CHARACTERIZATION OF VINEYARD-ASSOCIATED WINE YEASTS AND FLAVONOID PROFILES OF PINOT NOIR GRAPES IN THE OKANAGAN VALLEY OF BRITISH COLUMBIA, CANADA

by

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Abstract

Wine is a product of grape juice fermentation by yeast. *Terroir* is a term that encompasses all environmental factors and interactions at a specific geographical site, resulting in the development of regional-specific microbial strains and differences in grape phenotypes. The objectives of this study were to determine the distribution of vineyard-associated wine yeast strains, identify potential regional-specific wine yeast strains and characterize the flavonoid profile of Pinot Noir grapes among three different sub-regions in the Okanagan Valley (OV), a major wine region in British Columbia, Canada, in the 2017 vintage.

Grape samples were collected from thirteen vineyards among three sub-regions of the OV, namely Kelowna (KE), Naramata-Penticton (NP) and Oliver-Osoyoos (OO), within a week prior to the winery harvesting date, which occurred between September 2017 and October 2017. Vineyard-associated *S. cerevisiae* and *S. uvarum* on the grape samples were enriched and isolated from spontaneous fermentations conducted in the lab. The isolates were genotyped by microsatellite analysis. Regional-specific wine yeast strains were differentiated from commercial wine yeast strains using Bruvo's genetic distance. The anthocyanin and flavonol profiles of Pinot Noir grapes were analyzed by HPLC/UV-vis/MS and the tannin profile was analyzed by a spectrophotometric method.

In total, 10 commercial *S. cerevisiae* strains, 22 potentially indigenous *S. cerevisiae* strains, 2 previously isolated *S. uvarum* strains from OV, 1 previously isolated *S. uvarum* strain from Hornby Island and 2 newly discovered *S. uvarum* strains were isolated in this study. *S. cerevisiae* strains is genetically more closely related within each sub-region as compared to between sub-regions. The population structure of the *S. cerevisiae* strains was significant at the regional level. The anthocyanin content were significantly lower in Pinot Noir grapes isolated from OO as compared to KE. Furthermore, the relative abundance of methoxylated anthocyanin and flavonol compounds were higher in Pinot Noir grapes collected in OO as compared to KE. Therefore, the flavonoid profile of Pinot Noir grapes was significantly affected by sub-regional *terroirs*.

Further research is required to determine how regional-specific wine yeast strains and the flavonoid profile of Pinot Noir grapes affect the quality of wine production in the OV.

Lay Summary

Wine is a product of grape juice fermentation by yeast. The objectives of this study were to determine the distribution of vineyard-associated wine yeast strains, identify potential regional-specific wine yeast strains and characterize the phenolic profile of Pinot Noir grapes among three different sub-regions in the Okanagan Valley, a major wine region in British Columbia, Canada, in the 2017 vintage. Identification of regional-specific wine yeast strains and phenolic profile differences of Pinot Noir grapes is the first step towards wine product differentiation within the OV and helping local wineries become more competitive in a globalized market.

Preface

Subsection 2.2.7, Microsatellite analysis of *S. cerevisiae* and subsection 2.2.8, Microsatellite analysis of *S. uvarum* were conducted by the UBC Sequencing + Bioinformatics Consortium in Vancouver, British Columbia. The author, Elaine Cheng, submitted the PCR product to be tested and analyzed the results using the Genemapper software.

Subsection 3.2.2, Processing of Pinot Noir grapes for total soluble solids, was conducted with the help of Eugene Kovalenko at the Castellarin Lab, Wine Research Center, University of British Columbia, Vancouver, British Columbia. The author, Elaine Cheng, completed the processing of Pinot Noir grapes except the measurement of total soluble solids.

Subsection 3.2.3, Anthocyanin and Flavonol analysis was conducted by Lina Madilao at the Mass Spectrometry Laboratory, Wine Research Center, University of British Columbia, Vancouver, British Columbia. The author, Elaine Cheng, submitted the extracted flavonoid samples to be tested and analyzed the results.

The rest of this research was completed exclusively by the author, Elaine Cheng, under the guidance of Dr. Vivien Measday and Dr. Simone Castellarin.

