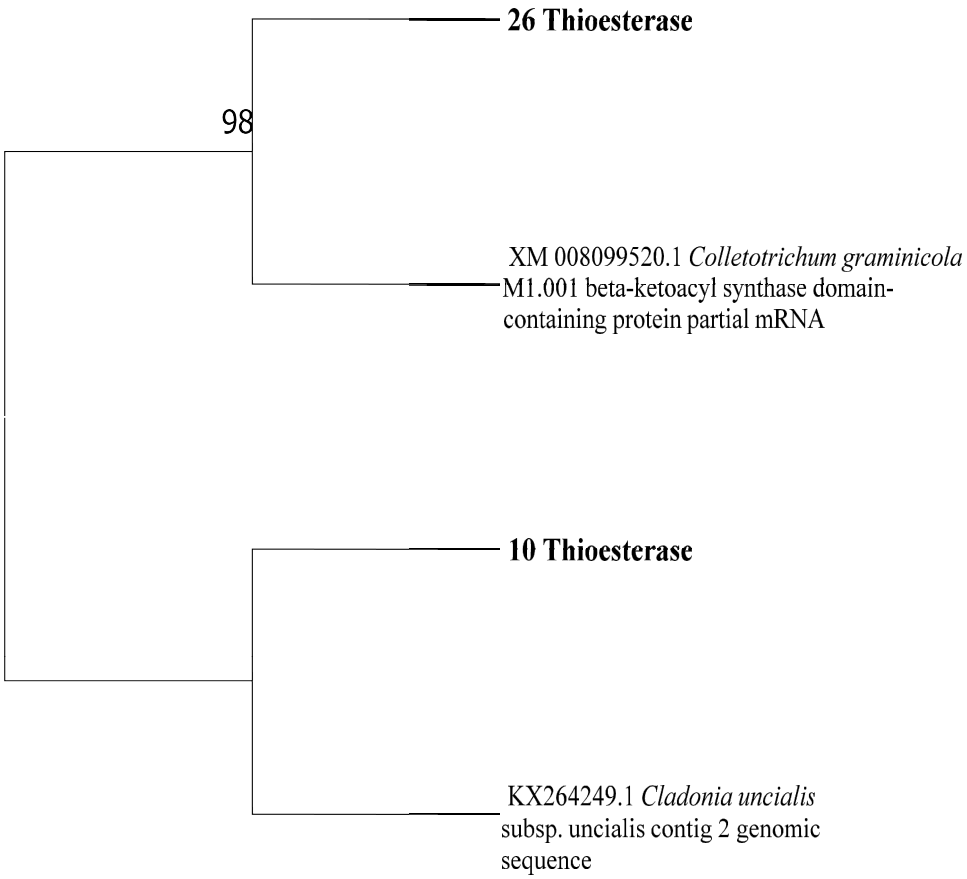


**Figure 13:** Phylogenetic analysis of the two Thioesterase target genes based on the BLAST searches performed in the NCBI GenBank database. Maximum Likelihood tree based on the 100 Bootstrap resamplings. The tree is not rooted.

Target gene 1 Beta-Ketoacyl Synthase and 23 Beta-Ketoacyl Synthase were most similarly related to *Cladonia rangiferina* putative synthase 1mRNA partial cds with a bootstrap support of >80% (Figure 14). 20 Beta-Ketoacyl Synthase was most related to *Cladonia uncialis* subsp. *uncialis* contig 10 genomic sequence with a bootstrap support of 100%. 26 Beta-Ketoacyl Synthase was closely related to *Cladonia uncialis* subsp. *uncialis* contig 12 genomic sequence with a low bootstrap support of 42%. 21 Beta-Ketoacyl Synthase and 10 Beta-Ketoacyl Synthase were closely related to each other but with a low bootstrap support of 37%.

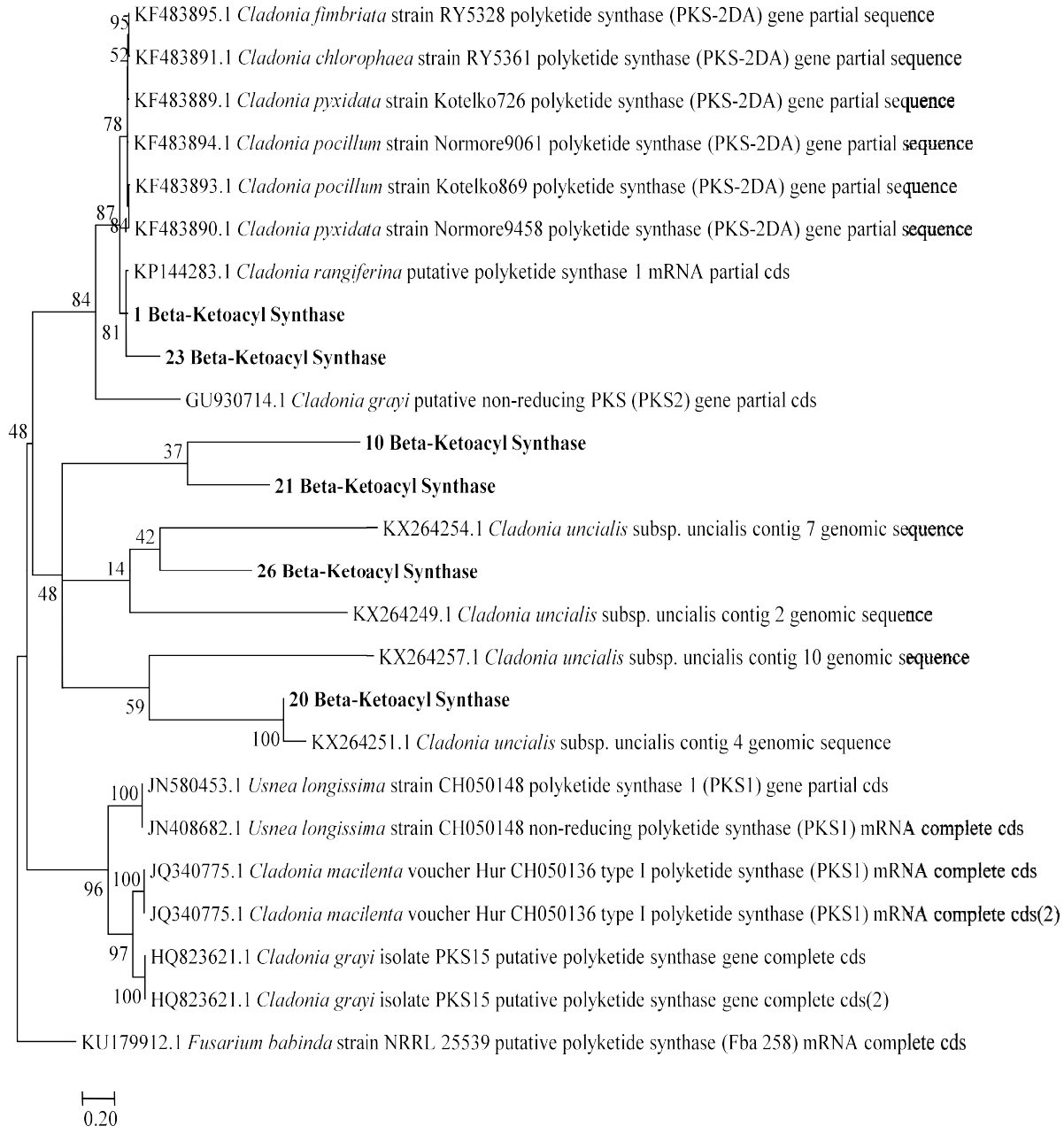


**Figure 14:** Phylogenetic analysis of the six Beta-ketoacyl Synthase target genes (shown in bold) based on the BLAST searches performed in the NCBI GenBank database. Maximum Likelihood tree using 100 Bootstrap resamplings. The root was set at *Fusarium babinda*.

### 4.7 Gene expression of PCR products

Of the 8 target genes (Figure 15), 10 Thioesterase domain showed the highest expression level with an expression value of 1.58E-14 (ng), followed by the 21 Beta Ketoacyl Synthase domain with an expression value of 1.40E-14 (ng) which was followed by the 10 Beta Ketoacyl Synthase domain which had an expression value of 1.12E-14 (ng), which was then followed by 26 Thioesterase domain with an expression value of 7.01E-15, followed by 1 Beta Ketoacyl Synthase domain with an expression value of 4.89E-15, and finally the 23 Beta Ketoacyl Synthase domain with an expression of 2.10E-15 (ng). Two target genes with the lowest expression values were 20 Beta-Ketoacyl Domain and 26 Beta-Ketoacyl Domain (Figure 16) with the expression values of 2.96E-20 (ng) and 7.19E-20 (ng), respectively.

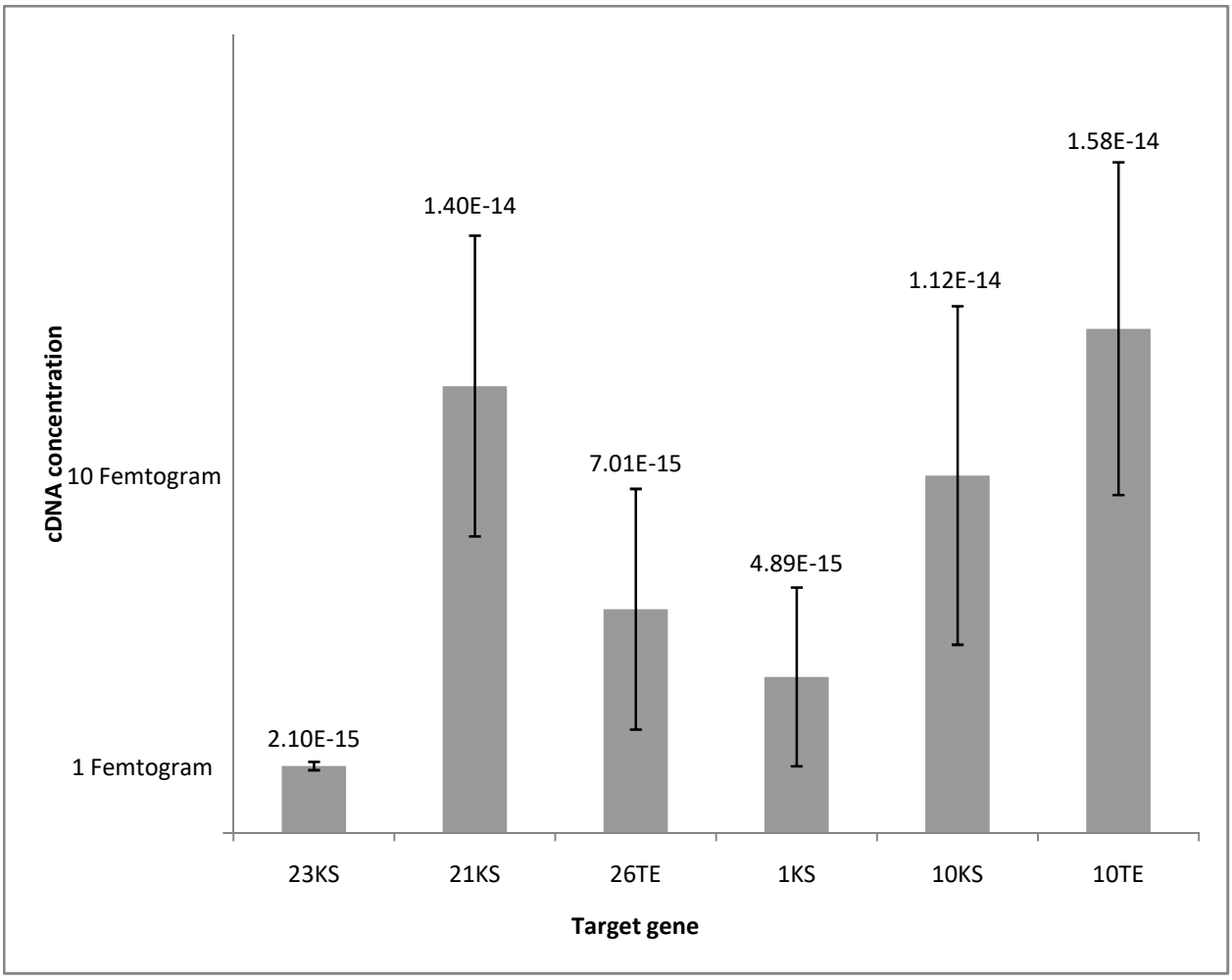


**Figure 15:** Expression of the six target genes relative to each other based on known cDNA dilutions of the PCR product of the target genes. The exact expression levels are above each bar and were obtained using standard curve equations for diluted cDNA PCR product for the target genes (Table 4). The vertical bars show the standard error of the mean (n=3 for all target genes). KS refers to the ketosynthase domain, TE refers to the thioesterase domain. The number before each domain abbreviation refers to the cluster number.

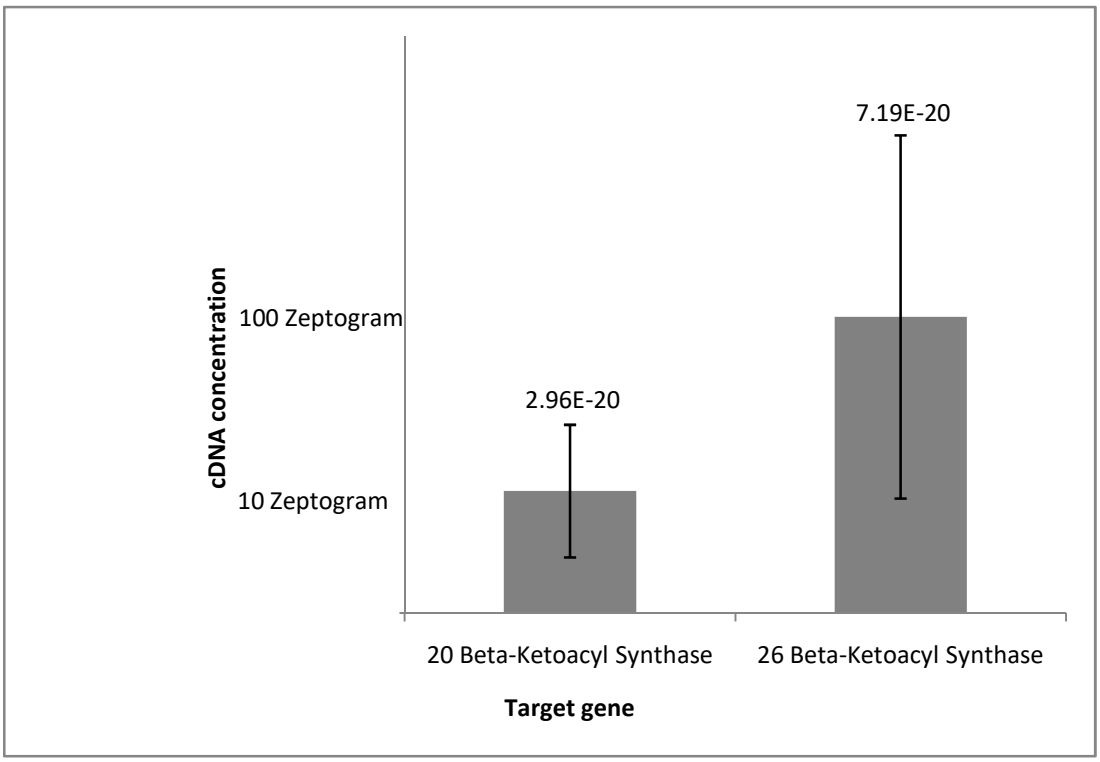


**Figure 16:** Expression of the two target genes relative to each other based on known cDNA dilutions of the PCR product of the target genes. The exact expression levels are above each bar and were obtained using standard curve equations for diluted cDNA PCR product for the target genes (Table 4). The vertical bars show the standard error of the mean (n=3 for all target genes). KS refers to the Beta Ketoacyl Synthase domain. The number before the domain abbreviation refers to the cluster number.

