A bioinformatics approach to the study of polyphenols and antioxidant capacity in Vaccinium vitis-idaea L.

by
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A thesis submitted to
School of Graduate Studies
in partial fulfilment of the
requirements for the degree of
Master of Biology

Faculty of Science
Memorial University of Newfoundland

St John's Newfoundland

Dedicated

to my Mom & Dad

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LIST OF ABBREVIATIONS

AA	Aspartate aminotransferase
AC	Antioxidant capacity
AFLP	Amplified fragment length polymorphism
ATP	Adenosine triphosphate
ASO	Allele specific oligonucleotide
Bp	Base Pair
CAP	Cut/cleaved amplified polymorphism
CF	Coastal vs inland,
CHS	Chalcone synthase
COG	Conserved oligomeric Golgi
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
DNA	Deoxyribonucleic acid
ER	endoplasmic reticulum
ETR	NCBI non-redundant
EYE	Embryo yellow
FW	Fresh weight
GAE	Gallic acid equivalents
GAUT	Galacturonosyltransferases
GBS	Genotyping-by-Sequencing
GL1	GLABRA1
GO	Gene Onthology
HRP	Horseradish peroxidase
ISSR	Inter simple sequence repeat
Kb	Kilobase pair
LSD	Least significant difference
MAF	Minor allele frequency
MAP	Mean annual precipitation
MAT	Mean annual temperature
Mbp	Megabase pair
MST	Mean summer temperature
MYB-TF	MYB transcription factor
NADPH	Nicotinamide adenine dinucleotide phosphate
NGS	Next generation sequencing
NR	Non-redundant
NTRB	NADPH-thioredoxin reductase
ORAC	Oxygen radical absorbance capacity
PAL	Phenylalanine ammonia lyase

PCR	Polymerase chain reaction
POD	Peroxidase
PPO	Polyphenol oxidase
RAPD	Random amplified polymorphism
Res	Restriction enzymes
RFLP	Restriction fragment length polymorphism
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSS	Reactive sulphur species
SAM	S-adenosylmethionine
SAR	Sensitivity to acid rain
SCAR	Sequence characterized amplified region
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variation
SSR	Simple sequence repeat
TE	Transcription factors
TPC	Total phenolic content
USDA	United States Department of Agriculture
UV	Ultraviolet
WER	WEREWOLF
4CL	4-coumarate: coenzyme A ligase

Acknowledgements

Grateful I am…

Alhamdulillah all praises are to ALLAH that I have successfully finished my research work. In this occasion, I would like to express my gratefulness and indebtedness to my parents for their never-ending prayer and efforts to educate me up to this level.

The author deeply owes to several contributors' assistance for this present research work. Without their patience and intelligence, I undoubtedly cannot present the thesis. First and foremost, I would like to acknowledge my advisers Dr. Julissa Roncal for her kind acceptance to supervise me. Throughout the course of my research, she provided me an opportunity to work with their laboratory and provide timely and valuable advices and excellent facilities. My honors and deep gratitude go to Dr. Lourdes Peña-Castillo for her constant inspiration, untiring supervision, scholastic guidance, constructive suggestions and intellectual instructions on all phases of research work. Without her sincere support, I would not be able to be experienced in this field.

I am also glad to expresses my gratefulness and indebtedness sense to Prof. Fereidoon Shahidi for guidance, assistance and let me use his laboratory. It was my pleasure to have such a kind people, who spend their valuable time for assistance. Thanks to Nishani Perera, Priya Ambigaipalan and JuDong Yeo for assisting in all aspects in the laboratory.

I would like to thanks all my well-wishers and friends who have assisted me during this research work directly or indirectly. I am thankful to my lab mates (Hugo, Boni and Fritz), this people adds color and spices to my life. I also like to gratefully acknowledge

The Research and Development Corporation (RDC) from the government of Newfoundland and Labrador in Canada for providing funding for this project.

Abstract

Lingonberry (*Vaccinium vitis-idaea* L.) rich in nutrients and biochemical properties beneficial for human health is one of the less studied crops of the *Ericaceae* family. In this study, we identified regions within Newfoundland and Labrador, Canada where wild berries contained a higher phenolic content and antioxidant capacity. Notably, berries within the North Shore forest and high temperatures had higher phenolic content. Having DNA markers to characterize lingonberry plants in terms of their metabolite production would be useful, however lingonberry's genomic sequences are unavailable. We applied next generation sequencing technology to discover single nucleotide polymorphisms (SNPs). Specifically, we investigated the association of SNPs with phenolic compound and antioxidant activity variation in lingonberry grown under different environmental conditions in Newfoundland and Labrador. After processing 144 million single-end DNA sequence reads, 1586 high-quality putative SNPs were identified. A Chi-square test identified 260 SNPs that were significantly correlated with at least one environmental variable, phenolic compound and/or antioxidant activity. Annotation of the 260 SNP-containing sequences, based on BLAST similarity searches, identified 128 genes that were involved in phenolic compound biosynthesis, transcription regulation, transport, drought and water pH resistance.

The SNP markers discovered in this study will provide a useful source for molecular genetic studies, such as genetic diversity and characterization, high-density quantitative trait locus (QTL) analysis, and association mapping. The identified candidate genes containing the SNPs need further studies on their adaptation ability in lingonberry.

1 Chapter one: Introduction

Lingonberry (*Vaccinium vitis-idaea* L.*: Ericaceae*), also commonly known as partridgeberry, is one of the least known small berry fruits in North America. Two subspecies of lingonberry are recognized: *Vaccinium vitis-idaea subsp. vitis-idaea,* which is native to Europe, and *Vaccinium vitis-idaea subsp. minus*, which is native to North America. Commercial cultivation of lingonberry is developed in Europe, it is on a developmental stage in the United States, and status is unknown for Canada.

Wild or cultivated berry fruits are a rich source of bioactive compounds (Szajdek and Borowska, 2008). Recent studies on berry fruits have revealed their positive effects on human diet and health (Mikulic- Petkovsek et al., 2012). Lingonberry has a wide range of bioactive compounds such as phenolic acids, flavonoids and tannins (Hokkanen et al., 2009). Lingonberry has been reported to be one of the top competitors with respect to the highest antioxidant activity, when compared to other berry fruits such as blackberries, blueberries, raspberries, strawberries, and cranberries (Wang and Jiao, 2000).

Major wild lingonberry harvesting countries include Sweden, Finland, Russia, Norway, Poland, Japan, Germany, Canada and U.S.A. Newfoundland exports more than 100 tons of lingonberry to Scandinavian countries to meet their processing needs (Burt and Penhallegon, 2003). Due to the increasing awareness of the benefits of lingonberry to human health, demand of fruit-based natural drinks and other processed products in the local and international markets has increased dramatically (Burt and Penhallegon, 2003; Debnath and Sion, 2009). Due to this increasing global demand of fresh partridgeberries

and processed products, Newfoundland has a unique opportunity to participate in the global market by producing high quality partridgeberries rich in nutrients, bioactive compounds and antioxidant capacity.

Lingonberry studies have mostly investigated the phenolic compound content, and a few others have addressed the genetic diversity of cultivars and wild populations (Garkava-Gustavsson et al., 2005; Foley and Debnath, 2007; Debnath and Sion, 2009; Debnath et al., 2012; Vyas et al., 2015). There is scarce research on how lingonberry regulates the biosynthesis of phenolic compounds. It is important to conduct genomic studies in this species to better understand the production of these biochemical compounds in order to achieve a consistent high quality and high value lingonberry production. A genetic study on wild lingonberry is important to establish a broad genetic basis for future use on crop improvement. A genotypic identification is especially important in plant species for germplasm characterization, mapping important genes, plant physical mapping, practical breeding purposes and evaluations of genetic diversity. One of the most important objectives of plant geneticists is to identify genes related to the biosynthesis of biochemical compounds and different important agronomical traits. The availability of genetic information gives us the possibility to use innovative approaches to increase our knowledge on a specific trait at a molecular level and develop new varieties with those useful traits or genes(Rao and Hodgkin, 2002; Fernie et al., 2006; Glaszmann et al., 2010; Govindaraj et al., 2015).

Single nucleotide polymorphisms (SNPs) are single nucleotide variations in the genomic DNA sequence of an individual, population or species. SNPs are the most abundant type of genetic polymorphism in most genomes. SNPs are currently a popular

marker choice due to their presence in large numbers in virtually all populations. Recent advances in next-generation sequencing (NGS) techniques have dramatically improved SNP discovery (Seeb et al., 2011). The applications of SNP genotyping include phylogenetic analysis, marker-assisted selection, genetic mapping of the gene underlying a quantitative trait (quantitative trait loci), and finding genetic variations associated with some particular traits (Kumar et al., 2012).

As part of the ongoing interest in nutritional and nutraceutical properties of locally produced fruits, the goals of this research is to determine the phenolic compound content and antioxidant capacity and discover genetic variations of wild lingonberry populations in Newfoundland and Labrador. Lingonberry research in Newfoundland had mainly focused on characterizing the phenolic content and antioxidant capacity of fruits from populations located in a few sites such as St. John's, Gambo, St. Pierre, Codroy Valley, St. Anthony and Eddies Cove (Debnath and Sion, 2009). No comparative research has been conducted on the influence of environmental factors on biochemical properties and genetic variations of lingonberry in the whole province. A biochemical analysis and genetic variation study on lingonberry could be a good way to get more in depth knowledge on the influence of environmental factors on biochemical properties and genetic variation.

1.1 Thesis outline

Chapter 1 introduces research topics and general outline of the thesis.

Chapter 2 Biochemical Analysis introduces the economic and nutritional importance of *Vaccinium vitis-idaea*. It evaluates the total phenolic compounds and antioxidant capacity of its fruit in relation to several environmental variables in Newfoundland and Labrador such as ecoregion, precipitation, temperature, proximity to the coast and surface water quality. Results from this chapter were published recently in the journal *Botany* (Alam et al., 2016). The motivation of this chapter was to obtain background information on these two commercially important biochemical properties of fruit in the wild that could aid future selection of clones suitable for breeding programs in the province.

Chapter 3 Bioinformatics Analysis builds on the knowledge of the total phenolics and antioxidant capacity of wild fruits. The introduction gives an overview of the genetic diversity of lingonberry (*Vaccinium vitis-idaea*), the molecular control on phenolic compound content, and the use of Genotyping-by-Sequencing (GBS) as a fingerprinting technique for genetic variation analysis. This chapter evaluates the presence of SNPs among individuals as revealed using the GBS technique. SNPs were analyzed for their correlations with the environmental factors, phenolic compound and antioxidant capacity of fruit. Finally, the SNP-containing sequences were searched for homologous gene sequences that were then searched in the Gene Ontology database and in the literature for their potential functional role in the plant.

Chapter 4 General Conclusions summarizes the outcome and impact of the work. Possible future studies as well as the practical implementation of this work are presented.

2 Chapter two: Biochemical analysis

2.1 Introduction

The genus *Vaccinium* from the family Ericaceae consists of more than 150 species (Yang et al., 2003). Some *Vaccinium* species bear edible fruits and are an important source of wild and cultivated berries. Juice, jam, jelly, and marmalade are the major products of industrial processing. The berries of these *Vaccinium* species constitute the highest proportion of Newfoundland wild berries collected for domestic consumption and export to Europe, especially Germany (Burt and Penhallegon, 2003). Lingonberry (*Vaccinium vitis-idaea* L.) is a perennial, cold adapted, small evergreen shrub, naturally growing in wild areas of circumboreal forests in Northern Eurasia and North America (Luby et al., 1991; Hjalmarsson and Ortiz, 2001; Yang et al., 2003). Lingonberry is naturally distributed throughout Newfoundland. The fruit are rich in natural antioxidants (Hakkinen et al., 1999). It is well reported that fruit and leaves of lingonberry have a well-established role in pharmacognosy, and are used as herbal medicine.

Phytochemicals are a large group of biologically active chemical compounds synthesized naturally in plants, which play an important role in plant growth, development and physiological activity. These chemicals have the capacity to protect plants under stress conditions. They also have beneficial health impacts in humans (Hurtado-Fernandez et al., 2010). They are considered antioxidants (Helmja et al., 2007) because they prevent oxidation by neutralizing free radicals, and in doing so they prevent different human

diseases such as cancer, diabetes, high blood pressure, inflammatory disorders, neurological degeneration and coronary heart diseases, among others (Herrero et al., 2005; Shahidi and Zhong, 2010). With this knowledge, antioxidant compounds are gaining greater interest especially in the consumption of food rich in antioxidants is increasing (Shahidi and Wanasundara, 1992; Shahidi et al., 1999).

Plants synthesize phytochemicals as primary and secondary metabolites. Primary metabolites are synthesized in larger amounts and are necessary for plant growth and development. Secondary metabolites are referred as secondary products synthesized naturally from primary metabolites, and are not directly involved in growth, development and reproduction (Bartwal et al., 2013). Secondary metabolites are synthesized in low concentrations, and the absence of secondary metabolites may cause long-term impacts or plant death. These compounds include pigments and are also responsible for plant chemical defense. There are three major groups of secondary plant metabolites: i) terpenes (terpenoids or isoprenoids), ii) phenolics (simple phenols, phenolic acids, flavonoids, tannins, lignans, etc) and iii) nitrogen-containing secondary metabolites (alkaloids such as cocaine, caffeine, and morphine; cyanogenic glycosides and glucosinolates, etc) (Michalak, 2006).

2.1.1 **Phenolic compounds**

Phenolics are one of the most ubiquitous groups of secondary metabolites, widespread throughout the plant kingdom (Harborne and Green, 1980; Boudet, 2007), and are of considerable physiological and morphological importance in plants. Phenolic

compounds have a large range of structures and functions, and encompass aromatic compounds characterized by at least one aromatic ring (phenyl ring) with at least one hydroxyl-group (-OH-) (Lattanzio et al., 2006). This definition of phenolic compounds is unsatisfactory, as it includes compounds such as oestrone, a female sex hormone that is principally terpenoid in origin. For this reason, an origin-based definition is preferred. Plant phenolic compounds are considered as those substances that are derived from the shikimate pathway and phenylpropanoid metabolism (Figure 2.1). Several compounds such as cinnamic acid, linoleic acid, shikimic acid, and quinic acid are considered phenolic compounds because of metabolic considerations; however, they lack the aromatic ring. Phenolic compounds are synthesized mainly from cinnamic acid, which is formed from phenylalanine by the action of phenylalanine ammonia-lyase (PAL), the branch-point enzyme between primary (shikimate pathway) and secondary (phenylopropanoid) metabolism (Bartwal et al., 2013). There are more than 9000 different known phenolic structures that have been identified in plants (Xiao et al., 2011).

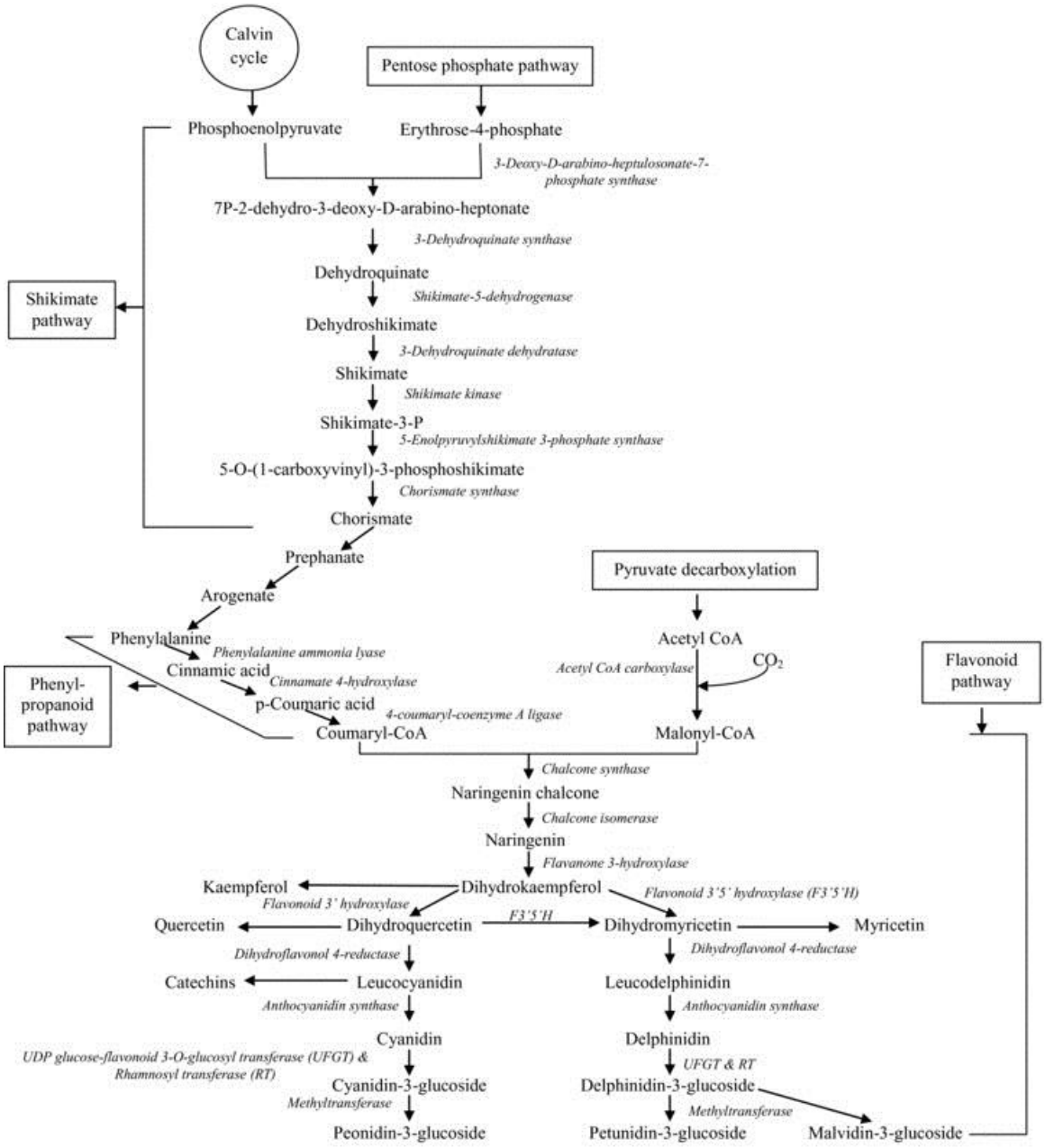


Figure 2.1 General biosynthesis pathway of some major anthocyanins present in blueberry (Routray & Orsat, 2011)

2.1.2 **Classification of phenolic compounds**

Phenolic compounds occur in plants in several forms. They can occur as soluble compounds in water, methanol, aqueous acetone, or in non-extractable form (Beart et al., 1985). Phenolic compounds range from simple, low molecular weight, single aromatic-ringed compounds to large and complex tannins and derived polyphenols (Kumar et al., 2014). Most phenolic compounds are synthesized from a common origin: the amino acid phenylalanine or tyrosine. In the phenolic compound biosynthesis pathway, these amino acids (phenylalanine or tyrosine) are divided into cinnamic acids, and subsequently enter into the phenylpropanoid pathways. There are two main classification systems of phenolic compounds. Phenolic compounds can be classified based on the number of carbon atoms present in the molecule (Crozier et al., 2006), or depending on the number of phenolic rings present (Kumar et al., 2014). In the latter system, two main groups are identified. The first is the flavonoid group, with C6–C3–C6 as the general structural backbone, in which the two C6 units (Ring A and Ring B) are of phenolic nature (Figure 2.2, 2.3). Flavonoids are further sub-categorized into flavones, flavonols, flavanones, flavanonols, flavanols or flavan-3-ols or catechins, anthocyanins, isoflavones, neoflavonoids and chalcones according to the function of each phenolic ring. The basic flavonoid skeleton can have numerous substituents. Hydroxyl groups are usually present at the 4, 5 and 7 positions (Figure 2.2). Sugars are very common in the majority of flavonoids existing naturally as glycosides (Kumar et al., 2014; Kabera et al., 2014). The second group is the non-flavonoid, with C1–C6 and C3–C6 as general structural backbones (Figure 2.3). Non-flavonoids are further sub-categorized onto simple phenols, phenolic acids, benzoic aldehydes,

hydrolysable tannins, acetophenones and phenylacetic acids, hydroxycinnamic acids, coumarins, benzophenones, xanthones, stilbenes, lignans and secoiridoids.

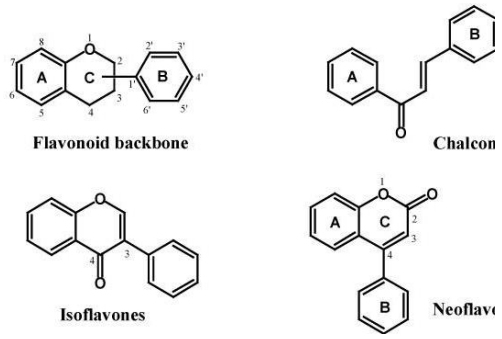


Figure 2.2 Basic flavonoid structures

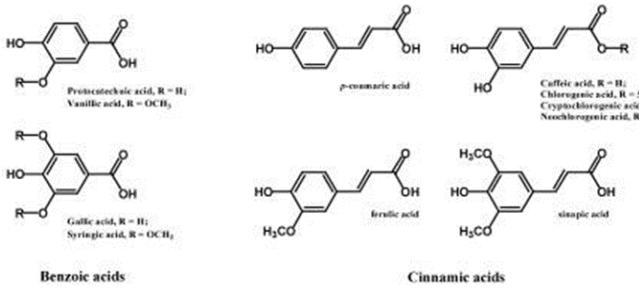


Figure 2.3 Basic non-flavonoid structures

2.1.3 **Role of phenolic compounds on plant physiology**

Because of their diverse chemical structure, phenolic compounds have a number of important functions in plants. They participate in seed dispersal, as insect attractants for pollination, in plant defense mechanisms, and in protection against biotic and abiotic stresses. Due to their antioxidant properties, they maintain a redox state in cells (Koes et al., 2005; Grotewold, 2006; Mierziak et al., 2014).

During plant development, plants are exposed to a variety of environmental conditions. All of these conditions might not be suitable for plant growth and development. With environmental stress, plants alter their basic metabolism (Bartwal et al., 2013). It has been reported that the accumulation of phenolic compounds under extreme stress condition can increase the concentration of PAL (Huang et al., 2010; Bartwal et al., 2013). Accumulation of phenolic compounds in plants can be induced by biotic and abiotic

stressors such as UV radiation, high light intensity, low temperature, wound, low nutrient, herbivory, and pathogen attack (Yamasaki et al., 1995). Flavonoids are capable of scavenging H_2O_2 by acting as electron donors for guaiacol peroxidases (GuPXs) such as horseradish peroxidase (HRP) (Sakihama et al., 2000). Tissue damage in plants cause oxidation of phenolic compounds (Sharma et al., 2012). Macromolecule associated copper and reactive oxygen generation may be important factors in the mechanism for inducing phenolic compounds in DNA damaged cells (Li and Trush, 1994). Phenolic compound biosynthesis in response to aluminum and nickel toxicity was reported in maize and wheat (Winkel-Shirley, 2002).

Phenolics can also induce resistance against pathogenic bacteria and fungi. They quench reactive oxygen species (ROS), which are generated both by the pathogens and the plant as a result of the infection (Mierziak et al., 2014). Phenolic compounds are involved in early defense by being transported to the infection site and induce a hypersensitivity reaction (Beckman, 2000). A study of barley mutants showed that proanthocyanidins and even small amounts of dihydroquercetin are involved in the defense against *Fusarium* species (Skadhauge et al., 1997). Salicylic acid, a simple phenolic acid, plays a key role in activating the immune pathway against wounding and necrotropic pathogens increasing disease resistance (Alvarez, 2000; Kumar, 2014). Salicylic acid also responds to a wide range of oxidative stressors (Shirasu et al., 1997).

2.1.4 **Role of phenolic compounds on human health**

In recent years, there has been a great deal of attention towards the health benefits of phenolic compounds and their antioxidant capacity. Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Benavente-Garcıa et al., 2000; Puupponen-Pimiä et al., 2001; Manach et al., 2005). Phenolic compounds in fruit are endorsed for nutritional intervention in Alzheimer's disease, since they are known to have extensive therapeutic properties apropos of brain health owing to their anti-oxidative effects against beta and neural reactive oxygen species (ROS). It is also reported that anthocyanins have immunostimulatory potential against immunosuppression induced by radiation (Fan et al., 2012).

The neuroprotective potential of lingonberry was investigated and found that fruit rich in flavan-3-ol and flavonol exhibited the strongest ability to maintain cell viability and prevent lactose dehydrogenase release (Bhullar and Rupasinghe, 2015). It is also reported that sugar reduced fruit spread containing different concentrations of lingonberry show natural preservation against fungal growth (Ermis et al., 2015). Lingonberry juice at small concentrations lowered blood pressure in a long-term treatment (Kivimaki et al., 2013).

2.1.5 Antioxidant activity of phenolic compounds

Antioxidants are molecules that slow down oxidation. They stop the chain reactions that occur during oxidation, by eliminating free radical intermediates. In plants, these free radicals are often derived from oxygen, nitrogen and sulphur, known as reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulphur species (RSS), respectively. ROS include free radicals such as superoxide anion ($O_2^{-\bullet}$), perhydroxyl radical ($HO_2^{\bullet}$), hydroxyl radical (•OH), nitric oxide, as well as nonradical molecules like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl) and peroxynitrite (ONOO−) (Lü et al., 2010; Sharma et al., 2012). These free radicals (ROS) are synthesized during metabolic processes and can induce oxidative stress. Oxidative stress and ROS play an important role in signalling and gene expression. ROS are highly reactive molecules that can affect carbohydrate, protein, lipid, RNA and DNA molecules, and can cause cellular damage and functional change (Birben et al., 2012). Excessive amount of free radicals can cause oxidative stress, which can lead to pathogenesis of inflammatory disease, neurodegenerative disorder, cardiovascular disease, cancer, diabetes, Alzheimer's disease, cataracts, autism and aging (Giles and Jacob, 2002; Rahman, 2007; Geier et al., 2009).

Plants produce ROS as side products of many metabolic processes, and are associated with signalling molecules involved in plant growth and development, plant defense, stress hormone synthesis, acclimation and programmed cell death (Foyer and Noctor, 2005; Tripathy and Oelmüller, 2012). Plants possess different enzymatic and non-enzymatic (glutathione, phenolic compounds, alkaloids, non-protein amino acids and α-

tocopherols) antioxidant defense systems, which protect them from oxidative damage by scavenging ROS. Phenolic compounds serve as potential non-enzymatic antioxidants within the cell to reduce the production of ROS through suppression of singlet oxygen and inhibition of ROS generating enzymes (Mierziak et al., 2014). The antioxidant activity of phenolic compounds depends on their metal reducing potential, chelating behavior, pH and solubility (Decker, 1997). The antioxidant activity of blueberry phenolic compounds has been well studied, supporting the use of this crop in the food industry (Kraujalyte et al., 2015; Bobinaite et al., 2015). Lingonberry has also a high potential in the food industry as a natural antioxidant since its fruit are rich in phenolic compounds and antioxidant activity (Lala, 2006; Vyas et al., 2015).

Total anthocyanin content, total phenolics, fruit surface area to volume ratio, genotype, environmental variation, fruit maturity and the post-harvest storage conditions can influence the antioxidant activity in fruit (Connor et al., 2002a; Connor et al., 2002b; Kalt et al., 2003). The correlation between antioxidant activity and total phenolics of *Vaccinium* species (e.g. highbush and lowbush blueberry, bilberry, cranberry) shows a linear positive correlation (Prior et al., 1998), which suggests that the antioxidant capacity of fruit is mainly related to the contribution of individual phenolic compounds (Abeywickrama et al., 2016). Smaller fruit has larger surface area to volume ratio, which gives higher total phenolic compounds and antioxidant activity because many of these characteristics are concentrated on the skin. Research has shown that more epidermal tissue per unit volume of smaller blueberries results in higher antioxidant activity than that of larger fruits (Ehlenfeldt and Prior, 2001; Howard et al., 2003). Blueberry cultivars producing smaller fruit, which have larger surface area to volume ratio, normally have

higher antioxidant activity as measured by ORAC values, and total phenolic levels than larger berries (Ehlenfeldt and Prior, 2001; Howard et al., 2003; Prior et al., 1998).

2.1.6 Phenolic compounds and environmental factors

The environment plays an important role in the biosynthesis of phenolic compounds. The world climate is changing and in a changing environment it is important to understand how plants respond to environmental conditions. The most common environmental factors that influence phenolic compound biosynthesis in plants are seasonal variations, sunlight and temperature, precipitation or water factors, pest or microbial stress, and nutrient status.