The study in this thesis is original and has not been previously issued.

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List of Abbreviations

AMOVA= Analysis of molecular variance

ANOVA= Analysis of variance

ATP= Adenosine triphosphate

BC= British Columbia

Bp= Base pairs

BPH= Biphenyl

BSA= Bovine serum albumin

C-3-G= Cyanidin-3-O-glucoside

D-3-G= Delphinidin-3-O-glucoside

ddH_2O= distilled-deionized water

Di= Di-substituted

CAN= Chloramphenicol

Ddwater= distilled-deionized water

DNA= Deoxyribonucleic acid

FeCl3= Iron(III) chloride

Gal= Galactoside

GDD= Growing degree days

Gld= Glucuronide

Glu= Glucoside

HCl= Hydrochloric acid

H. uvarum= Hanseniaspora uvarum

HPLC= High performance liquid chromatography

δ PCR= Interdelta polymerase chain reaction

Is= Isorhamnetin

Ka= Kaempferol

KE= Kelowna

KH_2PO_4= Monopotassium phosphate

KOH= Potassium hydroxide

La= Laricitrin

LC= Liquid chromatography

M-3-G= Malvidin-3-O-glucoside

m/z= Mass-to-charge ratio

Meth= Methoxylated

MLG= Multi-locus genotype

Mono= Mono-substituted

MS= Mass spectrophotometer

mitochondrial DNA= mtDNA

My= Myricetin

NaCl= Sodium Chloride

NaOH= Sodium Hydroxide

NAD+/NADH= Nicotinamide adenine dinucleotide

NHC= Nutrient holding capacity

NP= Naramata-Penticton

OO= Oliver-Osoyoos

ORF= Open Reading Frame

OV= Okanagan Valley

PCA= Principal Component Analysis

PCR= Polymerase chain reaction

Pn-3-G= Peonidin-3-O-Glucoside

Pt-3-G= Petunidin-3-O-Glucoside

PVDF= Polyvinylidene difluoride

Qu= Quercetin

RFLP= Restriction Fragment Length Polymorphism

RFLP-PCR= Restriction fragment length polymorphism-polymerase chain reaction

ROS= Reactive oxygen species

S. cerevisiae= Saccharomyces cerevisiae

S. uvarum= Saccharomyces uvarum

SDS= Sodium dodecyl sulfate

SSR= Simple sequence repeats

STR= Short tandem repeats

Sub-geographical indication= Sub-GI

Sy= Syringetin

TEA= Triethylamine

Tri= Tri-substituted

UBC= University of British Columbia

UBCO= University of British Columbia (Okanagan Campus)

UV= Ultraviolet

UVB= Ultraviolet B

UV-vis= Ultraviolet-Visible absorption

WHC= Water holding capacity

WNA= Wallerstein nutrient agar

YPD= Yeast extract-peptone-dextrose

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First of all, I would like to thank Dr. Vivien Measday and Dr. Simone Castellarin for being my biggest supporters, providing me with countless mentorship and supervision over these one and a half years. I would like to acknowledge them for giving me an opportunity to deepen my knowledge in Food Science by accepting me as a Master student under the degree of Master of Science in Food Science at the Wine Research Center. They have always encouraged me to challenge myself out of my comfort zone, given me practical guidance on any poster and oral presentations, provided assistances on conferences and scholarship applications, which substantially improved my academic writing skills and shaped my positive attitude towards scientific research.

Secondly, I would like to thank the thirteen participating vineyards in Okanagan Valley for donating Pinot Noir grapes in the 2017 vintage as the sampling materials in this study. Also, I would like to thank the British Columbia Wine Grape Council and Mitacs Accelerate for the funding in carrying out this study. This project would not be possible without their generous supports and trust upon me as the major researcher.

Thirdly, I would like to recognize my other committee members, Dr. Siyun Wang and Dr. Abel Rosado. Throughout these one and a half years, they have been very approachable and given me suggestions during the committee meetings to improve my presentation style and the content in my thesis. With their advices, I am able to achieve a critical and knowledgeable thesis project.