# CHAPTER 5 Discussion

## 5.1 Characterizing PKS genes in the genome of *Cladonia rangiferina*

There were 41 clusters of genes responsible for secondary metabolites identified by antiSMASH (Table 5) in the genome of *Cladonia rangiferina*. The 15 Type 1 PKS clusters are of interest for lichen fungi (Stocker-Wörgötter 2008; Keller et al. 2005) and the two non-reducing type 1 PKS genes are particularly significant for *C. stygia* in this study as it produces two polyketides which lack reduction in its side chains, atranorin and fumarprotocetraric acid. While the linkage between the gene clusters and the polyketide are unknown, this study provides evidence to suggest that clusters 21 and 26 may be responsible for the synthesis of atranorin and fumarprotocetraric acid (see next section).

The subdivisions of PKS genes are based on the identity of the domains present in the gene cluster. The type 1 non-reducing PKS cluster has three core domains which are Acyl Transferase (AT), Acyl carrier protein (ACP), and Ketoreductase (KS) domains (Figure 5) (Miao et al. 2001; Shen 2003; Wang et al. 2012). In addition to these three core domains, a Thioesterase (TE) domain can also be present which is responsible for either the release of the bound enzyme intermediates (Gokhale et al. 1998) or drives the final cyclization reaction hence releasing the final product (Korman et al. 2010). Once the growing polyketide chain reaches its full length, it is released from the PKS. Typically, TE domains have a conserved catalytic triad, Ser-His-Asp, which catalyzes the release of polyketide products (Yu et al. 2013). Previous studies have observed NR type 1 PKS in different lichen species (Valarmathi et al. 2009; Wang et al. 2012; Yu et al. 2013; Jeong et al. 2015). Partially reducing type 1 PKS clusters have the three core domains present in addition to the reducing domains, β-keto reductase (KR) and/or Dehydratase (DH) domains (Figure 6). Highly reducing type 1 PKS genes contain the three core domains,

both KR and DH reducing domains, and an enoyl reductase domain (ER) as well (Figure 7) (Jeong et al. 2015; Kroken et al. 2003). In a phylogenetic study conducted by Kroken et al. (2003) the PKS ketosynthase (KS) domain was used to construct the phylogenetic relationship among lichen-fungal PKS genes. It was observed that the bulk of the genes encoding fungal PKS were divided into two main groups: reducing PKS and non-reducing PKS. The reducing PKS clades consisted of genes with the three core domains (KS, AT, ACP) and also the reducing domains such as DH, ER and KR. The non-reducing domains consisted of the three core domains (KS, AT, ACP) and the Me/ Cyclic domains (Kroken et al. 2003). In this study, the partially reducing domains had an additional DH and KR domain with the core domains (Table 6; Figure 6). The highly reducing domain consisted of an additional ER domain with the core domains (Table 6; Figure 7). Every fungal and lichen species, which has been studied with respect to PKS genes, is known to have large numbers of secondary metabolite clusters present in their genomes (Chooi et al. 2008; Brunauer et al. 2009; Gagunashvili et al. 2009; Valarmathi et al. 2009; Armaleo et al. 2011; Wang et al. 2011; Yu et al. 2013). In *Aspergillus nidulans,* genomic analysis identified 27 different polyketide synthase (PKS) genes but only seven known secondary metabolites have been found (Eisendle et al. 2003; Bok et al. 2006; Scherlach et al. 2006; Bergmann et al. 2007). Abdel-Hameed et al. (2016) identified 32 putative PKS genes by antiSMASH in *Cladonia uncialis,* but only five were non-reducing PKS genes and contained the methyl transferase (CMet) domain which was required for the production of usnic acid in *Cladonia uncialis*. These five clusters lacked any of the reducing domains. This is comparable to this study where antiSMASH found 41 total PKS clusters but only two of them were non-reducing type 1 PKS (Table 5; Table 6).

In a study conducted on *Cladonia macilenta,* it was observed that the gene *CmaPKS1* contained the three core domains KS, AT, ACP and a TE domain, which indicated that the *CmaPKS1* gene is a non-reducing PKS type (Jeong et al. 2015). In another study conducted on *Usnea longissima,* the gene *U1PKS1* was shown to contain the three core domains and also the TE domain which made the gene a non-reducing Type 1 PKS (Wang et al. 2012). Valarmathi et al. (2009) examined the PKS genes in the lichen *Dirinairia applanata* and found that the gene *DnPKS* consisted of the domains KS, AT, two ACP domains and TE domains making it a non-reducing PKS. Another study focused on the lichen forming fungus *Hypogymnia physodes* and found two putative PKS genes: *Hypopks1* and *Hypopks2* both containing five catalytic domains which were KS, AT, ACP, ME and R domains instead of the TE domain, making both these genes non-reducing PKS type (Yu et al. 2013). Another study looked at the PKS gene in the lichen *Xanthoparmelia semiviridis* and found that the PKS gene *xsepks1* contains the KS, AT, ACP and CMet domains and lacked any reducing domains making this gene a non-reducing type PKS. In this study, it was observed that both cluster 21 (Table 6; Figure 5) and cluster 26 (Table 6; Figure 5) are non-reducing Type 1 PKS. Based on antiSMASH results, the focus of this study was also on three partially reducing PKS which were clusters 1, 10 and 14 since they only contained a DH domain and lacked any KR domain (Table 6; Figure 6). This was important because studies conducted on different fungal species have shown partially reducing PKS to be responsible for production of some polyketides (Fujii et al. 1996; Moriguchi et al. 2006). It has been observed that the *atX* gene from *Aspergillus terreus* encodes for the PKS of 6-methylsalicyclic acid and contains the domains KS, AT, ACP, DH and KR making it a partially reducing PKS (Beck et al. 1990; Fujii et al. 1996; Bhetariya et al. 2016). Highly reducing PKS are found in fungi but to date no study has found any polyketide produced by a highly reducing

PKS in lichens. In *Aspergillus terreus,* the polyketide Lovastatin is produced by the polyketide gene *LovB* which contains the KS, AT, DH, MT, ER, KR and ACP domains making it a highly reducing polyketide.

### 5.2 Upregulation of clusters 21 and 26

Cluster 21 Beta Ketoacyl Synthase and cluster 26 thioesterase domain showed significant differences in the gene expression with location (Figure 12) which corresponded with the significant difference in quantity of polyketide by location (Figure 10). Both of these clusters were identified as non-reducing type 1 PKS clusters (Table 6 and Figure 5). The significant expression levels may indicate that upregulation of these two clusters was affected by environmental differences between Athapap and Sherridan. Two environmental variables examined in this study were the pH level and canopy cover at the two locations. Canopy cover was variable but pH differed between locations. It is well known that polyketide synthesis is triggered by certain environmental variables (Begora and Fahselt 2001; Geisen 2004; Hager et al. 2008; Stocker-Wörgötter 2008) including the effect of different carbohydrates on PKS gene expression (Geisen 2004; Valarmathi et al. 2009; Wang et al 2012; Elshobary et al. 2016). A review by Penalva and Arst (2002) talked about the effect of pH on gene expression in fungi. It is hypothesized that the transcription factor (pacC) regulates the gene expression by pH. Members present in the *Cladonia chlorophaea* complex share similar morphological characters but have different secondary metabolites which might be due to the fact that some members such as *Cladonia grayi* and *Cladonia merochlorophaea* grow at lower pH than *Cladonia chlorophaea sensu stricto* or some other members of the complex (Culberson 1986; Culberson et al. 1998).

In a study conducted on *Cladonia macilenta,* it was observed that the expression on the *CmaPKS1* gene was upregulated after a 12 hour UV treatment (Jeong et al. 2015) *CmaPKS1* gene falls within a non-reducing PKS clade, in which other genes are involved in melanin synthesis (Langfelder et al 1998, Schmitt et al, 2005) explaining the high expression of *CmaPKS 1* under 12 hours of high UV treatment (Jeong et al. 2015). Valarmathi et al. (2016) found that different pH levels can also affect the expression of *DnPKS* gene in *Dirinaria applanata*. In this study both Athapap and Sherridan had different pH levels (Table 1) so based on previous studies the pH might have been responsible for upregulation of both 21 Beta-Ketoacyl Synthase and 26 Thioesterase (TE) domain. Cluster 21 is the most likely candidate for the expression of fumarprotocetraric acid based on the following results: Firstly, Fumarprotocetraric acid had a higher concentration present in Both Athapap and Sherridan compared to concentrations of atranorin present in these two locations (Figure 10). This corresponds with the data obtained from the semi-quantitative analysis where the ratio of Intensity (Volume INT*$mm^2$) was much higher for 21 Beta-Ketoacyl Synthase relative to the control when compared with the ratio of Intensity (Volume INT*$mm^2$) of the 26 TE domain (Figure 12). Secondly, fumarprotocetraric acid showed a significant difference in the concentration between Sherridan and Athapap (Figure 10), which coincides with the significant difference observed in the expression of 21 Beta-Ketoacyl Synthase between the two sites (Figure 12). Thirdly, the gene expression data based on cDNA concentrations showed that, compared to the 26 TE domain, 21 Beta-Ketoacyl Synthase had a higher concentration of cDNA expressed at similar conditions (Figure 15). All these observations provide support that 21 Beta-Ketoacyl Synthase might be the most likely candidate to encode the PKS that produces fumarprotocetraric acid in *Cladonia stygia* thalli. For atranorin, the most likely candidate is cluster 26. Atronorin had an overall lower concentration produced in

both locations (Figure 10) compared to fumarprotocetraric acid, which coincided with the ratio of Intensity (Volume INT*mm$^2$) observed for 26 TE domain compared to the 21 Beta-Ketoacyl domain (Figure 12). Based on the HPLC results, the difference in atranorin between the two locations was almost significant (Figure 10), which was also observed for the semi-quantitative analysis where the difference between two locations was significant compared to other target genes (Figure 12). The expression of the 26 TE domain was much lower than that of the 21 Beta-Ketoacyl Synthase (Figure 15), which corresponds with the concentration of atranorin compared to Sherridan (Figure 10). Based on these results, the 26 TE domain might be affecting the concentration of atranorin in the *Cladonia stygia* thalli.

From cluster 26, 26 Beta-Ketoacyl Synthase domain did not show any significant difference in expression between the two locations (Figure 12) and had an extremely low expression of cDNA concentration compared to other target genes (Figure 16). Previous studies have shown that domains in a cluster are co-regulated (Fox and Howlett 2008). TE domain has been known to catalyze the final transformation of both PKSs and Fatty acid synthases (Korman et al. 2010). Therefore, in cluster 26 the TE domain might have been responsible for the release of the final product. Gokhale et al. (1998) observed that theTE domain played a key role in the release of the enzyme bound intermediates in erythromycin polyketide synthase. This phenomenon was also observed in a study on aflatoxins conducted by Korman et al. (2010) where the TE domain drove the final cyclization reaction and released noranthrone after accepting the partially cyclised, ACP-bound intermediate. Based on these previous studies, the TE domain might also be responsible for releasing the final polyketide product in the PKS clusters identified in *Cladonia rangiferina* genome because it was present in both the non-reducing type 1 PKS (Figure 5) and the partially reducing type PKS 1 (Figure 6).

### 5.3 Quantity of polyketide differs by location

The concentrations of both fumarprotocetraric acid and atranorin were higher in Sherridan when compared to their concentrations present in Athapap (Figure 10). Sherridan soil is more acidic than Athapap which might have influenced the concentration of polyketides produced by *Cladonia stygia* in these two locations. Brzonkalik et al. (2012), examined how pH influences the production of mycotoxin by *Alternaria alternata*. They found that acidic pH values ranging between 4.0-4.5 were optimal for mycotoxin production where pH values higher than 5.5 led to a decrease in mycotoxin production. At pH 8.0 the production of mycotoxin was completely inhibited (Brzonkalik et al. 2012). In another study, Timsina et al. (2013) tested the effect of pH levels on secondary metabolite production in *Ramalina dilacerata*. It was observed that the optimal growth rate for *R. dilacerata* was between pH levels of 4.5-6.5 and the best conditions for highest secondary metabolite production was at pH levels above 6.5 and around 8.5 where the number of metabolites produced was 7 compared to pH 3.5 where only 3-4 metabolites were produced. This indicated that secondary metabolite production is highest when the species is under stress and growing under non-optimal conditions (Timsina et al. 2013). Species in the genera *Lassalia* and *Umbilicaria* were shown to produce their secondary metabolites under acidic conditions (Stocker-Wörgötter 2001). Based on these studies, *Cladonia stygia* may be under more stress in Sherridan hence producing a higher concentration of these two polyketides and the pH levels may be optimal for the production the polyketides (Stocker-Wörgötter 2001; Brzonkalik et al. 2012). This might be the case as to why there is higher expression of both atranorin and fumarprotocetraric acid in Sherridan where the pH level is acidic compared to Athapap. All three sites in Athapap had a pH value between 5.5 – 6.0 whereas the pH values in all Sherridan sites were between 5.0 – 5.5 (Table 1).

Atranorin has been shown to be affected by UV exposure and light gradients (Rundel 1978; Begore and Fahselt 2001). Five of 12 quadrats in Sheridan had 0% canopy cover (Table 1) which means the lichens growing in those quadrats were experiencing a higher UV exposure than in quadrats with canopy cover. Therefore, the higher concentration of atranorin present in Sherridan (Figure 10) might be helping *C. stygia* to cope with the higher light and UV exposure. There is also much more frequency of occurrence of both lichens and bryophytes in Sherridan compared to Athapap (Figure 8 and 9). Studies have noted that secondary metabolites produced by lichens provide them with a competitive advantage in their environment (Rundel 1978; Hager et al. 2007). Having a higher number of both bryophytes and lichens in Sherridan means there is going to be more competition for resources. Both Atranorin and Fumarprotocetraric acid have shown anti-microbial properties (Asplund et al. 2010; Lawrey 1984), so by having a higher concentration of these two compounds will help the lichen by increasing its fitness compared to other vegetation present in the site which will affected by microbes.

The overall concentration of fumarprotocetraric acid was higher than atranorin in both Athapap and Sherridan (Figure 10). This might be due to the fact that fumarprotocetraric acid has been shown to provide multiple benefits for the lichen. Fumarprotocetraric acid acts as an anti-herbivore compound (Rundel 1948; Reutimann and Scheidegger 1987). In a study conducted on two mites species (*Fuscozetes setosus* and *Carabodes intermedius),* it was observed that both species consumed the thallus of *Cladonia symphycarpa,* which contained atranorin but lacked fumarprotocetraric acid. However, they refused to eat *Cetraria islandica* which contains fumarprotocetraric acid. It was also observed that *F. setosus* started feeding on *C. islandica* samples as the concentration of fumarprotocetraric acid was reduced via acetone extractions. When acetone extractions were perfomed to completely remove fumarprotocetraric acid from *C.*

*islandica* samples then it was fed on by both *F. setosus* and *C. intermedius* (Reutimann and Scheidegger 1987). Thererfore, a larger amount of fumarprotocetraric acid instead of atranorin in the *C. stygia* thalli might be more beneficial to the lichen. Various authors have reported that reindeer avoid *Cladonia* species with fumarprotocetraric acid with high concentrations but they will feed on species that lack fumarprotocetraric acid completely (Rundel 1978). Fumarprotocetraric acid has also been shown to chelate iron from rocks and help the lichen tolerate heavy metals (Hauck et al. 2007; Hauck and Huneck 2007). There was a higher concentration of fumarprotocetraric acid in Sherridan 219.80 µg/ml compared to Athapap which had a concentration of 137.86 µg/ml (Figure 10). The difference might be due to the fact that Sherridan has predominately granite bedrock. Syers (1969) examined the total percent change in absorbance (A% absorbanse) over the visible wavelength range on *Parmelia conspersa* which contains fumarprotocetraric acid. Three different supernatent mixtures were created for this study. The first mixture contained ground granite with pH 7.4 phosphate buffer, second mixture contained an additional 0.050g fumarprotocetraric acid and the third mixture contained an additional 0.5g dry *P. conspersa* instead of fumarprotocetraric acid. It was observed that granite reacted in both the mixtures containing fumarprotocetraric acid and *P. conspersa.* Fumarprotocetraric acid was moderately soluble in the mixture. In addition, the absorbance curves at different wavelengths for both the lichen and the lichen acid were similar indicating that fumarprotocetraric acid might be acting as a chelating agent due to it having certain side groups (-OH, -COOH or –CHO and –OH). These side groups can act as electron donors and interact with the metals present in the rock (Syers 1969).