2.1.6.1 Geographical location

It has been reported by Davik et al. (2006) that long days and cool night temperatures in northern latitudes increase the production of aromatic compounds compared with the same plant species growing in the south of Norway. It was also reported that some vegetables contained higher amounts of carotenes, ascorbic acid, sugar, aroma and flavor compounds when grown in northern latitudes than in southern latitudes (Hårdh et al., 1977). A study showed that in bilberry (*Vaccinium myrtillus*) synthesis of anthocyanins was higher in northern latitudes than southern latitudes in Finland (Latti, Riihinen & Kainulainen 2008). Similar results were reported by Yang (2013), who found that berries of *Ribes* spp. cultivars grown at higher latitudes contained higher amounts of total phenolic compounds than those grown at lower latitudes in Finland, although this

might be an indirect effect of temperature. A study by Connor et al. (2002a), showed that anthocyanin content and activity were significantly different between the same cultivars grown in different locations, and between different cultivars grown in the same location. These results proved genotypic and environmental effects on anthocyanin content and activity. *Calluna vulgaris* (L.) Hull (heather) grown in different altitudes showed differences in flavonols and increased levels of quercetin glycosides (Monschein et al., 2010). Akerstrom et al. (2010) studied the phenolic compound variation of bilberry based on growing location from northern Germany to northern Finland, and based on parental origin. They found that phenolic content and antioxidant activity were under strong influence by genetic factors and less so by environmental factors. They reported that anthocyanidin content differed between latitudes and between plants with different parental origins. A recent study by Vyas et al. (2015) showed that lingonberry collected from different locations in Atlantic Canada significantly differed in terms of total phenolic content, flavonoid content, total anthocyanin and proanthocyanidin levels, and total antioxidant capacity.

2.1.6.2 Temperature

Temperature is an important factor that affects the biosynthesis of plant metabolites. The difference between day and night temperatures can have a marked effect on the accumulation of anthocyanin pigments in apple, berries, plums and grape skin (Tomas-Barberan and Espin, 2001). Lower temperature especially between 5 to 10 °C seemed to induce flavonol accumulation in *Arabidopsis thaliana* (Lea et al., 2007). A field experiment

demonstrated that anthocyanin synthesis in grape berries was affected by temperature through the regulation of PAL activity (Kataoka et al., 1984). This study found that at 20 °C day/15 °C night higher anthocyanin synthesis and PAL regulation occurred than at 30 °C day/20 °C night. Similar results were also reported by Leyva et al (1995) where both PAL and CHS (chalcone synthase) accumulated in leaves of *Arabidopsis thaliana* more readily in plants grown at 20 °C. Gene expression of anthocyanin biosynthesis proteins is induced at low temperature and suppressed at high temperature in various plants, such as apple (Ubi et al., 2006), *Arabidopsis* (Leyva et al., 1995), and grape (Mori et al., 2005; Yamane et al., 2006). Temperature thus affects the expression of genes involved in the biosynthesis of anthocyanins (Shaked-Sachray et al., 2002). In bilberries (*Vaccinium myrtillus)*, low temperature produced significantly higher content of total phenolic compound, acid and sugar (Martinussen et al., 2009). Similarly, high temperature induces several phenolic compound accumulations as a defense mechanism (Oh, 2008). In strawberry, cultivation at high temperature induced the biosynthesis of anthocyanins and *p*-coumaroyl glucose (Treutter, 2010), and promoted the accumulation of phenolic compounds in tomato (Dannehl et al., 2014). Thermal stress (temperature) in tomato and watermelon induced the accumulation of phenolics in the plant by activating their biosynthesis as well as inhibiting their oxidation, which increased the resistance against cold and heat stress (Rivero et al., 2001).

2.1.6.3 Light

Exposure to sun is an important environmental factor that can influence plant metabolism (Jackson and Lombard, 1993). There is an increase of temperature as the sun exposure increases (Spayd et al., 2002) rendering difficult to understand whether this physiological change is because of sunlight or temperature variation. It was also observed that extreme sun exposure could increase berry temperature, which inhibits or causes degradation of anthocyanin production (Spayd et al., 2002; Downey et al., 2004). It is reported that most of the change caused by light is hormonal in nature (Zucker, 1965; Cohen and Kennedy, 2010). PAL (phenylalanine ammonia lyase) is an initial precursor for chlorogenic acid biosynthesis, and it was found that PAL biosynthesis is induced by white light (Zucker, 1965). Other studies have shown that an increase in sun exposure increases flavonol biosynthesis (e.g. glucosides of quercetin, myricetin and kaempferol) (Spayd et al., 2002). Several studies have shown that artificially reducing ambient UV irradiation in *Vaccinium uliginosum* in Greenland can cause a 23% reduction of photosynthesis. UV enhances the flavonoid pool strongly, which is of prime importance for antiradical activity and for pathogen defense (Smirnoff, 2005). It has been reported that plants exposed to direct sunlight produced higher amount of phenolic compounds than those exposed to partial shade, full shade or grown under a high canopy covered plantation (high UV-B exposure increased jasmonate acid synthesis) (Ballare et al., 2012).

2.1.6.4 Annual precipitation

Yang et al. (2013) reported that high precipitation is associated with lower contents of anthocyanins, flavonol glycosides, and hydroxycinnamic acid conjugates in *Ribes spp*. On the other hand, olive fruits grown in Turkey reported a decrease in total phenolic content with a decrease in rainfall (Arslan and Ozcan, 2011). In lingonberry, annual precipitation exhibited positive correlation with antioxidant activity, flavonoid and tannin contents (Vyas et al., 2015).

2.1.6.5 Coastal proximity

Environmental stress such as salinity can lead to the accumulation of polyphenol constituents, and excessive amount of salinity can limit the growth and development of plants. High salinity in soil or irrigation water cause nutritional imbalance, osmotic stress, water deficiency and oxidative stress (ROS), which affect leaf expansion, carbon metabolism and many secondary metabolites including terpenoids and phenolic compounds (Parida and Das, 2005; Rezazadeh et al., 2012). High levels of oxidative stress during environmental stress in a plant may cause cellular damage through lipid peroxidation, oxidation of proteins, damage nucleic acids and inhibit enzymes (Apel and Hirt, 2004; Habib et al., 2016). Plants synthesize ascorbate peroxidase, glutathione and carotenoids, or activate ROS scavenging enzymes to reduce or overcome the effects of salinity-induced oxidative stress (Apel and Hirt, 2004; Mudgal et al., 2010). Several studies have shown that coastal soil has higher salinity content (high Na^+ and Cl^-) than inland soil (Chartzoulakis and Klapaki, 2000).

2.1.7 **Research on *Vaccinium vitis-idaea***

Studies on lingonberry have mostly investigated their phenolic content, antioxidant capacity, and their beneficial effects on human health. Few others have addressed the genetic diversity of cultivars and wild populations (Debnath, 2007; Debnath and Sion, 2009; Balsdon et al., 2011). Very few studies have focused on the influence of environmental factors and geographical location on the biosynthesis of phenolic compounds in lingonberry (Szakiel et al., 2012; Vyas et al., 2015; Li et al., 2016a). *Vaccinium vitis-idaea* leaves showed an increase in phenolic compound as a strategy to avoid the damaging effect of UV-B light (Semerdjieva et al., 2003). An experiment on *V. vitis-idaea* leaves in Alaska by Roy and Mulder (2014) reported an increased level of phenolic compounds in colder soil, colder air temperature, less canopy cover, and less availability of nutrients. Hansen et al. (2006) found that the concentration of tannins in *V. vitis-idaea* leaves grown in Sweden increased with temperature, shading, and nutrient addition. A recent study by Vyas et al. (2015) observed that *V. vitis-idaea* leaves in higher elevations and reduced temperatures show a higher level of anthocyanins, proanthocyanidins and total antioxidant activity, whereas other flavonoids and tannins increased with precipitation. Most of these studies were conducted in Europe, and therefore baseline information on the biochemical variation of North American wild lingonberry populations as a function of abiotic and biotic factors is much needed. Research on these effects is important not only to determine the best growing conditions that enhance antioxidants in this species, but also for germplasm characterization and practical breeding purposes.

As part of the ongoing interest in nutritional and nutraceutical properties of locally produced fruits, one of the goals of this research is to determine the phenolic compound content and antioxidant capacity of wild lingonberry populations in Newfoundland and Labrador (NL), because this is one of the top lingonberry growing and harvesting provinces in Canada (Penney et al., 1996). Lingonberry research in NL had focused on characterizing the anthocyanin content and antioxidant activity of fruits from populations located in a few sites such as the St. John's vicinity, Gambo, St. Pierre, the Codroy Valley, St. Anthony and Eddies Cove (Debnath and Sion, 2009; Vyas et al., 2015). No comparative research has been conducted on the biochemical properties of fruit among ecoregions and other environmental factors in the island.

2.1.8 **Research objectives**

The main objective of the work presented in the rest of this chapter was to assess the variation of total phenolic content (TPC) and antioxidant capacity (AC) of wild lingonberry fruit in NL as a function of environmental factors. We had four expectations: 1) TPC and AC vary according to the different ecoregions in Newfoundland and Labrador, 2) higher TPC and AC will be found in areas of lower temperature and higher precipitation regimes based on previous studies on lingonberry and other species, 3) TPC and AC will be higher in coastal areas because of a higher soil salinity stress, 4) total phenolic content of fruits is positively correlated with their antioxidant capacity because the latter is a function of the former.

2.2 Materials and Methods

2.2.1 Site selection and sample collection

In this study, we have extended the sampling effort of wild lingonberry populations from previous biochemical studies (Debnath and Sion, 2009; Vyas et al., 2015). The province of NL is divided into 19 ecoregions based on patterns of vegetation and soil characteristics, which are determined and controlled by the local climate and geology. Meades (1991) identified nine ecoregions on the island of Newfoundland, and ten in Labrador. We selected eight ecoregions (Meades, 1991) in Newfoundland, and one from Labrador for sample collection (Appendix 1). We excluded the Avalon Forest Ecoregion (#5) from our sampling because of its small area and were difficult to separate the area in the field. In NL lingonberry fruit ripens in the months of August and September, thus a field trip was conducted from mid-August to mid-September of 2014 to collect fruit samples. We selected a total of 54 sites from nine ecoregions (Table 2.1), with four to seven sites per ecoregion. For each sampling site, GPS coordinates were taken to construct a map showing the sampling distribution (Figure 2.4, Table 2.1). Since boundaries among ecoregions are not fixed, it was challenging to identify ecoregion limits in the field and therefore sample sizes were different. We collected fruits from healthy and vigorous adult plants at each sampling site. To avoid variation among maturity levels, which has been proven to influence the amount of antioxidants in different berry fruits (Mahmood et al., 2012), we collected fruits at roughly the same developmental stage. Fruits were half red and half green, thus not completely ripe (Figure 2.5). Fruits were collected in 50 ml

centrifuge tubes and stored in a cooler containing ice bags to avoid the loss of antioxidant capacity until samples were stored at -20^0 C.

2.2.2 Environmental variables

In order to evaluate how TPC and AC vary according to the different environmental conditions, we grouped the 54 samples in categories within the following eight variables: ecoregion, mean annual precipitation, mean annual temperature, mean summer temperature, annual runoff, surface water pH, surface water sensitivity to acid rain, and proximity to the coast. Using data from the Department of Natural Resources of Newfoundland and Labrador (Ullah, 1992) we partitioned the province into three precipitation areas: low (700-1100 mm), medium (1100-1300 mm) and high (1300-1500 mm). For the mean annual temperature, we partitioned the province into three categories: low (1.0-2.9°C), medium (3.0-3.9°C), and high (4.0-7.9°C). The mean summer temperature was also of interest because this is the time when *V. vitis-idaea* produces berries. Samples were divided into three summer temperature categories: low (6-11.9°C), medium (12-13.9°C), and high (14-19.9°C). Newfoundland and Labrador were categorized into 500-800 mm, 800-1000 mm, 1000-1300 mm, 1300-1800 mm and 1800-2200 mm zones based on mean annual runoff. Surface water pH is also an important parameter because its association with soil acidity is considered to be limiting for plants growth and development. The study area was divided into <6.1, 6.2-6, 6.6-7.and >7.0). Surface water alkalinity confers the ability to resist pH changes, particularly acidification. The study area was categorized into extreme (<60 μeq/l), high (60-100 μeq/l), moderate (100-200 μeq/l) and

low (>200 μeq/l) based on the sensitivity to acid rain (Ullah, 1992). Finally, samples were divided into coastal if located within a 2 km distance from the coast, versus inland if further away from the coast based on field observations (Appendix 1).

2.2.3 Sample extraction for biochemical tests

Frozen lingonberry fruits were ground to powder in liquid nitrogen. A total of three grams of fine fruit powder was homogenized with 30 ml of 70% aqueous acetone. To homogenize the plant material, we exposed samples to sonication using a 300 ultrasonic water bath (Blackstone-NEY Ultrasonic, Jamestown, NY) at an amplitude of 60 Hz, at 40°C for 30 minutes. Centrifugation at 4,000 rpm (1431 g) for 10 minutes at 4°C followed the sonication. The liquid supernatant was transferred to a new 50 ml centrifuge tube using a Whatman No. 1, 90 mm filter paper and diluted the sample to a final volume of 70 v/vol. These plant extracts were stored at -20° C until further analyses of TPC and AC.

2.2.4 Chemicals

DPPH (2,2-diphenyl-1-picrylhydrazyl radical), Folin-Ciocalteu's phenol reagent, gallic acid, sodium carbonate, and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Canada Co (Oakville, ON). Acetone and ethanol were purchased from Merck Canada Inc (Kirkland, QC). All chemicals used in the study were of analytical grade.

2.2.5 Total phenolic content measurement

Total phenolic content for each sample was determined using the Folin–Ciocalteu method described in McDonald et al. (2001), with minor modifications. Briefly, 50 µl of diluted plant extract were added to 50 µl of 2N Folin–Ciocalteu reagent and mixed thoroughly for three minutes. One hundred microliters of 5% sodium carbonate were added to the mixture and the volume was adjusted to 1 ml with distilled water. The mixture was allowed to stand for 30 minutes at dark, followed by a one-minute vortexing. The absorbance of the mixture was measured at 760 nm against the blank using a UV–VIS spectrophotometer (Ultrospec 4300 pro, Jasco, Tokyo, Japan). A standard curve was established using gallic acid solutions (0-2 mg/ml). TPC was expressed as milligrams of gallic acid equivalents per 100 grams of fresh fruit weight (mg GAE/100g FW).

2.2.6 Antioxidant capacity measurement

The antioxidant capacity of fruit extracts was measured using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method of Chandrasekara and Shahidi (2011) with slight modifications. A stock solution of 300 µM DPPH was freshly prepared by dissolving 29.9 mg of DPPH in 250 ml of ethanol. Two hundred fifty microliters of plant extract were mixed with 750 µl of freshly prepared DPPH solution. This mixture in the dark for 30 minutes at room temperature. The decrease in the absorbance was measured at 517 nm using an electron paramagnetic resonance spectrometer (Bruker-Biospin, Karlsruhe, Germany). The scavenging capacity was expressed as % inhibition of DPPH consumption. The percentage inhibition of radicals was calculated using the following formula:

% inhibition=($A^{control}$–A^{sample})×100/$A^{control}$ (Astadi et al., 2009)

Where $A^{control}$ is the absorbance of DPPH solution without extract, and A^{sample} is the absorbance of the sample with DPPH solution. Measurements were performed in triplicate, and the mean value of the three determinations was plotted against a Trolox standard curve to express the results as milligrams of Trolox equivalents per 100 grams of fresh fruit weight (mg TE/100g FW).

2.2.7 Statistical analyses

All chemical measurements were done in triplicate. These values were subsequently averaged and their standard derivations calculated. Statistical comparisons of TPC and AC means among the eight environmental factors described above were performed using a one-way analysis of variance (ANOVA), with a significance level α of 0.05. Post-hoc pairwise comparisons followed using Fisher's Least Significant Difference (LSD). Non-parametric Kruskal-Wallis tests were conducted when samples violated the equality of variances assumption and normality tests. In addition, we tested the effects of variable interactions on TPC and AC, however, our small sample sizes for each variable combination violated the assumptions of the two-way analyses of variance, and therefore results were not presented here. A Pearson correlation between TPC and AC to test the hypothesis that phenolic contents positively influence the antioxidant capacity of fruits collected in the wild was established. Data were analyzed using the IBM SPSS Statistics 23.0 for Mac (SPSS, Chicago, IL).

2.3 Results

2.3.1 Influence of ecoregion on TPC and AC of lingonberry fruits

TPC content of lingonberry fruits was significantly different among ecoregions ($F_{(8,45)} = 5.808$, p-value<0.001). The highest TPC was found in fruits harvested from the North Shore Forest (1082 ± 70 mg GAE/100g FW) and the lowest TPC was present in fruits from the Long Range Barrens (509 ± 76 mg GAE/100g FW) (Table 2.1). The simultaneous pair wise comparisons (LSD) indicated that the North Shore Forest ecoregion produced fruits with significantly higher TPC than the Western Newfoundland Forest, the Northern Peninsula Forest, the Long Range and Forteau Barrens (Table 2.1). No significant variation was observed on AC of lingonberry fruits with respect to ecoregions ($F_{(8,45)} = 0.986$, p-value=0.460, Table 2.1).

2.3.2 Influence of mean annual temperature on TPC and AC

Temperature is an important environmental factor that influences the growth, development and biochemical properties of plants. The results so obtained show a significant influence of annual temperature on TPC ($F_{(2,51)} = 7.174$, p-value=0.002). The highest TPC (817 ± 36 mg GAE/100g FW) was found at 4.0-7.9 °C, and the lowest TPC (562 ± 58 mg GAE/100g FW) was noted at 3.0-3.9 °C (Table 2.2). The pairwise comparison (LSD) did not show any significant difference in the TPC of fruits grown at 4.0-7.9 °C and

1.0-2.9 °C (Table 2.2). Mean annual temperature also showed a significant variation on AC ($F_{(2,51)}$ = 7.417, p-value=0.001) of lingonberry fruits. Mean annual temperature showed the highest AC (703 ± 51 mg TE/100g FW) at 1.0-2.9 °C, and the lowest AC (444 ± 46 mg TE/100g FW) was found at 3.0-3.9 °C (Table 2.2). Similar to the pairwise comparisons for TPC, the LSD tests did not show any significant difference in AC of fruits grown at 4.0-7.9 °C and 1.0-2.9 °C. Therefore, the moderate temperature (3.0-3.9 °C) was the least suitable for TPC biosynthesis and AC of lingonberry fruits.

2.3.3 Influence of mean summer temperature on TPC and AC

Mean summer temperature showed a significant influence on TPC of lingonberry fruits ($F_{(2,51)}$ = 6.056, p-value=0.004). TPC was highest (854 ± 44 mg GAE/100g FW) at 14-19.9 °C, whereas the lowest TPC (645 ± 43 mg GAE/100g FW) was observed at 12-13.9 °C (Table 2.2). The highest and lowest mean values were significantly different as revealed by the pairwise comparison test. AC of fruits also showed significant variation with respect to mean summer temperature ($F_{(2,51)}$ = 3.925, p-value=0.026) (Table 2.2). The highest AC (727 ± 57 mg TE/100g FW) was obtained for the 6-11.9 °C mean summer temperature range, and the lowest AC (551 ± 36 mg TE/100g FW) was recorded for the 14-19.9 °C mean summer temperature range (Table 2.2). Significant LSD values were observed in the 6-11.9 °C mean summer temperature ranges with respect to the other two higher ranges.

2.3.4 Influence of mean annual precipitation on TPC and AC

TPC ($F_{(2,51)}$ = 0.723, p-value=0.490) and AC ($F_{(2,51)}$ = 1.235, p-value=0.299) of lingonberry fruits showed no significant difference across mean annual precipitation regions (Table 2.3).

2.3.5 Influence of ocean proximity on TPC and AC

Coastal vs. inland areas showed no significant difference on TPC of lingonberry fruits ($t_{(1,52)}$ = 1.27, p-value=0.211, Table 2.4). On the other hand, ocean proximity showed significant influence on AC of lingonberry fruits ($t_{(1,52)}$ = -2.59, p-value=0.013). The highest AC (642 ± 33 mg TE/100g FW) was observed in fruits collected from inland areas more than 2 km away from the coast, while the lowest AC (522 ± 33 mg TE/100g FW) was observed in coastal areas (Table 2.4).

2.3.6 Effect of mean annual runoff on TPC and AC

Mean annual runoff showed a significant influence on TPC of lingonberry fruits ($F_{(1,52)}$ = 4.26, p-value=0.01). The highest TPC (859 ± 53 GAE mg/100g FW) was observed at the low annual runoff category (500-800 mm), while the lowest TPC (604 ± 51 GAE mg/100g FW) was observed at the high annual runoff category (1800-2200 mm) (Table 2.3). Mean annual runoff did not show any significant influence on AC of lingonberry fruits ($F_{(1,52)}$ = 1.13, p-value=0.35).

2.3.7 Effect of surface water quality on TPC and AC

Surface water pH did not show a significant difference on TPC ($F_{(1,52)} = 2.17$, p-value=0.10) and AC values ($F_{(1,52)} = 0.53$, p-value=0.67) of lingonberry fruits (Table 2.5). Surface water sensitivity to acid rain showed a significant effect on TPC ($F_{(4,49)} = 3.50$, p-value=0.02) of lingonberry fruits. The highest TPC (949 ± 63 GAE mg/100g FW) was observed at the highly sensitive category (60-100 μeq/l). Sensitivity to acid rain did not show any significant influence on AC of lingonberry fruits ($F_{(4,49)} = 0.183$, p-value=0.91) (Table 2.5).

2.3.8 Correlation test

In order to evaluate the correlation between the TPC and AC of wild lingonberry fruits, a Pearson`s correlation coefficient was used. This showed no significant correlation (R^2=0.0006, P-value = 0.584). Therefore, total phenolic content, as measured in this study, did not positively influence the antioxidant capacity of fruits collected in the wild.

Table 2.1 Effect of ecoregion on TPC and AC of lingonberry fruits

Ecoregion #	Ecoregion name	Sample size (N)	TPC (mg GAE/100g FW) mean ± SE	AC (mg TE /100g FW) mean ± SE
1	Western Newfoundland Forest (WNF)	7	624.91 ± 64.61 a	559.63± 67.66 a
2	Central Newfoundland Forest (CNF)	8	832.43 ± 60.43 ab	528.59 ± 63.29 a
3	North Shore Forest (NSF)	6	1081.71 ± 69.78 b	573.80 ± 73.08 a
4	Northern Peninsula Forest (NPF)	6	581.23 ± 69.781 a	528.02 ± 73.08 a
6	Maritime Barrens (MB)	7	833.42 ± 64.61 ab	605.05 ± 67.66 a
7	Eastern Hyper Oceanic Barrens (EHOB)	6	748.40 ± 69.784 ab	674.12 ± 73.08 a
8	Long Range Barrens (LRB)	5	508.77 ± 76.44 a	461.87 ± 80.05 a
9	Strait of Belle Isle Barrens (SBIB)	4	825.29 ± 85.47 ab	655.05 ± 89.50 a
10	Forteau Barrens (FB)	5	728.68 ± 76.44 a	691.81 ± 81.05 a

Different letters within the same column indicate significant differences among ecoregions (one-way ANOVA and LSD test, $p<0.05$).

Table 2.2 Effect of mean annual temperature and mean summer temperature on TPC and AC of lingonberry fruits

	Sample size (N)	TPC (GAE mg/100g FW) mean ± SE	AC (TE mg/100g FW) mean ± SE
Mean annual temperature (°C)			
1.0-2.9	10	795.71 ± 63.95 a	703.29 ± 50.74 a
3.0-3.9	12	561.54 ± 58.38 b	443.97 ± 46.32 b
4.0-7.9	32	816.52 ± 35.75 a	595.78 ± 28.36 a
Mean summer temperature (°C)			
6-11.9	10	799.83 ± 68.59 ab	726.55 ± 56.56 a
12-13.9	24	644.96 ± 42.91 a	555.25 ± 35.38 b
14-19.9	20	854.17 ± 43.87 b	550.72 ± 36.18 b

Different letters within the same column indicate significant differences among mean annual temperature and mean annual summer temperature categories (one-way ANOVA and LSD test, $p<0.05$).

Table 2.3 Influence of mean annual precipitation and mean annual runoff on TPC and AC of lingonberry fruits

	Sample size (N)	TPC (GAE mg/100g FW) mean ± SE	AC (TE mg/100g FW) mean ± SE
Mean annual precipitation (mm)			
700-1100	24	771.40 ± 46.08 a	587.92 ± 36.34 a
1100-1300	17	783.91 ± 54.75 a	532.95 ± 43.18 a
1300-1500	13	691.09 ± 62.61 a	635.02 ± 49.38 a
Mean annual runoff (mm)			
500-800	15	859 ± 53.4 a	563 ± 81.3 a
800-1000	11	854 ± 69.0 a	666 ± 52.7 a
1000-1300	8	614 ± 72.1 b	514 ± 72.9 a
1300-1800	12	734 ± 61.9 ab	551 ± 54.6 a
1800-2200	8	604 ± 50.9 b	630 ± 67.8 a

Different letters within the same column indicate significant differences among mean annual precipitation and mean annual runoff categories (one-way ANOVA and LSD test, $p<0.05$).

Table 2.4 Effect of ocean proximity on TPC and AC of lingonberry fruits

Ocean proximity	Sample size (N)	TPC (GAE mg/100g FW) mean ± SE	AC (TE mg/100g FW) mean ± SE
Coastal	27	794.47 ± 42.97 a	522.18 ± 32.70 a
Inland	27	717.54 ± 42.97 a	641.73 ± 32.70 b

Same letters within the same column indicate no significant difference between coastal and inland categories as revealed by a T-test.

Table 2.5 Effect of surface water pH and surface water sensitivity to acid rain on TPC and AC of lingonberry fruits

	Sample size (N)	TPC (GAE mg/100g FW) mean ± SE	AC (TE mg/100g FW) mean ± SE
Surface water pH			
<6.1	15	802 ± 53.1 a	578 ± 50.6 a
6.2-6.5	9	855 ± 75.5 a	546 ± 64.0 a
6.6-7.0	24	733 ± 75.5 a	578 ± 35.9 a
>7.0	6	585 ± 55.6 a	663 ± 56.7 a
Surface water sensitivity to acid rain (μeq/l)			
Extremely sensitive (<60)	13	775 ± 57.2 ab	562 ± 51.7 a
Highly sensitive (60-100)	10	949 ± 63.2 a	612 ± 70.2 a
Moderately sensitive (100-200)	14	690 ± 51.0 b	569 ± 44.6 a
Low (>200)	18	698 ± 56.3 b	592 ± 42.3 a

Same letters within the same column indicate no significant difference between surface water pH and surface water sensitivity to acid rain categories (one-way ANOVA and LSD test, $p<0.05$).

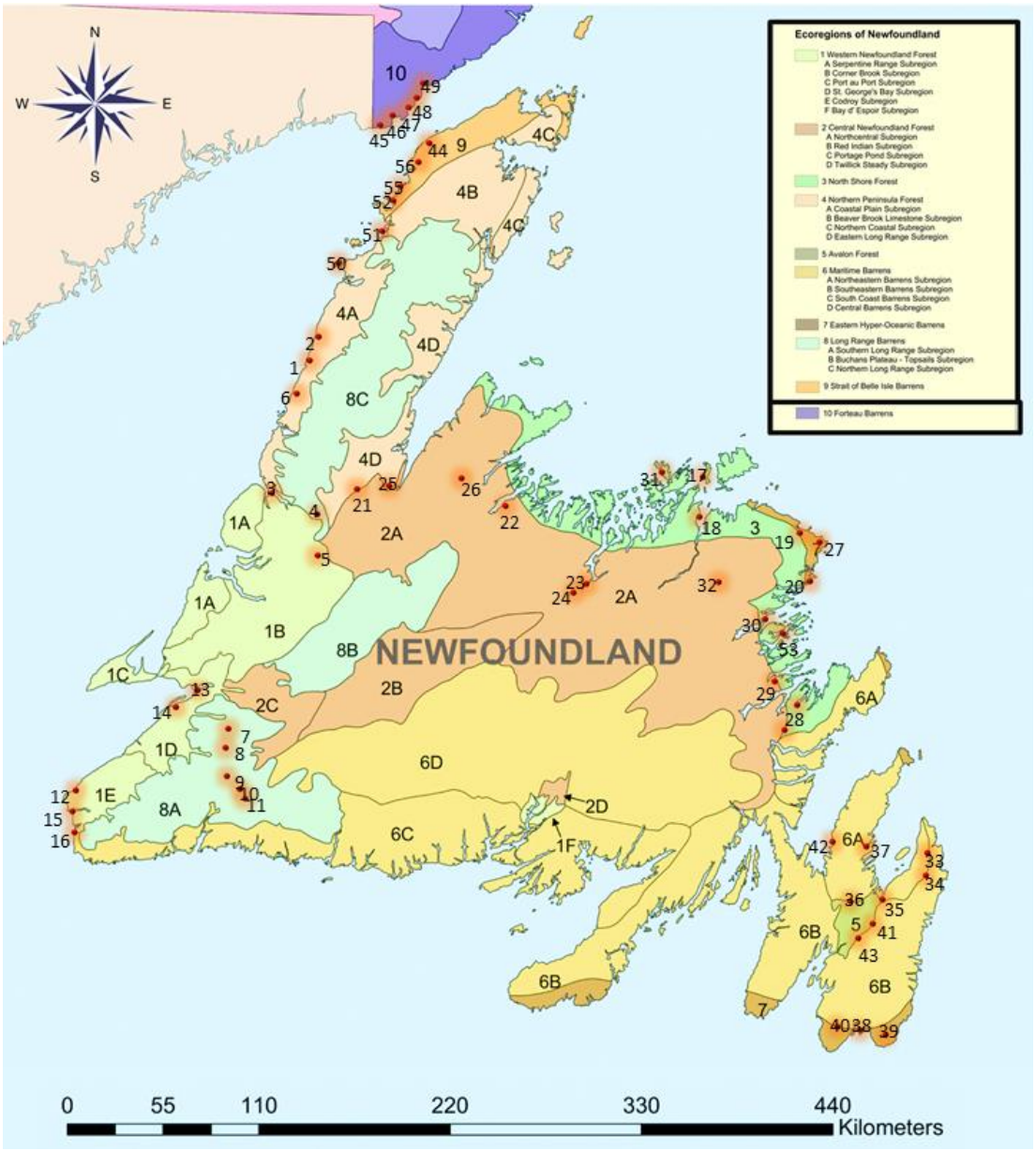


Figure 2.4 Distribution map of lingonberry fruits sample collected from Newfoundland and Labrador. Dots 1-56 represent location of sampling sites with coordinates in Appendix 1. Ecoregion data in map available from Parks and Natural Areas Division, Department of Environment and Conservation, Government of Newfoundland and Labrador. Location of each ecoregion is shown in the map by numbers 1–10. Map credit: Hugo Morales.