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Last but not the least, I would like to sincerely thank all my friends and families for being so supportive on what I am doing. I hope to make all of you proud with the completion of this Master project and I wish to spend more valuable times with you onwards.

1 Chapter 1- Literature Review and Research Purpose

1.1 Terroir

Terroir is a term that encompasses all environmental factors and interactions at a specific geographical site, resulting in the development of regional-specific microbial strains and differences in grape phenotypes (Knight *et al.*, 2015). Although debatable, all constituents of *terroir* should be native, such as temperature, water availability and soil structure and composition, and have limited influence from the viticultural management (Gladstones *et al.*, 2015a). The interactions between different constituents of *terroir* result in a unique regional climate (macroclimate), vineyard mesoclimate, and even a vine microclimate, causing variation in wine quality. In the Old World, the concept of *terroir* is well established and attributed to the geographical indication (i.e. appellation) system and the determination of cultivated varieties at vineyards (refer to section 1.3) (Remaud and Couderc, 2006). In the New World, however, some researchers debate as to the extent that *terroir* influences wine quality and others have tried to use different viticultural and vinicultural techniques to mimic the influence of *terroir* (Gladstones et al., 2015a).

1.1.1 Temperature

Temperature is a major climatological component that influences grapevine development (Gladstones and Webb, 2015). The temperature of grapevines is altered by factors including elevation and grapevine positioning (Tomasi et al., 2013). The average air temperature can impact the enzymatic activity, fruiting ability and growth rate of grapevines. The growing degree days (GDD) index is used to measure the average heat accumulation in a particular geographical site and is a tool to predict grapevine development (Washington State University, 2016). For grapevines, GDD is calculated as the sum of the daily average temperature minus 10 ºC from the beginning to the end of the growing season (i.e. from 1st April to 31st October in the Northern hemisphere). Generally, the average air temperature for grapevine development should be above 10ºC and below 32ºC to maintain the enzymatic activities necessary for regularly physiological phenomena (Tomasi et al., 2013). In addition, an extremely high soil temperature can negatively

impact hormone synthesis and nutrient absorption of the grape plants and affect berry development (Gladstones and Webb, 2015; Tomasi et al., 2013).

1.1.2 Soil

Soil is composed of mineral particles, smaller than 2mm, developed from the weathering of bedrock and sedimentation from water masses (Table 1-1) (Gladstones et al., 2015b; White, 2015). Soil serves as an anchor for the plant and as a nutrient and water source, which is necessary for grapevine and berry development (Gladstones et al., 2015b).

Table 1-1 Soil Types

Types of soil	Particles diameter (mm)
Clay	< 0.002
Silt	0.002 - 0.02
Fine sand	0.02 - 0.2
Coarse sand	0.2 - 2

The information is retrieved from Gladstones et al., 2015b and White, 2015.

1.1.2.1 Soil Texture

Clay is the smallest form of soil particle; it has a larger surface area compared to other types of soil, resulting in higher water holding capacity (WHC) and nutrient holding capacity (NHC) (Gladstones et al., 2015b). In comparison, sand has a lower WHC and NHC; requiring more frequent irrigation and fertilization. Generally, for grapevine growing, the soil should have a reasonable WHC, good drainage and air penetration properties to optimize root development (Gladstones et al., 2015b; White, 2015). Generally, loam (mixture of clay, slit and sand) is considered to be the best soil for the growth of grapevines because of adequate WHC and NHC along with abundant calcium content (Gladstones et al., 2015b). Yet, the most desirable soil texture of each vineyards varies with the geographical climate (e.g. water evaporation rate).

1.1.3 Water

Water is necessary for grapevine development. Precipitation (i.e. rainfall) and neighboring water sources (i.e. rivers, lakes) that provide irrigation water are natural water sources in vineyards. Water availability for the plant varies with the soil type; as mentioned in

1.1.2, clay-dominated soil has a high WHC as opposed to sand-dominated soil (White, 2015). Furthermore, the water requirement of grapevines differs by the phenological stage. For instance, during flowering, an adequate amount of water is required for the development of leaves and stems while a water surplus can lead to vigorous vegetative growth and lower crop yield due to a large shaded canopy (Gladstones et al., 2015c). After veraison, however, moderate water deficit can favor the concentration of phenolic (including pigments) and aromatic compounds in the berries, improving wine quality (see section 1.2).