### 5.4 Species diversity around *C. stygia* thalli differs with location

Since the quadrats were placed on thalli of *Cladonia stygia*, *C. stygia* was present in 100% of the quadrats. Another reindeer lichen that had a high frequency of occurrence was *Cladonia arbuscula,* which was present in 100% of the quadrats in Athapap and 90% of quadrats in Sherridan (Figure 8). The similarity in occurrence of both species might be due to the fact that both *C. stygia* and *C. arbuscula* are reindeer lichens and have similar growth rates (Ahti 1964). The response of *Cladonia* lichen communities to fire and logging is such that the biomass of reindeer lichens was higher in logged sites compared to burned sites (Lafleur et al. 2016). They observed that *Cladonia arbuscula* was present in 6 out of 8 total sites and *Cladonia stygia* was present in 5 out of 8 total sites. Similarly, Kotelko et al. (2008) showed that *Cladonia rangiferina* was present in 56% of the quadrats and had a maximum cover of 80% while *Cladonia arbuscula* was present 52.1% of the time with a maximum cover of 95%. These results support the co-occurrence of these two species and support this study where *Cladonia stygia* was present 100% of the time in both locations (Figure 8) with a maximum cover of 75% (Table 7) and *Cladonia arbuscula* was present 100% in Athapap and 90% of time in Sherridan (Figure 8) with a maximum cover 31% (Table 7). Kotelko et al. (2008) also found *Pleurozium schreberi* to be the most prevalent bryophyte species present 48% of the time with a maximum cover of 99% (Kotelko et al. 2008), which also supports this study where the most frequent bryophyte was *Pleurozium schreberi* present 100% in Athapap and 40% in Sherridan (Figure 9) with a maximum cover of 45% in Athapap and 40% in Sherridan (Table 8). The reindeer lichens and feathermosses are slow growing, but they also have a large size. Secondary metabolites produced by reindeer lichens also provide them with a competitive advantage over other organisms present in the same habitat (Rundel 1978). *Pleurozium schreberi* is a feather moss which are competitive

due to the mat forming growth pattern which can overgrow other mosses (Kotelko et al. 2008; During 1979; Rydin 1997). Reindeer lichens are competitive due to the rapid growth and their ability to produce secondary metabolites which provide them benefits in the environment in which they are found (Ahti 1959). The higher number of lichens in the study sites compared to bryophytes might be due to the fact that different lichens produce secondary metabolites which help them compete with other organisms and protect them from microbes, harmful UV rays and herbivory (Rundel 1978; Stocker-Wörgötter 2007). In a study conducted on sand dunes in Estonia by Jüriado et al. (2016), it was found that lichens are present in areas with both high and low pH levels. In total 66 lichenized fungi were identified out of which there were 43 *Cladonia* representatives. It was observed that *Cladonia* species were positively correlated with variable pH levels. Lichens that contain apothecia, richly branched thallus and lichens lacking soredia preferred higher pH levels whereas lichens containing soredia, lichens with infrequent apothecia and lichens that have sparsely branched fruticose thallus preferred soil with lower pH levels (Jüriado et al. 2016). Six of the 11 lichen species that were present only in Sherridan are from the genus *Cladonia* (Figure 8). Some species in the genus *Cladonia* such as *C. coniocraea, C. chlorophaea,* and *C. macilenta* are referred to as acidophytes (Wolsely et al. 2005) due to the fact that these species can easily thrive under highly acidic conditions (Wolseley et al. 2005; Wolseley et al. 2006). In a study conducted by van Herk (2002) on epiphytes it was found that *Cetraria cholorophylla* was also an acidophyte, which supports this study where both the *Cetraria* species (*C. ericetorum* and *C. islandica*) (Figure 8) were found only in Sherridan with more acidic pH soils compared to Athapap (Table 1).

There was also a significant difference in frequency of occurrence of bryophytes between the two locations. Five of 13 bryophyte species were present only in Athapap whereas only three

were present in Sherridan (Figure 9). It was also observed that bryophyte species that were present in both locations had a higher frequency of occurrence in Athapap compared to Sherridan except *Dicranum polysetum* which had a higher occurrence in Sherridan (Figure 9). This might be due to that fact that Athapap had a higher pH than Sherridan and, in general, some moss species have been shown to prefer higher pH values for growth (Pereira et al. 2014). In a study conducted on two different *Nothofagus* trees (*N. dombeyi* and *N. oblique* ) it was observed that the bryophyte richness was higher on *N. oblique* which had a mean pH range of 5.06 - 6.25 compared to *N. dombeyi* which had a mean pH range of 4.06 - 5.17. In this study, it was observed that the liverwort, *Ptilidium pulcherrimum,* was present only in Sherridan (Figure 9) which has an acidic pH (Table 1). This was also observed in a study conducted by Pereira et al. (2014) where they found that the liverwort species richness was higher on *N. dombeyi* which had a lower pH in both locations tested. Wiklund (2003) reported that spore germination of *Buxbaumia viridis* increased in relation to higher pH. When the pH was lowered to 3 or 4, the germination rate was significantly reduced. The higher species richness in Athapap compared to Sherridan is supported by studies which have found that bryophyte richness increases as the pH level increases (Hydbom et al. 2012; Pereira et al. 2014). Hydbom et al. (2012) conducted their study in sandy and calcareous grasslands where it was observed that bryophyte species richness increased in sites with higher pH levels at both experimental and natural sites.

### 5.5 Effect of carbohydrates on gene induction

The overall number of lichen species present in Sherridan (Figure 8) and the overall number of bryophyte species in Sherridan (Figure 9) exceeded the number of lichens and bryophytes present in Athapap. This may suggest a higher diversity of carbohydrates present in the soil since it has been widely accepted that carbohydrates and lipids in plant litter form a big

proportion of the soil organic matter (Lorenz et al. 2007; Marschner et al. 2008). The major carbohydrates found in soil are glucose, galactose, mannose, arabinose and xylose (Kögel-Knabner, 2002; 2017). The lichen forming fungus, *Cladonia macilenta,* produced two different mycobionts when isolated in culture: a purple mycobiont which produced biruloquinone and a white mycobiont which lacked biruloquinone (Jeong et al. 2015). The *CmaPKS1* expression was highly expressed in the purple mycobiont and it was also influenced by different culture conditions of 2% glucose, 2% sucrose and 8% sucrose treatments (Jeong et al. 2015). In a different study on *Usnea longissima,* media conditions which contained sucrose, glucose, inositol, sorbitol or fructose as a carbon source were used to examine changes in expression of the *U1PKS1* gene (Wang et al. 2012). They reported that both sucrose and glucose stimulated U1PKS1 gene expression whereas inositol, sorbitol and fructose induced U1PKS1 gene expression at 2% but inhibited transcription at 10% (Wang et al 2012). The same study showed that *U1PKS1* was closely related to the non-reducing fungal PKS clade 1 based on a phylogeny. The effect of different carbon sources was also examined on *Dirinaira applanta*. A non-reducing gene *DnPKS* produced by *D. applanta* showed different expression levels depending on the culture conditions (Valarmthi et al. 2009). When the concentration of sucrose increased, the *DnPKS1*-like expression was down regulated. DnPKS1-like expression was highly induced in the media containing mannitol, in the presence of glucose the expression was moderately induced, and in the presence of fructose the expression only showed a slight induction. The study also found a slight decrease in *DnpKS1*-like expression when alanine and proline were added to the culture medium (Valarmthi et al. 2009). Geisen (2004) examined the expression of the orchratoxin A producing gene *otapksPN* in *Penicillium nordicum* and how it is affected by different media conditions. Three different treatments were used; 1) YES medium which was

rich medium containing yeast, 2) one minimal medium containing glycerol as a carbon source, and 3) a second minimal medium which suppresses ochratoxin A production containing glucose as a carbon source. RT-PCR was performed to check the expression of *otapksPN* gene and HPLC was performed to obtain the concentration of Oochratoxin A produced. It was observed that the YES medium produced the highest concentration of Ochratoxin A and it had the highest *otapksPN* expression, followed by the minimal medium containing glycerol, and then the lowest levels of production and expression occurred in the minimal medium containing glucose (Geisen. 2004). Elshobary et al. (2016) showed that concentration of certain carbohydrates greatly influenced the PKS gene expression. Their study focused on four PKS genes from *Cladonia rangiferina* (*CrPKS1, CrPKS3, CrPKS7* and *CrpKS16*) and how they are affected by carbohydrate sources (glucose, sorbitol and ribitol) and three concentrations of each (1%, 5% or 10%). It was observed that transcription of all four genes was higher at 30 days when compared to 20 days. *CrPKS1* and *CrPKS16* were upregulated when the media contained 1% ribitol and low sorbitol concentrations induced *CrPKS3* and *CrPKS7* gene transcription. These previous studies on gene expression based on different carbohydrates support the findings in this study in the naturally collected thalli from Sherridan and Athapap and the difference in gene expression (Figure 10) and the concentration of polyketides produced (Figure 12).

**5.6 Environmental effect on gene expression**

PKS gene expression of *CmaPKS* was induced in *Cladonia macilenta* when UV light was applied to the fungal culture (Jeong et al. 2015). The four domains in *CmaPKS,* Ketoacyl synthase (KS), Acyl transferase (TE) acyl carrier protein (ACP) and a Thioesterase (TE) domain, suggests that the *CmaPKS1* gene is a non-reducing PKS because it lacks the reducing domains (Jeong et al. 2015). Phylogenetic analysis also placed *CmaPKS1* in a non-reducing PKS clade.

The effect of UV exposure on *DnpKS1* gene expression in the lichen *Dirinaria applanata*. induced the *DnPKS*-like transcript for 90 days compared to the control with no UV treatment (Valarmathi et al. 2009). Any exposure more than 90 days was lethal for the cells (Valarmathi et al. 2009). Another study examined structural genes *stcU* in *Aspergillus nidulans* and ver-1 in *Aspergillus parasiticus* and the expression of the homologous aflatoxin and sterigmatocystin structural genes. Aflatoxin production by *Aspergillus parasiticus* increased in acidic environments. The same phenomenon was recorded for *A. nidulans* where sterigmatocystin production was highest at pH 4 when compared to pH 8 (Keller et al. 1997). This phenomenon was also recorded in a study by Begora and Fashalt (2001) where levels of atranorin and usnic acid increased under high or low intensity UV-A and visible light.

Based on the HPLC data it was observed that the average concentration of atranorin present in both Athapap (18.22 µg/ml) and Sherridan (60.67 µg/ml) (Figure 10) was significantly lower than the average concentration of fumarprotocetraric acid in Athapap (137.86 µg/ml) and Sherridan (219.80 µg/ml) (Figure 10). This difference might have been due to the different functions that these secondary metabolites provide to the lichen. Atranorin has been shown to protect the lichen from harmful UV rays (Rao and Leblanc. 1965). The average canopy cover was 44.2% in Athapap and 39.6% in Sherridan (Table 1) suggesting that the canopy is providing some protection to the lichens present in those quadrats. However, since pH is thought to influence the production of polyketides (Hamada 1989; Yammamto et al. 1995; Fox and Howlett 2008; Bizukojc et al. 2012), the interaction between pH and the canopy cover may explain different levels of atranorin in the sites.

### 5.7 Relationship between *Cladonia stygia* and *C. rangiferina*

*Cladonia stygia* and *C. rangiferina* are very similar species morphologically but the morphology can vary depending on the habitat in which they are found. The features used to distinguish them include a black basal thallus and pink pycnidial jelly in *C. stygia*, while a gray or browned basal thallus and clear pycnidial jelly is diagnostic of *C. rangiferina* (Ruoss and Ahti 1989). Both *C. rangiferina* and *C. stygia* form mats over large areas of ground cover in the northern ecosystems (Auclair and Rencz 1982; Shaver and Chapin 1991), and they provide an important source of winter food for reindeer and caribou (Svihus and Holand 2000; den Herder et al. 2003), but *C. stygia* is more common in the open wet bogs and fens while *C. rangiferina* prefers drier sites.

Even with morphological and habitat differences, Athukorala et al. (2016) showed that one haplotype was shared between *C. rangiferina* and *C. stygia* and they were conspecific based on a phylogenetic tree using the ITS rDNA. The BLAST comparison for the MrSSU gene showed a strong E-value and Identity percentage to both the *Cladonia stygia* and *Cladonia rangiferina* mitochondrial complete genome (Table 9) providing support that the control gene is indeed the MrSSU gene. A phylogeny of the genus *Cladonia* using sequences based on internal transcribed spacer 1 (ITS1) and the internal transcribed spacer 2 (ITS2) regions showed that *Cladonia stygia* and *Cladonia rangiferina* are sister species and hence share a common ancestor (Stenroos et al. 2002). Despite the morphological and habitat differences between *C. rangiferina* and *C. stygia,* they are considered to be closely related and have the same polyketides (atranorin and fumarprotocetraric acid) (Ruoss and Ahti 1989).

Phylogenetic anylysis for all the target genes provided strong support that these target genes were the expected polyketide synthase domains (Figures 13 and 14). There were no thioestersae domains in GenBank but the two sequences that showed a match with BLAST, were shown to be similar to a PKS gene in a non-lichenized fungus and similar to a genome contig from a lichen fungus. However, a larger number of sequences were available in GenBank for the Beta-ketoacyl synthase gene. The six target genes fell within clusters that contained other PKS KS regions from both lichenized and non-lichenized fungi, confirming the identity of these genes.

### 5.8 Gene transcription relative to each other

It has been well documented that lichens and fungi produce multiple secondary metabolite genes which express differently under different conditions (Feng and Leonard 1998; Valarmathi et al. 2009; Elshobary et al. 2016). In this study, it was observed that different genes were being transcribed differently under similar natural field conditions, whereas some genes showed a higher transcription and others showed a lower expression level (Figure 15; Figure 16). The gene for the 21 Beta-Ketoacyl synthase domain had a higher transcription level than the 26 Thioesterase domain suggesting that conditions may have been more favourable for the transcription of this particular gene. This would be beneficial for the lichen because production of secondary metabolites is costly and occurs when the conditions are not optimal for growth. However, an increase in the expression of genes that provide benefits to the lichen might be favourable for the overall fitness of the lichen.

### 5.9 Semi-quantitative PCR vs Quantitative PCR

Polymerase chain reaction (PCR) is a technique used to amplify a single or few copies of DNA and make millions of copies of the targeted gene. The benefit of PCR is that by using it with electrophoresis, bands can be observed which represent DNA amplification. The cycle number and the denaturing, annealing, and extension steps can then be further optimized to obtain more intense bands. The main limitation to regular PCR is that once the reaction reaches a plateau the end point, quantification of the PCR product is unreliable (Staggemeirer et al 2015). To get past this problem in previous years semi-quantitative PCR has been used. In semi quantitative PCR, instead of observing the samples at one particular cycle number which is the case in conventional PCR, the samples are observed at multiple cycle numbers. This allows the observer to view the logarithmic change in the electrophoresis gel image. One limitation of semi-quantitative PCR includes the use of gel electrophoresis with reagents including ethidium bromide which are harmful to humans and the risk of carry over because multiple apparatuses are being used (Paiva-Cavalcanti et al 2010).