Figure 2.5 Maturity stage of *Vaccinium vitis-idaea* fruits in Newfoundland and Labrador, Canada during sample collection

2.4 **Discussion**

Newfoundland and Labrador is a suitable place to grow lingonberry. It is shown in Figure 2.4 that lingonberry is commonly found on the island. The health benefits of fruits are mainly attributed to their vitamins, carotenoids, and phenolic compounds. It is well reported that lingonberry is rich in phenolic compounds and that the AC of this fruit is higher than that in some other available berry fruits (e.g. highbush blueberry, blackberry) (Mikulic-Petkovsek et al., 2012). In our study, we found that the average TPC and AC of wild lingonberry collected from Newfoundland and Labrador was 756 mg GAE/100 g FW and 581.96 mg TE/100g FW, respectively. A study by Wang et al. (2005) reported similar results to ours with lingonberry grown in USA and Europe synthesizing 773.7 mg GAE/100g FW of total phenolics. Earlier studies have reported that wild *Vaccinium vitis-idaea ssp. minus* populations possessed up to 2-3 fold higher TPC and AC than those of *Vaccinium vitis-idaea ssp. vitis-idaea* cultivars (Kalt et al., 2007; Debnath and Sion, 2009; Vyas et al., 2015). A comparative study on some commonly edible fruits including lingonberry cultivated in Poland reported that lingonberry produced more phenolic compounds (410.73 ± 14.53 mg GAE/100 g FW) and had higher antioxidant activity (4.82 ± 3.85 μmol TE/g fruits) than other commonly available fruits such as blackberry, blueberry, cranberry (Podsedek et al., 2014). Our results show that lingonberry growing in Newfoundland and Labrador produce more TPC and AC than the European lingonberry. Commercial cultivation of lingonberry in Newfoundland has not been reported and no suitable cultivar has been introduced in Newfoundland. Our results thus show the potential

for selecting wild Newfoundland lingonberry populations for use in future commercial cultivations.

The biosynthesis of phenolic compounds is different for a crop under different growing conditions. It is reported that phenolic compound biosynthesis and AC is influenced by light, temperature, mineral nutrition, water management, grafting, elevated atmospheric CO_2, growth and differentiation of the plant, and application of elicitors, stimulating agents and plant activators (Kataoka et al., 1984; Tomas-Barberan and Espin, 2001; Kouki and Manetas, 2002; Treutter, 2010). Up to date, the effect of environmental factors and geographical location on phenolic compound biosynthesis and AC has not been thoroughly studied for lingonberry.

2.4.1 Influence of ecoregion on TPC and AC of lingonberry fruit

A recent study by Vyas et al. (2015) discussed the influence of environmental factors on phenolic compounds and antioxidant capacity of lingonberry growing in different provinces of Canada. In our study, the ecoregions of Newfoundland and Labrador showed a significant influence on TPC biosynthesis in lingonberry. We found that lingonberries growing in the north shore forest, maritime barrens, Strait of Belle Isle barrens and central Newfoundland forest had higher TPC than the other ecoregions tested. This is in agreement with the findings of Connor et al. (2002a; 2005) who found that antioxidant content varied significantly in blueberry fruits harvested from different locations in Minnesota, Michigan, and Oregon over two years. Dennath and Sion (2009) reported similar results to those in this work. Lingonberries grown in the Strait of Belle Isle

barrens and Avalon forest had higher TPC and anthocyanin content. However, not all previous studies have shown a significant influence of growing location on TPC. A previous study by Wang et al. (2005) showed that TPC of lingonberry did not vary significantly under different growing locations in USA. TPC variation in lingonberry at different ecoregions of Newfoundland and Labrador may be due to climatic conditions. The ecoregion with the highest TPC (North Shore Forest) had the highest mean annual temperature (4.0-7.9 °C), the highest mean summer temperature (14-19.9 °C), medium rainfall (1100-1300 mm), low water pH and highly sensitive to acid rain. On the other hand, the ecoregion with the lowest TPC (Northern Peninsula Forest) had medium mean annual temperature of 3.0-3.9 °C, medium mean summer temperature of 12-13.9 °C, low (700-1100 mm) annual precipitation, high water pH, and low sensitivity to acid rain. The observed variation in TPC may also be due to variations in population genetics or differential mineral nutrients availability. Studies by Foley and Debnath, (2007) and Debnath et al. (2012) reported that TPC and AC of berries grown in different locations vary because of their genotypic differences.

Antioxidant capacity of lingonberry showed no dependency on ecoregion. This is in agreement with previous work reporting that the AC of different *Vaccinium* berries was not influenced by growing location (Prior et al., 1998). This may be because of the different antioxidant ability of different phenolic compounds, as it has been reported that the same amount of total phenolic content has different antioxidant capacity because of the variation in individual phenolic compounds (Cao et al., 1997; Wang et al., 1997; Shahidi and Chandrasekara, 2010).

2.4.2 Influence of temperature on TPC and AC of lingonberry fruits

Both mean annual temperature and mean summer temperature showed an effect on TPC. The highest amount of TPC was observed at the highest mean annual temperature (4.0-7.9 °C), and highest mean summer temperature (14-19.9 °C). The reasons for the variation in TPC are unknown. A possible explanation could be that as photosynthesis increase with increasing temperature, a part of this photosynthesis could be allocated to the synthesis of phenolic compounds and carbohydrates (Jonasson et al., 1986). On the other hand, in the present study, the antioxidant activity increased with decreasing temperature. A similar relationship between AC and temperature was reported by Boo et al. (2011) and Li et al. (2010) for *Lactuca sativa* L. Larrauri et al. (1998) found a negative relationship between temperature and AC (measured as DPPH scavenging effect) for grape pomace peels, and Islam et al. (2003) reported similar observations for *Ipomoea batatas* L. The lower DPPH activity at higher temperatures may be related to a reduction in the amount of anthocyanins (He et al., 2010; Boo et al., 2011). Jaakola and Hohtola (2010) reported that cold temperature increased the rate of photosynthesis compared to warm temperature, which enabled the plant to increase the amount of fixed carbon available for secondary metabolite biosynthesis. Some studies have shown that lowering the temperature increased the synthesis of anthocyanins in tomato (Løvdal et al., 2010), apple (Ubi et al., 2006), and grape (Mori et al., 2005), and also reported to have higher AC. In other studies, high

temperature stress induced the accumulation of TPC in tomato and watermelon, as well as inhibited their antioxidant capacity (Rivero et al., 2001).

It is reported that flavonols increased with light irradiation showing that the synthesis of this group of flavonoids is temperature dependent at least indirectly. The quantity and quality of phenolics depend on the irradiation temperatures. The optimum temperature for the synthesis of phenolic acids, anthocyanins and flavonols was 24 °C in apple regardless of the maturity stage and variety (Bakhshi and Arakawa, 2006). Activation of the expression of the flavonoid biosynthetic genes by enhanced light has recently been reported in bilberry (Lancaster and Dougall, 1992; Jaakola et al., 2004). Although low temperatures have been suggested to induce PAL activity (Lancaster and Dougall, 1992), phenolic acids might be synthesized due to the induction of PAL by UV-B.

2.4.3 Influence of precipitation and runoff on TPC and AC of lingonberry fruits

Annual precipitation in Newfoundland varies depending on the location. Mean annual precipitation in Newfoundland varies from 700 to 1500 mm. Although not statistically significant, our results show a trend of increasing TPC with decreasing annual precipitation. Our results were not supported by those of Arslan and Ozcan (2011) who reported an increase in phenolic contents with an increase of annual precipitation in Turkish olives. Annual precipitation showed no significant effect on AC of partridgeberries, although a previous study on partridgeberries by Vyas et al. (2015) reported a negative correlation between annual precipitation and AC.

Runoff is that portion of precipitation that flows into rivers, lakes and oceans by surface drainage and through the ground. This available water from precipitation may play an important role in plant growth, development and synthesis of bioactive compounds. Our results showed that lingonberry growing under low annual runoff had higher TPC than populations under high annual runoff. Some studies have reported in some studies that water stress can induce the biosynthesis of phenolic compounds. King (2010) reported that drought often causes oxidative stress, and showed an increase in the amounts of flavonoids and phenolic acids in willow leaves. Plant tissues containing anthocyanins are usually resistant to drought. Anthocyanins are reported to accumulate under drought stress and at cold temperatures (Solíz-Guerrero et al., 2002). In the sampling locations in our study, low annual runoff conditions may create water deficiency activating the phenolic biosynthesis genes to resist drought stress. This hypothesis however needs to be confirmed. The mechanism of drought resistance is not well understood, hence further research is needed to corroborate that the high TPC biosynthesis responds to water deficiency and not to other soil and environmental factors.

2.4.4 **Influence of proximity to ocean on TPC and AC of lingonberry fruits**

Salinity is an important abiotic factor that can cause unfavourable environmental conditions that restrict the normal growth and development of plants. About 53% of the coastal areas are affected by salinity (Rasel et al., 2013) and assumed to have higher salinity than inland. In this present study, we measured the effects of coastal proximity on

antioxidant capacity of lingonberry. The AC of Newfoundland's lingonberries varied significantly according to their proximity to the ocean. Our results showed that fruits collected inland have higher AC than fruits from the coastal area. Most of the previous studies however, reported an opposite trend, where salinity could induce oxidative stress in plants and could lead to formation of ROS (Apel and Hirt, 2004). Plants overcome this oxidative stress by synthesizing antioxidants and ROS-scavenging enzymes (Mudgal et al., 2010) including many flavonoids and other phenolic compounds. One possible explanation for our results disagreement with the literature is that the 2 km threshold defining samples subject to salt exposure might be inappropriate, and an empirical evaluation of salinity in wild populations awaits investigation.

2.4.5 Influence of surface water quality on TPC and AC of lingonberry fruits

The present study also evaluated the effects of surface water quality using pH and sensitivity to acid rain. Our study showed no significant effect of surface water pH on TPC and AC on lingonberry fruits. To the best of our knowledge, no other study has tested the effect of water pH on the synthesis of secondary compounds, but the effect of soil pH on phenolic compound biosynthesis and antioxidant activity has received some att. In *Potentilla fruticosa* L no correlation was observed between antioxidant activity and soil pH (Liu et al., 2016). However, in *Morus alba*, an increase in antioxidant enzyme activity was observed under increased soil salt concentration and pH (Ahmad et al., 2014). Further

studies are needed to understand the direction and strength of the effect of surface water pH or soil pH on secondary metabolites biosynthesis and antioxidant activity.

Acid rain can damage plants through change in leaf anatomy, leaf necrosis and reduction in biomass, which can decline the photosynthetic capacity of plants (Neufeld et al., 1985; Sant'Anna-Santos et al., 2006). In our study, TPC in lingonberry fruits changed significantly by sensitivity to acid rain. The lowest TPC were found in waters with moderate to low sensitivity (high alkalinity, >100 μequiv/L). In support of our findings, earlier studies have reported that very high alkalinity impairs the detoxifying response (i.e., antioxidant activity) of plants to reactive oxygen species (Cartmill et al., 2008). In the Brazilian coastal ecosystem species, *Sophora tomentosa* exposed to acid mist deposition enhanced the activities of antioxidant enzymes such as catalases and superoxide dismutase as a mechanism to partially neutralize oxidative stress (Kuki et al., 2008). Similar responses were observed in other tropical tree species in response to simulated acid rain where phenolic compounds were accumulated in necrotic areas, probably by rupture of the vacuolar membrane (Sant'Anna-Santos et al., 2006).

2.4.6 TPC and AC correlation

In fruits, TPC exhibited no correlation with AC. Skrovankova et al. (2015) evaluated the AC of different cultivars of blackberry, bilberry, and strawberry and verified that the content of TPC correlated significantly with AC. The results found in the present study are not similar to those reported by Skrovankova et al. (2015), or Cheel et al. (2007) in strawberries, or Jiao and Wang, (2000) in blackberries. Several studies have reported

that the antioxidant activity has been correlated to TPC and flavan-3-ol contents, as well as to the content of specific compounds such as vanillic acid, gallic acid and catechin (Heim et al., 2002; De Beer et al., 2002). However, our understanding of the contribution of phenolics to the antioxidant activity of different species of fruits and vegetables is still basic and insufficient (Skrovankova et al., 2015). Some authors have suggested that the AC is a consequence of synergism between different phenolic compounds, and it cannot be attributed specifically to one constituent (Rice-Evans et al., 1997; Arnous et al., 2002; Lee et al., 2003). Fukumoto and Mazza (2000) analyzed the antioxidant activity of phenolic compounds and concluded that it increases with the increase in the number of hydroxyl groups, and the decrease in glycosylated groups. For example, *o*-dihydroxyl groups in the molecules results in higher antioxidant activity because of the formation of intramolecular hydrogen bonds during the reaction with free radicals (Rogaie et al., 2013). In support of this, hydroxytyrosol was found to exert the strongest antioxidant capacity compared with tyrosol, elenolic acid, deacetoxy (Carrasco-Pancorbo et al., 2005). It was also observed that o-methylation (-OCH_3) of the hydroxyl group of the benzoic rings caused a decrease in the antioxidant capacity, because this group was not able to form a hydrogen bond in comparison with molecules having a catecholic group in their structure (Carrasco-Pancorbo et al., 2005).

TPC and AC may vary based on the extraction method employed. A method may be suitable for TPC but give low AC. Based on the extraction method TPC and AC may not be correlated (Fernandes de Oliveira et al., 2012). In studies by Alviano et al. (2008) and da Silva et al. (2011) aqueous methanol extract was shown to be more effective than ethanol extract in the DPPH assay of antioxidant capacity. Aqueous organic solvent may

facilitate the extraction of various compounds of different polarity, which may contribute to the increased concentration of antioxidants in the extract (Do et al., 2014). A negative correlation between TPC and AC was reported by Rumbaoa et al. (2009) in five varieties of *Ipomoea batatas.* A study by Malenčić et al. (2008) used acetone extracts for twenty hybrids of *Glycine max* L. and showed no correlation between AC and TPC. It is possible that TPC and AC of Newfoundland's lingonberries may be influenced by the phenolic extraction method used in this work, or the phenolic compounds extracted had different antioxidant activities, or because the differences in the amounts of phenolics and AC obtained from the 54 samples is not large enough to show a pattern of correlation (Alam et al., 2016).

2.5 Concluding remarks

On the basis of our results, ecoregion, temperature, runoff, and proximity to the coast seemed to be important factors regulating TPC and AC of Newfoundland wild lingonberry fruits. Conclusions reached here await further corroboration upon repetitions in multiple years. It is important to maintain size and ripeness uniform while selecting fruit samples, as it is well reported that different maturity stages vary in terms of the content of phenolic compounds. A study on highbush blueberry (*Vaccinium corymbosum* L.) showed that TPC and AC in unripe green and fully ripe fruits was higher in the former and lower in the latter (Ribera et al., 2010). It is also reported that fruit size is an important factor for phenolic compounds and AC. In a recent study, it was found that plum skin possessed higher TPC than pulp and whole fruits (Cosmulescu et al., 2015). Similar results were

shown in highbush blueberry, where the skin had six times higher AC than whole fruits (Ribera et al., 2010). Smaller fruits have more surface area (skin) than larger fruits, which can influence TPC and AC. Therefore, it is possible that fruit size selection in our study may have influenced TPC and AC. The environmental factors under which we collected lingonberry fruits were uncontrolled, which may also influence the experimental results. Our results need to be verified with investigations that use replicated sites under controlled experimental conditions, such as in a greenhouse, to see which environmental factors influence TPC and AC the most. Our study however, provides good baseline information for these kinds of experiments to follow and compare, since biochemical synthesis in plants in the wild are often unknown. In the present study we only measured TPC and AC of lingonberry fruits, and measurement of individual phenolic compounds can provide better insight on the direction and magnitude of the environmental effects.

3 Chapter three: Bioinformatics Analysis

3.1 Introduction

Plants are an important source of food, fibers, construction material, renewable energy, and important resource for bioremediation. Researchers are trying to manipulate plant genetic resources at the molecular level for the production of transgenic plants, molecular breeding, genome mapping, and for obtaining biopharmaceuticals. To conduct genetic engineering, plant genomes (i.e the complete set of DNA of an organism, including all of its) and their variations have to be analyzed.

3.1.1 Molecular controls of phenolic content in plants

The phenolic content of plants depends both quantitatively and qualitatively on their genetic information (Tomas-Barberan and Espin, 2001). It has been shown that phenolic content differs among species from the same genus or/and among different cultivars of the same species. For example, individual citrus species produce a characteristic flavanone profile that can be used to know the origin of processed citrus products (Garcia-Viguera et al., 1993). It has been reported that total antioxidant capacity and total phenolic content of blueberry progenies, which includes anthocyanin is moderately to highly heritable (Connor et al., 2002b; Scalzo et al., 2008; Routray and Orsat, 2011). Phenolic content in apple also differs among cultivars. It has been shown that red apple accumulates more anthocyanin and other phenolic compounds than yellow apple (Slatnar et al., 2012; Kalinowska et al.,

2014). Susceptibility to browning in apple differs among cultivars, which is correlated to the synthesis of phenolic compounds and polyphenol oxidase (PPO) activity (Amiot et al., 1992). Genomic DNA cloning has been done for PPO and peroxidase (POD) to better understand their functional activity on different cultivars and leaf developmental stages, and to develop a novel strategy for preventing browning (Steffens and Zabeau, 1994). The expression of POD and PPO genes has been studied to obtain cultivars with less susceptibility to browning.

In another example, the effect of the excess and deficiency of phenylalanine ammonia lyase (*PAL*) activity has been studied in tobacco plants showing that down-regulation of the phenylpropanoid biosynthesis pathway, by suppressing the accumulation of transcripts encoded by *PAL* genes, caused the appearance of a series of unusual phenotypes, including altered leaf shape and texture, reduced lignification, stunted growth, altered pigmentation and flower morphology, and reduced pollen viability (Kawamata et al., 1997). Leaves of *PAL*-overexpressing plants contained increased levels of the hydroxycinnamic acid ester, chlorogenic acid, but not of the flavonoid rutin, indicating that *PAL* is the key control point for flux into chlorogenic acid (Howles et al., 1996). In addition, levels of glucoside of 4-coumaric acid increased in the overexpressing plants, suggesting that the 4-coumarate coenzyme A ligase or coumarate hydroxylase reactions might have become limiting (Howles et al., 1996). It has been well studied that modification of the phenolic biosynthesis pathway can lead to the inhibition or increase of the phenolic properties of plants. The use of prohexadione-calcium or phostrade-Ca, a growth regulator, can inhibit or induce the anthocyanin biosynthesis in apple fruits by modifying the flavanone 3-hydroxylase (FHT) pathway in phenol biosynthesis. This can increase or

decrease the susceptibility of the plant to apple scab (Bizjak et al., 2013a; Bizjak et al., 2013b).

Phenolic compound biosynthesis genes have been identified in different crops, this information could aid breeding programs specifically oriented towards the increase phenolic compounds and antioxidant activity. Jaakola et al. (2002) identified genes involved in phenolic biosynthesis under different developmental stages of bilberry fruit, and traced their activities. Kaga et al. (2012) conducted an experiment to characterize 1603 soybean samples from Japan and Asia using 191 SNP markers, which shows a clear genetic variation among Japanese soybean based on population diversification and clustering analysis. A follow up by Phommalath et al. (2014) of the Kaga et al. (2012) results showed a wide genetic variation in phenolic compounds contents of black soybean seed coat. It has also been found that, in wheat, nine phenolic compound biosynthesis pathway genes exhibited three distinct expression patterns during grain filling, which may be related to different levels of phenolic compounds (Ma et al., 2016). Several of the phenolic compound biosynthesis enzyme-encoding genes are present as small gene families in plants (Huang et al., 2010). For example, the *PAL* gene family comprises one to five genes in most plant genomes (Huang et al., 2010). The variation in gene sequence and copy number may act as a source of variation in the phenolic compound output in different species. It was also reported that the expression of the *PtPAL1* gene isolated from *Populus tremuloides* varied with condensed tannins accumulation along with primary and secondary growth. However, the *PtPAL2* gene was involved in lignin biosynthesis (Kao et al., 2002). The 4-coumarate: coenzyme A ligase (*4CL)* genes found in plants are categorized into class I and class II. Most of the *4CL* genes belong to class I, which are generally known for their function in

lignin biosynthesis, while class II are more likely to biosynthesize flavonoids (Wagner et al., 2009). The phylogenetic analysis of *4CL* genes expressed in the stem of silver birch showed that two of them belong to class I and one *4CL* gene belongs to class II (Sutela et al., 2014). Extensive analysis of duplicated gene copies is thus essential for elucidating the species-specific complexities of phenolic acid metabolism.

3.1.2 Molecular markers measure genetic diversity

Plant biologists have learned that genetic diversity is an important asset for humanity. The presence of genetic variation within plant species is of great importance because it facilitates the sustainable genetic improvement of crops, and it permits adaptation under various environmental conditions (Govindaraj et al., 2015). Knowledge of the molecular basis of essential biological phenomena in plants is important for improving plant adaptation, conservation actions, management, and utilization of plant genetic resources. Plant domestication is a long process and depends on a continuous mutation with wild relatives, varieties and use of modern breeding techniques (Breseghello and Coelho, 2013). To select and improve plant varieties, an assessment of the species genetic diversity is required. Different molecular laboratory techniques are currently used to assess the genetic diversity within and among populations.

Gregor Mendel used phenotype-based markers in his experiments. Although phenotype-based marker selection was popular among conventional plant breeders, it was a time consuming process. This limitation of phenotype-based markers led to the development of genetic-based markers. The molecular basis of different biological

functions in plants can be understood through an analysis of genetic variation. Molecular markers are the biological features that are determined by allelic forms of genes or genetic loci and can be transmitted from one generation to another (Jiang, 2013). The development and use of molecular markers for the study of DNA polymorphism is one of the most significant developments in the field of molecular genetics. These marker identification techniques can identify genetic variation ranging from single nucleotide variation (SNV) to complete duplication or complete deletion of a chromosome (Hu et al., 2015).

In the 1980's different kinds of molecular makers such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphism (RAPD), simple sequence repeat (SSR), sequence characterized amplified region (SCAR), cut/cleaved amplified polymorphism (CAP) were used because of their accuracy, reproducibility, repeatability, speed and inexpensive development (Idrees and Irshad, 2014). In addition to the molecular markers developed in the 80's, more recent techniques include high throughput sequencing for the identification of single nucleotide polymorphism (SNP). These techniques were and are intensively used in population genetics, and in the ecological, evolutionary, taxonomical, and phylogenetical study of plants (Agarwal et al., 2008).

3.1.3 Single nucleotide polymorphism and genotyping-by-sequencing

SNPs are the most abundant source of genetic variation within the genome and are linked to heritable differences between two individuals. A SNP is a variation at a single base pair in a DNA sequence among individuals (Brookes, 1999). SNP variation occurs everywhere in the genome including coding and noncoding regions of genes, regulatory regions and so on. If this variation occurs in the coding region of a gene, then the gene is described as having more than one allele (Hrdlickova et al., 2014). This kind of SNPs may cause variation in the amino acid sequence, which might be associated with different important traits. However, not all SNPs may cause a difference in phenotype. Phenotype (trait) SNP associations allow a researcher to think more about this SNP, and evaluate the putative functional consequence of that SNP (Zhao et al., 2011). Researchers can also examine stretches of DNA near SNPs in order to identify the gene or genes responsible for the trait. A genome-wide polymorphism database of rice has been constructed which shows approximately 1 SNP per 268 bp (Shen et al., 2004). SNP frequency varies from 3 SNPs per 1000 bp (1kb) in coding sequences to 27.6 SNPs per kb in transposable elements, with a genome wide measure of 1 SNP per 66bp (Oraguzie et al., 2007). Frequency of SNPs in barley varies from 1 in 130 bp to 1 in 2 kb, and are significantly more frequent than the estimated average for plant genomes of 2 SNPs per kb (Pattemore, 2011). SNPs are important markers for the construction of genetic and physical maps, genome sequencing,

and marker-assisted selection (You et al., 2011). SNPs found in promoters and expressed regions that affect the function of a gene are considered good markers (Paris et al., 2003).

Today several methods are available to discover SNPs and the choice of methods depend on the scale of the study and question to be answered. Most of the SNPs genotyping technologies have been developed based on hybridization methods such as southern hybridization, allele specific oligonucleotide (ASO) probes, and polymerase chain reaction (PCR). In the 1990's primer extension technique known as minisequencing was introduced for SNPs genotyping (Minnehan, 2010). Today, there are several high-throughput SNP genotyping techniques available with the capacity of rendering several million genotypes per day. It is proven that next generation sequencing (NGS) is a very effective tool for large scale, genome wide discovery of markers including SNPs (Altshuler et al., 2000). Several studies in plants have applied NGS techniques to achieve high sequencing coverage of the genome, which is a prerequisite for high-quality genome-wide SNP discovery (You et al., 2011).

The advances in high throughput and continuously decreasing cost of sequencing technologies led to the development of a genome-wide SNP genotyping method called Genotyping-by-Sequencing (GBS) (Elshire et al., 2011). GBS is a fairly recent technique applicable to species with high diversity and large genome sizes using restriction enzymes (REs) (Elshire et al., 2011). The advantage of GBS is that it reduces genome complexity through an enzyme-based genomic complexity reduction step to attain high-density SNP discovery and genotyping. It is suitable for trait mapping in diverse populations, genetic diversity, breeding, population studies, genomic selection, molecular marker discovery, and germplasm characterization (Elshire et al., 2011). Among the advantages of using GBS

are its ease, speed, specificity, reduced sample handling, and fewer PCR and purification steps producing fewer technical errors that bias sequencing of certain DNA regions. GBS can be performed either through a reduced representation or a whole genome sequencing approach. The choice to sequence the whole genome or a reduced portion of it is generally dictated by several factors, including repetitive content, ploidy, and presence or absence of homologs (Deschamps et al., 2012). The reduced representation sequence-based marker discovery technique has been successfully used in important cereal crops including wheat and barley to develop high-density genetic maps (Elshire et al., 2011; Poland et al., 2012). SNP detection in plants is challenging because of their large genome size and polyploidy nature. However, double restriction endonucleases like *MspI* and *PstI* are used to generate digested fragments whose ligation to universal adapters allowed for the specific amplification of PstI-MspI digested DNA fragments (Deschamps et al., 2012).

3.1.4 Genetics of *Vaccinium vitis-idaea*

Lingonberry (*Vaccinium vitis-idaea* spp. minus, Ericaceae) is a perennial evergreen shrub distributed in temperate and boreal regions of North America. The demand of lingonberry at the international market is increasing because of its nutritional value and health beneficial properties. However, this berry crop is poorly exploited commercially in North America. For example, the average harvest of lingonberry in Newfoundland is around 96,500 kilograms per year, which is only 10% of its total wild production (Penney et al., 1996). Producers cannot meet the lingonberry demand at the local nor international markets because fruit harvest is manual from the wild. For commercial cultivation,

selection of good quality fruit and its improvement are necessary. Plants with high-quality characteristics can be identified by their phytochemical content or phenotypic traits, and by genotyping methods, whereby the detected molecular polymorphisms are correlated to phenotypic traits of interest (Zoratti et al., 2015). Unfortunately, very little research has been done on lingonberry genetics. A study on the genetic diversity of 15 lingonberry populations from seven countries showed a pronounced geographical grouping in most cases, and a significant correlation between geographical and genetic distances for all populations (Garkava-Gustavsson et al., 2005). A study of 129 plants from two lingonberry populations collected in Sweden identified 29 different genotypes using RAPD technique (Persson and Gustavsson, 2001). Inter simple sequence repeat (ISSR) variation has been widely used for genetic diversity studies of wild *Vaccinium* species mainly *V. vitis-idaea* (Debnath, 2007). In another study of 43 wild lingonberry clones collected from four Canadian provinces showed a substantial degree of genetic diversity using ISSR-based markers. Geographical distribution of these wild clones could explain 10% of total genetic variation (Debnath, 2007). However, the drawback of ISSR markers is that the primer designed to anneal to the di- or trinucleotide repeats can lack specificity in the PCR causing a lack of reproducibility (Potter et al., 2002).