1.1.4 Other Factors considered in *Terroir*

The constitutes of *terroir* are not limited to the factors mentioned above and several other critical factors will be briefly described in this section. Sunlight provides a form of radiant energy, which is necessary to initiate photosynthesis, production of sugar molecules in berries and many biochemical processes (e.g. respiration) in grapevines (Gladstones and Smart, 2015a). Moreover, multiple features of the land surface are important in establishing *terroir*. Hill and valley slopes provide thermal zones at moderate elevation through turbulent surface air, which benefits viticulture practices in cool climates by promoting the ripening of berries and extending the frost-free length of the season (Gladstones and Smart, 2015b). Moreover, the aspect of a slope can modify the sunlight exposure and temperature of a vineyard. For example, in North America, southerly faced slopes are the warmest since they are exposed to sunshine for the longest period in a day whereas northerly faced slopes are the coolest since they are hidden away from the sun (Gladstones and Smart, 2015b).

1.2 Red Grape Berry Development

The berry originates from the flower ovary after fertilization. The grape berry development is divided into three stages, including a double sigmoid growth period and a lag phase in between, after flowering (Figure 1-1) (Christensen, 2000; El-Esawi, 2017). The initial stage (stage I) begins with fruit set, which denotes the beginning of ovary swelling after flower fertilization. Following fruit set, the ovary develops and the berry grows by rapid cell division and expansion, which lasts from three to four weeks, between May and July in the Northern Hemisphere (Christensen, 2000). At this stage, the berries grow in volume and accumulate

metabolites such as tartaric acid, malic acid and tannins (El-Esawi, 2017). These compounds are unpalatable, and protect the grape berry against herbivory at this early stage of grape development (Gould and Lister, 2005). Also, the seed embryos are formed during this stage (El-Esawi, 2017; Kennedy, 2002, Serrano et al., 2017).