Real time quantitative PCR, which is also known as qPCR, is similar to traditional PCR but the main difference is that qPCR is measured after each round of amplification. The most important parameter of qPCR is the Ct (threshold cycle) value which is used to quantify the amount of DNA in the samples. The threshold value is set within the exponential increase phase also known as the log phase. qPCR performs the quantification of the target gene in the exponential amplification of log phase which reduces the problems that are associated with conventional PCR (Staggemeirer et al. 2015). Previous reviews conducted on qPCR have shown that during the exponential phase of the PCR it is possible to determine the starting template (Ginzinger 2002). Another benefit of qPCR is that only a lower concentration of target genomic

DNA is required (Ginzinger 2002). In a study conducted on water and sediments for detection of human adenoviruses it was found that qPCR showed a higher detection rate than conventional PCR (Staggemeier et al. 2015). The main drawbacks of qPCR are firstly to interpret the data, a high level of expertise is required and the assays and the reagents used are much more expensive than conventional PCR (Donaldson et al. 2002; Wolf et al. 2010).

During this study the main obstacle was getting rid of the primer dimers. Even at a high annealing temperature (62°C), the primer dimers were still visible making the qPCR method not useful. This is because the qPCR thermal cycler cannot differentiate between the primer dimmers and the template PCR product. If the annealing temperature was increased, the high temperature degraded the cDNA product. Therefore, semi-quantitative PCR was performed instead of qPCR where the bands were visible on the gel making it easy to distinguish them from the primer dimmers.

# CHAPTER 6 Conclusions and Future Work

This study provided insight into the secondary metabolite clusters present in the *Cladonia rangiferina* genome which was represented by the closely related *Cladonia stygia* in this study. There were a total of 41 different secondary metabolite clusters found in *C. rangiferina* genome, out of which 15 were Type 1 PKS clusters and out of those 15, two were non-reducing Type 1 PKS clusters (Cluster 21 and Cluster 26). There was a significant difference in the concentration of fumarprotocetraric acid between Athapap and Sherridan and a close significant difference in the concentration of atranorin in both locations. Fumarprotocetraric acid was also found to have a much higher overall concentration in Sherridan compared to Athapap. The 21 Beta-Ketoacyl Synthase gene showed a significant difference in gene transcription between the two locations and the 26 Thioesterase domain also showed a significant difference compared to other target genes being tested. Based on the HPLC, semi-quantitative PCR and gene expression data, Cluster 21 may be involved in the production of fumarprotocetraric acid and 26 Thioesterase domain may play a role in the production of ataranorin in *C. stygia*.

Improvements to this study include the use of a pH strip which ranged from 5.0-14.0 hence the lowest pH observed being between 5.0 - 5.5. To get a better representation of the pH in the field a pH strip ranging from 1-14 should been used to check if the pH of any of the sites was actually below pH 5 which was observed. Another way to check the pH of the sites would be to obtain soil samples and using a pH meter to obtain the pH readings would provide more accurate readings. A better alternative to using canopy cover would be to use a light intensity meter to measure if there is any change in the UV and light intensity in the different sites. However, this method also has problems depending on the time of day and the extent of cloud cover, so many ecologists continue to use canopy cover to measure transmitted light. Future research should

focus on the other clusters which were determined as partially reducing PKS by the antiSMASH software because the software does not identify the gene clusters correctly 100% of the time and there are always some clusters that are identified incorrectly (Medema et al. 2011). Studies should also focus on the two non-reducing PKS clusters and check their expression at extreme conditions Future work should also focus on observing how changing the pH and or UV levels will affect the expression of 26 Beta-Ketoacyl Synthase and the 26 Thioesterase domain. Further work need to be done to confirm if these two clusters are actually responsible for the production of the two secondary metabolites present in *Cladonia stygia*. One way to achieve this is to grow the lichen forming fungus in culture under different conditions such as pH levels and carbohydrate sources and concentration. At each treatment level gene expression and the concentration of the polyketide should be obtained. If there is a strong correlation between the upregulation of the gene and the increase in the concentration of the polyketide that would provide strong evidence that there is a relationship between that cluster and the polyketide produced.

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**Appendix A:** antiSMASH results obtained for *Cladonia rangiferina* genome.

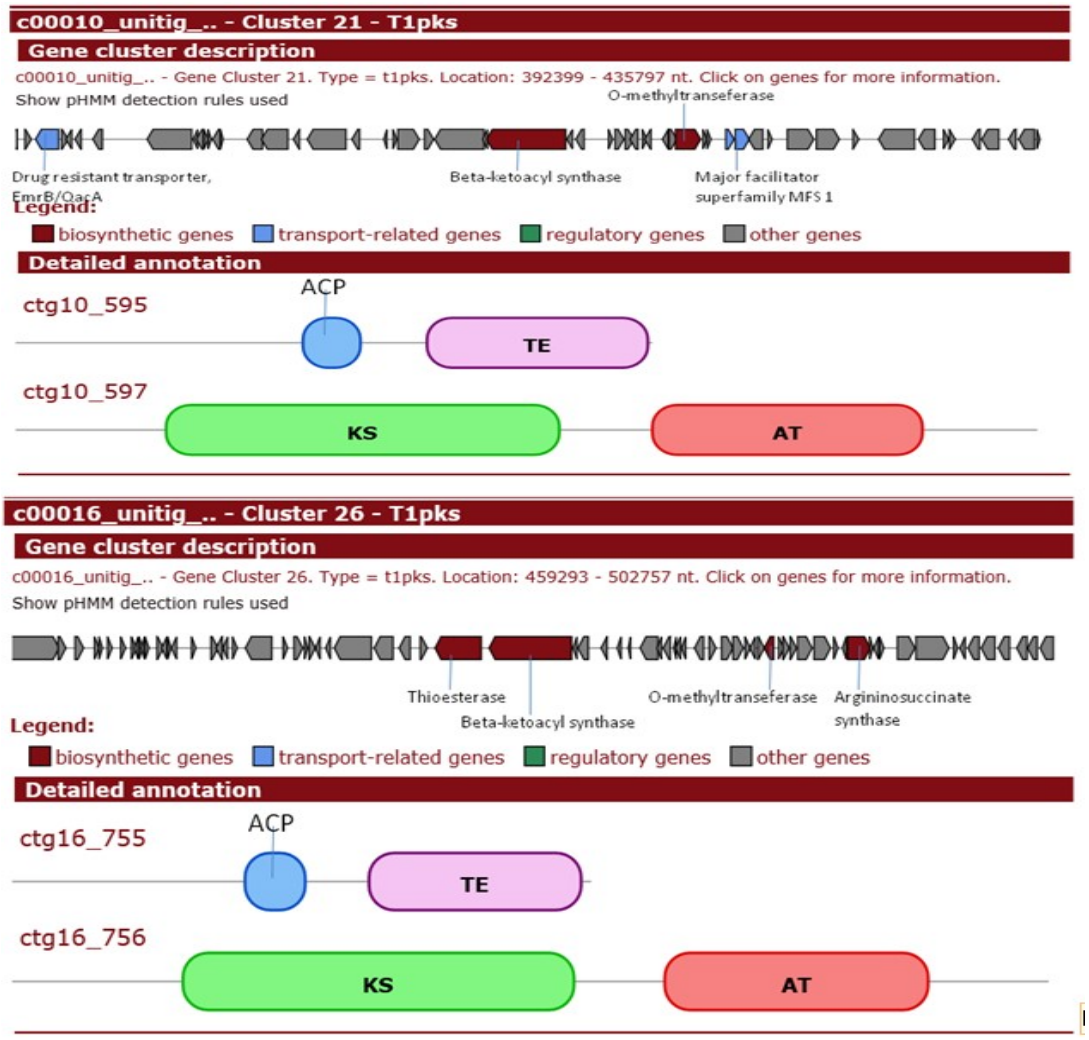



**Figure A1:** Two non-reducing Type 1 PKS clusters (Cluster 21 and Cluster 26) obtained from *Cladonia rangiferina* whole genome using antiSMASH. Last accessed on 17 June 2017.

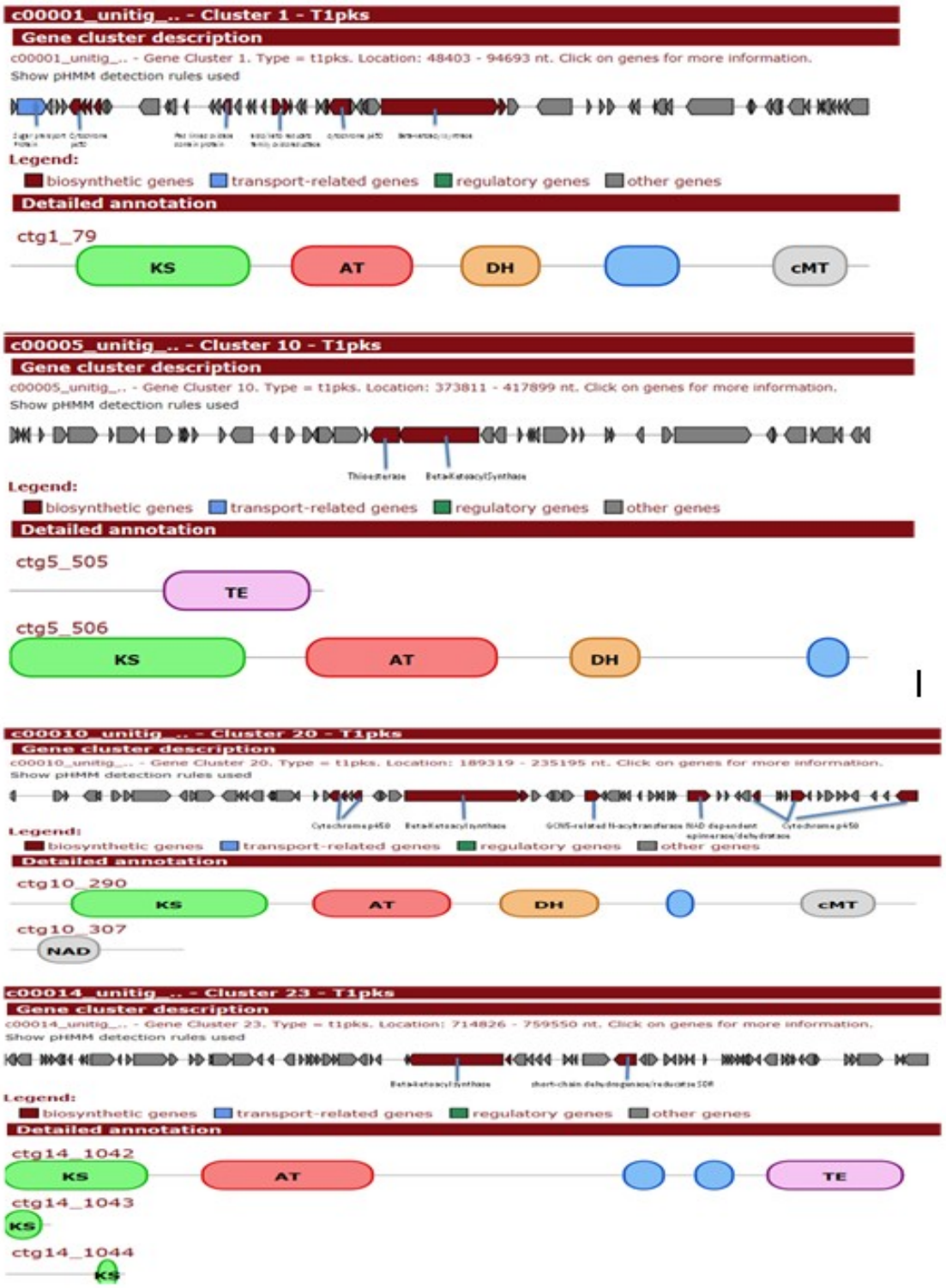



**Figure A2:** Four partially reducing Type 1 PKS clusters (cluster 1, cluster 10, cluster 20 and cluster 23) obtained from *Cladonia rangiferina* genome using antiSMASH. Last accessed on 17 June 2017.

**Appendix B:** TLC sample image and HPLC standard regression lines

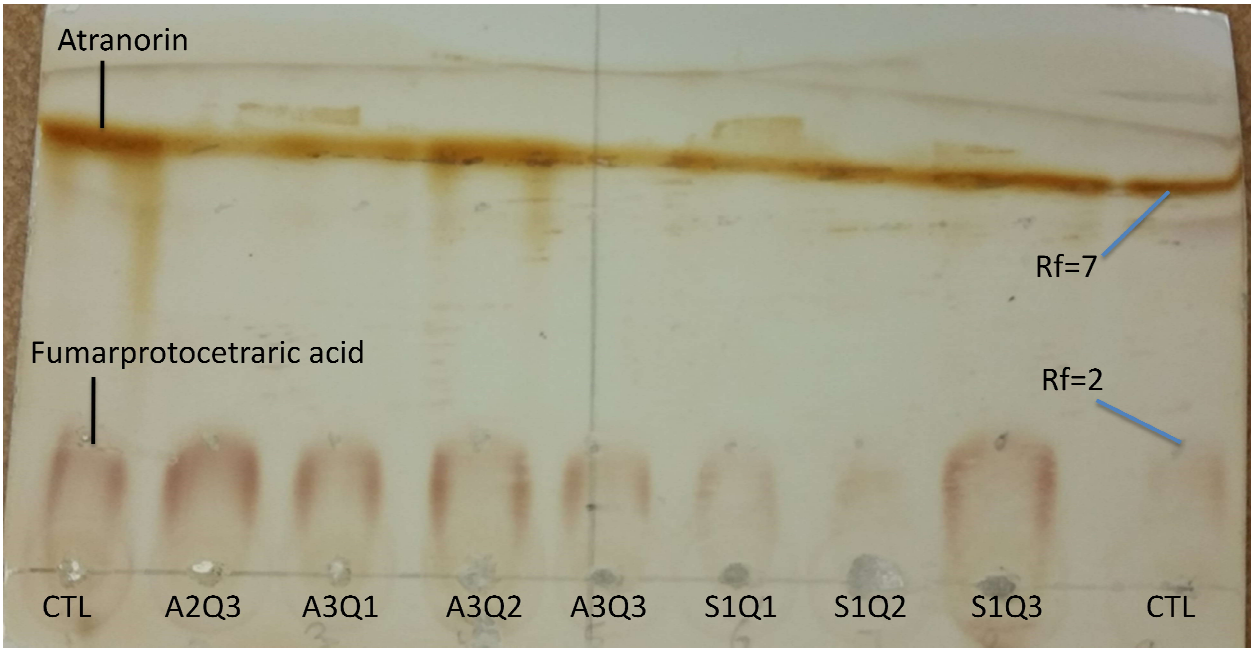
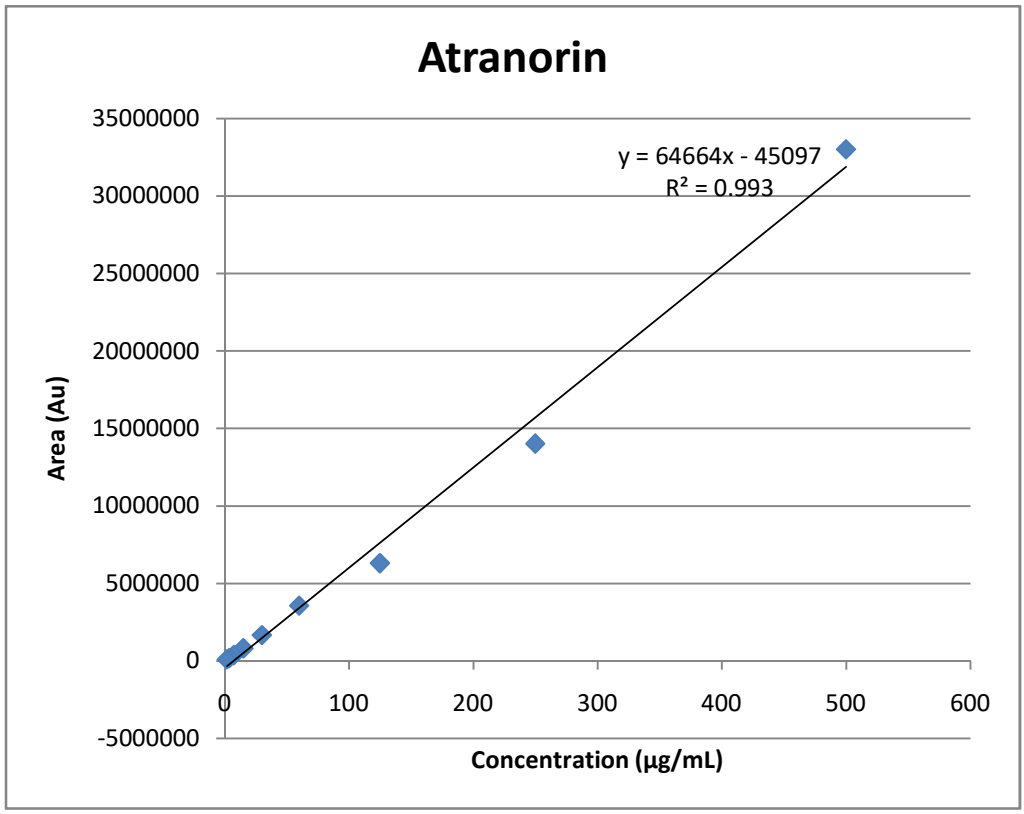



**Figure B1:** Secondary metabolite data obtained via TLC analysis. Control was the acetone extract from *Cladonia rangiferina* (Voucher specimen Collection Number: Normore 1547 deposited in WIN).