To date, the complete nuclear genome or the plastome of *V. vitis-idaea* have not been sequenced. Researchers at the Plants for Human Health Institute at North Carolina State University are sequencing the complete genome of *V. corymbosum* (blueberry) but the results were not publicly available at the time this research was conducted. Researchers at the USDA, Agriculture Research Service, Genetic Improvement of Fruits and Vegetables are sequencing the complete genome of American cranberry (*Vaccinium macrocarpon* Ait.)

(Polashock et al., 2014). The genome size of lingonberry is unknown, but it was reported that the genome size of the diploid American cranberry is about 470 Mbp (Polashock et al., 2014) and the size of the haploid blueberry (*Vaccinium corymbosum*) is approximately 500 Mbp (Bian et al., 2014). Ploidy level of lingonberry is diploid and tetrapoid.

3.1.5 **Research objectives**

Among the molecular marker techniques mentioned before, we chose to discover SNPs discovery using GBS since this method is relatively inexpensive and scans the entire genome for DNA differences. In the present study we address the following objectives:

I. to discover SNPs among 56 lingonberry samples from Newfoundland and Labrador,
II. to assess which SNP (if any) is correlated with total phenolic, antioxidant capacity, and environmental variables.
III. to annotate functionally the reads with SNPs which can elucidate which gene regions might play a role in the differential phenolic compound content and different environmental conditions, as well as other plant metabolic functions.

3.2 Materials and Methods

The workflow for SNPs discovery in lingonberry was as follows (Figure 3.1).

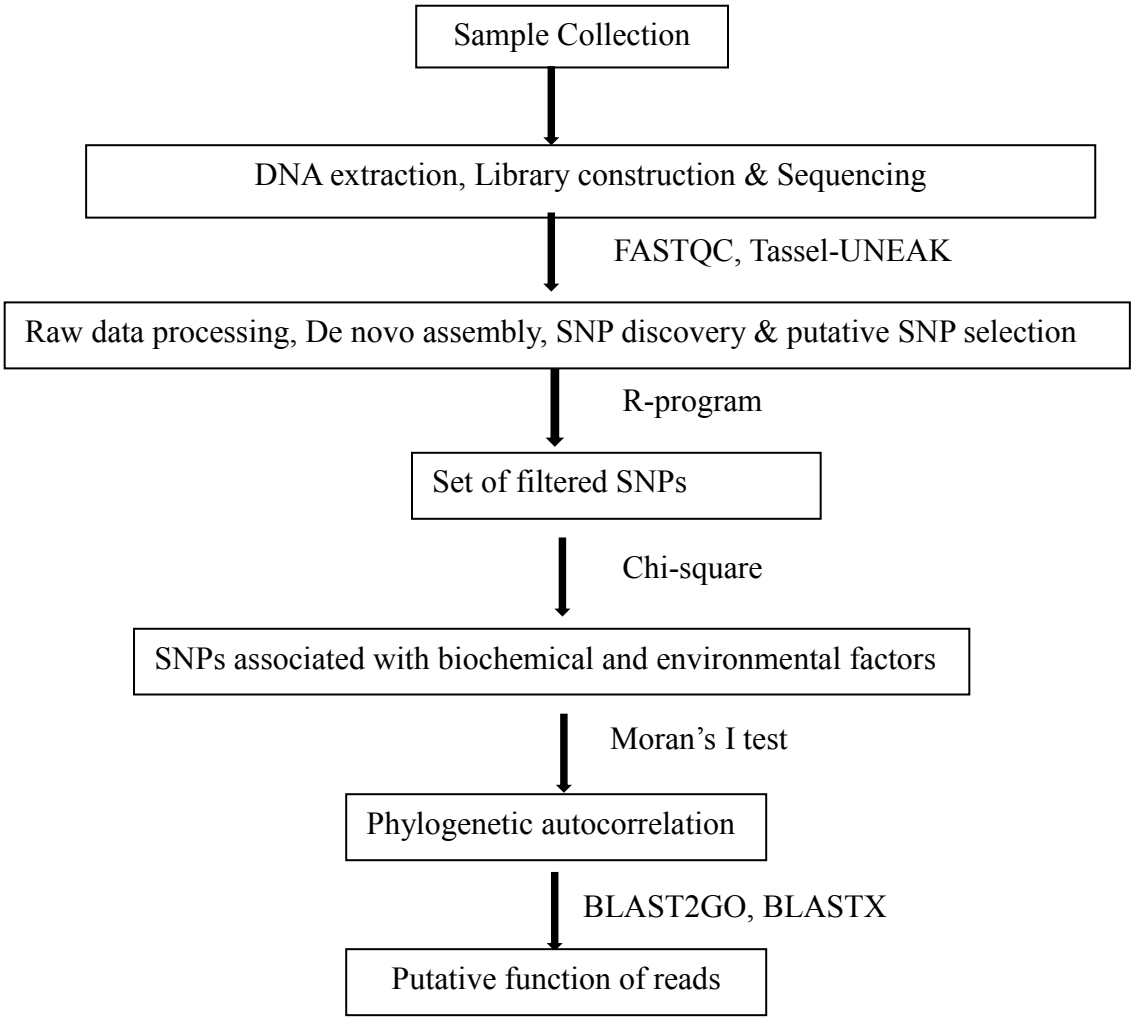


Figure 3.1 Bioinformatics pipeline for SNPs discovery and functional annotations

3.2.1 **Sample collection, DNA extraction and quantification**

Lingonberry leaves were collected for DNA extraction from 56 sites in Newfoundland and Labrador in 2014 (Appendix 1). To avoid variation, leaf samples were collected from healthy adult plants at each sampling site. Leaves were stored in silica gel to dry the plant material.

Genomic DNA isolation was done using DNeasy Plant mini kit (Qiagen, cat. no. 69181; Valencia, CA, USA) following the manufacturer's protocol with some modifications. Approximately 45 mg of leaf material was ground to a fine powder using TissueLyserLT (Valencia, CA, USA) and to which 600 µL of buffer AP1 and 4 µL of RNase were added and subsequently thoroughly mixed. The solution was incubated for 10 minutes at 65 ºC while mixing 2-4 times. Approximately 195 µL of P3 buffer were added to the lysate and incubated for 5 minutes on ice. The lysate was then centrifuged for 5 minutes at 14,000 rpm (18000 g), transferred the lysate into the QIAshredder mini spin column, and centrifuged it again for 2 minutes at 14,000 rpm (18000 g). The lysate was collected in a new Eppendorf tube and to the cleared lysate added 1.5 volumes of buffer AW1 were added and mixed by pipetting. The mixture was pipetted into a DNeasy mini spin column placed in a 2 mL collection tube and then centrifuged for one minute at 8,000 rpm (9900 g). The lysate was discarded and placed the DNeasy mini spin column into a new collection tube. Five hundred microliters of buffer AW2 were then added, centrifuged for one minute at 8,000 rpm (9900 g), the flow was discarded and again 500 µL of buffer AW2 were added on the DNeasy mini spin column and centrifuged for two minutes at 14,000 rpm (18000 g). The DNeasy mini spin column was transferred to a 1.5 mL

Eppendorf tube, and 30 μL of EB buffer were added directly to DNeasy mini spin column. The incubate was allowed to stand for 15 minutes at room temperature and then centrifuged for one minute at 8,000 rpm (9900 g) to collect the eluate. The genomic DNA was placed immediately on ice and stored at -20 ºC. The total quantity of the isolated genomic DNA was determined using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) and NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). DNA quality was assessed visually by running 5uL of the extraction on a 1% agarose gel in 1.0X Tris/Acetate/EDTA (TAE) buffer at 115V for 30 minutes to ensure a lack of degradation. DNA concentration of each individual was standardized at 10 ng/μL in EB buffer.

3.2.2 Library construction and Genotyping-by-Sequencing

A total of 100 ng of genomic DNA from each individual in 10 μL of EB buffer was sent to University of Laval's library construction service. The library was constructed using a genotyping-by-sequencing (GBS) approach. The basic scheme of the GBS protocol is shown in Figure 3.2 (Elshire et al., 2011). GBS libraries were prepared for the 56 genomic DNA samples using a double-digest restriction protocol with enzymes Pstl and Mstl as described in Poland et al. (2012). The Pst1 overhang corresponds to the barcoded forward adapter, Adapter 1, and the Msp1 overhang corresponds to the common Y-adapter, Adapter 2 (Poland et al., 2012).

The University of Laval designed a set of 56 barcoded adapters with different nucleotide lengths (4-7 bp). Genotypes were multiplexed with no biological replication.

Barcode and common adapter were diluted in TE, annealed in a thermocycler and quantified using an intercalating dye and diluted in water.

A mixture of each adapter was pipetted into a 96-well PCR plate. In each well, total genomic DNA was digested using a 20 ul reaction volume of NEB Buffer 4 with PstI (High-Fidelity) and MspI (New England BioLabs Inc., Ipswich, MA). The digestion was conducted at 37°C for 2 h and then at 65°C for 20 minutes to inactivate the enzymes in a Bio-Rad Dyad Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, CA).

The ligation reaction was carried out in the same plate to ligate the adapter with individual samples using a mixture of NEB Buffer 4 (1× final), ATP (1 mM final), 200 U T4 ligase (NEB T4 DNA Ligase #M0202), and water. The ligation master mix was mixed with the restriction digest, Adapter 1, and Adapter 2. The ligation was completed at 22°C for 2 hours and at 65°C for 20 minutes in a Bio-Rad Dyad Peltier Thermal Cycler.

A 56-plex library was prepared by pooling all the ligated samples into a single tube. PCR reaction was performed for the ligated library using NEB MasterMix (5 µl), 10 µM Illumina F and R (5 µl each) primers, and water (8 µl) with the following conditions: 95°C for 30 seconds; 16 cycles of 95°C for 30 seconds, 62°C for 20 seconds, and 68°C for 90 seconds; and 72°C for 5 minutes. PCR amplified libraries were pooled, purified, and re-suspended in Buffer EB. The fragment size of samples was evaluated using electrophoresis. The 56-plex libraries were sequenced using Illumina Hiseq2500, adopting the methods for standard single end sequence (100 bp) preparation protocol on a single lane at McGill University-Génome Québec Innovation Center in Montreal, Canada. A table of the 56 genotypes used in this analysis along with their assigned barcodes can be found in Table 3.1.

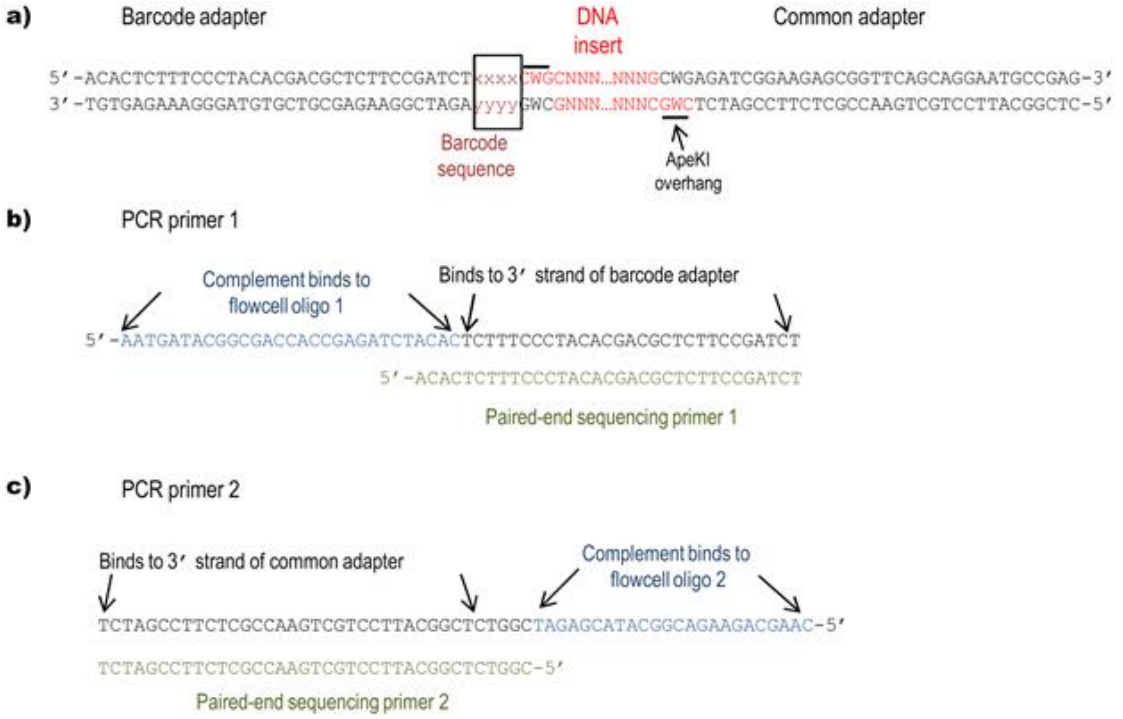


Figure 3.2 GBS adapters, PCR and sequencing primers

(a) Sequences of double-stranded barcode and common adapters. Adapters are shown ligated to ApeKI-cut genomic DNA. Positions of the barcode sequence and ApeKI overhangs are shown relative to the insert DNA; (b) Sequences of PCR primer 1 and paired end sequencing primer 1 (PE-1). Binding sites for flowcell oligonucleotide 1 and barcode adapter are indicated; (c) Sequences of PCR primer 2 and paired end sequencing primer 2 (PE-2). Binding sites for flowcell oligonucleotide 2 and common adapter are indicated (reproduced from Elshire et al., 2011).

Table 3.1 Illumina single end short read summary of each *Vaccinium vitis-idaea* sample. GBS ID=genotyping-by-sequencing identification. Sampling location ID as in appendix 1

GBS ID	Sample ID	Barcode	Total no. Of reads	Good barcode reads
VF0104	4	CAGA	2002830	1630030
VF0105	5	AACT	2652423	2197993
VF0112	12	TGCGA	4263160	2533624
VF0113	13	CGCTT	6025458	3414788
VF0114	14	TCACG	5051455	4715386
VF0115	15	CTAGG	1173382	893288
VF0116	16	ACAAA	2619973	2079727
VF0221	21	ACCGT	2846141	1595540
VF0222	22	GCTTA	2562129	1731669
VF0223	23	GGTGT	2305724	1482797
VF0224	24	AGGAT	3503462	2345697
VF0226	26	CATCT	2560094	2058102
VF0227	27	CCTAG	1528113	1233045
VF0228	28	GAGGA	1951255	1159512
VF0252	52	GTACTT	6041356	3531737
VF0256	56	TATTTTT	5735437	4766056
VF0317	17	TTCTG	1867008	1203405
VF0318	18	AGCCG	1457394	1144130
VF0329	29	GGAAG	1226691	820649
VF0330	30	GTCAA	1820456	1216929
VF0331	31	TAATA	4603534	3624200
VF0332	32	TACAT	4060114	3258419
VF0401	1	CTCG	2276595	1566887
VF0402	2	TGCA	1683886	1270173
VF0403	3	ACTA	1646706	1415262
VF0406	6	GCGT	2391988	1725397
VF0425	25	ATTGA	1900481	1024365
VF0454	54	TAACGA	3887627	3289283
VF0533	33	TCGTT	3977516	2388448
VF0534	34	GGTTGT	2587034	1365408
VF0535	35	CCACGT	1630613	992043
VF0536	36	TTCAGA	948094	732396
VF0537	37	TAGGAA	2131484	1268827
VF0541	41	GAGATA	1818652	1286026
VF0542	42	ATGCCT	1518961	1163134
VF0619	19	GTATT	2098182	1217959
VF0620	20	CTGTA	2072330	1444016
VF0638	38	GCTCTA	988001	733174
VF0639	39	CCACAA	1659342	1415392
VF0640	40	CTTCCA	1542618	1239516
VF0643	43	AGTGGA	2930853	1770780
VF0707	7	CGAT	1938608	1251868
VF0708	8	GTAA	2361182	1371867
VF0709	9	AGCG	1391194	1043773
VF0710	10	GATG	3194743	1861042

VF0711	11	TCAG	1895650	1255021
VF0844	44	ACCTAA	1434036	1132533
VF0850	50	GCCAGT	4863468	3555122
VF0851	51	GGAAGA	2419714	1854652
VF0853	53	GTTGAA	1940748	1292224
VF0855	55	TGGCTA	1482273	1069896
VF0945	45	ATATGT	2907497	1820486
VF0946	46	ATCGTA	2239527	1368738
VF0947	47	CATCGT	1905492	1610722
VF0948	48	CGCGGT	1886443	889457
VF0949	49	CTATTA	2649803	2025306

3.2.3 **Processing of Illumina Raw Data**

The raw Illumina data were processed following the protocol outlined in Poland et al. (2012). The quality of the raw Illumina DNA reads was investigated using FASTQC (Banraham Bioinformatics, Cambridge, England) for per base sequence quality, per sequence quality scores, per base sequence content, per sequence GC content and sequence duplication levels. The high quality FASTQ sequence reads generated from Illumina were de-multiplexed according to their unique barcodes, allowing one mismatch in their barcode to still be consider as good reads. The adapter/barcode sequence were removed using the Fastx-toolkits, Fastx_barcode_spliter (Hannon Lab, Cold Spring Harbor Laboratory, USA).

3.2.4 **De novo assembly and SNP discovery**

The recent development of TASSEL-UNEAK pipeline (Lu et al., 2013) gives an opportunity to analyse large scale GBS data and genome wide SNP discovery without a reference genome. All single-end raw sequences obtained from the GBS libraries were processed using the Network-Based de novo SNP discovery protocol with non-reference genome (TASSEL-UNEAK v.3.0) to identify accurate and high quality SNPs (Lu et al.,

2013). The TASSEL-UNEAK pipeline was run using the following six Plugins 1) UQseqToTagCountPlugin, 2) UMergeTaxaTagCountPlugin, 3) UTagCountToTagPairPlugin, 4) UTagPairToTBTPlugin, 5) UTBTToMapInfoPlugin and 6) UMapInfoToHapMapPlugin.

In the Universal Network Enabled Analysis Kits (UNEAK), the multiplexed Illumina reads were sorted out into unique sequence tags by compiling exactly matching reads with at least ≥3X read depth observed in the corresponding FASTQ file using UQseqToTagCountPlugin. The sequences were trimmed to 64 bases including the corresponding restriction enzyme site and barcode to eliminate chimeric sequences. All FASTQ information was converted to bit format to reduce the amount of storage space and to enable relatively fast computation. A taglist (identical reads are classified as a tag) was created for each sample and merged all taglist files into a single tag file using UMergeTaxaTagCountPlugin. To reduce the rare or singleton tag from sequencing error, the taglist was sorted out with ≥5X tag depth. A pairwise alignment was conducted by allowing 1bp mismatch using UTagCountToTagPairPlugin. Tag Pairs with 1bp mismatch were considered as putative SNPs. A homology network was constructed by joining all of the tags that differed by a single base, and then a network filter (Figure 3.3) is applied to find the reciprocal tag pairs (Lu et al., 2013). UNEAK uses a network filtering which is the key step for identifying and removing paralogs. In this filtering, a network was created between nearly matching tags and rare tags considering one bp mismatch, and filtered out the complex network based on a sequencing error tolerance rate (ETR, Lu et al., 2013). ETR is an important parameter to call SNPs. Lu et al. (2013) reported that the frequency of most sequencing errors is less than 3%. Therefore, the ETR was specified as 0.05 in this

study, allowing reciprocal tags to be called with 5% sequence divergence. Tags that pass through the UNEAK filtering process are used to analyze the distribution of tags in each sample using UTagPairToTBTPlugin. The tags were then sorted out in order, assign genotypes to each sample and convert to HapMap using UTBTToMapInfoPlugin. The HapMap file is then filtered out using the default minor allele frequency (0.05) and a maximum allele frequency (0.5) using UMapInfoToHapMapPlugin (see figure 3.3 for network filtering procedure) to call SNPs. The HapMap file with SNPs was used for further analysis.

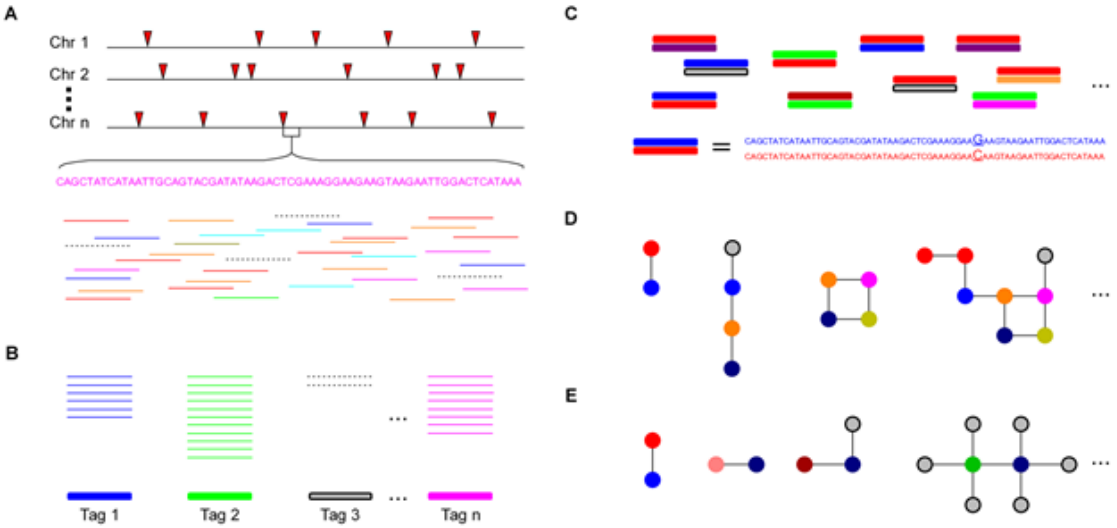


Figure 3.3 The analytical framework of UNEAK

(A) Multiple DNA samples are digested and sequenced using GBS (red arrows represent cut sites). The inputs of UNEAK are Illumina Qseq or Fastq files. All of the reads are computationally trimmed to 64 bp. The solid colored lines represent error-free ("real") reads, while the dashed lines are reads containing one or more sequencing errors. (B) Identical reads are classified as a tag. The colored bars are real tags, whereas the shaded bar is a rarer error tag. (C) Pairwise alignment is performed to find tag pairs differing by only a single bp mismatch. (D) Topology of tag networks. The colored circles are real tags. The shaded circles are error tags.

Lines ("edges") are drawn only between tags that differ by a single bp mismatch. (E) Only reciprocal, real tag pairs are retained as SNPs (Lu et al., 2013).

3.2.5 SNPs Filtering

SNPs generated through TASSEL-UNEAK pipeline need filtering to reduce the amount of false variable sites. PCR error and sequencing error frequently cause false SNP calling. A set of strict filtering parameters was employed to increase SNP quality. Using a custom R script, putative SNPs were filtered out by setting the minimum coverage threshold at 3X, minimum minor allele frequency at 0.30 and accepted only those SNPs for which genotypes were called for at least 50% of the samples.

3.2.6 SNPs associated with biochemical and environmental factors

The procedure for lingonberry fruit sample collection and measurement of TPC and AC was described in Chapter 1, and results on the variation of these biochemical variables according to different environmental conditions were discussed on chapter one, sections 1.3 and 1.4. The identified putative SNPs were analysed for a significant association with TPC, AC, ecoregion and seven other environmental variables defined in chapter one. TPC of lingonberry fruit was categorized into three groups, low (< 650 mg GAE/100g FW), medium (650-850 mg GAE/100g FW) and high (> 850 mg GAE/100g FW). AC of fruit was categorized into two groups, low (<650 mg TE/100g FW) and high (> 650 mg TE/100g FW). A Chi-square test (McHugh, 2013) calculating p-values by Monte Carlo simulation

(10,000 replicates) was conducted to identify any significant association of SNPs with each of the variables (ecoregion, seven environmental variables, TPC and AC). A p-value of 0.01 was used to call significant associations between SNPs and each variable.

3.2.7 Phylogenetic autocorrelation with respect to biochemical and environmental factors

Identified SNPs by Chi-square test were used to construct a phylogenetic tree. A distance matrix of the identified putative SNPs were calculated using the function dist.gene available in the R package 'ape' (Paradis et al., 2004). In short, the distance between two given samples is the percentage of variables sites (SNPs) with different alleles between these two samples. The resulting distance matrix was used to reconstruct a phylogenetic tree based on neighbour joining using the R library 'ape' (Paradis et al., 2004). Support values for each tree were obtained by Bootstrap analysis using 1000 replicates. Furthermore, a Moran's I test (Paradis, 2016) was performed using the dist.gene to analyze whether a given biochemical or environmental variable has a significant phylogenetic autocorrelation using a cutoff p-value of <0.05. If the observed Moran's I value is significantly greater than the expected I value, then it is considered as positively autocorrelated. Whereas if the observed Moran's I value is smaller than the expected I value, then it is negatively autocorrelated (Paradis, 2016).

3.2.8 **Putative function of reads with SNPs**

For determining the putative functions of filtered SNPs, the HapMap file containing read sequences for each of the filtered SNPs was used. To obtain a putative functional annotation of the genomic regions were SNPs were found, we performed BLAST searches against the NCBI non-redundant (NR) protein database using BLAST2GO PRO (Conesa et al., 2005) with an e-value of 0.001 (e-value<= 0.001). The first step was to find proteins with the highest sequence similarity to our query sequence and retrieve them for further analysis. The next step was the InterPro function, within BLAST2GO, which provides a functional analysis of proteins by classifying them into families and predicting domains. Mapping was performed to retrieve gene ontology (GO) terms associated to the hits obtained after a BLAST search. An annotation process was performed to assign functional terms to query sequences from the pool of GO terms gathered in the mapping step. Function assignment is based on the GO and annotation score. The BLAST2GO provide functional (GO term) annotation for very few SNP containing genomic regions. Therefore, we decided to perform a BLASTX search against the non-redundant protein database using the same parameters as before. The identified gene accession numbers were uploaded to Batch Entrez to retrieve gene annotation. A functional annotation of the identified protein was performed based on a literature search.

3.3 Results

3.3.1 Processing the Illumina raw data, de novo assembly, SNP discovery and selection

For this study, a single lane of Illumina HiSeq 2000 generated a total of approximately 144 million reads 100 bp long (Table 3.2). All low quality reads with more than one mismatch and/or with unreadable bases ('N') on each barcode were discarded. Approximately 98.53 % (142,058,930 reads) reads were separated with none or one mismatch in each barcode using Fastx-tool kits. A quality analysis using FastQC, reported that the average Phred quality score was 34, and % GC content was 53.

After discarding the reads with more than one mismatch, average number of reads per sample was about 2.53 million, but this number was highly variable among samples (~0.94 to ~6.02 million reads, Table 3.2 and Figure 3.4). The UNEAK-TASSEL pipeline was run to trim the reads to 64 bp and discard the low quality reads. After trimming, analysis of GBS data with the UNEAK pipeline retained ~99,347,916 sequences devoid of ambiguities. These reads were grouped into ~1,288,835 distinct total tags, of which ~79,529 tag pairs were supported by more than five reads. Nearly 76,529 reciprocal tag pairs that differ by a single nucleotide and ETR of more than 0.05 were identified, including 76,354 SNP loci putatively located at a single locus. A subset of 75,555 SNP loci with minor allele frequency (MAF) of 0.05 were identified. Using an in-house R program, a set of 1,586 SNPs was identified and selected for further genetic characterization analysis (Table 3.2). Each of these selected SNPs has a read depth coverage of at least 3X was

genotyped in at least half of the sample and its minor allele frequency was greater than 0.30.