A)




B)

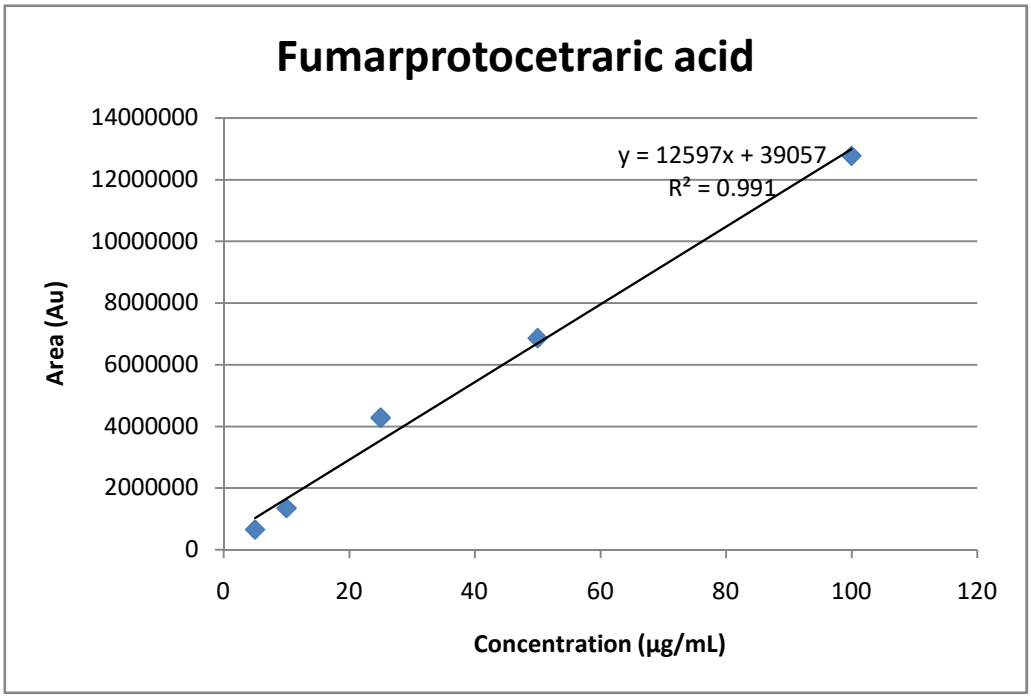



**Figure B2:** Regression line graphs based on the standard concentration of a) atranorin and b) fumarprotocetraric acid. Area (Au) under the chromatograph peaks was obtained using HPLC analysis.

**Appendix C:** PCR cycles and gel electrophoresis images.

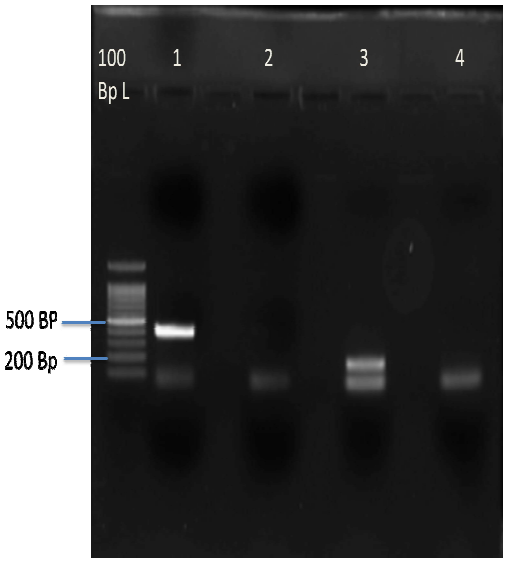



**Figure C1:** Gel electrophoresis image to test primers. 10µl reactions were prepared. 5µl SYBER, 1µl of 10µM forward primer, 1µl of 10µM Reverse primer and 3µl cDNA (88ng/µl) was added to each sample. For the negative control, 3µl sdH2O (Fisher Scientific). PCR conditions [34 cycles of 5 min 95ºC, (95ºC for 15 seconds, 62ºC for 15 seconds and 72ºC for 15 seconds), 72ºC for 2 minutes and ending with 25ºC for infinite hold]. Lane 1 (MrSSU control gene), Lane 2 (negative), Lane 3 (26 Beta-Ketoacyl synthase target gene), Lane 4 (Negative). Image obtained by Quantity One-4.6.9 software.

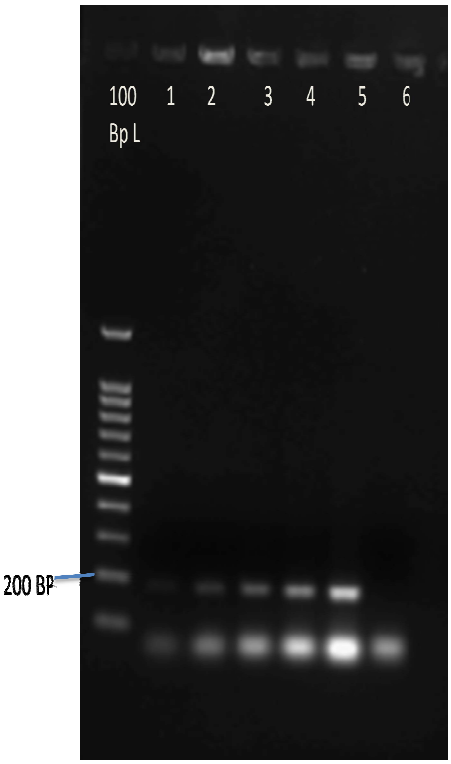



**Figure C2**: Gel electrophoresis image to test PCR cycle number for the increasing expression of the target genes. Reaction volume 15µl of SYBER, 3µl of forward primer and 3µl of reverse primer and 8µl of cDNA. The negative consisted of 5µl SYBER, 1µl 26KSF, 1µl 26KSR and 3µl sdH2O. PCR [40 cycles of 5 min 95ºC, (95ºC for 15 seconds, 62ºC for 15 seconds and 72ºC for 15 seconds), 72ºC for 2 minutes and ending with 25ºC for infinite hold]. From lanes 1 to 5: target gene (26 Beta-Ketoacyl synthase). Lane 1 cycle #32, Lane 2 cycle #34, Lane 3 cycle #36, Lane 4 cycle #38, Lane 5 cycle #40 and Lane 6 negative at cycle number 40. Image obtained by Quantity One-4.6.9 software.

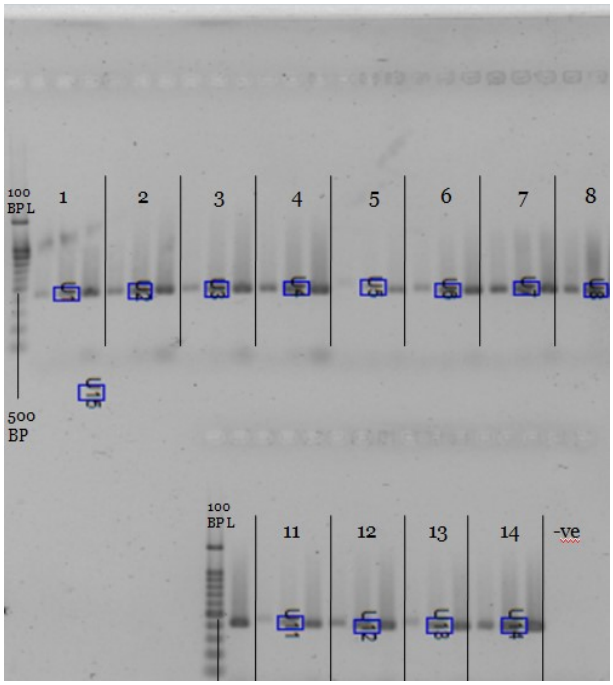



**Figure C3:** Gel electrophoresis image showing the semi-quantitavie image obtained of the control gene (MrSSU). Reaction volume of 22 µL consisting of 10µl of SYBER, 2µl of MrSSU1 primer, 2µl of MrSSU2R and 8µl cDNA. The PCR cycle was [34 cycles of 5 min 95ºC, (95ºC for 15 seconds, 62ºC for 15 seconds and 72ºC for 15seconds), 72ºC for 2 minutes and ending with 25ºC for infinite hold]. 7µl aliquots were taken out at cycle number 20, 22, 24 for the control gene (MrSSU). Image obtained by Quantity One-4.6.9 software. Blue boxes indicate the band that was used for obtaining the band intensity values.

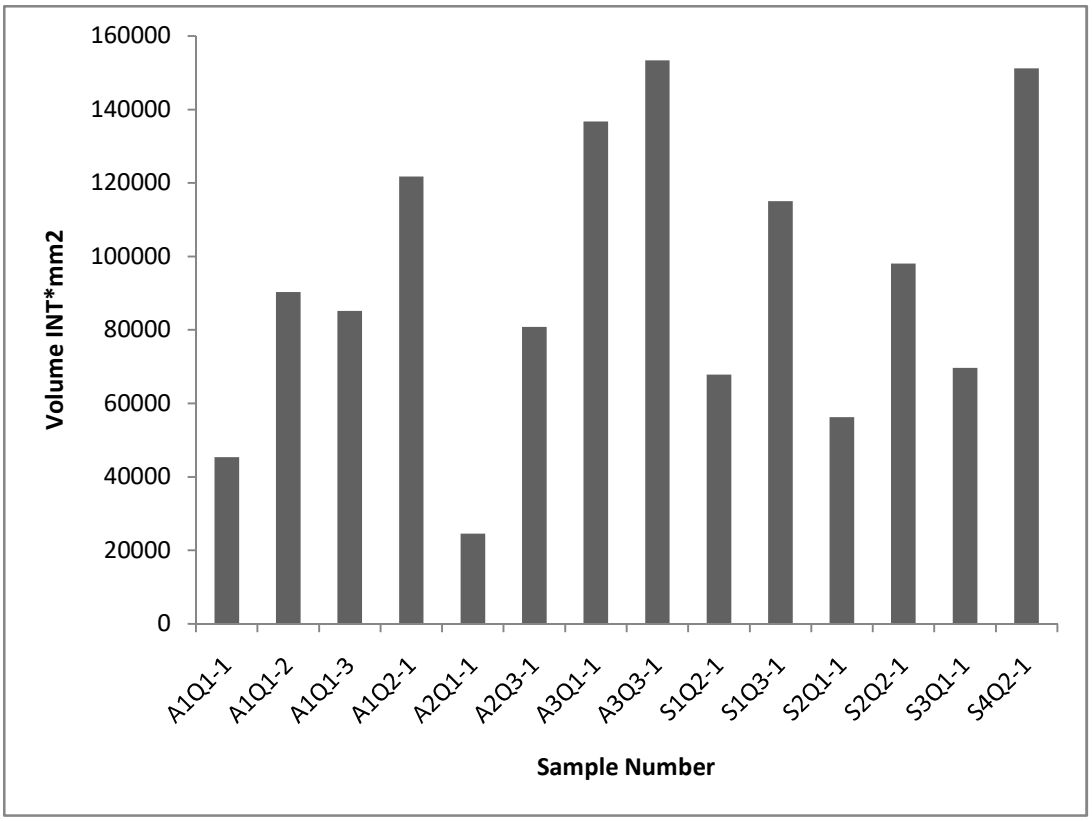



**Figure C4:** Intensity (Volume INT*mm$^2$) of cycle number 22 (middle band) in Figure 6. Density values obtained using Quantity One-4.6.9 software. PCR reaction was [34 cycles of 5 min 95ºC, (95ºC for 15 seconds, 62ºC for 15 seconds and 72ºC for 15seconds), 72ºC for 2 minutes and ending with 25ºC for infinite hold]. Gel electrophoresis image of the mitochondrial small subunit gene of the mitochondrial ribosomal DNA (MrSSU) was used to obtain the density values of 8 samples from Athapap and 6 samples from Sherridan. These values were used as a control. Samples names refer to Location (A= Athapap, S=Sherridan) and Quadrat (Q).

The highest and lowest band density for Athapap was for sample A3Q3-1 with a value of 153367.65 Volume INT*mm$^2$ followed by sample A3Q1-1 with a value of 136715.54 Volume INT*mm$^2$, respectively. The lowest value for Athapap was for sample A2Q1-1 with a value of 24543.95 Volume INT*mm$^2$. The highest density for Sherridan was for the sample S4Q2-1 with a value of 151183.20 Volume INT*mm$^2$ followed by S1Q3-1 with a value of 115047.04 Volume INT*mm$^2$. The lowest Density for Sherridan was for sample S2Q1-1 with a value of 56208.55 Volume INT*mm$^2$.

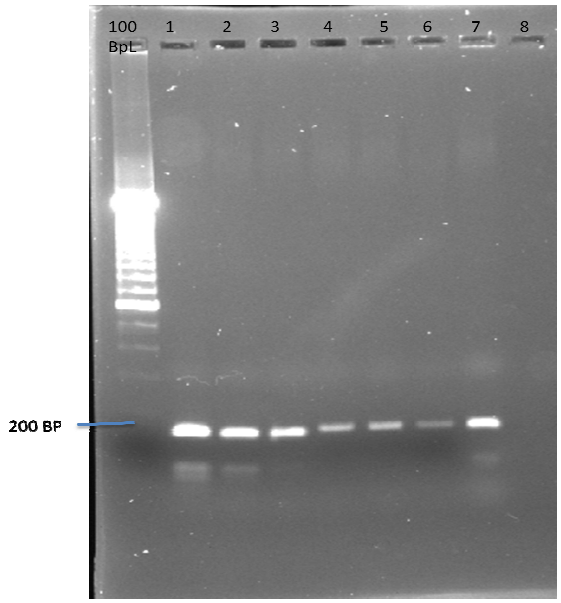
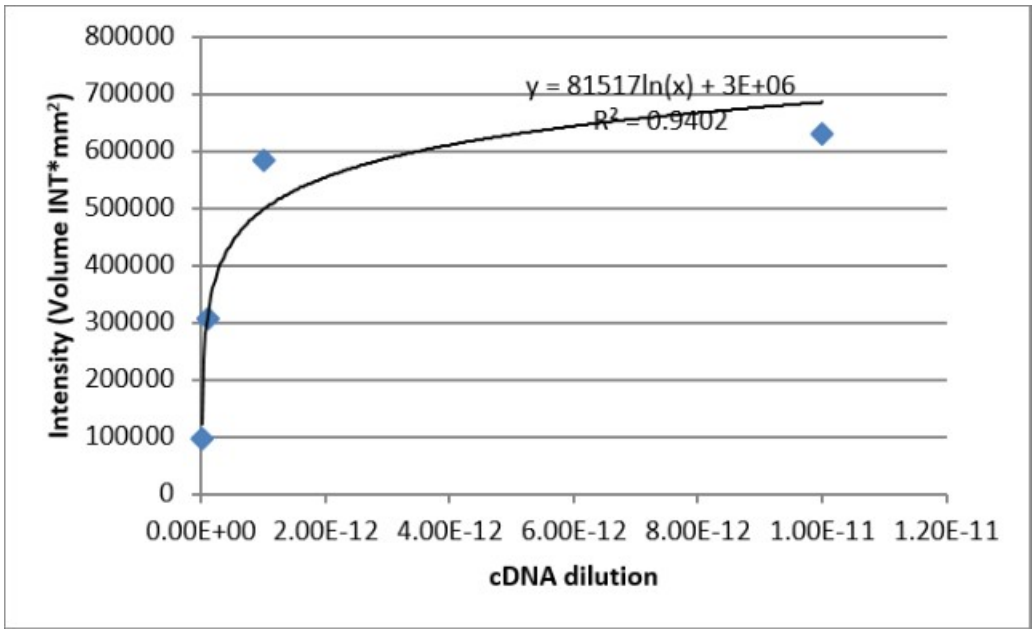
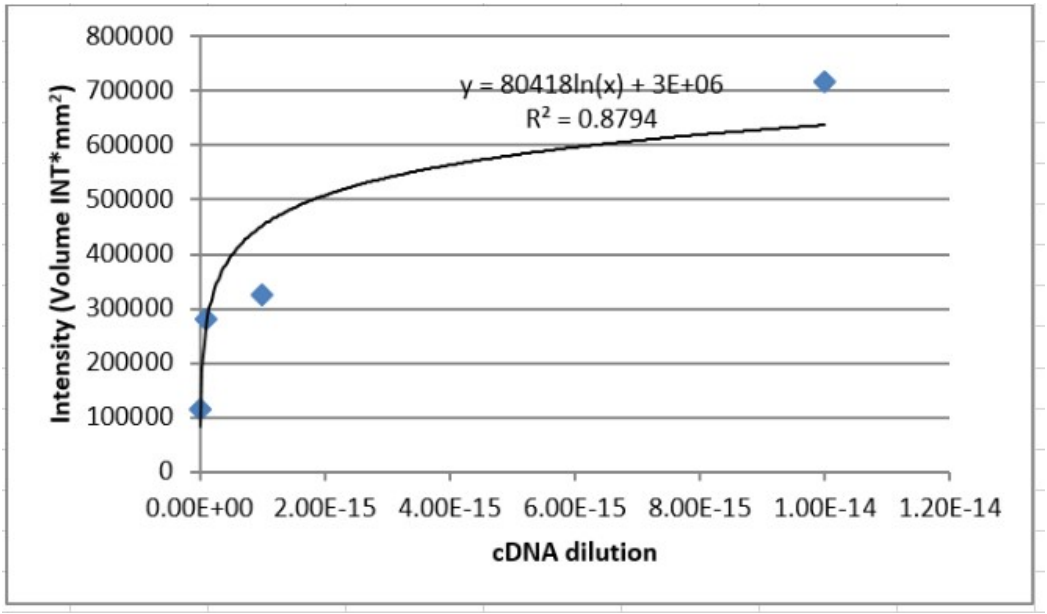



**Figure C5:** Gel electrophoresis image showing the expression of cDNA by 23 Beta-Ketoacyl Synthase target primers relative to its known dilutions. 23 Beta-Ketoacyl Synthase was amplified, excised and purified to get known concentration then dilutions were prepared. The PCR reaction was 10µl containing 5µl SYBER, 1µl 23KS 270F, 1µl 23 KS 428R and 3 µl of either of the dilution or the cDNA. The PCR cycle was [30 cycles of 5 min 95ºC, (95ºC for 15 seconds, 62ºC for 15 seconds and 72ºC for 15seconds), 72ºC for 2 minutes and ending with 25ºC for infinite hold]. Lane 1=1.0E-13, Lane 2=1.0E-14, Lane 3=1.0E-15, Lane 4=1.0E-16, Lane 5=1.0E-17, Lane 6=1.0E-17, and Lane 7=cDNA. Lane 8 was negative control.

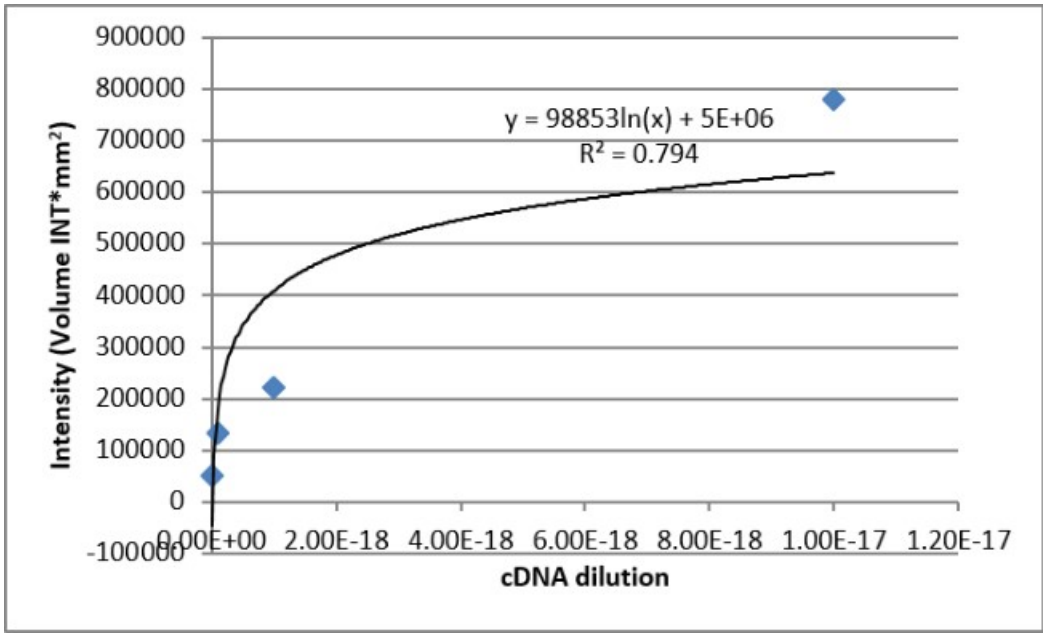



a)MrSSU

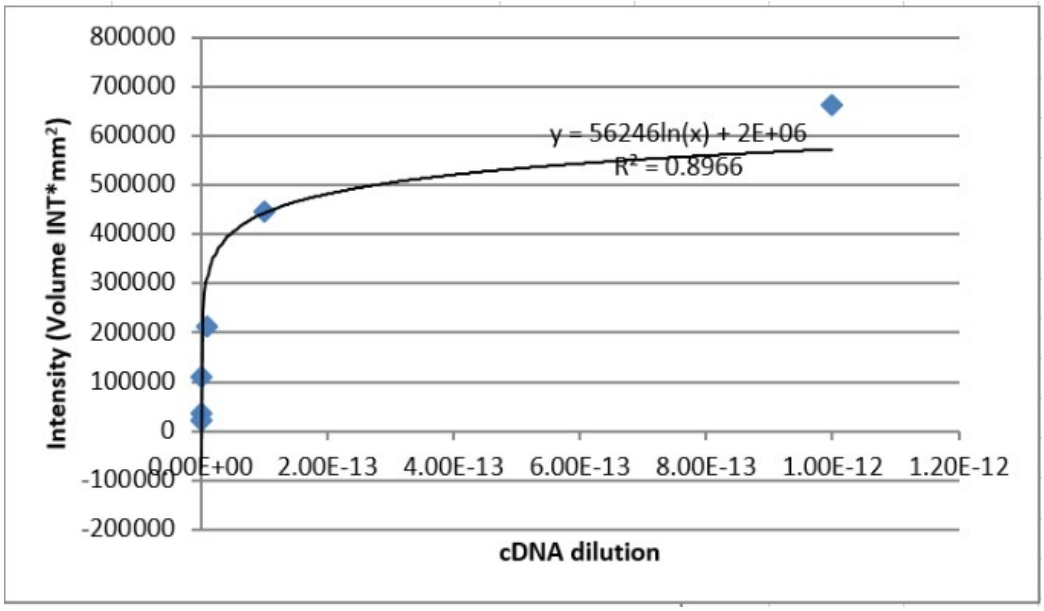


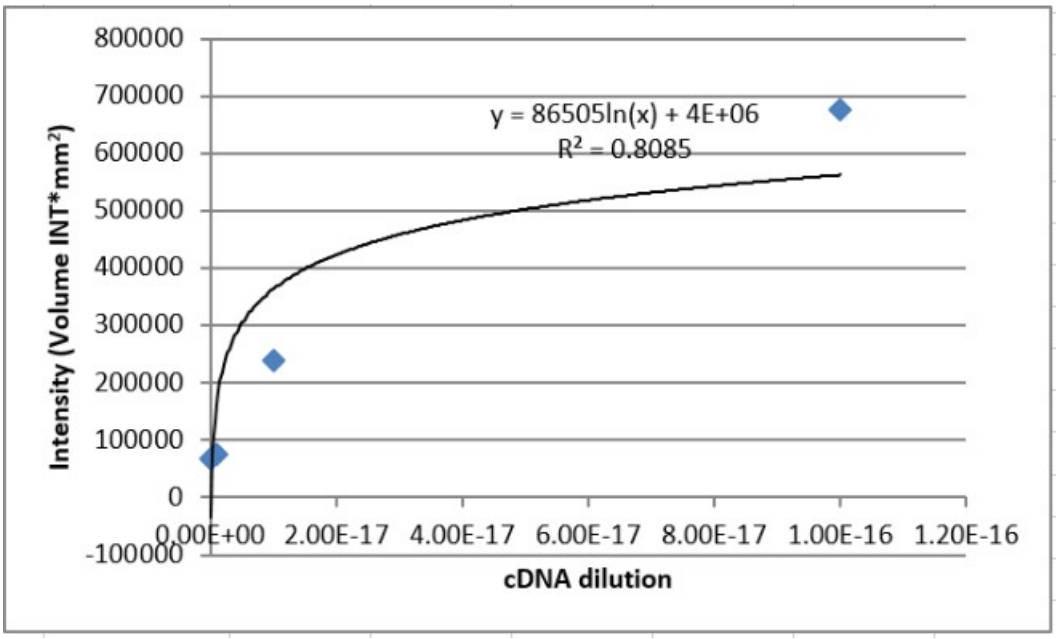

b) 23KS

c) 26KS

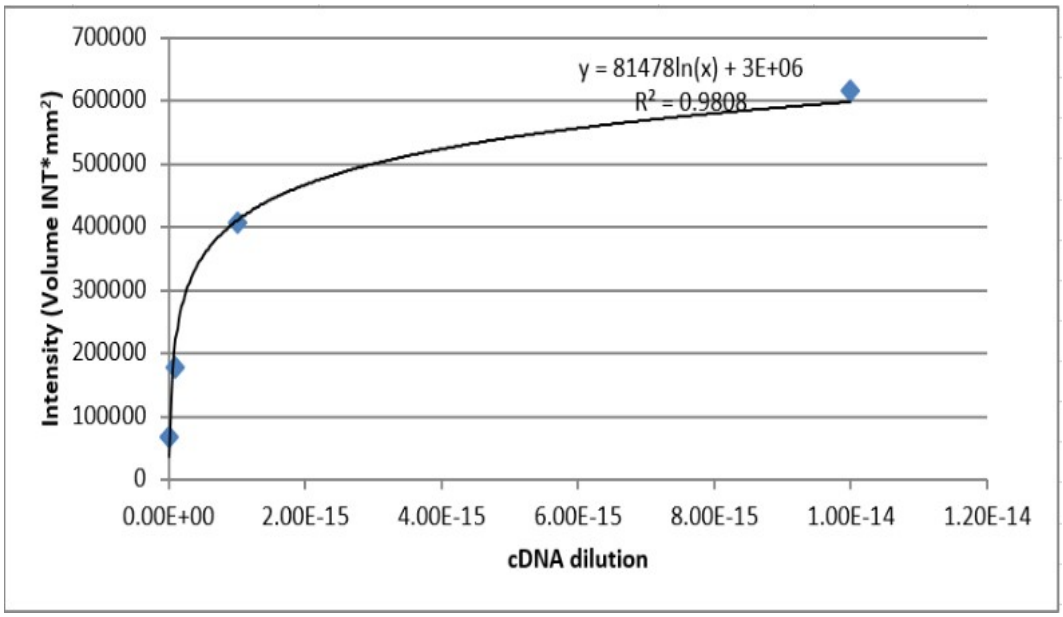

y = 98853ln(x) + 5E+06
R² = 0.794
Intensity (Volume INT*mm²)
cDNA dilution
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
-100000
0.00E+00
2.00E-18
4.00E-18
6.00E-18
8.00E-18
1.00E-17
1.20E-17

d) 21KS


y = 56246ln(x) + 2E+06
R² = 0.8966
Intensity (Volume INT*mm²)
cDNA dilution
800000
700000
600000
500000
400000
300000
200000
100000
0
-100000
-200000
0.00E+00
2.00E-13
4.00E-13
6.00E-13
8.00E-13
1.00E-12
1.20E-12