Table 3.2 Summary of Genotyping-by-Sequencing data from 56 lingonberry samples using Illumina HiSeq 2000 and analyzed in TASSEL-UNEAK

Illumina sequencing and UNEAK analysis	Number
Total reads	144’179,623
Total reads with at most one mismatch in the barcode	142’058,930
Total reads with barcodes without “N”	139’409,127
Total tags	1’288,835
Good tags (≥5X tag depth)	228,809
Tag Pairs	79,529
Reciprocal tag pairs (ETR ≥0.05)	76,529
SNP loci at a single locus	76,354
SNP loci (MAF 0.05)	75,555
SNP loci (with coverage above 3X, frequency above> 0.5 and minor allele frequency above> 0.30)	1,586

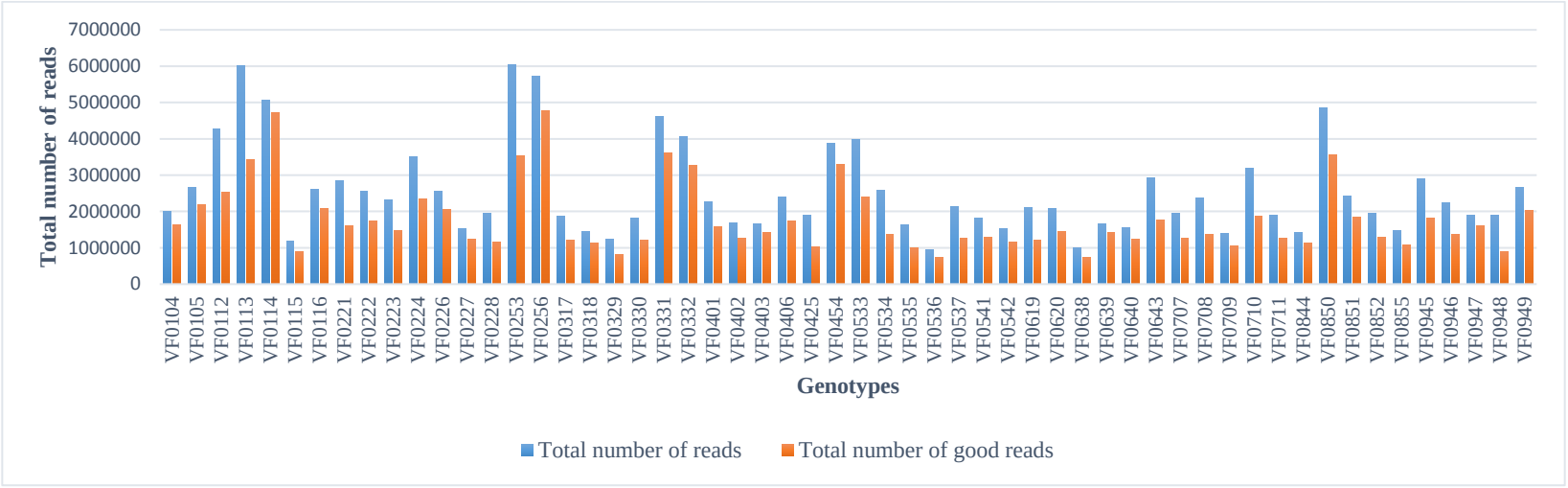


Figure 3.4 Total number of reads and total number of good reads (i.e. with at most one mismatch and no "N" in the barcode) for each of the 56 *Vaccinium vitis-idaea* genotypes.

3.3.2 Significant association of SNPs with environmental variables and biochemical properties

The Chi-square statistical test identified a total of 260 SNPs significantly associated with biochemical and environmental variables. The maximum number of associated SNPs (44) was identified for the variable ecoregion, and the minimum for the variable TPC with 11 SNPs (Table 3.3). It was also observed that some of the SNPs were correlated with more than one variable (Table 3.4). Ecoregion shared SNPs with all other environmental variables, while the biochemical variables did not share SNPs between them or with any other environmental variable.

3.3.3 Phylogenetic autocorrelation with respect to biochemical and environmental factors

In our study, the phylogenetic trees reconstructed using the associated SNPs showed some clustering of individuals with respect to environmental and biochemical variables (Figures 3.5-3.14). The 21 SNPs that were significantly associated with mean annual mean temperature were useful to group 27 out of 32 individuals growing under 4.0 to 7.9°C (Figure 3.5). This indicates that samples growing on similar mean annual temperature are more genetically similar considering these 21 SNPs than samples growing on different mean annual temperature. Individuals from the Central Newfoundland Forest, Northern Peninsula Forest, Maritime Barrens, Eastern Hyper Oceanic Barrens and Forteau Barrens formed clusters using the 44 SNPs associated with ecoregion (Figure 3.6). The 26 SNPs

that were significantly associated with mean summer temperature were useful to group 14 out of 23 individuals growing under 12-13.9°C and two groups of 11 and 7 individuals growing at 14-19.9°C (Figure 3.7). The phylogenetic tree for mean annual precipitation showed a group of 10 out of 13 individuals growing in high (1300-1500 mm) annual precipitation (Figure 3.8). The 18 SNPs significantly associated with coastal proximity did not cluster individuals into coastal and inland (Figure 3.9). We observed from the phylogenetic tree of mean annual runoff, that seven out of eight individuals (grey) in the runoff category 1800-2200mm clustered together (Figure 3.10). Newfoundland and Labrador was categorized into four surface water pH zones; 10 out of 15 individuals from pH zone < 6.1 formed a cluster (Figure 3.11). SNPs were useful to cluster 10 out of 13 individuals growing under extremely sensitive (<60 uel/L) to acid rain conditions (Figure 3.12). TPC did not show any clear clustering of individuals to their corresponding groups (Figure 3.13). However, 11 out of 16 individuals with AC higher than 650 mg GE/100g FW formed a group, and 32 out of 38 individuals with AC less than 650 mg GE/100g FW appeared in three groups (Figure 3.14).

In our study, Moran's *I* statistics indicated a positive phylogenetic autocorrelation (p-value < 0.05) for MAP ($I_{Observed}$=-0.0166, $I_{Expected}$=-0.0189) and marginal for MAR ($I_{Observed}$=-0.0168, $I_{Expected}$=-0.0189).

Table 3.3 List of SNP ID that showed significant association with each environmental and biochemical variables. MAT=mean annual temperature, MST=mean summer temperature, MAP=mean annual precipitation, CF=coastal versus inland, SAR=surface water sensitivity to acid rain, PH=surface water pH, MAR=mean annual runoff, TPC=total phenolic content and AC=antioxidant capacity.

Variable	Total number of SNPs	SNP ID
Ecoregion	44	TP146, TP864, TP2381, TP4025, TP4351, TP6393, TP10396, TP10935, TP15319, TP16158, TP17460, TP18101, TP24645, TP24648, TP25931, TP26282, TP29433, TP31324, TP35894, TP37224, TP37582, TP38879, TP39309, TP39366, TP39539, TP39657, TP39662, TP43227, TP49169, TP51204, TP60283, TP62542, TP65112, TP70998, TP73887, TP75290, TP77042, TP77345, TP77483, TP77640, TP77841, TP77976, TP78753, TP79490
MAT	21	TP146, TP414, TP539, TP2294, TP4351, TP10963, TP15558, TP21266, TP24645, TP29433, TP37224, TP39309, TP49117, TP53183, TP57307, TP60073, TP64029, TP69947, TP72740, TP77042, TP77841
MST	26	TP146, TP575, TP864, TP965, TP3807, TP7981, TP9984, TP14573, TP23015, TP24119, TP24645, TP24776, TP28826, TP43227, TP44612, TP46659, TP52380, TP70833, TP70841, TP70994, TP72738, TP75125, TP75238, TP75290, TP76448, TP77511
MAP	25	TP581, TP2842, TP3585, TP3735, TP11976, TP14604, TP18101, TP25049, TP28717, TP29832, TP40359, TP41989, TP43227, TP44483, TP44612, TP64913, TP66318, TP69829, TP71127, TP73887, TP74902, TP76886, TP77529, TP78719, TP79490
CF	18	TP4696, TP9763, TP11397, TP18101, TP24945, TP30961, TP31453, TP33048, TP45859, TP59955, TP60743, TP61961, TP65693, TP67677, TP72508, TP73401, TP78871, TP78957
PH	39	TP4, TP581, TP3549, TP3569, TP3585, TP3744, TP4351, TP6042, TP7404, TP7428, TP7929, TP10662, TP15810, TP22508, TP24035, TP31324, TP36505, TP38879, TP39309, TP39415, TP42526, TP43228, TP44156, TP45867, TP47066, TP49727, TP50878, TP51204, TP55838, TP60257, TP68101, TP69020, TP72740, TP73811, TP77483, TP77542, TP77607, TP77841, TP79490
SAR	30	TP89, TP3361, TP4351, TP8161, TP9925, TP14721, TP15151, TP15319, TP17155, TP22913, TP37224, TP39415, TP45867, TP47917, TP49727, TP51204, TP53661, TP60257, TP64018, TP70609, TP70716, TP70739, TP70998, TP71043, TP75314, TP77455, TP77483, TP77505, TP78719, TP79120

MAR	43	TP4, TP89, TP581, TP864, TP3549, TP3735, TP3846, TP3966, TP6551, TP10165, TP10460, TP12963, TP13763, TP18028, TP21266, TP23135, TP25049, TP25366, TP29544, TP40359, TP41989, TP44612, TP50294, TP51204, TP53661, TP55824, TP58992, TP60133, TP63576, TP69971, TP70849, TP71702, TP71842, TP74023, TP74922, TP75131, TP76886, TP76974, TP77483, TP77841, TP77852, TP77976, TP78864
TPC	12	TP3361, TP3683, TP4734, TP4832, TP14763, TP16392, TP17823, TP50346, TP57174, TP69825, TP70889, TP72048
AC	11	TP1684, TP2391, TP7760, TP15472, TP21688, TP36838, TP44814, TP51044, TP65645, TP70833, TP72119

Table 3.4 Number of SNPs shared between different environmental and biochemical variables. Variable acronyms as in Table 3.3

SNPs ID	Ecoregions	MAT	MST	MAP	CF	PH	SAR	MAR	TPC	AC
Ecoregions	--									
MAT	8	--								
MST	5	2	--							
MAP	4		2	--						
CF	1			1	--					
PH	8	4		3		--				
SAR	6	2		1		7	--			
MAR	5	2	2	7		5	3	--		
TPC									--	
AC										--

3.3.4 **Functional annotation by search similarity, Gene Ontology assignments and analysis**

In order to predict protein coding genes and their putative function among the reads containing SNPs, the complete set of 1,586 putative SNPs (3,172 alleles sequences) were aligned against the NCBI non-redundant protein (NR) database using BLASTX search with an E-value threshold of 10^{-3}, and using BLAST2GO for their functional annotation. The summary of the data distribution (including the top hit species) of unigenes similar to *V.*

vitis-idaea against the NR database showed that the highest number of best hits have 14 unigenes matches to both *Ricinus communis*, and *Gossypium raimondii*, 13 unigenes matches with *Vitis vinifera*, and 13 unigenes matches with *Mimulus guttatus*. A large proportion (2,869 allele sequences) of lingonberry sequences containing SNPs had no significant sequence alignment or hits in the NCBI NR database, which suggested that they may contain novel sequences, non protein coding sequences, or lingonberry specific sequences. Gene names and GO terms were assigned to the genomic sequences based on sequence homologies (E-value of 10^{-6}). Homologous annotated sequences were utilized to assign functional terms (GO terms) to query (Figure 3.15). Mapped sequences are those assigned GO terms based on the hits obtained after a BLAST search. This BLAST2GO annotation produced 15 annotated sequences out of the 64 genomic sequences with homologous genes.

The Gene Ontology (GO) encompasses terms describing genes and gene products roles in cells (Ashburner et al., 2000). These terms are in three categories: 1) biological process- referring to the biological objective of the genes or gene products; 2) cellular component – referring to the place in the cell where the gene product is active; and 3) molecular function – referred to the biochemical activity of the genes or gene products. The GO annotation using BLAST2GO of lingonberry sequences containing SNPs significantly associated with our variables of interest are shown in Figure 3.15. In the biological process category (Figure 3.15A), a total of 13 annotated unigenes were related to organic substance metabolic process, another dominant subcategory was primary metabolic process with 11 annotated unigenes, cellular metabolic process (10 unigenes), single-organism metabolic process (6 unigenes), single-organism cellular process (6 unigenes), cellular component

organization (4 unigenes) and nitrogen compound metabolic process (4 unigenes). The cellular component (Figure 3.15B) was dominated by intrinsic components of the membrane (5 unigenes), plasma membrane (4 unigenes), intracellular (4 unigenes), cell periphery (4 unigenes), intracellular organelle (2 unigenes), membrane-bounded organelle (2 unigenes) and endomembrane system (2 unigenes). The molecular function category (Figure 3.15C) was comprised of protein coding genes involved in organic cyclic compound binding (8 unigenes), heterocyclic compound binding (8 unigenes), ion binding (6 unigenes), transferase activity (6 unigenes), small molecule binding (4 unigenes), carbohydrate derivative binding (4 unigenes), guanyl-nucleotide exchange factor activity (2 unigenes), protein binding (2 unigenes), hydrolase activity (2 unigenes) and oxidoreductase activity (1 unigene). This Gene Ontology analysis suggested that lingonberry SNP containing sequences code for diverse proteins involved in structural, regulatory, metabolic and stress response mechanisms.

Because of the few BLAST hits and GO annotation obtained from BLAST2GO, another BLAST search was made using a local NCBI database. The complete set of genomic sequences containing the 1,586 putative SNPs (3,172 allele sequences) from all lingonberry samples were aligned against the NCBI NR protein database using BLASTX with an E-value threshold of 1e-3 for the identification of homologous sequences. A total of 150 SNP-containing sequences (9.46%) showed protein matches (BLASTX) with a minimum of 80% similarity over the full length of the read. A total of 2,017 homologous sequence matches was observed in NCBI NR protein database, which were used to find the function of the gene using Batch Entrez. A total of 1,120 proteins were identified. It was observed that a large number (67.41 %) of these proteins have homologous with the same

annotation. After extracting unique protein annotation, a total of 241 (21.52 %) unigenes were identified (Appendix 2).

According to the e-value distribution of the top hits in the NR database, 18.69 % of the matched sequences showed high sequence similarity with an e-value <1e-8, while 81.30 % of the matched sequences showed moderate sequence similarity with an e-value >1e-8. The top BLAST hits against the NR database showed that these genes had the greatest number of matches with genes of *Theobroma cacao*, *Solanum lycopersicum*, *Medicago spp*., *Ricinus communis*, *Morus notabilis*, *Nelumbo nucifera*, *Brassica napus*, *Glycine max*, *Vitis vinifera*, *Gossypium arboretum*, *Nicotiana spp*., *Elaeis guineensis*, *Solanum tuberosum*, and *Prunus mume*. The other 57.37 % of genes had hits with others species. This indicates that the genomic DNA of lingonberry is more closely related to that of *Theobroma cacao* than to other plant genomes currently present in the NCBI protein database.

The functional annotation of the 241 unigenes showed that 69% of them had a known protein function, 18% had a hypothetical protein function, 12% were uncharacterized proteins, and only 1% were unnamed proteins. To identify the function of the known genes, a literature search was made, which is shown in Appendix 2.

Functional annotation of the 260 SNP-containing sequences that were significantly correlated with the 10 environmental and biochemical variables identified a total of 128 hits of similar protein sequences with a threshold e-value of 1e-3. The identified gene IDs were imported into Batch-Entrez to search for their protein function. Batch Entrez removed the duplicate accessions number and rendered 88 unigenes accession IDs, of which only 50 unigenes had protein functions. These 50 protein functions belong to genomic sequences

containing 9 SNPs (Table 3.5). The identified functions of these unigenes are dominated by hypothetical proteins, followed by transcription factors, nutrient transport proteins, uncharacterised proteins and metabolic proteins (Table 3.5).

Table 3.5 Functional annotations of 9 SNP-bearing sequences significantly associated with biochemical and environmental variables based on their sequence similarity (using BLASTX). Acronyms for environmental and biochemical variables as in Table 3.3.

SNPs ID	Environmental variable	BLASTX hits	Functional Annotation from Batch-Entrez
TP3846	MAR	GI:657983582 GI:674952706 GI:674859503	PREDICTED: protein transport protein Sec61 subunit alpha-like
TP14763	TPC	GI:694373907 GI:595819833	PREDICTED: trichome differentiation protein GL1-like
		GI:645275423	PREDICTED: transcription factor WER-like
		GI:658054507	PREDICTED: myb-related protein 305-like
		GI:720079076	PREDICTED: transcription factor MYB82-like
		GI:828301767	PREDICTED: transcription factor MYB114-like
		GI:914408355	R2R3-MYB protein
TP15510	Ecoregion	GI:641847217	hypothetical protein CISIN_1g0165811mg, partial
		GI:641806635	hypothetical protein CISIN_1g024250mg
TP40359	AP, MAR	GI:345293837	AT5G60780-like protein, partial
		GI:568869667	PREDICTED: high affinity nitrate transporter 2.6-like
		GI:604301483	hypothetical protein MIMGU_mgv1a022931mg
		GI:641841285	hypothetical protein CISIN_1g040770mg
		GI:643738009	hypothetical protein JCGZ_05464
		GI:734376937	High affinity nitrate transporter 2.4
		GI:920700937	hypothetical protein LR48_Vigan05g176700
		GI:947076238	hypothetical protein GLYMA_12G078900
TP49117	MAT, TPC	GI:731376510	PREDICTED: calmodulin-binding transcription activator 4 isoform X1
		GI:703077622	Calmodulin-binding transcription activator 4

TP72119	AC	GI:567922764	hypothetical protein CICLE_v10009891mg
		GI:586703911	PREDICTED: uncharacterized protein LOC18435512
		GI:697099161	PREDICTED: uncharacterized protein LOC104120366
		GI:698490682	PREDICTED: uncharacterized protein LOC104238979
		GI:743767724	PREDICTED: uncharacterized protein LOC105039620
		GI:747065582	PREDICTED: uncharacterized protein LOC105162951
TP72740	MAT, PH	GI:661892540	unnamed protein product
		GI:674923097	BnaC05g04690D
		GI:674923210	BnaC08g44950D
		GI:685369222	PREDICTED: probable galacturonosyltransferase 6 isoform X1
		GI:685369225	REDICTED: probable galacturonosyltransferase 6 isoform X2
		GI:685369227	PREDICTED: probable galacturonosyltransferase 6 isoform X3
		GI:685369231	PREDICTED: probable galacturonosyltransferase 6 isoform X4
		GI:922545007	PREDICTED: probable galacturonosyltransferase 6
TP73887	Ecoregion	GI:255558824	glycogen phosphorylase, putative
		GI:743790326	PREDICTED: LOW QUALITY PROTEIN: alpha-glucan phosphorylase, H isozyme-like
		GI:702271122	PREDICTED: alpha-glucan phosphorylase, H isozyme
		GI:763816868	hypothetical protein B456_013G260900
		GI:695039956	PREDICTED: alpha-glucan phosphorylase, H isozyme
		GI:661887364	unnamed protein product
		GI:703130705	Alpha-glucan phosphorylase, H isozyme
		GI:848852446	PREDICTED: alpha-glucan phosphorylase, H isozyme
		GI:920711254	hypothetical protein LR48_Vigan09g116200
TP78719	SAR, MAP	GI:720072744	PREDICTED: conserved oligomeric Golgi complex subunit 5
		GI:590655128	Golgi transport complex protein-related
		GI:697131716	PREDICTED: conserved oligomeric Golgi complex subunit 5

A literature search was carried out to find the function of the 241 identified unigenes. The search results showed that 44 genes were annotated as transcription factors, DNA binding/transduction and signalling. These sequences likely represent expressed transcription factors involved in the regulation of gene expression during plant development, flowering, fruit ripening or metabolic processes. 47 proteins were associated with primary or secondary metabolic processes. Of these, 4 were involved in lipid or fatty acid metabolic processes, and 6 were involved in carbohydrate or sugar metabolism. Another 34 proteins were associated with transportation of nutrients or plant metabolites within different cells. It was also observed that 19 proteins played a role on plant defense. The literature search annotation identified 39 candidate genes that are involved in regulation and synthesis of phenolic compounds and their antioxidant activity. For example, the sequences containing SNPs TP36904 and TP77354 were homologous to sequences like pyridine nucleotide-disulfide oxidoreductase and L-ascorbate oxidase, respectively. Pyridine nucleotide-disulfide oxidoreductase, which is involved in detoxification of ROS, were lower in drought-stressed plants (Kim et al., 2015). L-ascorbate oxidase function as a plant defense that decreases the availability of ascorbate to insects. In the absence of sufficient ascorbate, plant produces elevated levels of ROS, which is associated with oxidative damage to nutrients and oxidative stress in tissues. Ascorbate oxidase simultaneously act with phenolics to increase their pro-oxidant effects on herbivores (Barbehenn et al., 2008) (Appendix 2).

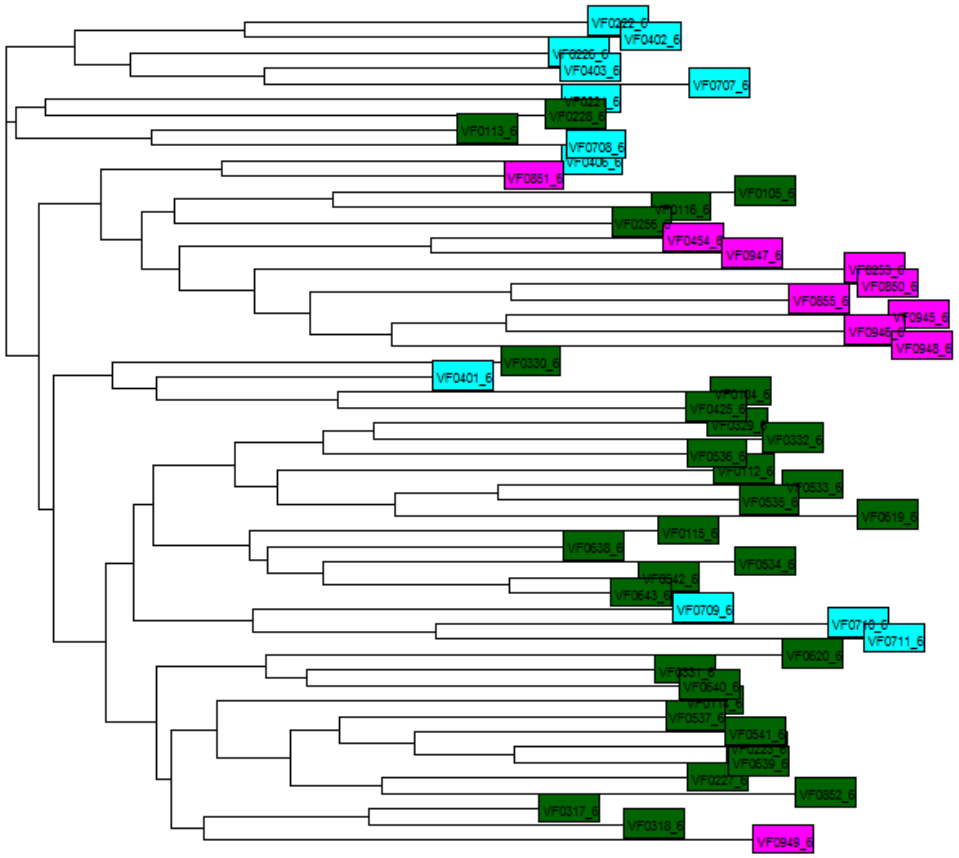


Figure 3.5 A phylogenetic tree based on 20 the significantly correlated SNPs with mean annual temperature. Color codes denote individuals growing under different mean annual temperatures as follows: purple=1.0-2.9 °C, light blue=3.0-3.9 °C and green= 4.0-7.9 °C. Moran's I = -0.0172, p-value=0.115.

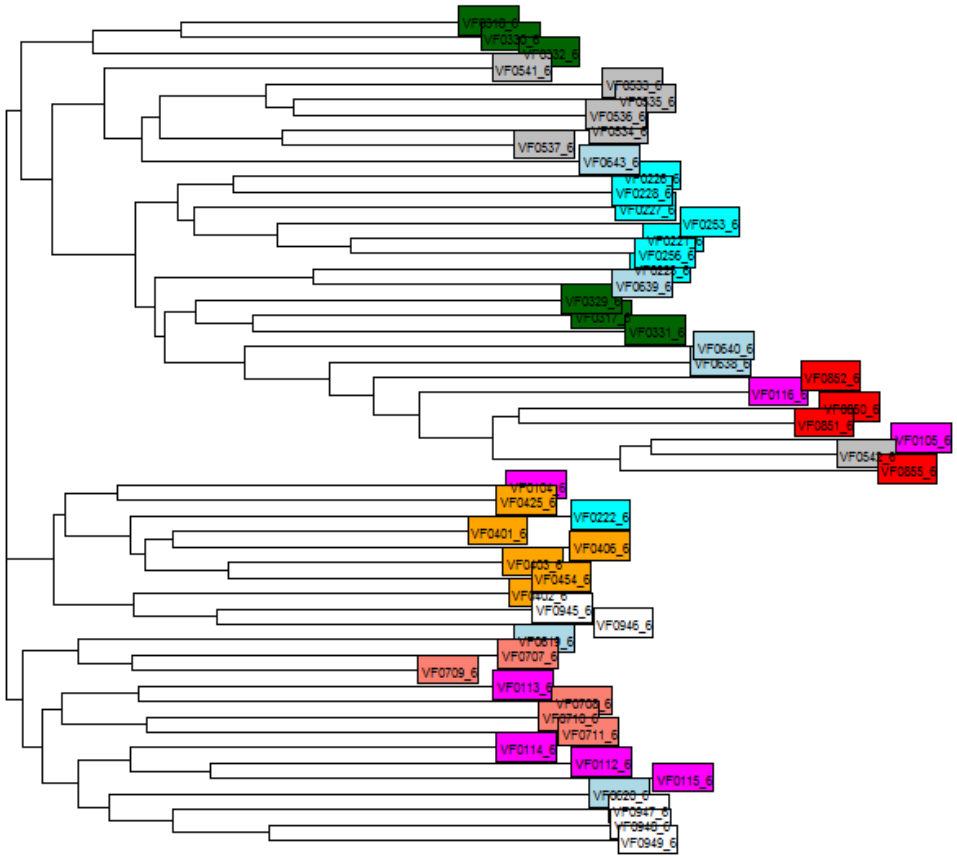


Figure 3.6 A phylogenetic tree based on the 40 significantly correlated SNPs with ecoregion. Color codes denote individuals growing under different ecoregions as follows: purple= Western Newfoundland Forest, blue= Central Newfoundland Forest, green= North Shore Forest, orange= Northern Peninsula Forest, grey= Maritime Barrens, light blue= Eastern Hyper Oceanic Barrens, salmon= Long Range Barrens, red= Strait of Belle Isle Barrens, White= Forteau Barrens. Moran's I = -0.0194, p-value=0.648

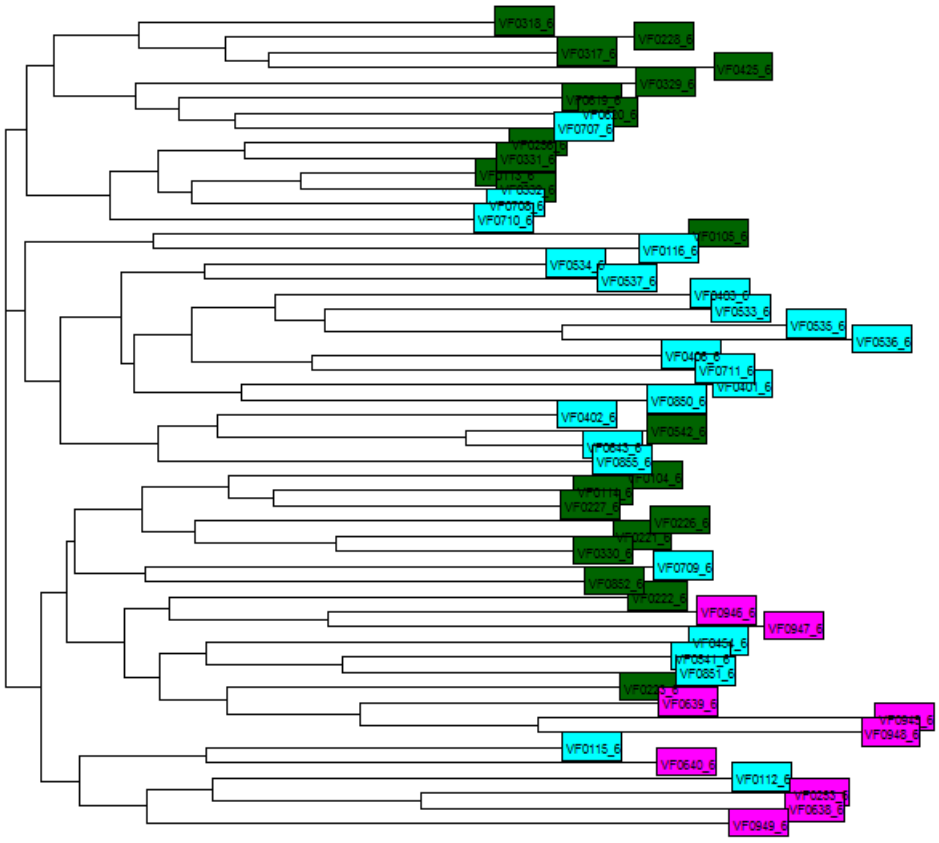


Figure 3.7 A phylogenetic tree based on the 28 significantly correlated SNPs with mean annual summer temperature. Purple=6-11.9°C, light blue=12-13.9°C and green= 14-19.9°C. Moran's I = -0.0191, p-value=0.826.