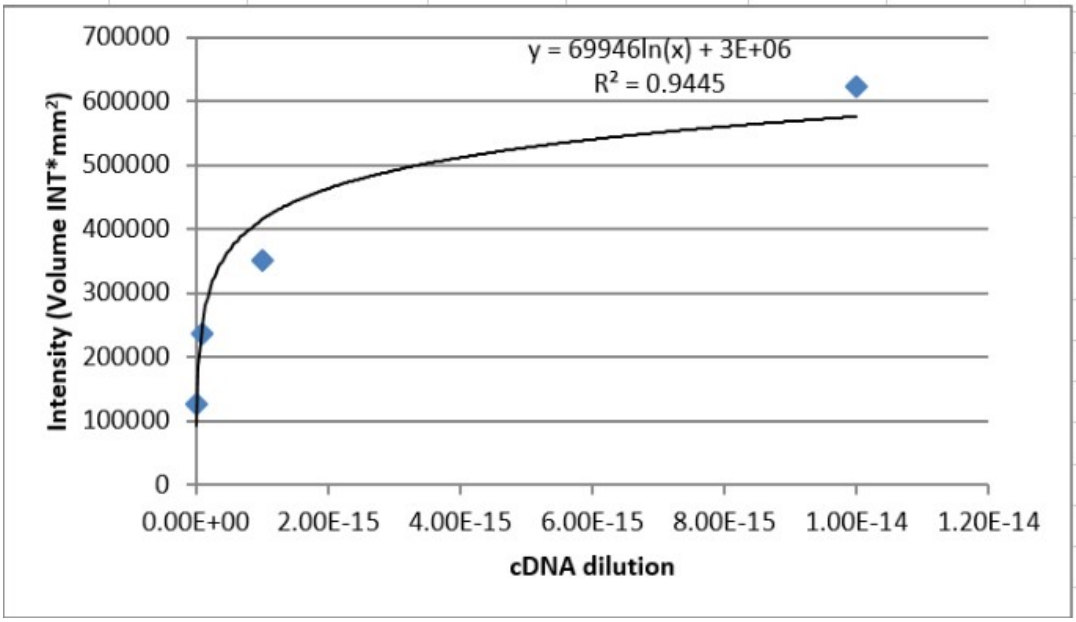

800000
700000
600000
500000
400000
300000
200000
100000
0
-100000
Intensity (Volume INT*mm²)
y = 86505ln(x) + 4E+06
R² = 0.8085
0.00E+00
2.00E-17
4.00E-17
6.00E-17
8.00E-17
1.00E-16
1.20E-16
cDNA dilution

e) 20KS

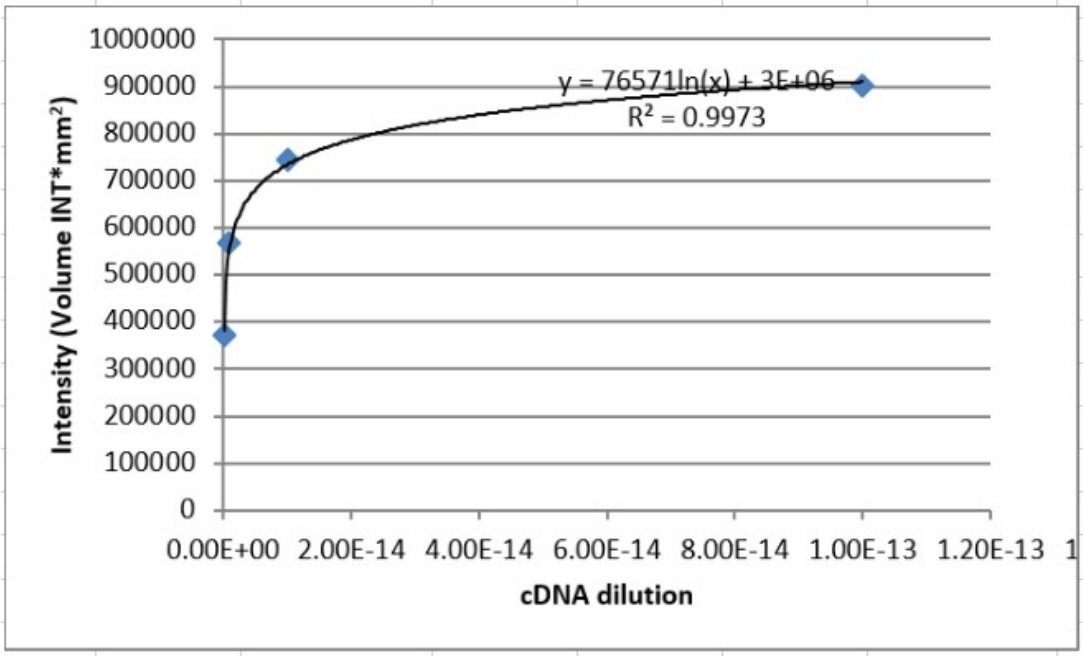

700000
600000
500000
400000
300000
200000
100000
0
Intensity (Volume INT*mm²)
y = 81478ln(x) + 3E+06
R² = 0.9808
0.00E+00
2.00E-15
4.00E-15
6.00E-15
8.00E-15
1.00E-14
1.20E-14
cDNA dilution

f) 26TE

g) 1KS


y = 69946ln(x) + 3E+06
R² = 0.9445
Intensity (Volume INT*mm²)
700000
600000
500000
400000
300000
200000
100000
0
0.00E+00
2.00E-15
4.00E-15
6.00E-15
8.00E-15
1.00E-14
1.20E-14
cDNA dilution

h) 10KS


y = 76571ln(x) + 3E+06
R² = 0.9973
Intensity (Volume INT*mm²)
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
0.00E+00
2.00E-14
4.00E-14
6.00E-14
8.00E-14
1.00E-13
1.20E-13
cDNA dilution

i) 10TE

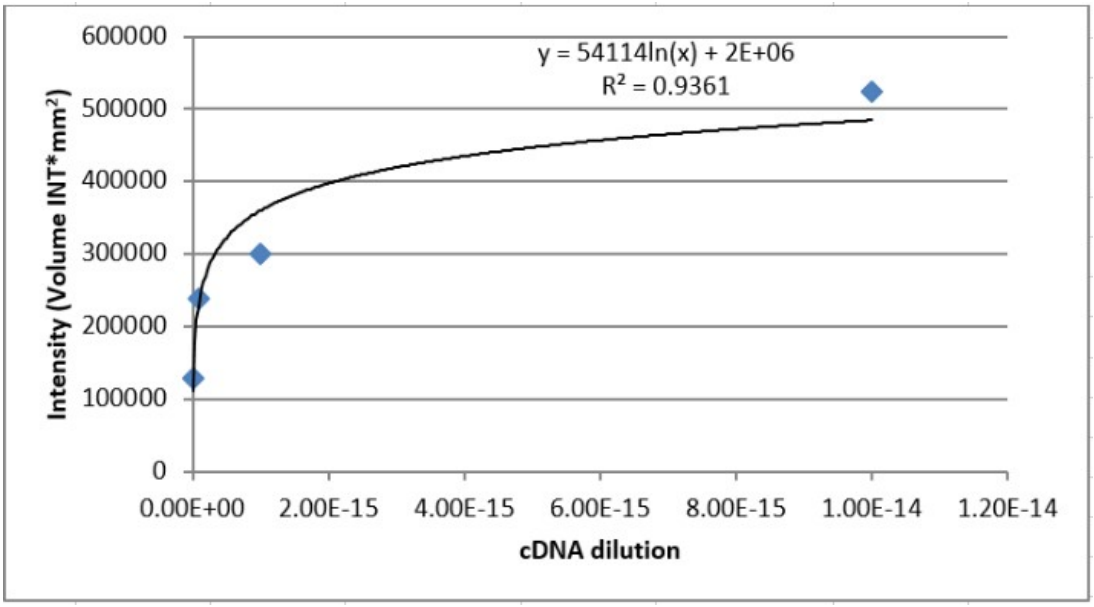



**Figure C6:** Regression line graphs based on the known PCR concentration dilutions of the control a) (MrSSU) and the target genes, b) 23 Beta Ketoacyl Synthase, c) 26 Beta Ketoacyl Synthase, d) 21 Beta Ketoacyl Synthase, e) 20 Beta Ketoacyl Synthase, f) 26 Thioesterase, g) 1 Beta Ketoacyl Synthase, h) 10 Beta Ketoacyl Synthase and i) 10 Thioesterase domain. Y axis represents Intensity Intensity (Volume INT*mm$^2$) and x axis represents cDNA dilution concentrations of the PCR product.

**Appendix D:** Field work spreadsheet describing the species obtained during field work in summer of 2016. Total number of quadrats was 21 with 9 quadrats from Athapap and 12 Quadrats from Sherridan. Spreadsheet showing the species of lichens and bryophytes, habitat type and surrounding tree types, site and GPS readings from each quadrat.

| Collection number | Species | Habitat | Site | GPS | Collector | Date |
|---|---|---|---|---|---|---|
| A1Q1-1 | *Cladonia stygia* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q1-2 | *Pleurozium schreberi* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q1-3 | *Dicranum polysetum* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q1-4 | Vascular plant | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q2-1 | *Cladonia stygia* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q2-2 | *Dicranum majus* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q2-3 | *Pleurozium schreberi* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q2-4 | *Cladonia arbuscula* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q2-5 | *Cladonia multiformis* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q3-1 | *Cladonia stygia* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q3-2 | *Cladonia arbuscula* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q3-3 | *Pleurozium schreberi* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q3-4 | *Cladonia chlorophea* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q3-5 | *Cladonia pyxidata* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q3-6 | *Dicranum majus* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q3-7 | *Cladonia cornuta* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q3-8 | *Polytrichum juniperinu* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A1Q3-9 | *Thuidium delicatulum* | Limestone Table, Abies balsamea, Aralia nudicaulis, Betula papyrifera, Linnaea borealis, Picea glauca, Populus tremuloides, F | Site 1 12.6Km east from Provincial Truck Hwy 10. Parallel to | N54°30'40.2" W101° 32' 43.3" | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q1-1 | *Cladonia stygia* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q1-2 | *Pleurozium schreberi* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q1-3 | *Cladonia arbuscula* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q1-4 | *Vascular plant* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q2-1 | *Cladonia stygia* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q2-2 | *Cladonia arbuscula* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q2-3 | *Pleurozium schreberi* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q2-4 | *Cladonia crispata* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q2-5 | *Brachythecium campes* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q3-1 | *Cladonia stygia* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q3-2 | *Cladonia arbuscula* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q3-3 | *Hylocomium splendens* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q3-4 | *Pleurozium schreberi* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q3-5 | *Cladonia pyxidata* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q3-6 | *Cladonia multiformis* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q3-7 | *Dicranum polysetum* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A2Q3-8 | *Dicranella sp* | Limestone Table, Abies balsamea, Alnus glutinosa, Aralia nudicaulis, Betula papyrifera, Cornus canadensis, Empetrum nigrum, | Site 2 11.2Km east from Provincial Truck Hwy 10. Parallel to | 54°31'03.6"N 101°31'38.5"W | Kamaldeep Chhoker | 11-Jun-16 |
| A3Q1-1 | *Cladonia stygia* | Limestone Table, Abies balsamea, Betula papyrifera, Empetrum nigrum, Fragaria virginiana, Kalmia sp, Linnaea borealis, Pice | Site 3 9.7Km east from Provincial Truck Hwy 10. Parallel to A | N54°31'26.5" W101°30'31.5" | Kamaldeep Chhoker | 11-Jun-16 |
| A3Q1-2 | *Cladonia arbuscula* | Limestone Table, Abies balsamea, Betula papyrifera, Empetrum nigrum, Fragaria virginiana, Kalmia sp, Linnaea borealis, Pice | Site 3 9.7Km east from Provincial Truck Hwy 10. Parallel to A | N54°31'26.5" W101°30'31.5" | Kamaldeep Chhoker | 11-Jun-16 |
| A3Q1-3 | *Pleurozium schreberi* | Limestone Table, Abies balsamea, Betula papyrifera, Empetrum nigrum, Fragaria virginiana, Kalmia sp, Linnaea borealis, Pice | Site 3 9.7Km east from Provincial Truck Hwy 10. Parallel to A | N54°31'26.5" W101°30'31.5" | Kamaldeep Chhoker | 11-Jun-16 |
| A3Q1-4 | *Cladonia phyllophora* | Limestone Table, Abies balsamea, Betula papyrifera, Empetrum nigrum, Fragaria virginiana, Kalmia sp, Linnaea borealis, Pice | Site 3 9.7Km east from Provincial Truck Hwy 10. Parallel to A | N54°31'26.5" W101°30'31.5" | Kamaldeep Chhoker | 11-Jun-16 |
| A3Q2-1 | *Cladonia stygia* | Limestone Table, Abies balsamea, Betula papyrifera, Empetrum nigrum, Fragaria virginiana, Kalmia sp, Linnaea borealis, Pice | Site 3 9.7Km east from Provincial Truck Hwy 10. Parallel to A | N54°31'26.5" W101°30'31.5" | Kamaldeep Chhoker | 11-Jun-16 |
| A3Q2-2 | *Cladonia arbuscula* | Limestone Table, Abies balsamea, Betula papyrifera, Empetrum nigrum, Fragaria virginiana, Kalmia sp, Linnaea borealis, Pice | Site 3 9.7Km east from Provincial Truck Hwy 10. Parallel to A | N54°31'26.5" W101°30'31.5" | Kamaldeep Chhoker | 11-Jun-16 |
| A3Q2-3 | *Pleurozium schreberi* | Limestone Table, Abies balsamea, Betula papyrifera, Empetrum nigrum, Fragaria virginiana, Kalmia sp, Linnaea borealis, Pice | Site 3 9.7Km east from Provincial Truck Hwy 10. Parallel to A | N54°31'26.5" W101°30'31.5" | Kamaldeep Chhoker | 11-Jun-16 |
| A3Q2-4 | *Dicranum majus* | Limestone Table, Abies balsamea, Betula papyrifera, Empetrum nigrum, Fragaria virginiana, Kalmia sp, Linnaea borealis, Pice | Site 3 9.7Km east from Provincial Truck Hwy 10. Parallel to A | N54°31'26.5" W101°30'31.5" | Kamaldeep Chhoker | 11-Jun-16 |
| A3Q2-5 | *Cladonia phyllophora* | Limestone Table, Abies balsamea, Betula papyrifera, Empetrum nigrum, Fragaria virginiana, Kalmia sp, Linnaea borealis, Pice | Site 3 9.7Km east from Provincial Truck Hwy 10. Parallel to A | N54°31'26.5" W101°30'31.5" | Kamaldeep Chhoker | 11-Jun-16 |
| A3Q3-1 | *Cladonia stygia* | Limestone Table, Abies balsamea, Betula papyrifera, Empetrum nigrum, Fragaria virginiana, Kalmia sp, Linnaea borealis, Pice | Site 3 9.7Km east from Provincial Truck Hwy 10. Parallel to A | N54°31'26.5" W101°30'31.5" | Kamaldeep Chhoker | 11-Jun-16 |
| A3Q3-2 | *Cladonia arbuscula* | Limestone Table, Abies balsamea, Betula papyrifera, Empetrum nigrum, Fragaria virginiana, Kalmia sp, Linnaea borealis, Pice | Site 3 9.7Km east from Provincial Truck Hwy 10. Parallel to A | N54°31'26.5" W101°30'31.5" | Kamaldeep Chhoker | 11-Jun-16 |
| A3Q3-3 | *Pleurozium schreberi* | Limestone Table, Abies balsamea, Betula papyrifera, Empetrum nigrum, Fragaria virginiana, Kalmia sp, Linnaea borealis, Pice | Site 3 9.7Km east from Provincial Truck Hwy 10. Parallel to A | N54°31'26.5" W101°30'31.5" | Kamaldeep Chhoker | 11-Jun-16 |
| A3Q3-4 | *Cladonia multiformis* | Limestone Table, Abies balsamea, Betula papyrifera, Empetrum nigrum, Fragaria virginiana, Kalmia sp, Linnaea borealis, Pice | Site 3 9.7Km east from Provincial Truck Hwy 10. Parallel to A | N54°31'26.5" W101°30'31.5" | Kamaldeep Chhoker | 11-Jun-16 |
| S1Q1-1 | *Cladonia stygia* | Granodiorite rock Mat forming lichens and mosses, east facing granite rock ridge, Picea glauca, Pinus banksiana. | Sherridan site 1, Neosap 1 west, approximately 28 Km from Hwy 10 | N54°50'38.4", W101°28'45.6" | Kamaldeep Chhoker | 12-Jun-16 |
| S1Q1-2 | *Cladonia arbuscula* | Granodiorite rock Mat forming lichens and mosses, east facing granite rock ridge, Picea glauca, Pinus banksiana. | Sherridan site 1, Neosap 1 west, approximately 28 Km from Hwy 10 | N54°50'38.4", W101°28'45.6" | Kamaldeep Chhoker | 12-Jun-16 |
| S1Q1-3 | *Pleurozium schreberi* | Granodiorite rock Mat forming lichens and mosses, east facing granite rock ridge, Picea glauca, Pinus banksiana. | Sherridan site 1, Neosap 1 west, approximately 28 Km from Hwy 10 | N54°50'38.4", W101°28'45.6" | Kamaldeep Chhoker | 12-Jun-16 |
| S1Q2-1 | *Cladonia stygia* | Granodiorite rock Mat forming lichens and mosses, east facing granite rock ridge, Picea glauca, Pinus banksiana. | Sherridan site 1, Neosap 1 west, approximately 28 Km from Hwy 10 | N54°50'38.4", W101°28'45.6" | Kamaldeep Chhoker | 12-Jun-16 |
| S1Q2-2 | *Cladonia arbuscula* | Granodiorite rock Mat forming lichens and mosses, east facing granite rock ridge, Picea glauca, Pinus banksiana. | Sherridan site 1, Neosap 1 west, approximately 28 Km from Hwy 10 | N54°50'38.4", W101°28'45.6" | Kamaldeep Chhoker | 12-Jun-16 |
| S1Q2-3 | *Dicranum polysetum* | Granodiorite rock Mat forming lichens and mosses, east facing granite rock ridge, Picea glauca, Pinus banksiana. | Sherridan site 1, Neosap 1 west, approximately 28 Km from Hwy 10 | N54°50'38.4", W101°28'45.6" | Kamaldeep Chhoker | 12-Jun-16 |
| S1Q2-3 | *Hylocomium splendens* | Granodiorite rock Mat forming lichens and mosses, east facing granite rock ridge, Picea glauca, Pinus banksiana. | Sherridan site 1, Neosap 1 west, approximately 28 Km from Hwy 10 | N54°50'38.4", W101°28'45.6" | Kamaldeep Chhoker | 12-Jun-16 |
| S1Q2-4 | *Peltigera leucophlebia* | Granodiorite rock Mat forming lichens and mosses, east facing granite rock ridge, Picea glauca, Pinus banksiana. | Sherridan site 1, Neosap 1 west, approximately 28 Km from Hwy 10 | N54°50'38.4", W101°28'45.6" | Kamaldeep Chhoker | 12-Jun-16 |
| S1Q2-5 | *Pleurozium schreberi* | Granodiorite rock Mat forming lichens and mosses, east facing granite rock ridge, Picea glauca, Pinus banksiana. | Sherridan site 1, Neosap 1 west, approximately 28 Km from Hwy 10 | N54°50'38.4", W101°28'45.6" | Kamaldeep Chhoker | 12-Jun-16 |
| S1Q3-1 | *Cladonia stygia* | Granodiorite rock Mat forming lichens and mosses, east facing granite rock ridge, Picea glauca, Pinus banksiana. | Sherridan site 1, Neosap 1 west, approximately 28 Km from Hwy 10 | N54°50'38.4", W101°28'45.6" | Kamaldeep Chhoker | 12-Jun-16 |
| S1Q3-2 | *Cladonia arbuscula* | Granodiorite rock Mat forming lichens and mosses, east facing granite rock ridge, Picea glauca, Pinus banksiana. | Sherridan site 1, Neosap 1 west, approximately 28 Km from Hwy 10 | N54°50'38.4", W101°28'45.6" | Kamaldeep Chhoker | 12-Jun-16 |
| S1Q3-3 | *Pleurozium schreberi* | Granodiorite rock Mat forming lichens and mosses, east facing granite rock ridge, Picea glauca, Pinus banksiana. | Sherridan site 1, Neosap 1 west, approximately 28 Km from Hwy 10 | N54°50'38.4", W101°28'45.6" | Kamaldeep Chhoker | 12-Jun-16 |
| S1Q3-4 | *Cladonia cornuta* | Granodiorite rock Mat forming lichens and mosses, east facing granite rock ridge, Picea glauca, Pinus banksiana. | Sherridan site 1, Neosap 1 west, approximately 28 Km from Hwy 10 | N54°50'38.4", W101°28'45.6" | Kamaldeep Chhoker | 12-Jun-16 |
| S1Q3-5 | *Cladonia phyllophora* | Granodiorite rock Mat forming lichens and mosses, east facing granite rock ridge, Picea glauca, Pinus banksiana. | Sherridan site 1, Neosap 1 west, approximately 28 Km from Hwy 10 | N54°50'38.4", W101°28'45.6" | Kamaldeep Chhoker | 12-Jun-16 |
| S1Q3-6 | *Dicranum polysetum* | Granodiorite rock Mat forming lichens and mosses, east facing granite rock ridge, Picea glauca, Pinus banksiana. | Sherridan site 1, Neosap 1 west, approximately 28 Km from Hwy 10 | N54°50'38.4", W101°28'45.6" | Kamaldeep Chhoker | 12-Jun-16 |

| | | | | | | |
|---|---|---|---|---|---|---|
| S2Q1-3 | *Vulpicida pinastri* | Sandy Plain between granite ridges; Gaultheria, Pinus banksiana, Vaccinium spp. | Sherridan site 2, Sandy Plain, approximately 21-22 km from HWY 10 | N54°47'50.3", W101°27'44.3" | Kamaldeep Chhoker | 12-Jun-16 |
| S2Q1-4 | *Cladonia gracili spp tur* | Sandy Plain between granite ridges; Gaultheria, Pinus banksiana, Vaccinium spp. | Sherridan site 2, Sandy Plain, approximately 21-22 km from HWY 10 | N54°47'50.3", W101°27'44.3" | Kamaldeep Chhoker | 12-Jun-16 |
| S2Q1-5 | *Vascular plant* | Sandy Plain between granite ridges; Gaultheria, Pinus banksiana, Vaccinium spp. | Sherridan site 2, Sandy Plain, approximately 21-22 km from HWY 10 | N54°47'50.3", W101°27'44.3" | Kamaldeep Chhoker | 12-Jun-16 |
| S2Q2-1 | *Cladonia stygia* | Sandy Plain between granite ridges; Gaultheria, Pinus banksiana, Vaccinium spp. | Sherridan site 2, Sandy Plain, approximately 21-22 km from HWY 10 | N54°47'50.3", W101°27'44.3" | Kamaldeep Chhoker | 12-Jun-16 |
| S2Q2-2 | *Cladonia stellaris* | Sandy Plain between granite ridges; Gaultheria, Pinus banksiana, Vaccinium spp. | Sherridan site 2, Sandy Plain, approximately 21-22 km from HWY 10 | N54°47'50.3", W101°27'44.3" | Kamaldeep Chhoker | 12-Jun-16 |
| S2Q2-3 | *Cladonia arbuscula* | Sandy Plain between granite ridges; Gaultheria, Pinus banksiana, Vaccinium spp. | Sherridan site 2, Sandy Plain, approximately 21-22 km from HWY 10 | N54°47'50.3", W101°27'44.3" | Kamaldeep Chhoker | 12-Jun-16 |
| S2Q2-4 | *Dicranum majus* | Sandy Plain between granite ridges; Gaultheria, Pinus banksiana, Vaccinium spp. | Sherridan site 2, Sandy Plain, approximately 21-22 km from HWY 10 | N54°47'50.3", W101°27'44.3" | Kamaldeep Chhoker | 12-Jun-16 |
| S2Q2-5 | *Vascular plant* | Sandy Plain between granite ridges; Gaultheria, Pinus banksiana, Vaccinium spp. | Sherridan site 2, Sandy Plain, approximately 21-22 km from HWY 10 | N54°47'50.3", W101°27'44.3" | Kamaldeep Chhoker | 12-Jun-16 |
| S2Q3-1 | *Cladonia stygia* | Sandy Plain between granite ridges; Gaultheria, Pinus banksiana, Vaccinium spp. | Sherridan site 2, Sandy Plain, approximately 21-22 km from HWY 10 | N54°47'50.3", W101°27'44.3" | Kamaldeep Chhoker | 12-Jun-16 |
| S2Q3-2 | *Pleurozium schreberi* | Sandy Plain between granite ridges; Gaultheria, Pinus banksiana, Vaccinium spp. | Sherridan site 2, Sandy Plain, approximately 21-22 km from HWY 10 | N54°47'50.3", W101°27'44.3" | Kamaldeep Chhoker | 12-Jun-16 |
| S2Q3-3 | *Dicranum majus* | Sandy Plain between granite ridges; Gaultheria, Pinus banksiana, Vaccinium spp. | Sherridan site 2, Sandy Plain, approximately 21-22 km from HWY 10 | N54°47'50.3", W101°27'44.3" | Kamaldeep Chhoker | 12-Jun-16 |
| S2Q3-4 | *Cladonia gracili spp tur* | Sandy Plain between granite ridges; Gaultheria, Pinus banksiana, Vaccinium spp. | Sherridan site 2, Sandy Plain, approximately 21-22 km from HWY 10 | N54°47'50.3", W101°27'44.3" | Kamaldeep Chhoker | 12-Jun-16 |
| S2Q3-5 | *Cladonia cornuta* | Sandy Plain between granite ridges; Gaultheria, Pinus banksiana, Vaccinium spp. | Sherridan site 2, Sandy Plain, approximately 21-22 km from HWY 10 | N54°47'50.3", W101°27'44.3" | Kamaldeep Chhoker | 12-Jun-16 |
| S2Q3-6 | *Cladonia arbuscula* | Sandy Plain between granite ridges; Gaultheria, Pinus banksiana, Vaccinium spp. | Sherridan site 2, Sandy Plain, approximately 21-22 km from HWY 10 | N54°47'50.3", W101°27'44.3" | Kamaldeep Chhoker | 12-Jun-16 |
| S2Q3-7 | *Vascular plant* | Sandy Plain between granite ridges; Gaultheria, Pinus banksiana, Vaccinium spp. | Sherridan site 2, Sandy Plain, approximately 21-22 km from HWY 10 | N54°47'50.3", W101°27'44.3" | Kamaldeep Chhoker | 12-Jun-16 |
| S3Q1-1 | *Cladonia stygia* | Granite rock table, Betula papyrifera, Linnaea borealis, Picea glauca Pinus banksiana, Vaccinium spp. | Sherridan site 3, Rock table, approximately 18-19 km from HWY 10 | N54°46'24.9", W101°28'40.4" | Kamaldeep Chhoker | 12-Jun-16 |
| S3Q1-2 | *Cladonia arbuscula* | Granite rock table, Betula papyrifera, Linnaea borealis, Picea glauca Pinus banksiana, Vaccinium spp. | Sherridan site 3, Rock table, approximately 18-19 km from HWY 10 | N54°46'24.9", W101°28'40.4" | Kamaldeep Chhoker | 12-Jun-16 |
| S3Q1-3 | *Vascular plant* | Granite rock table, Betula papyrifera, Linnaea borealis, Picea glauca Pinus banksiana, Vaccinium spp. | Sherridan site 3, Rock table, approximately 18-19 km from HWY 10 | N54°46'24.9", W101°28'40.4" | Kamaldeep Chhoker | 12-Jun-16 |
| S3Q2-1 | *Cladonia stygia* | Granite rock table, Betula papyrifera, Linnaea borealis, Picea glauca Pinus banksiana, Vaccinium spp. | Sherridan site 3, Rock table, approximately 18-19 km from HWY 10 | N54°46'24.9", W101°28'40.4" | Kamaldeep Chhoker | 12-Jun-16 |
| S3Q2-2 | *Cladonia arbuscula* | Granite rock table, Betula papyrifera, Linnaea borealis, Picea glauca Pinus banksiana, Vaccinium spp. | Sherridan site 3, Rock table, approximately 18-19 km from HWY 10 | N54°46'24.9", W101°28'40.4" | Kamaldeep Chhoker | 12-Jun-16 |
| S3Q2-3 | *Pleurozium schreberi* | Granite rock table, Betula papyrifera, Linnaea borealis, Picea glauca Pinus banksiana, Vaccinium spp. | Sherridan site 3, Rock table, approximately 18-19 km from HWY 10 | N54°46'24.9", W101°28'40.4" | Kamaldeep Chhoker | 12-Jun-16 |
| S3Q2-4 | *Cladonia cornuta* | Granite rock table, Betula papyrifera, Linnaea borealis, Picea glauca Pinus banksiana, Vaccinium spp. | Sherridan site 3, Rock table, approximately 18-19 km from HWY 10 | N54°46'24.9", W101°28'40.4" | Kamaldeep Chhoker | 12-Jun-16 |
| S3Q2-5 | *Cladonia pyxidata* | Granite rock table, Betula papyrifera, Linnaea borealis, Picea glauca Pinus banksiana, Vaccinium spp. | Sherridan site 3, Rock table, approximately 18-19 km from HWY 10 | N54°46'24.9", W101°28'40.4" | Kamaldeep Chhoker | 12-Jun-16 |
| S3Q2-6 | *Cladonia crispata* | Granite rock table, Betula papyrifera, Linnaea borealis, Picea glauca Pinus banksiana, Vaccinium spp. | Sherridan site 3, Rock table, approximately 18-19 km from HWY 10 | N54°46'24.9", W101°28'40.4" | Kamaldeep Chhoker | 12-Jun-16 |
| S3Q2-7 | *Cladonia uncialis* | Granite rock table, Betula papyrifera, Linnaea borealis, Picea glauca Pinus banksiana, Vaccinium spp. | Sherridan site 3, Rock table, approximately 18-19 km from HWY 10 | N54°46'24.9", W101°28'40.4" | Kamaldeep Chhoker | 12-Jun-16 |
| S3Q2-8 | *Dicranum polycetum* | Granite rock table, Betula papyrifera, Linnaea borealis, Picea glauca Pinus banksiana, Vaccinium spp. | Sherridan site 3, Rock table, approximately 18-19 km from HWY 10 | N54°46'24.9", W101°28'40.4" | Kamaldeep Chhoker | 12-Jun-16 |
| S3Q2-9 | *Vascular plant* | Granite rock table, Betula papyrifera, Linnaea borealis, Picea glauca Pinus banksiana, Vaccinium spp. | Sherridan site 3, Rock table, approximately 18-19 km from HWY 10 | N54°46'24.9", W101°28'40.4" | Kamaldeep Chhoker | 12-Jun-16 |
| S3Q3-1 | *Cladonia stygia* | Granite rock table, Betula papyrifera, Linnaea borealis, Picea glauca Pinus banksiana, Vaccinium spp. | Sherridan site 3, Rock table, approximately 18-19 km from HWY 10 | N54°46'24.9", W101°28'40.4" | Kamaldeep Chhoker | 12-Jun-16 |
| S3Q3-2 | *Cladonia arbuscula* | Granite rock table, Betula papyrifera, Linnaea borealis, Picea glauca Pinus banksiana, Vaccinium spp. | Sherridan site 3, Rock table, approximately 18-19 km from HWY 10 | N54°46'24.9", W101°28'40.4" | Kamaldeep Chhoker | 12-Jun-16 |
| S3Q3-3 | *Cetraria islandica* | Granite rock table, Betula papyrifera, Linnaea borealis, Picea glauca Pinus banksiana, Vaccinium spp. | Sherridan site 3, Rock table, approximately 18-19 km from HWY 10 | N54°46'24.9", W101°28'40.4" | Kamaldeep Chhoker | 12-Jun-16 |
| S3Q3-4 | *Vascular plant* | Granite rock table, Betula papyrifera, Linnaea borealis, Picea glauca Pinus banksiana, Vaccinium spp. | Sherridan site 3, Rock table, approximately 18-19 km from HWY 10 | N54°46'24.9", W101°28'40.4" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q1-1 | *Cladonia stygia* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q1-2 | *Cladonia arbuscula* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q1-3 | *Cladonia gracili spp tur* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q1-4 | *Cetraria ericetorum* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q1-5 | *Cladonia cornuta* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q1-6 | *Pohlia Sp* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q1-7 | *Cladonia cornuta* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q1-8 | *Vascular plant* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q2-1 | *Cladonia stygia* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q2-2 | *Cladonia arbuscula* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q2-3 | *Cladonia deformis* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q2-4 | *Cladonia pyxidata* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q2-5 | *Cladonia cornuta* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q2-6 | *Dicranum polycetum* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q2-7 | *Cladonia gracili spp tur* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q2-8 | *Pohlia nutans* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q3-1 | *Cladonia stygia* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q3-2 | *Cladonia arbuscula* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q3-3 | *Polytrichum juniperinu* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q3-4 | *Cladonia cornuta* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q3-5 | *Cladonia gracili spp tur* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q3-6 | *Cladonia deformis* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q3-7 | *Cladonia phyllophora* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q3-8 | *Cladonia pyxidata* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
| S4Q3-9 | *Diploschistes scruposus* | Granite rock table, Picea glauca, Pinus banksiana, Rhododendron tomentosum, Vaccinium spp. | Sherridan site 4, Rock Table, approximately 16-17 km from HWY 10 | N54°45'46.5" W101° 30'10.1" | Kamaldeep Chhoker | 12-Jun-16 |
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