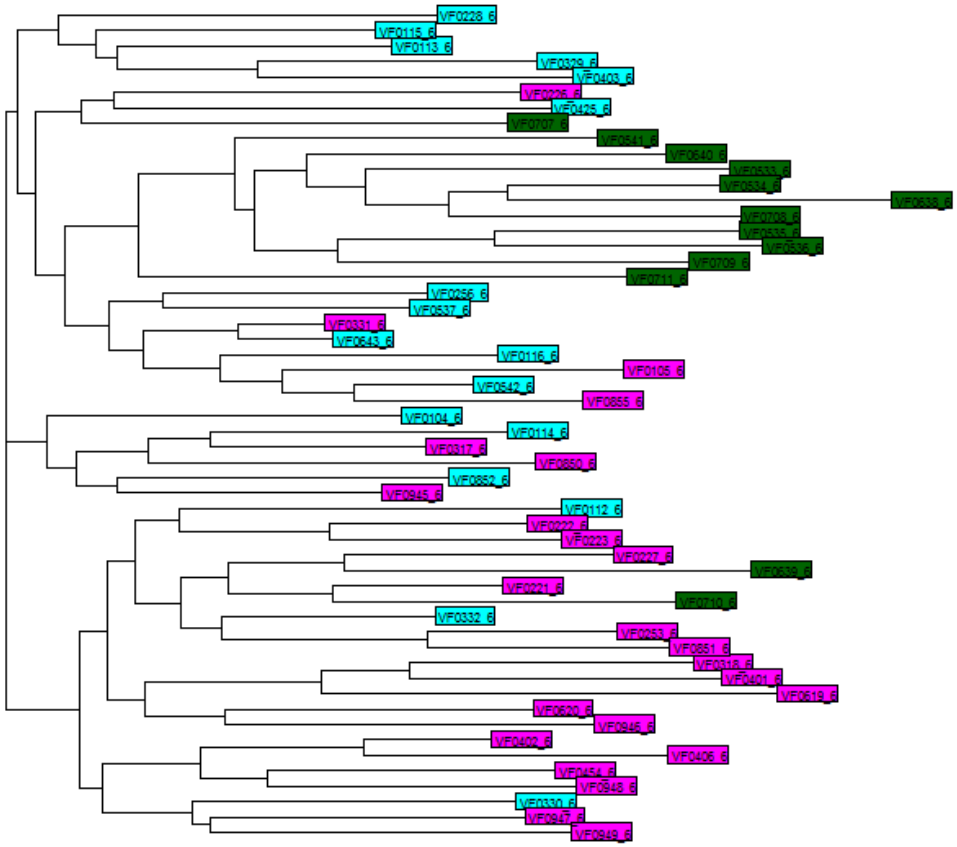


Figure 3.8 A phylogenetic tree based on the 23 significantly correlated SNPs with mean annual precipitation. Purple=700-1100 mm, light blue=1100-1300 mm, and green= 1300-1500 mm. Moran's I = -0.0166, p-value=0.0342.

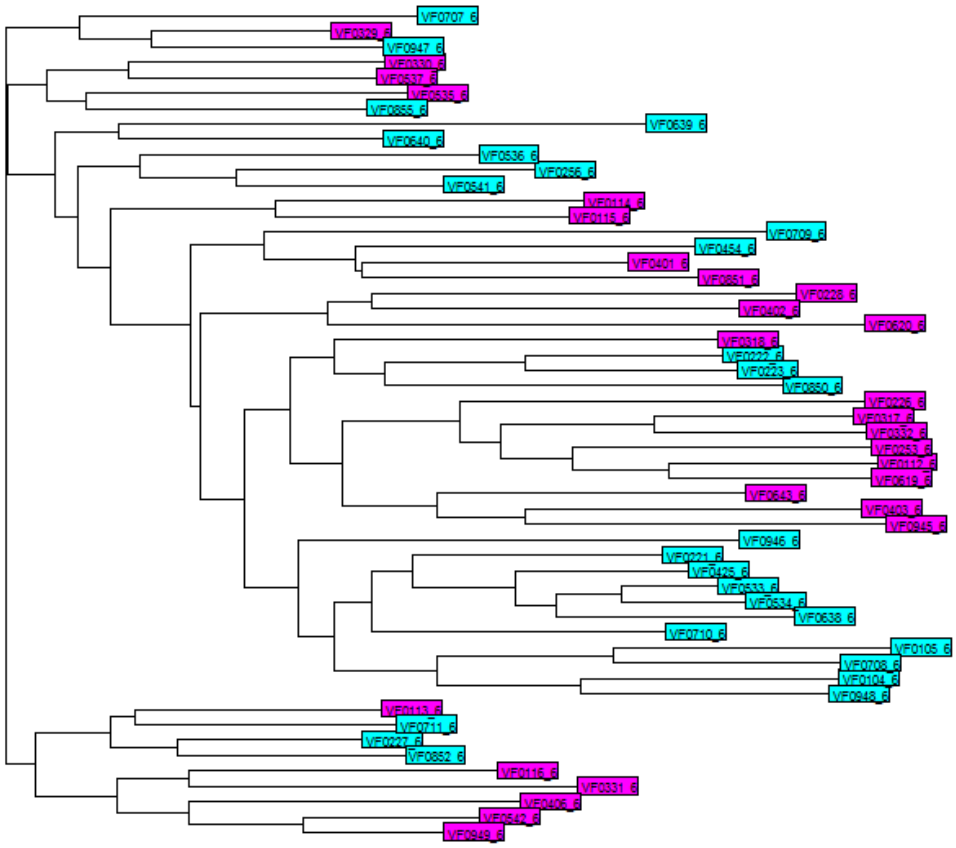


Figure 3.9 A phylogenetic tree based on the 18 significantly correlated SNPs with coastal vs inland. Purple=coastal and light blue=inland. Moran's I = -0.0191, p-value=0.829.

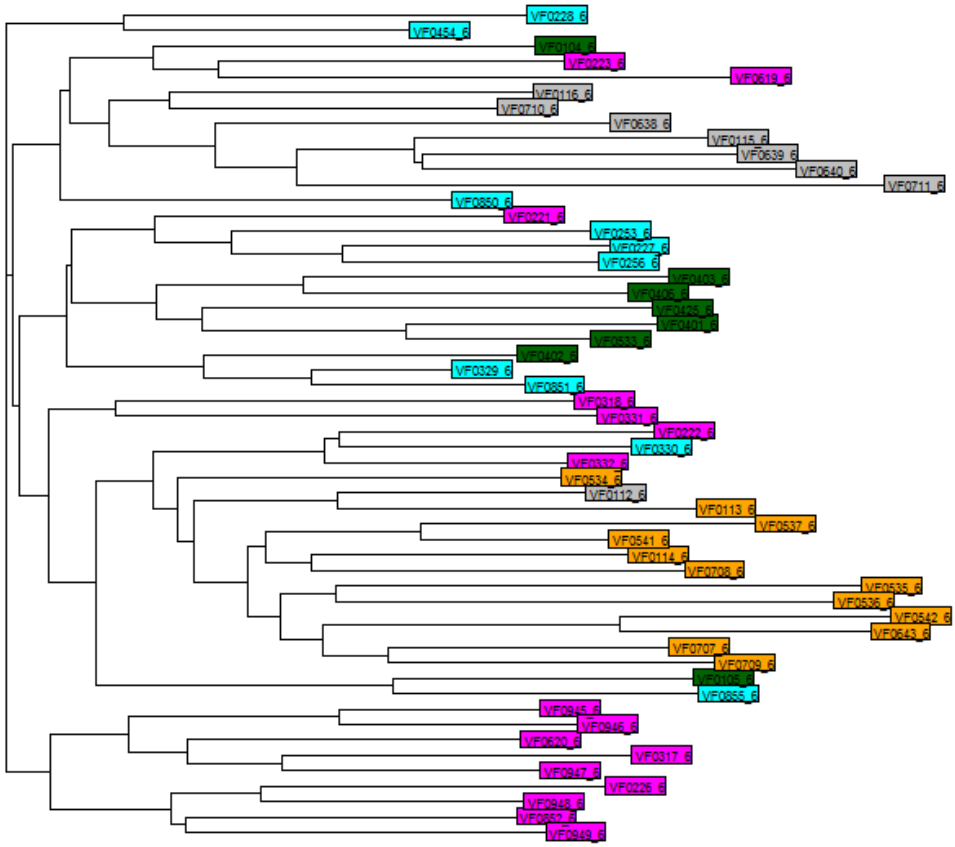


Figure 3.10 A phylogenetic tree based on the 24 significantly correlated SNPs with mean annual runoff. Purple=500-800 mm, light blue=800-1000 mm, green= 1000-1300 mm, yellow=1300-1800 mm, grey= 1800-2200 mm. Moran's I = -0.0168, p-value=0.057.

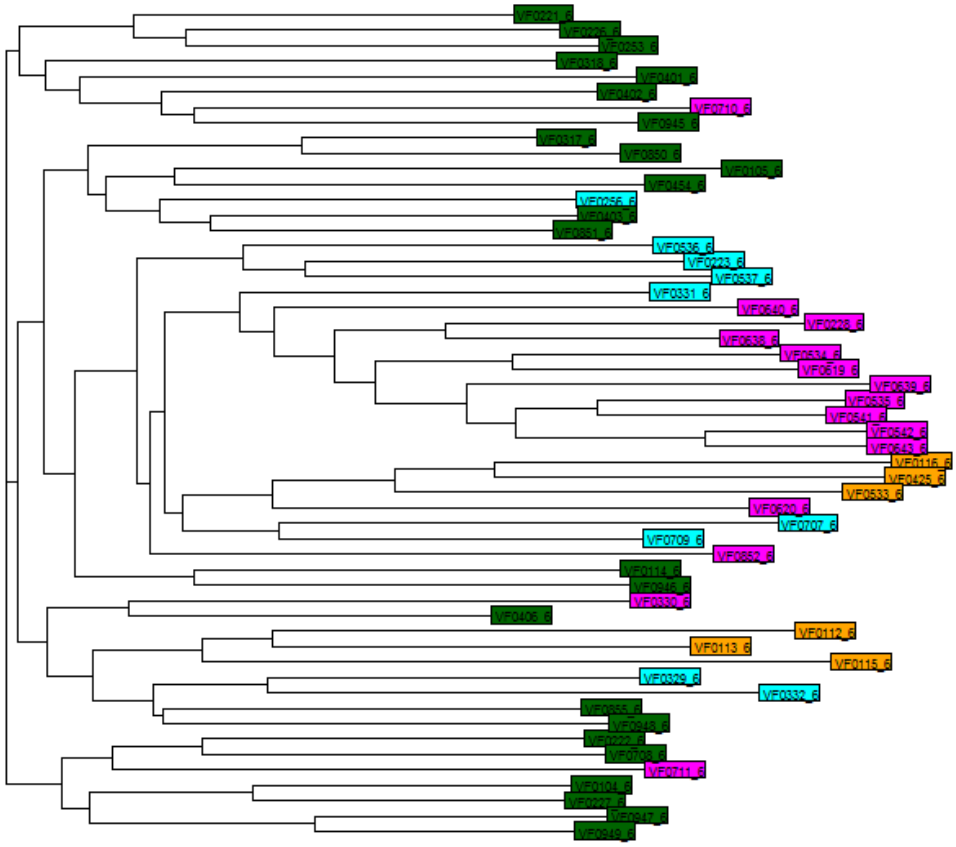


Figure 3.11 A phylogenetic tree based on the 28 significantly correlated SNPs with soil pH. Purple=<6.1, light blue=6.2-6.5, green= 6.6-7.0, and yellow=>7.0. Moran's I = -0.0175, p-value=0.217.

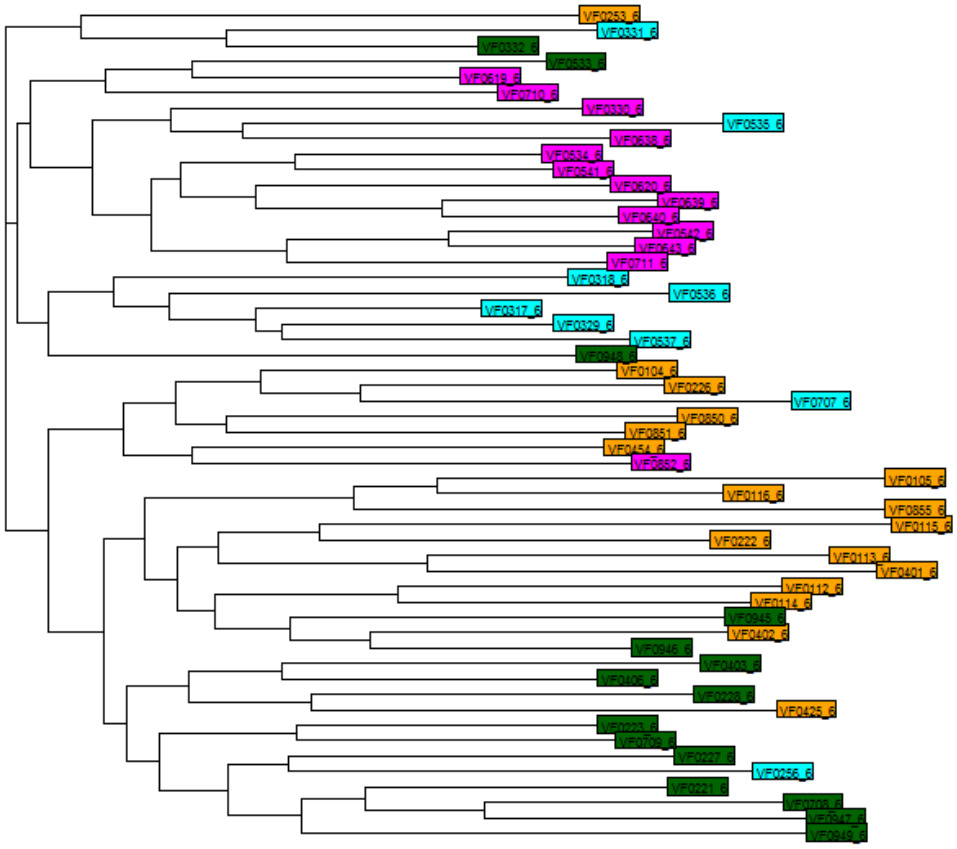


Figure 3.12 A phylogenetic tree based on the 20 significantly correlated SNPs with sensitivity to acid rain. Purple= extremely sensitive (<60 μeq/l), light blue= highly sensitive (60-100 μeq/l), green= moderate (100-200 μeq/l), and yellow= low (>200 μeq/l). Moran's I = -0.0174, p-value=0.169.

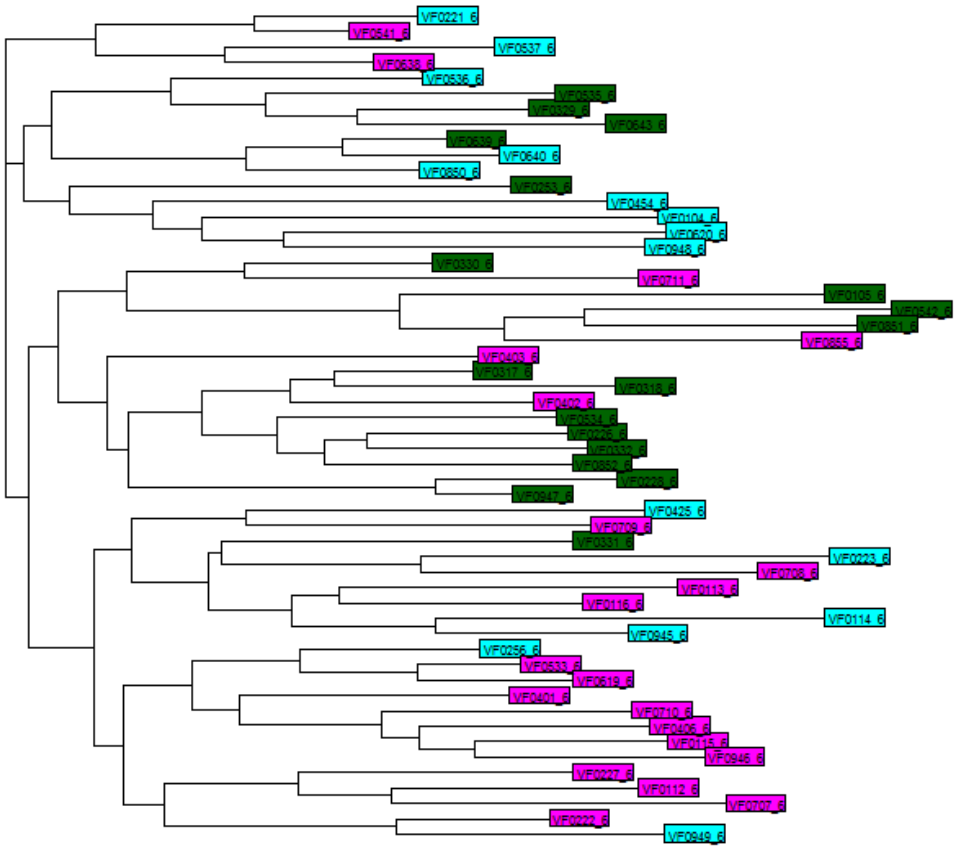


Figure 3.13 A phylogenetic tree based on the significantly correlated SNPs with TPC. Purple=650 (mg GE/100g FW), light blue=650-850 (mg GE/100g FW), and green= >850 (mg GE/100g FW). Moran's I= 0.0185, p-value=0.711.

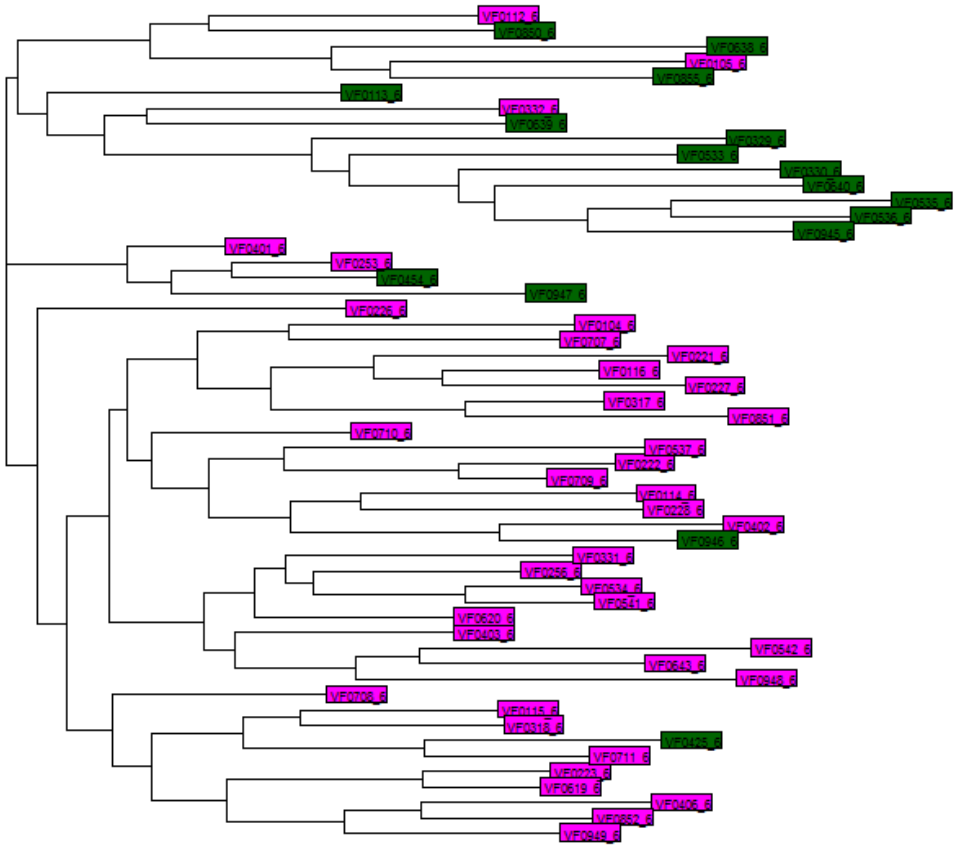
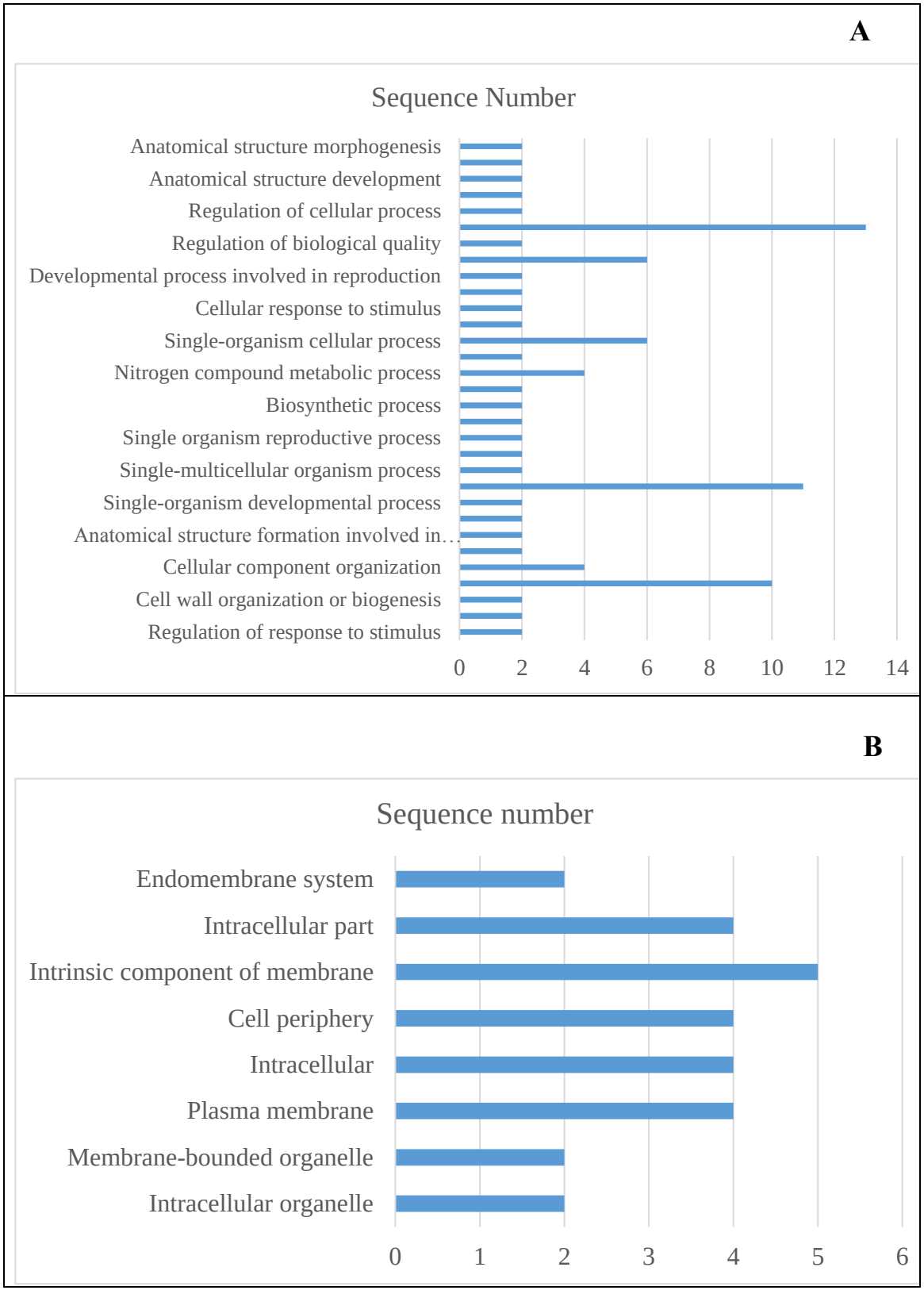


Figure 3.14 A phylogenetic tree based on the 14 significantly correlated SNPs with antioxidant capacity. Purple=<650 (mg GE/100g FW), and green=>650 (mg GE/100g FW). Moran's I = -0.0176, p-value=0.251.


A
Sequence Number
Anatomical structure morphogenesis
Anatomical structure development
Regulation of cellular process
Regulation of biological quality
Developmental process involved in reproduction
Cellular response to stimulus
Single-organism cellular process
Nitrogen compound metabolic process
Biosynthetic process
Single organism reproductive process
Single-multicellular organism process
Single-organism developmental process
Anatomical structure formation involved in…
Cellular component organization
Cell wall organization or biogenesis
Regulation of response to stimulus
0
2
4
6
8
10
12
14
B
Sequence number
Endomembrane system
Intracellular part
Intrinsic component of membrane
Cell periphery
Intracellular
Plasma membrane
Membrane-bounded organelle
Intracellular organelle
0
1
2
3
4
5
6

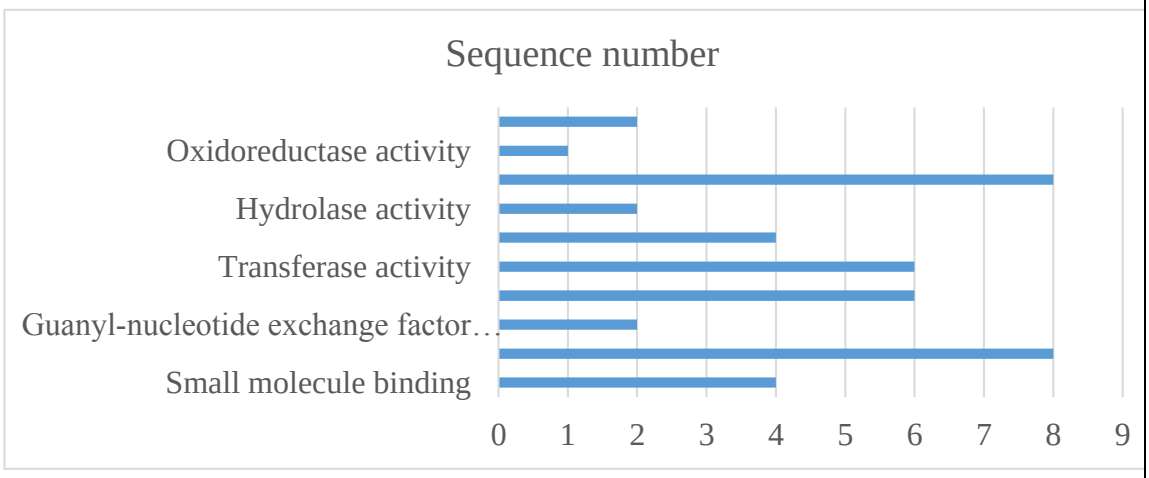


Figure 3.15 Distributions of Gene Ontology annotations for *Vaccinium vitis-idaea* genomic sequences using BLAST2GO. The results are summarized as follows A) Biological process, B) Cellular component, C) Molecular function

3.4 Discussion

3.4.1 SNP discovery in lingonberry samples from Newfoundland and Labrador

In this study, we identified genome-wide SNPs in lingonberry (*Vaccinium vitis-idaea spp. minus*) using the GBS method. To the best of our knowledge, this is the first study on SNP discovery in this plant species. The GBS approach allowed us to discover a significant number of novel SNPs (76,354) before filtering and 1,586 SNPs after filtering. A study on tetraploid kiwiberry (*Actinidia arguta*) discovered 1,160 SNPs (Melo et al., 2016) with 75 percent population coverage using TASSEL-UNEAK, which is similar to our results. A study on pea RIL (F8 Recombinant Inbred Lines) discovered 419,024 SNPs using discoSnp tools (Boutet et al., 2016). The lower number of SNPs discovery using TASSEL-UNEAK compared to discoSnp might be due to the complex network filtering step implemented in TASSEL-UNEAK. In cranberry transcriptome, 602,000 SNPs were discovered in an assembly of 420 Mbp (Polashock et al., 2014).

3.4.2 Functional annotation of all SNP-containing sequences

The function of the identified SNP containing proteins are mainly transcription factors, regulators of biosynthesis, transportation, signal transduction, DNA binding, stress response and pathogenicity (Appendix 2). Among common metabolic regulator proteins were S-adenosylmethionine (SAM, TP9982), E3 ubiquitin protein ligase (ATL31,

TP71890), aspartate aminotransferase (AAT, TP10440), auxin response factor 3 (TP33726), glucose-1-phosphate adenylytransferase (TP19923), acetyl-CoA carboxylase carboxyl transferase beta subunit (TP65734, TP30852), protein COBRA precursor (TP64237), terminal flower 1 (TP15782), 3-ketoacyl-CoA synthase 8 (TP24839). The aspartate aminotransferase is a key enzyme for the biosynthesis of aspartate, which is a key precursor for amino acid biosynthesis. Aspartate aminotransferase affects nitrogen and carbon metabolism mainly in legumes (Gantt et al., 1992). It was also reported that aspartate within the plant chloroplast in combination with prephenate generate arogenate, which is a direct precursor of phenylalanine and, to a lesser extent, tyrosine. Phenylalanine is the first compound in phenolic compound biosynthesis. A recent study also showed that suppression of AAT in *Arabidopsis* led to several fold decrease of chlorophyll and lignin, which led to a reduction in growth and chlorosis symptoms (de la Torre et al., 2014).

Identified proteins related to the biosynthesis or metabolism of plant biochemical properties include multicopper oxidase (TP77354), putative peroxidase (TP73525), protein trichome birefringence like 10 (TP73646), cellulose synthase-like protein G3 (TP77277), homoserine O-acetyltransferase (TP66366), malic enzyme (TP31548), detoxification 41-like protein (TP46191), 4-coumarate coenzyme A ligase 4 (4CL, TP79304), allene oxide cyclase 4 (TP44917), 2-methylene-furan-3-one reductase (TP36838). The 4-coumarate coenzyme A ligase is an important enzyme for the biosynthesis of a diverse group of phenylpropanoid products naturally occurring in plants (Rastogi et al., 2013) mainly cinnamic acid, flavonoid and lignin (Chowdhury et al., 2013), which control various physiological functions in plants and facilitate adaptation to different environmental conditions (Dixon and Paiva, 1995). Studies on tobacco, *Arabidopsis*, and aspen showed

that down regulation of 4CL-reduced lignin biosynthesis, increased cellulose, and increased plant growth (Hu et al., 1999). Anthocyanins are important phenolic compounds responsible for flower and fruit colour whose biosynthesis is regulated by 4CL. It was reported that low temperature and high red light results in darker flowers and fruit because of an increase in anthocyanin content (Stiles et al., 2007). It was also reported that acid irrigation water (low pH) could cause light flower and fruit colour because of a significant reduction of anthocyanin content (Zhao and Tao, 2015).

In our study we have identified a list of potential adaptive genes to different environmental conditions including peroxidase and thioredoxin reductase NTRB-like. Peroxidases (TP73525) are ubiquitous plant enzymes present as large multigene families that oxidize several different substrates in the presence of H_2O_2. The main function of peroxidases in plants is its specialized role in lignification. Overexpression of a chimeric anionic peroxidase in tobacco resulted in high levels of lignin compared to control plants (Lagrimini, 1991). Peroxidase genes were also involved in metal and salt stress, thirteen of these genes were associated with aluminum stress, and one gene was involved in both aluminum and cadmium stress in roots (Cosio and Dunand, 2009). Plants may tolerate this metal stress by the antioxidant activity of peroxidase (Chiang et al., 2006; Kumari et al., 2008). In strawberry salt stress increased peroxidase activity (Gulen et al., 2006). It was also reported that NADPH-thioredoxin reductase NTRB like protein is important for plant development and adaptation to environmental change and stress (Meyer et al., 2008). NTRB plays a role in the regulation of photoperiod dependent metabolic and developmental processes in *Arabidopsis*, including carbon metabolism, auxin metabolism, chlorophyll biosynthesis and the Shikimate pathway in chloroplasts, but it is more efficient

in antioxidant defense (Meyer et al., 2008; Tognetti et al., 2012). A NTRB mutant showed a higher level of flavonoids accumulation and overexpression of several genes of the flavonoid pathway. A mutation of the first enzyme (transparent testa 4) of flavonoid biosynthesis showed that NTRB mutant plants are more resistant to UV-C than wild plants, suggesting that flavonoids in NTRB mutants protects plants against UV-light (Bashandy et al., 2009; Cha et al., 2015).

Homologous sequences for, L-ascorbate oxidase (TP77354) and pyridine nucleotide-disulfide oxidoreductase (TP36904) were identified, which are known for their antioxidant capacity, playing an important role in mitigation of excessive cellular reactive oxygen species activities caused by abiotic stress (Venkatesh and Park, 2014). For example, high temperature down regulated the expression of L-ascorbate oxidase (Jeong et al., 2011), whereas light up regulated its expression in tobacco (Pignocchi et al., 2003).

Transcription factors function as regulators of biochemical and cellular processes in plants. Among the transcription factors identified in this study were myeloblastosis (MYB) (TP11064, TP14763, TP36904), bZIP128 (tP72930), beta glucosidase (TP40196), trichome differentiation protein GL1 like/kinase like protein TMKL1 (TP14763), glucosyltranferase (TP70388), and actin related protein 9 (TP14733). The MYB transcription factor (MYB-TF) is involved in various functions including cell development and cell cycle, primary and secondary metabolism, hormone synthesis, signal transduction, various biotic and abiotic stresses and defense (Hichri et al., 2011; Ambawat et al., 2013). Another category of homologous genes found were those that play a role as transporters of metabolic compounds and nutrients such as the high affinity nitrate transport protein like

(NRT2.3, TP 40350). Down regulation of NRT2 resulted in a decrease in high affinity nitrate uptake (Lezhneva et al., 2014).

3.4.3 SNPs associated with environmental variables and biochemical properties

Recent advances of high-throughput DNA sequencing technology provide an opportunity to investigate adaptations or environmental effects on plant genetics (Demanèche et al., 2013). Among the 150 putative SNPs that were found associated with environmental or biochemical variables, only 9 SNP-containing DNA sequences (6%) yielded hits in the NCBI NR protein database. These results suggest that most of the putative SNPs identified in lingonberry were located in unknown proteins or non-coding genomic regions. Below we discuss the gene identity and function of each of the 9 SNP-containing sequences as revealed from Batch Entrez and the literature search.

SNP ID TP 3846 was associated with MAR and the sequence containing it was predicted to be homologous to the protein transport protein Sec61 subunit alpha-like. We did not find any function of this gene that could be related to annual runoff. Sec61 is an endoplasmic reticulum (ER) membrane protein translocator that participates in the translocation of newly synthesized proteins into the lumen of the ER. Sec61 coimmunoprecipitates with certain protein substrates destined for elimination (ER-associated degradation, (Liu and Howell, 2010). A recent study on *Citrus sinensis* shows that induction of Sec61 subunit β in Al rich roots increase adaptability by maintaining ER

homeostasis. Sec61 was also found to decrease the plant susceptibility to powdery mildew fungus (Zhang et al., 2013).

SNP ID TP 14763 was associated with TPC and the sequence containing it has homologous sequences coded for genes involved in trichome differentiation such as GLABRA1 (GL1), and transcription factors such as WEREWOLF (WER, which regulates hairless cell differentiation), and MYB (305, 114, 82 and R2R3-like involved in phenolic compound biosynthesis). It was reported that MYB305 transcription factor activates the phenylpropanoid metabolism through the phenylalanine ammonia lyase gene (Moyano et al., 1996). Overexpression of MYB114 results in substantial increases in pigment (anthocyanin) production (Gonzalez et al., 2008). It was also reported that loss of the MYB82 function did not disrupt trichome development, but overexpression of this gene led to abnormal development of trichomes (Liang et al., 2014)**.** R2R3-MYB transcription factor is known for its role in tryptophan biosynthesis (Bender and Fink 1998), and also may control homeostasis between indolic glucosinolate and Indole-3-acetic acid biosynthesis (Celenza et al 2005). In *Vaccinium* species such as bilberry, highbush blueberry and bog bilberry, an involvement of R2R3 MYB transcription genes in flavonoid biosynthesis was found (Karppinen et al., 2016). This SNP-bearing DNA region is worth of more in depth studies on the genetic changes related with different phenolic compound phenotypes.

SNP ID TP15510 was associated with ecoregion and significant BLASTX hits to the sequence containing it were hypothetical proteins CISIN_1g0165811mg or 1g024250mg from *Citrus sinensis.* This gene has a similar function to the 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase protein. This

enzyme is responsible for the regeneration of S-adenosylmethionine (SAM), which serves as the methyl donor for the biosynthesis of compatible osmolytes (Zou et al., 2016). Salt stress induced the expression of enzymes in the SAM regeneration pathway. In *Solanum chilense*, three enzymes including methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, S-adenosylmethionine synthetase 1, and adenosylhomocysteinase were induced by salt treatment in roots (Zhou et al., 2011). The connection between ecoregions where lingonberry grow and the amount of salt stress on each of them need to be further evaluated, and how this activates the hypothetical enzymes in the SAM pathway encoded by this particular SNP.

The SNP ID TP40359 is associated with two water-related environmental variables (MAP and MAR) and the sequence containing it has homologous sequences that have high affinity with nitrate transporter genes. In *Arabidopsis*, the high-affinity transport system (HATS) is encoded by the NRT2 gene family (Hu and Schmidhalter, 2005). It has been reported that the expressions of NRT2.1, NRT2.2, and NRT2.3 decreased with nitrate supply increased (Ping et al., 2011). The expression of the NRT2 gene is induced by nitrate and more efficiently by nitrite, but down regulated by ammonium (Quesada et al., 1998). Thus, the beneficial effects of overexpressing NO_3 or NH_4 transporters would be greatest under conditions where the external NO_3 or NH_4 levels are low, which is true for drought conditions (Villar Salvador, 2013). This SNP constitutes a genetic variation among individuals linked to drought tolerance, which deserves further exploration. This was quite surprising for us because minimum and maximum values of MAP (700-1,500 mm) and MAR (500-2,200 mm) do not have a wide range, yet we found a DNA region that might be subject to drought adaptation within Newfoundland.

SNP ID TP49117 was associated with MAT and TPC and the sequence containing it was annotated as calmodulin-binding transcription activator (CAMPTA) 4 isoform X1, which has been reported to play an important role in biotic and abiotic stress response (Galon et al., 2008). It has been shown that the tobacco early ethylene-responsive gene NtER1 encodes a calmodulin-binding protein (Yang and Poovaiah 2000). In *Arabidopsis*, one NtER1 homolog and five related genes are rapidly and differentially induced by environmental signals such as temperature extremes, UVB, salt, wounding, and hormones. Hence, they were designated as AtSR1-6 (*Arabidopsis thaliana* signal-responsive genes). The AtSR gene family encodes a family of calmodulin-binding/DNA-binding proteins involved in multiple plant signal transduction pathways, however, their physiological roles are unknown (Yang and Poovaiah 2002), and this SNP-containing region might provide a hint towards this goal, since it was associated with MAT and TPC.

The functional annotation of the sequence containing SNP ID TP 72119 associated with AC rendered a protein of unknown function and we therefore cannot further comment. NCBI hits yielded from the sequence containing SNP ID TP72740 were annotated as galacturonosyltransferases (GAUT). GAUTs are among the enzymes responsible for pectin biosynthesis, a family of complex polysaccharides present in the cell walls of all land plants. Pectin is the most structurally complex plant cell wall polysaccharide (Abdel-Massih et al., 2003; Abdel-Massih et al., 2007). Pectin plays an important role in plant growth, development, and disease resistance including roles in cell–cell adhesion, wall porosity, cell elongation, and wall extensibility (Akita et al., 2002). This SNP was correlated to MAT and pH, therefore we highlight its potential relevance as follows. Plants acclimate to cold temperatures by increasing their cell wall thickness and altering their cell

wall ultrastructure and composition. Adaptation to environmental change has been associated with modifications in leaf stiffness, cell wall and pectin contents, pectin methylesterase activity, and the low-methylated pectin content (Wang et al., 2016). Pronounced modification of cell wall properties mainly pectin has also been found on pathogen resistant genotypes (Solecka et al., 2008).

The sequence containing SNP ID TP73887 correlated with ecoregion, was annotated as glycogen phosphorylase, and alpha-glucan phosphorylase H isozyme, which is a glucan degrading enzyme that catalyzes the production of glucose-1-phosphate (G1P, (Alonso-Casajus et al., 2006). Glucan phosphorylases metabolize starch, and therefore may also be called starch phosphorylases (Schupp et al., 2004). Glucan phosphorylases have been shown to play a pivotal role in tolerance to abiotic stress, flowering, and seed growth (Alonso-Casajús et al., 2006). Starch phosphorylase is regulated by pH, temperature, redox potential, oligosaccharides level, allosteric changes and covalent modification (Orzechowski, 2008). At high temperatures, glucan phosphorylases become inactive. Ecoregions differ in a number of environmental conditions ranging from temperature to soil type and vegetation, some of these conditions may be related to the putative function of this SNP bearing region.

Lastly, the sequence containing SNP ID TP78719 correlated with SAR and MAP was annotated as a conserved oligomeric Golgi (COG) complex subunit 5, which has unique morphological and functional characteristics such as transportation of cell building materials to energy rich compartments of the cell (Faso et al., 2009). However, we cannot comment on a direct and clear association of COG to SAR and MAP. A mutation in the *embryo yellow (EYE)* gene was identified in *Arabidopsis* that lead to the abnormal

coloration and morphology of embryos. The EYE mutation encodes a putative protein homologous to COG7, which is required for the normal morphology and function of the Golgi apparatus. The *EYE* mutation caused mislocalization of a Golgi protein and the size of the Golgi apparatus was also altered. Thus, EYE might be involved in the transport or retention of Golgi-localized proteins, and in the maintenance of Golgi morphology (Ishikawa et al., 2008). Several studies indicated that the COG complex has gained additional functions. For example, HvCOG3 is essential for the resistance to powdery mildew fungal penetration into the host cell of barley (Ostertag et al., 2013).

3.5 Concluding remarks

This study is the first to discover putative SNPs using GBS in lingonberry and to functionally annotate genome-wide lingonberry sequences that contain SNPs associated to the biosynthesis of phytochemicals and/or environmental conditions. We identified a number of putative SNPs that are correlated with the biosynthesis of phenolic compounds and adaptation to environmental conditions. In most cases we were able to establish a link between the gene identity and protein function and the biochemical or environmental variables these SNPs were associated with. Future research should focus on experimentally confirming these SNPs and analyzing the mechanism by which the specific nucleotide changes render the differential phenolic compound content or environmental preferences. The identified SNPs could be a good source to study the adaptation of lingonberry under environmental stress condition, and differential phenolic compound biosynthesis. The identified genes could be directly manipulated in order to enhance the production of these

phytochemical compounds and adaptation to environmental stress. The discovered genes associated with the biosynthesis and metabolism in lingonberry need further investigation. The SNPs present in this study require further investigation of the genome, proteome and metabolome under changing environmental conditions that stimulate production and biosynthesis of phytochemicals in lingonberry. The SNPs discovered in this study could be an excellent resource for agricultural application to improve lingonberry as a crop.

4 Chapter Four: General Conclusions

This thesis focuses on total phenolic compound content, antioxidant capacity and the application of a next generation sequencing approach to study the genetic variation of lingonberry.

This thesis explored the variation of phenolic compounds concentrations and antioxidant capacity of wild lingonberry fruit across several environmental factors in Newfoundland and southern Labrador. The results of this study demonstrate that the response of TPC and AC to environmental factors varies among different samples. The results show that, ecoregion, temperature, runoff, and proximity to the coast seemed to be important factors influencing the amount of TPC and AC of Newfoundland wild lingonberry fruit. The amount of TPC were observed in North Shore Forest followed by Maritime Barrens, Central Newfoundland Forest and Strait of Belle Isle Barrens. No significant variation was observed for AC. It is also observed that high mean annual temperature and mean summer temperature had highest TPC but highest AC was observed in Low mean annual and mean summer temperature. Medium (1100-1300 mm) mean annual precipitation show highest TPC but lowest AC. Generalizations about the mechanism of how phenolic compounds are affected by environmental factors are still difficult to formulate. Contrary to our expectations, this study did not show any significant correlation between TPC and AC. Among the potential explanations for this result is that the specific chemical concentration of each secondary metabolite can be equally or more important than the total concentration for determining the antioxidant capacity of a plant

extract. Some phenolic compounds have higher antioxidant capacity than others. Furthermore, the small variation in TPC and AC among samples from this Canadian province might mask a positive correlation.

Conclusions reached in this study await further corroboration upon repetitions in multiple years. It is important to maintain fruit size and ripeness uniform while selecting samples, as it is well reported that different maturity stages and fruit sizes vary in terms of the content of phenolic compounds. Smaller fruits have more surface area (skin) than larger fruits, which can influence TPC and AC. Therefore, it is possible that fruit size selection in our study may have influenced TPC and AC. The environmental factors under which we collected lingonberry fruits were uncontrolled, which may also influence the experimental results. Our results need to be verified with investigations that use replicated sites under controlled experimental conditions, such as in a greenhouse, to see which environmental factors influence TPC and AC the most. Our study however, provides good baseline information for these kinds of experiments to follow and compare, since biochemical synthesis in plants in the wild are often unknown. In the present study we only measured TPC and AC of lingonberry fruits, and measurement of individual phenolic compounds can provide better insight on the direction and magnitude of the environmental effects.

This study is the first to discover SNPs in lingonberry and to functionally annotate genome-wide lingonberry sequences that contain SNPs associated to the biosynthesis of phytochemicals and/or environmental conditions. We demonstrated here that high numbers of genome-wide SNPs can be discovered by sequencing random short DNA fragments of a complex genome with NGS platforms without a reference genome. Using the TASSEL-UNEAK pipeline, 1,586 putative SNPs were discovered among 56 samples of *Vaccinium*

vitis-idaea. We identified 260 putative SNPs that are correlated with the biosynthesis of phenolic compounds and environmental conditions. Note that our analyses are computationally oriented with no experimental validation. These SNPs remain to be experimentally validated in the future, either by experiments or by computational approaches that will support our findings. Functional annotation of SNP-containing sequences identified key enzymes that are involved in the biosynthesis of secondary plant metabolites, plant growth and development, resistance to biotic and abiotic stress, and genes related to adaptations to cold conditions, water shortage condition or different pH conditions. The identification of these genes gave insight on their potential function in plants. These results can be used to direct efforts to genetically manipulate plants in order to enhance their production of these bioactive phytochemicals or to grow under the desired environmental conditions. These predicted functional annotations also need to be experimentally validated. The majority of functional predictions inform whether a variation in a position is likely to be biologically relevant based on the presence of one or more biochemical signals. When a variant has a particular strong annotation related to SNPs, laboratory follow up of that variant is often required.

The structurally and functionally annotated SNP-containing sequences in diverse coding and non-coding gene regions could have potential to be utilized for various large-scale marker-based genotyping applications in lingonberry. However, in most cases we were able to establish a link between the gene identity and protein function and the biochemical or environmental variables these SNPs were associated with. Future research should focus on analyzing the mechanism by which the specific nucleotide changes render the differential phenolic compound content or environmental preferences. The identified

SNPs could be a good source to study the adaptation of lingonberry under environmental stress condition, and differential phenolic compound biosynthesis. The identified genes could be directly manipulated in order to enhance the production of these phytochemical compounds and adaptation to environmental stress. The discovered genes associated with the biosynthesis and metabolism in lingonberry need further investigation. The SNPs present in this study require further investigation of the genome, proteome and metabolome under changing environmental conditions that stimulate production and biosynthesis of phytochemicals in lingonberry. Lastly, the SNPs discovered in this study could be an excellent resource for agricultural application to improve lingonberry as a crop.

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6 Appendices

Appendix 1. Sampled *V. vitis-idaea* populations in Newfoundland and Labrador. Each population is coded by ecoregion, mean annual temperature, mean summer temperature, mean annual precipitation, mean annual runoff, surface water pH, surface water sensitivity to acid rain, and proximity to the coast (Government of Newfoundland and Labrador 1992).

Sampling ID	Sampling locations	Latitude	Longitude	Ecoregion	MAT	MASP	MAP	MAR	Surface water pH	Surface water sensitivity (SAR)	Coastal Proximity
1	Daniel's Harbour (DHB)	50.258204	-57.570914	4	2	2	1	3	3	4	1
2	Daniel's Harbour (DHB2)	50.29574	-57.554394	4	2	2	1	3	3	4	1
3	Cow Head (CWH)	49.921719	-57.807381	4	2	2	2	3	3	3	1
4	Cornier's Pond (CRP)	49.074177	-57.570234	1	3	3	2	3	3	4	2
5	Deer's Lake (DLK)	49.230142	-57.297187	1	3	3	1	3	3	4	2
6	Parsons Pond (PRP)	50.045723	-57.704007	4	2	2	1	3	3	3	1
7	Top Pond (TPD)	47.977643	-57.628318	8	2	2	3	4	2	2	2
8	King George's Park (KGP)	48.182911	-57.739559	8	2	2	3	4	3	3	2
9	King George's Park (KGP2)	48.022422	-57.673641	8	2	2	3	4	2	3	2
10	King George's Park (KGP3)	47.918525	-57.646175	8	2	2	3	5	1	1	2
11	Route 480 (R480)	47.829422	-57.67501	8	2	2	3	5	1	1	2
12	Codroy (COD)	47.882011	-59.393378	1	3	2	2	5	4	4	1
13	St. George's (STG)	48.439436	-58.453665	1	3	3	2	4	4	4	1
14	St. George's 2 (STGP)	48.379947	-58.588072	1	3	3	2	4	3	4	1
15	Codroy (COD2)	47.887137	-59.397337	1	3	2	2	5	4	4	1

16	Codroy (COD3)	47.852131	-59.343778	1	3	2	2	5	4	4	1
17	Twillingate (TWL)	49.652608	-54.762294	3	3	3	1	1	3	2	1
18	Gander Bay (GBY)	49.364175	-54.499142	3	3	3	1	1	3	2	1
19	Lumsden (LMS)	49.305873	-53.646194	7	3	3	1	1	1	1	1
20	New-Wes-valley (NWV1)	49.159781	-53.573217	7	3	3	1	1	1	1	1
21	Black Lake (BLK)	49.55919	-56.462041	2	2	3	1	1	3	3	2
22	St. Patricks (SPG 360)	49.573004	-55.993633	2	2	3	1	1	3	4	2
23	Bishop Falls (BF1)	49.019023	-55.490362	2	3	3	1	1	2	3	2
24	Bishop Falls (BF2)	48.975324	-55.568296	2	3	3	1	1	2	2	2
25	Rocky Pond (RC1)	49.474919	-57.142606	4	3	3	2	3	4	4	2
26	Springsdale (SPG)	49.522484	-56.003895	2	2	3	1	1	3	4	1
27	Sandylake (SLK)	49.22833	-57.076607	2	3	3	1	2	3	3	2
28	Terra Nova NP (TNN1)	48.41931	-54.134101	2	3	3	2	2	1	3	1
29	Sandy Beach Cove (SBC)	48.658204	-53.84607	3	3	3	2	2	2	2	1
30	Gambo (GMB1)	48.80791	-54.18204	3	3	3	2	2	1	1	1
31	Dauidsuille (DUT)	49.592478	-54.41205	3	3	3	1	1	2	2	1
32	Lewisporte (LWP)	49.259259	-55.033839	3	3	3	2	1	2	3	1
33	Avalon peninsula (AVA1)	47.583102	-52.758423	6	3	2	3	3	4	3	2
34	Avalon peninsula (AVA2)	47.427251	-52.766663	6	3	2	3	4	1	1	2
35	Avalon peninsula (AVA3)	47.095463	-52.958923	6	3	2	3	4	1	2	1
36	Avalon peninsula (AVA4)	47.194471	-53.381897	6	3	2	3	4	2	2	2
37	Avalon peninsula (AVA5)	47.668254	-53.272034	6	3	2	2	4	2	2	1
38	Avalon peninsula (AVA6)	46.739389	-53.34104	7	3	1	3	5	1	1	2
39	Avalon peninsula (AVA7)	46.650565	-53.18574	7	3	1	3	5	1	1	2

40	Avalon peninsula (AVA8)	46.709071	-53.486851	7	3	1	3	5	1	1	2
41	Avalon peninsula (AVA9)	47.167128	-52.908556	6	3	2	3	4	1	1	2
42	Avalon peninsula (AVA10)	47.813517	-53.457461	6	3	3	2	4	1	1	1
43	Avalon peninsula (AVA11)	46.922322	-54.171964	7	3	2	2	4	1	1	1
44	Flower Cove (FCV)	51.311631	-56.726437	9	1	1	1	2	3	4	1
45	Labrador (LAB1)	51.420523	-57.106342	10	1	1	1	1	3	3	1
46	Labrador (LAB2)	51.447211	-57.015381	10	1	1	1	1	3	3	2
47	Labrador (LAB3)	51.486962	-56.890261	10	1	1	1	1	3	3	2
48	Labrador (LAB4)	51.537334	-56.812801	10	1	1	1	1	3	3	2
49	Labrador (LAB5)	51.565912	-56.744939	10	1	1	1	1	3	3	1
50	St. Barbes (STBS)	50.814569	-57.060627	9	1	2	1	2	3	4	2
51	Bird Harbour (BHD)	51.056136	-56.920986	9	1	2	1	2	3	4	1
52	Bird Cove (BDC)	48.949858	-54.599919	2	3	3	2	1	1	1	2
53	Charlottetown (CHT)	51.221539	-56.754309	9	1	1	1	2	3	4	1
54	Gander (GND)	50.639775	-57.206044	4	1	2	1	2	3	4	2
55	Port Sanders (PSD)	50.727439	-57.185445	9	1	2	1	2	3	4	2
56	Port Sanders (PSD2)	48.443822	-54.013736	2	3	3	2	2	2	2	2

Appendix 2: Functional annotation of 241 uniproteins from 1,586 putative SNPs based on their sequence similarity on the NCBI NR protein database using BLASTX

Serial No.	SNP ID	BLAST hits	Putative Function	Biological process	Reference
1	TP9982	GI:1346523	S-adenosylmethionine synthase 1	Regulate ethylene metabolism	(Van Breusegem et al., 1994)
2	TP14093	GI:8778539	Putative telomere repeat-binding protein TRFL2	Telomere-binding specificity	(Karamysheva et al., 2004; Nelson et al., 2014)
3	TP63345	GI:13676402	Type I chlorophyll a/b-binding protein a, partial	Transcription factor for light harvest and pigment	(Canas and Valenzuela, 1989; Krol et al., 1995)
4	TP71890	GI:15240924	E3 ubiquitin-protein ligase ATL31	Regulate and signalling for growth and ABA	(Fu et al., 2014)
5	TP71276	GI:16905223	Putative retrotransposon-related protein	Stress related protein coding gene for gene and genome evolution	(Todorovska, 2007; Jiang et al., 2016)
6	TP24839	GI:18447765	Fiddlehead-like protein	Biosynthesis cuticular wax and suberin	(Pruitt et al., 2000; Lee et al., 2009a)
7	TP71890	GI:21536625	RING-H2 zinc finger protein-like	Seed development	(Xu and Li, 2003)
8	TP23265	GI:24412728	Phosphoenolpyruvate carboxylase, 9partial	Involved in anaplerotic metabolism, pH regulation, and stomatal opening.	(Chollet et al., 1996; Cousins et al., 2007)
9	TP66508	GI:28412309	Amino acid transporter AAP2, partial	Transport growth and development related amino acid	(Ortiz-Lopez et al., 2000)
10	TP40359	GI:30171283	High affinity nitrate transporter, partial	Involved in the high and low affinity nitrate transport	(Orsel et al., 2002)

11	TP64237	GI:38194917	Phytochelatin synthetase-like protein	Stress response to heavy meatal ditoxification	(Tsuji et al., 2004)
12	TP10440	GI:38453854	Aspartate aminotransferase, partial	Regulate metabolism of phenylalanine and lignin	(de la Torre et al., 2014)
13	TP24769	GI:47027061	HUELLENLOS-like protein	Growth and development	(Skinner et al., 2001)
14	TP51270	GI:60657604	Secondary cell wall-related glycosyltransferase family 14	Transfer sugar	(Hansen et al., 2010)
15	TP77277	GI:73624747	Cellulose synthase-like protein CslG	Metabolism of plant cell walls	(Richmond and Somerville, 2000)
16	TP11064	GI:110931796	Transcription factor MYB148, partial	Transcription factor for plant growth, development, metabolism and stress responses	(Liao et al., 2008)
17	TP72930	GI:113367156	Transcription factor bZIP128, partial	Transcription factor regulate pathogen defence, light and stress signalling	(Jakoby et al., 2002)
18	TP65748	GI:118430826	Chloroplast phosphate transporter, partial	Transport phosphate	(Versaw and Harrison, 2002)
19	TP76719	GI:148250189	SecY protein, partial	Transport protein to endoplasmic reticulum (ER)	(Park and Rapoport, 2012)
20	TP18843	GI:150022290	Alpha-expansin 6, partial	Cell-wall hydrolysis activity	(Sampedro and Cosgrove, 2005)
21	TP79435	GI:167019276	DND1, partial	Disease resistance	(Clough et al., 2000)
22	TP70388	GI:182634659	Glycosyl transferase family 2	Transfer of polysaccharides	(Lairson et al., 2008)

23	TP44921	GI:198400323	Nam-like protein	DNA binding	(Nuruzzaman et al., 2013)
24	TP51860	GI:217502671	Glycyl-tRNA synthetase, alpha subunit	ATP binding and transfer RNA molecule	(Uwer et al., 1998)
25	TP24008	GI:224078630	ATP-binding-cassette transporter family protein	Transport and/or regulate other transporters	(Rea, 2007)
26	TP44464	GI:224086345	ATPase 6 family protein	ATP hydrolysis to the active transport of cations or other compounds	(Morsomme and Boutry, 2000)
27	TP14641	GI:224096918	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48kDa subunit	Transfer of a high mannose oligosaccharide	(Qin et al., 2013)
28	TP4578	GI:225425760	PREDICTED: UDP-galactose/UDP-glucose transporter 3	Transport non-cellulosic polysaccharides and glycoproteins	(Norambuena et al., 2002)
29	TP73525	GI:225431330	PREDICTED: peroxidase 64	Signalling in lignification, suberization, auxin catabolism and defense	(Hiraga et al., 2001)
30		GI:225432318	PREDICTED: probable histone-arginine methyltransferase 1.3	Transcriptional regulation of arginine methylation and signal transduction	(Niu et al., 2008)
31	TP19017	GI:238617558	70 kDa heat shock protein, partial	Stress response on cold, UV light, wound healing	(Su and Li, 2008)
32	TP23086	GI:254777812	DnaK, partial	Stress response on cold, UV light, wound healing	(Neumann et al., 1993; Su and Li, 2008)
33	TP9995	GI:255537884	Receptor protein kinase CLAVATA1 precursor, putative	Signal transduction for cell differentiation	(Clark et al., 1997)
34	TP40196	GI:255542149	Beta-glucosidase, putative	Transcriptional factor to hydrolysis of cell wall-derived oligosaccharides	(Morant et al., 2008)
35	TP72930	GI:255542297	DNA binding protein, putative	Sequence-specific DNA-binding proteins	(Strauch, 2001)

36	TP64237	GI:255547141	Protein COBRA precursor, putative	Regulate cell expansion	(Roudier et al., 2002)
37	TP52840	GI:255548956	ADP, ATP carrier protein, putative	Transporter protein for starch biosynthesis	(Soliman et al., 2014; Haferkamp and Schmitz-Esser, 2014)
38	TP72794	GI:255550469	Maintenance of ploidy protein mob1, putative	Transcription factor for mitosis and maintenance of Ploidy	(Luca and Winey, 1998)
39	TP19017	GI:255551749	Protein arginine n-methyltransferase, putative	Cell proliferation, signal transduction, and protein trafficking	(Pawlak et al., 2000)
40	TP18874	GI:255553635	26S proteasome regulatory subunit rpn1, putative	Regulatory protein involved in developmental processes	(Brukhin et al., 2005)
41	TP73887	GI:255558824	Glycogen phosphorylase, putative	Metabolite of glycogen synthase	(Schupp and Ziegler, 2004)
42	TP63250	GI:255560229	Serine/threonine-protein kinase PBS1, putative	Pathogenesis related	(Swiderski and Innes, 2001)
43	TP77354	GI:255567689	Multicopper oxidase, putative	Metabolism of lignin degradation	(Hoegger et al., 2006)
44	TP14763	GI:255572479	r2r3-myb transcription factor, putative	R2R3-type MYB genes control many aspects of plant secondary metabolism	(Stracke et al., 2001)
45	TP75434	GI:255575762	Expansin-like B1//Expansin-like protein /Major pollen allergen Ory s 1 precursor, putative	Regulate growth of organ through altering cell wall structure	(Russell et al., 2008; Chen et al., 2016)
46	TP25317	GI:255577467	Arsenite inducuble RNA associated protein aip-1, putative	Binding protein for cell division and growth	(Ketelaar et al., 2004)

47	TP33726	GI:259027684	Putative ETTIN protein, partial	DNA binding proteins for auxin response elements	(Sessions et al., 1997)
48	TP73525	GI:259414641	Putative peroxidase	Biosynthesis of lignin and stress response	(Vicuna, 2005)
49	TP45674	GI:294715576	Chloride channel protein	Transporter activity	(Guo et al., 2014)
50	TP26669	GI:294819072	Spermidine/putrescine transport system ATP-binding protein	ATP binding protein for growth	(Fujita and Shinozaki, 2014)
51	TP71276	GI:297724777	Os06g0316600	Nucleic acid binding	http://www.uniprot.org/uniprot/C7J398
52	TP40359	GI:297818992	ATNRT2.6	High-affinity nitrate transport	(Nandanavanam, 2011)
53	TP33726	GI:302190092	Auxin response factor 3	Regulate auxin-response	(Liu et al., 2014)
54	TP63345	GI:310696692	Putative chlorophyll A/B binding protein, partial	Transfer light energy for photochemical reaction.	(Green et al., 1991)
55	TP19923	GI:313574423	Glucose-1-phosphate adenylyltransferase, partial	Starch regulatory enzyme	(Bejar et al., 2006)
56	TP23086	GI:327358433	Heat shock 70kDa protein 5, partial	Protect cell from stress	(Ferradini et al., 2015)
57	TP22838	GI:334187912	Pathogenesis-related thaumatin-like protein	Plant defense	(Wang et al., 2010)
58	TP65734	GI:339836239	Acetyl-CoA carboxylase, carboxyl transferase, beta subunit	Fatty acid metabolism	(Sasaki and Nagano, 2004)
59	TP40359	GI:345293837	AT5G60780-like protein, partial	High-affinity nitrate transporter protein	(Armengaud et al., 2004)
60	TP15810	GI:353441064	Putative Phospholipid-Diacylglycerol acyltransferase, partial	Triacylglycerol metabolism	(Fan et al., 2013)
61	TP44921	GI:354992035	NAC domain protein NAC1	Lateral root formation	(Xie et al., 2000)

62	TP79304	GI:356497397	PREDICTED: probable acyl-activating enzyme 1, peroxisomal	Signalling for developmental and defense-related process	(Koo et al., 2006)
63	TP10387	GI:356543391	PREDICTED: MACPF domain-containing protein At4g24290	Involved in innate immunity	(Morita-Yamamuro et al., 2005)
64	TP52840	GI:356563859	PREDICTED: adenine nucleotide transporter BT1, chloroplastic/mitochondrial	Many metabolic reactions in the endoplasmic reticulum (ER)	(Leroch et al., 2008)
65	TP14426	GI:357446231	F-box protein interaction domain protein	Floral meristem and floral organ development	(Jain et al., 2007)
66	TP15782	GI:357511039	Terminal flower 1	Regulate flowering time and development	(Hanano and Goto, 2011)
67	TP65734	GI:373098621	Acetyl-CoA carboxylase subunit beta	Regulate fatty acid metabolism	(Berg et al., 2002)
68		GI:375057900	Radical SAM domain protein, partial	Catalyze diverse reactions, including methylations, anaerobic oxidation and protein radical formation.	(Sofia et al., 2001)
69	TP37873	GI:375316664	Translation initiation factor 2	Down regulation of protein synthesis in response to stress	(Immanuel et al., 2012)
70	TP75434	GI:377824016	Expansin L	Modifying the cell wall matrix during growth and development	(Li et al., 2002)
71	TP14266	GI:409129268 / EKM99128.1	Glycogen debranching protein GlgX	Regulate glycogen synthesis and breakdown	(Zmasek and Godzik, 2014)
72	TP15782	GI:410442699	Centroradialis	Growth and development of flower	(Cremer et al., 2001; Huang et al., 2012)
73	TP75547	GI:413943661	Putative ribosomal protein S4 (RPS4A) family protein	Ribosome assembly and rRNA binding	(Sudhamalla et al., 2013)

74	TP3846	GI:414865502 / DAA44059.1	TPA: hypothetical protein ZEAMMB73_205158	Protein transport protein	
75	TP51007	GI:429183183	Pyruvate dehydrogenase E1 component subunit beta	Glycolysis metabolic pathway	(Tovar- Méndez et al., 2003)
76	TP44921	GI:449449759	PREDICTED: NAC domain-containing protein 2-like	Regulates a wide range of developmental processes	(Nuruzzaman et al., 2013)
77	TP77354	GI:449453507	PREDICTED: L-ascorbate oxidase homolog	Antioxidant activity and defense	(Barbehenn et al., 2008)
78	TP13826	GI:449461607	PREDICTED: coatomer subunit alpha-1	Protein transport	(Wang et al., 2015a)
79	TP40359	GI:459464865	putative nitrate transporter, partial	Transport nitrate (NH4+ and NO3−)	(Li et al., 2012)
80	TP11478	GI:460380941	PREDICTED: iron-sulfur cluster assembly protein 1-like	Regulate gene expression	(Couturier et al., 2013)
81	TP73466	GI:460393468	PREDICTED: protein trichome birefringence-like 10	Cellulose biosynthesis	(Bischoff et al., 2010)
82	TP77471	GI:460393688	PREDICTED: MIP18 family protein At1g68310-like	DNA metabolism	(Couturier et al., 2013)
83	TP10437	GI:460394082	PREDICTED: eukaryotic peptide chain release factor subunit 1-3-like	Signal transduction for growth and development	(Petsch et al., 2005)
84	TP77277	GI:460399944	PREDICTED: cellulose synthase-like protein G3	Cell wall synthesis	(Plett et al., 2014)
85	TP78719	GI:460405503	PREDICTED: conserved oligomeric Golgi complex subunit 5	Cell wall development	(Ishikawa et al., 2008)
86	TP33726	GI:460413335	PREDICTED: auxin response factor 6-like isoform X1	Transcription factor for adventitious root initiation	(Kulcheski et al., 2015)
87	TP25958	GI:469469380	Auxin response factor 10	Transcription factor for development	(Liu et al., 2007)

88	TP14763	GI:470104806	PREDICTED: trichome differentiation protein GL1-like /kinase-like protein TMKL1	Trichome development	(Payne et al., 2000)
89	TP63250	GI:470112326	PREDICTED: putative kinase-like protein TMKL1	Regulates organ development	(Valon et al., 1993; Rameneni et al., 2015)
90	TP47085	GI:470122442	PREDICTED: protein KTI12 homolog	Roots and leaves development	(Nelissen et al., 2003)
91	TP40359	GI:475616851	Nitrate transporter	Nitrate transporters	(Hu and Schmidhalter, 2005)
92	TP24839	GI:482679618	3-ketoacyl-CoA synthase 8	Regulate fatty acid biosynthesis	(Weidenbach et al., 2014)
93	TP9086	GI:489696039	MULTISPECIES: molecular chaperone DnaK	Degradation and maturation of all cellular compartments.	(Boston et al., 1996; Aguilar-Rodriguez et al., 2016)
94	TP30852	GI:495822505	MULTISPECIES: acetyl-CoA carboxylase biotin carboxylase subunit	Regulate fatty acid synthesis	(Berg et al., 2002; Sasaki and Nagano, 2004)
95	TP34691	GI:496166775	1-deoxy-D-xylulose-5-phosphate synthase	Regulate photosynthesis, respiration, growth, cell cycle	(Wright and Phillips, 2014)
96	TP51860	GI:497450570	Glycine--tRNA ligase subunit alpha	Ligates the amino acid glycine to its cognate tRNA species	(Uwer et al., 1998)
97	TP26669	GI:498344380	Spermidine/putrescine ABC transporter ATP-binding protein	Transport ATP-binding cassette (ABC) proteins.	(Davies and Coleman, 2000)

98	TP63667	GI:501351948	homoserine O-acetyltransferase	Cysteine biosynthesis	(Mulet et al., 2004)
99	TP23468	GI:501353159	Pyruvate, phosphate dikinase	Bifunctional regulatory protein	(Podesta et al., 1990; Chastain et al., 2002)
100	TP44464	GI:502114188	PREDICTED: ATPase 10, plasma membrane-type	Activate secondary transport	(Morsomme and Boutry, 2000)
101	TP72794	GI:502165230	PREDICTED: MOB kinase activator-like 1A [Cicer arietinum]	Regulation of cell expansion and cell division control of root growth	(Vitulo et al., 2007; Pinosa et al., 2013)
102	TP36990	GI:506397591	3-hydroxybutyryl-CoA dehydrogenase	Regulate blanched chain amino acid degradation	(Kamada et al., 2003)
103	TP11176	GI:506411246	Omega amino acid--pyruvate aminotransferase	Transport	(Yun et al., 2004)
104	TP25358	GI:514762111	PREDICTED: RING-H2 finger protein ATL32-like	Seed development	(Guzmán, 2012)
105	TP75547	GI:514801217	PREDICTED: chaperone protein ClpD2, chloroplastic	Degradation and maturation of all cellular compartments.	(Boston et al., 1996; Aguilar-Rodriguez et al., 2016)
106	TP69971	GI:515487377	Ferredoxin--NADP(+) reductase	Electron transfer metabolic processes	(Musumeci et al., 2012)
107	TP7256	GI:516615412	Radical SAM protein	Catalyze diverse reactions, including methylations, anaerobic oxidation and protein radical formation.	(Sofia et al., 2001)
108	TP31548	GI:517078404	Malic enzyme	Metabolism various metabolic pathways	(Casati et al., 1999)
109	TP14266	GI:517576982	Glycogen debranching enzyme	Carbohydrate metabolism	(Nakayama et al., 2001)

110		GI:518232202	MULTISPECIES: two-component system response regulator	Signaling for ethylene perception and transduction	(Lohrmann and Harter, 2002)
111	TP15510	GI:527200628	methionine synthase	Cellular metabolism	(Hesse et al., 2004)
112	TP65038	GI:543177062	protein GPR107-like isoform 1	Roles in signalling	(Urano and Jones, 2014)
113	TP26669	GI:543965436	ABC transporter ATP-binding protein	Transporters involved in detoxification processes	(Kang et al., 2011)
114	TP31548	GI:556755935	PREDICTED: NADP-dependent malic enzyme, chloroplastic-like		(Wheeler et al., 2005)
115	TP63632	GI:564590704	Oxidoreductase	Electron transporting	(Lüthje et al., 1997)
116	TP69875	GI:565353651	PREDICTED: fimbrin-2-like	Pollination	(Inglis et al., 2014)
117	TP25317	GI:565369391	PREDICTED: zinc finger AN1 and C2H2 domain-containing stress-associated protein 13	Plant growth and development	(Gupta et al., 2012)
118	TP46523	GI:565389761	PREDICTED: zinc finger protein GIS2 isoform X1	Floral development	(Zhou et al., 2012)
119	TP27313	GI:565393942	PREDICTED: ACT domain-containing protein ACR2	Plant hormone regulator/arsenic reductase	(Hayakawa et al., 2006)
120	TP37737	GI:567402656	RNA polymerase sigma-54 factor		
121	TP46191	GI:568829621	PREDICTED: protein DETOXIFICATION 41-like	Flavonoid/H^+-metabolic process	
122	TP187	GI:568849650	PREDICTED: polygalacturonase-like	Metabolic process of pectin degradation	(de la Paz Celorio-Mancera, Maria et al., 2008)
123	TP72826	GI:571433543	PREDICTED: receptor-like protein kinase 5	Polypeptide growth factor/cell wall signaling	(Afzal et al., 2008)

124	TP27313	GI:590572310	ACT-like superfamily protein	Metabolic enzymes/	(Feller et al., 2006)
125	TP15121	GI:590601706	Leucine-rich receptor-like protein kinase family protein isoform 2	Developmental and defense-related processes including cell proliferation, stem cell maintenance, hormone perception	(Torii, 2004)
126	TP77490	GI:590605738	Multidrug resistance-associated protein 3 isoform 3	Transport gene *Mrp3* expression is controlled by the regulators of anthocyanin	(Sodani et al., 2012)
127	TP53658	GI:590621549	P-loop containing nucleoside triphosphate hydrolases superfamily protein		
128	TP72708	GI:590623782	Trichome birefringence-like 37 isoform 1	Cellulose biosynthesis	(Bischoff et al., 2010)
129	TP56121	GI:590642527	Ankyrin repeat family protein	Signal transductions in anthocyanin biosynthesis	(Mou et al., 2013)
130	TP11478	GI:590663500	SufE/NifU family protein	Biosynthesis of the Iron-Molybdenum Cofactor of Nitrogenase	(Zhao et al., 2007)
131	TP10387	GI:590681941	MAC/Perforin domain-containing protein	Function in immunity, invasion and development.	(Rosado et al., 2008)
132	TP77252	GI:590699741	Sec7 domain-containing protein		
133	TP18843	GI:590700043	Expansin A1, ALPHA 1.2,EXPA1		
134	TP79304	GI:592923297	4-coumarate:coenzyme A ligase 4	Phenylpropanoid pathway	(Ehlting et al., 2001)
135	TP72708	GI:602183257	TBL1	Putative transcriptional regulator	(Finlayson, 2007)
136	TP38764	GI:610510280	Dna binding response regulator PrrA (RegA)	Regulation of photosynthesis gene expression in response to oxygen	(Eraso and Kaplan, 1994)
137	TP51007	GI:639288462	pyruvate dehydrogenase complex E1 component subunit beta	Cellular metabolism	(Millar et al., 1998)

138	TP70388	GI:643026628	CAZy families GT2 protein, partial	Biosynthesis of polysaccharides	
139	TP53658	GI:645245421	PREDICTED: ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic		(He et al., 1997)
140	TP42618	GI:645259362	PREDICTED: phospholipase A1-Igamma2	Photosynthesis/photoperiodic control of flowering	(Dai et al., 2015)
141	TP72263	GI:645269294	PREDICTED: cyclic dof factor 3	Transcription factor	(Ma et al., 2015)
142	TP14763	GI:645275423	PREDICTED: transcription factor WER-like	Transcription factor	(Tominaga-Wada and Nukumizu, 2012)
143	TP38764	GI:648414219	Chemotaxis protein CheY		
144	TP70388	GI:651611982	Glucosyltransferase	Transcription factor for flavonol-3-*O*-sophorosides/phytohormon biosynthesis	(Trapero et al., 2012)
145	TP2889	GI:654277417	MULTISPECIES: membrane protein	Transport protein	(Chrispeels et al., 1999)
146	TP14763	GI:658054507	PREDICTED: myb-related protein 305-like	Phenylpropanoid metabolism, phenylalanine ammonia lyase (PAL)	(Ambawat et al., 2013)
147	TP33726	GI:658058225	PREDICTED: auxin response factor 12-like	Transcription factor	(Jones et al., 2002)
148	TP24008	GI:659067752	PREDICTED: UPF0051 protein ABCI8, chloroplastic	Light signaling	
149	TP14093	GI:672114454	PREDICTED: telomere repeat-binding protein 5-like isoform X1	Telomere-binding specificity	(Karamysheva et al., 2004; Nelson et al., 2014)

150	TP15088	GI:672119187	PREDICTED: thioredoxin reductase NTRB-like	Regulation of cellular processes	(Cha et al., 2015)
151	TP69848	GI:672142899	PREDICTED: probable serine/threonine-protein kinase At1g54610	Signaling pathway	(Afzal et al., 2008)
152	TP10354	GI:674781082	Aldehyde dehydrogenase	Metabolic pathway	(Brocker et al., 2013)
153	TP18857	GI:674887978	BnaA05g28820D		
154	TP23086	GI:675383932	Heat shock protein cognate 4, partial	Pollen development	(Lee et al., 2009b)
155	TP18857	GI:685312673	PREDICTED: superkiller viralicidic activity 2-like 2	Regulating melanoblast proliferation	(Yang et al., 2007)
156	TP72740	GI:685369222	PREDICTED: probable galacturonosyltransferase 6 isoform X1	Pectin biosynthesis	(Caffall et al., 2009)
157	TP64086	GI:694317216	PREDICTED: lipase 1-like	Plant immunity	(Kwon et al., 2009)
158	TP44464	GI:694410600	PREDICTED: ATPase 11, plasma membrane-type-like	Signals regulating plant growth	(Falhof et al., 2016)
159	TP75434	GI:694427179	PREDICTED: expansin-like B1	Regulators of wall extension during growth	(Lu et al., 2015)
160	TP72806	GI:694443634	PREDICTED: multidrug and toxin extrusion protein 1-like	Transporters	(Shoji et al., 2009)
161		GI:695020245	PREDICTED: glucose-6-phosphate/phosphate translocator 2, chloroplastic-like	Metabolism and photosynthesis/starch	(Dyson et al., 2015)
162		GI:697174262	PREDICTED: S-adenosylmethionine synthase 2	Ethylene biosynthesis	(Mao et al., 2015)

163		GI:698448324	PREDICTED: protein phosphatase 2C 37-like	Plant signal transduction processes	(Rodriguez, 1998)
164		GI:698497153	PREDICTED: chlorophyll a-b binding protein 7, chloroplastic-like, partial	Photosynthesis	(Silva et al., 2016)
165		GI:698498292	PREDICTED: phosphatidylinositol 4-kinase alpha 2-like, partial	Signal transduction in higher plants	(Vermeer et al., 2009)
166		GI:702345959	PREDICTED: protein trichome birefringence-like 38	Regulate cell wall polysaccharides	(Gille and Pauly, 2007)
167		GI:703064151	Phospholipase A1-Igamma1	Involved in early wound response/phospholipid-based signal transduction	(Canonne et al., 2011)
168	TP3846	GI:703079072	Protein transport protein Sec61 subunit alpha	Pathogenicity/post-translational protein translocation	(Zhang et al., 2013)
169	TP51270	GI:703102264	Xylosyltransferase 1		
170	TP24839	GI:703106039	3-ketoacyl-CoA synthase 10	Fatty acid biosynthesis in vegetative tissues	(Todd et al., 1999)
171	TP14659	GI:703117832	Homeobox-leucine zipper protein MERISTEM L1	Transcription factor	(Lu et al., 1996)
172	TP77490	GI:703134224	ABC transporter C family member 3	Transport of glutathione conjugates	(Kang et al., 2011)
173		GI:703138334	Receptor-like protein kinase HSL1	Signaling for post-pollination abscission of floral organs	(Taylor et al., 2016)
174	TP54303	GI:703147528	K(+) efflux antiporter 5		
175	TP14763	GI:703160293	Transcription factor	DNA buinding	(Yanagisawa, 1998)
176	TP8223	GI:719979828	PREDICTED: E3 ubiquitin-protein ligase ATL42		

177	TP8223	GI:719993936	PREDICTED: putative RING-H2 finger protein ATL12	Roles in defense responses	(Aguilar-Hernández et al., 2011)
178	TP72794	GI:720018797	PREDICTED: MOB kinase activator-like 1 isoform X3	Signaling in cell proliferation and programmed cell death	(Ye, 2010)
179	TP72053	GI:720055989	PREDICTED: probable RNA 3'-terminal phosphate cyclase-like protein	RNA splicing	(Filipowicz et al., 1985)
180	TP54303	GI:720069481	PREDICTED: cysteine and histidine-rich domain-containing protein RAR1	Plant innate immunity	(Sadanandom et al., 2004)
181	TP14763	GI:720079076	PREDICTED: transcription factor MYB82-like	Transcription factor for developmental function	(Liang et al., 2014)
182	TP69875	GI:720087119	PREDICTED: LOW QUALITY PROTEIN: fimbrin-5	Pollen development	(Wu et al., 2010)
183	TP72930	GI:720098363	PREDICTED: ABSCISIC ACID-INSENSITIVE 5-like protein 2 isoform X2	Transcription factor	(Brocard et al., 2002)
184	TP33726	GI:723747273	PREDICTED: auxin response factor 8-like isoform X2	Transcriptional activator	(Li et al., 2016b)
185	TP70316	GI:727430353	PREDICTED: probable voltage-gated potassium channel subunit beta	Transport systems for K(+) accumulation and release	(Dreyer and Uozumi, 2011)
186	TP44464	GI:727463810	PREDICTED: ATPase 7, plasma membrane-type-like	Plant growth/activate and secondary transport	(Morsomme and Boutry, 2000)
187	TP13406	GI:727620566	PREDICTED: formate--tetrahydrofolate ligase-like	Response to cadmium ion, folic acid and derivative biosynthetic process	
188	TP46191	GI:728810428	Transparent testa 12 -like protein	Transporter of proanthocyanidins	(Gao et al., 2016)
189	TP69875	GI:728821903	Fimbrin-like protein 2	Cell development and an actin binding protein	(Eshel and Beeckman, 2013)

190	TP50316	GI:728831838	Putative C15orf43		
191	TP15088	GI:728837883	Thioredoxin reductase 2 -like protein	Growth and development of seed	(Shahpiri et al., 2008)
192	TP72708	GI:728840841	Enolase	Plant metabolism	(Voll et al., 2009)
193	TP51270	GI:728845856	Xylosyltransferase 2	Biosynthesis of N-glycoproteins	(Kajiura et al., 2012)
194	TP14733	GI:729349532	PREDICTED: actin-related protein 9	Embryogenesis and plant development	(Kandasamy et al., 2005)
195	TP64086	GI:731367668	PREDICTED: gastric triacylglycerol lipase isoform X2	Plant growth and development	(Messaoudi et al., 2011)
196	TP64237	GI:731418153	PREDICTED: COBRA-like protein 4	Secondary wall cellulose synthesis or lignin deposition	(McNair, 2015)
197	TP8631	GI:731419168	PREDICTED: methyl-CpG-binding domain-containing protein 9 isoform X3	Epigenetic control of plant growth and development	(Clouaire and Stancheva, 2008)
198	TP7312	GI:734415398	2-hydroxyacyl-CoA lyase	Participates in peroxisomal fatty acid degradation	(Prinsi et al., 2016)
199	TP72053	GI:734419450	Putative RNA 3'-terminal phosphate cyclase-like protein, partial	Responsible for production of the cyclic phosphate	(Bischoff et al., 2010)
200	TP45585	GI:734420199	Proteasome subunit alpha type-4	Defense response to bacterium	(Bahrami and Gray, 1999)
201	TP44577	GI:739379324	Outer membrane assembly lipoprotein YfiO		
202	TP14093	GI:743786953	PREDICTED: telomere repeat-binding protein 2-like	Plant telomere metabolism	(Kuchař and Fajkus, 2004)

203	TP14093	GI:743818640	PREDICTED: telomere repeat-binding protein 3-like isoform X1		
204	TP14093	GI:743818656	PREDICTED: telomere repeat-binding protein 3-like isoform X4		
205	TP44464	GI:743903334	PREDICTED: plasma membrane ATPase 4 isoform X2		
206	TP77252	GI:743921998	PREDICTED: ARF guanine-nucleotide exchange factor GNOM-like	Development	(Anders et al., 2008)
207	TP23086	GI:748340225	DnaK family protein	Heat stress	(Lin et al., 2001)
208	TP70055	GI:752610649	Transposase	Role in the evolution of many genomes.	(Muñoz-López and García-Pérez, 2010)
209	TP18874	GI:764604880	PREDICTED: 26S proteasome non-ATPase regulatory subunit 2 homolog A isoform X1	Protein degradation	(Wang et al., 2009)
210	TP26200	GI:768806869	GPT2-b	Imbibed seeds and developing seedlings	(Dyson et al., 2014)
211	TP26200	GI:769795317	PREDICTED: glucose-6-phosphate/phosphate translocator 1, chloroplastic	Starch biosynthesis	(Kunz et al., 2010)
212	TP14641	GI:769797758	PREDICTED: dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	Growth and development of roots cell	(Qin et al., 2013)
213	TP16066	GI:769813944	PREDICTED: serine/threonine-protein kinase D6PKL1	Regulates the auxin transport	(Rademacher and Offringa, 2012)
214	TP24769	GI:769820200	PREDICTED: 50S ribosomal protein HLP, mitochondrial	Organ/tissue development	(Mizumoto et al., 2004)

215	TP2889	GI:773015677	Low affinity iron permease family protein	Transporter protein	(Dix et al., 1994)Dix, D. R., Bridgham
216	TP25425	GI:778250001	Argininosuccinate synthase	Signal	(Patel et al., 2010)
217	TP70055	GI:778253378	ISH4 transposase		
218	TP44917	GI:808175934	Allene oxide cyclase 4	Biosynthesis of octadecanoids and jasmonate (JA)	(Stenzel et al., 2003)
219	TP30852	GI:809269968	Biotin carboxylase	Precursor for de novo fatty acid biosynthesis	(Nikolau et al., 2003)
220	TP36838	GI:823630988	2-methylene-furan-3-one reductase	Plant secondary metabolism biosynthesis	
221	TP14763	GI:828301767	PREDICTED: transcription factor MYB114-like	Regulation of anthocyanin biosynthesis	(Wang et al., 2015b)
222	TP73466	GI:848850078	PREDICTED: protein trichome birefringence-like 11	Cell wall polymers	
223	TP73887	GI:848852446	PREDICTED: alpha-glucan phosphorylase, H isozyme	Carbohydrate metabolism	(Rathore et al., 2009)
224	TP66508	GI:848864970	PREDICTED: amino acid permease 6-like	Transport a broad spectrum of amino acids,	(Marella et al., 2013)
225	TP6001	GI:860497821	L-asparaginase	Major nitrogen transport and storage amino acid during germination	(Chagas and Sodek, 2001)
226	TP26669	GI:861559451	ABC-type nitrate/sulfonate/bicarbonate transport system	Involved in thiamine biosynthetic pathway and desulfurization	
227	TP25823	GI:889925008	5-oxoprolinase	Catalyzes the ATP-dependent hydrolysis of 5-oxo-L-proline to glutamate.	(Mazelis and Creveling, 1978)
228	TP37873	GI:896526845	Translation initiation factor IF-2	Translation regulation	(Munoz and Castellano, 2012)

229	TP69695	GI:901817853	Methionine adenosyltransferase	Metabolism	(Kotb and Geller, 1993)
230	TP14763	GI:914408355	R2R3-MYB protein	Involved in the control of the cell cycle in plants	(Stracke et al., 2001)
231	TP36904	GI:922329791	Pyridine nucleotide-disulfide oxidoreductase	Antioxidant machinery, glutathione reductase. Play important role in the protection of cell against oxidative damage	(Trivedi et al., 2013)
232	TP64237	GI:922350519	COBRA-like protein 2 precursor		
233	TP26200	GI:922364296	Glucose 6-phosphate/phosphate translocator 1		
234	TP54303	GI:922396531	Binding protein		
235	TP33726	GI:922436249	PREDICTED: auxin response factor 19-like	Ethylene biosynthesis	(Li et al., 2006)
236	TP18857	GI:922496631	PREDICTED: protein HUA ENHANCER 2		(Han et al., 2004)
237	TP22838	GI:923532326	PREDICTED: pathogenesis-related protein 5-like	Plant disease resistance and antifungal activity	(El-Kereamy et al., 2011)
238	TP63632	GI:927913423	Putative aldo/keto reductase, NAD(P)-binding	Plant metabolic reactions including reactive aldehyde detoxification, biosynthesis of osmolytes, secondary metabolism	(Sengupta et al., 2015)
239	TP9086	GI:946966604	MULTISPECIES: RNA polymerase-binding protein DksA	Transcription factor	(Wippel and Long, 2016)
240	TP14266	GI:948255929	Glycogen debranching protein		
241	TP13826	GI:951024120	PREDICTED: coatomer subunit alpha-2-like	COPI coatomer forms a coat around vesicles budding from the Golgi.	(Cevher-Keskin, 2013)