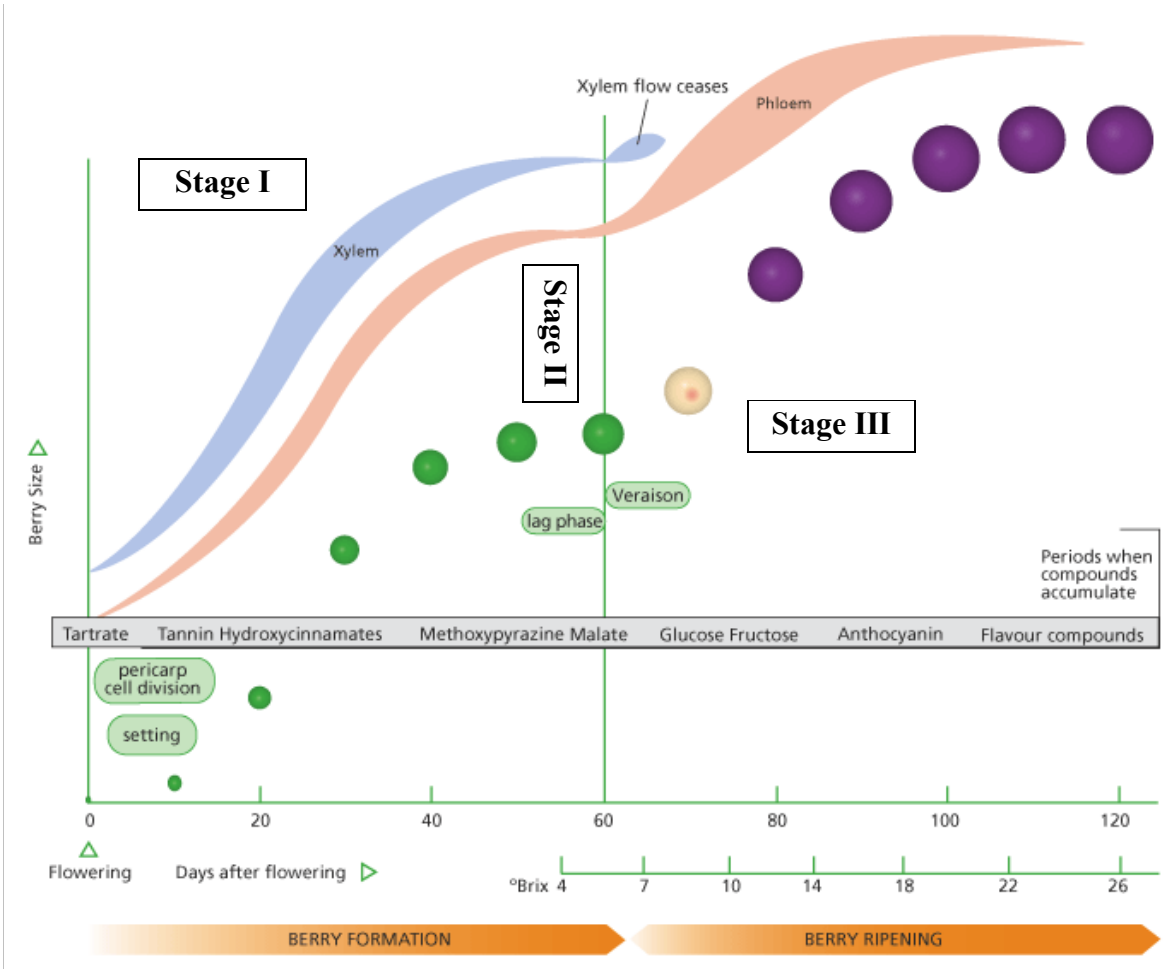


Figure 1-1 Grape Berry Development after Flowering at 10-day Intervals.
Major developmental events (green ovals) and developmental stages are labelled. The accumulation of sugars (°Brix) and different compounds (grey boxes) are also shown. The inflow rate of xylem and phloem vascular saps into the grape berry is indicated by the blue and red curves. The figure is illustrated by Jordan Koutroumanidis, Winetitles and additional labels of developmental stages have been added.

The intermediate stage (stage II) of berry development is characterized by a lag phase of berry growth associated with the termination of cell division and a pause of cell expansion (Christensen, 2000; El-Esawi, 2017). Meanwhile, the seed embryos begin to rapidly increase in size and mature, achieving their ultimate seed size at ten to fifteen days before veraison (i.e. end of stage II, discussed in the next paragraph) (Serrano et al., 2017). Also, acid and tannin

accumulation continues in the berries until veraison (Christensen, 2000; Serrano et al., 2017). The intermediate stage typically lasts from two to three weeks and varies by multiple factors including the geographical location and weather conditions (Christensen, 2000).

Finally, the terminal stage of berry development (stage III) begins with veraison, which is defined as the onset of grape ripening (Christensen, 2000; El-Esawi, 2017; Kennedy, 2002; Serrano et al., 2017). During veraison, there is development of berry pigmentation and accumulation of anthocyanin (in red grape varieties), aroma and flavor compounds within the grape skin. There is also significant sugar accumulation in the berry flesh, associated with berry cell expansion, and a doubling in size of the wine grapes. Meanwhile, the berries become soft and malic acid is metabolized. Also, the total tannin concentration accumulated before veraison is reduced through dilution associated with the berry cell expansion (see 1.7.2) (Downey et al., 2003; Kennedy, 2002). With these transformations in the stage III, the berries become palatable and attractive to herbivory, which facilitates grape seed dispersion for reproduction (Gould and Lister, 2005). Stage III typically lasts from one to two months for wine grapes and occurs around August to October in the Northern Hemisphere, depending on the harvesting time (Kennedy, 2002).

A more detailed description of flavonoid accumulation in red grape berries is reported in section 1.7.

1.3 The Okanagan Valley Wine Region

Wine, a popular beverage that has historical significance, is a product of grape juice fermentation by wine yeasts, including *Saccharomyces cerevisiae*, *Saccharomyces uvarum* and non-*Saccharomyces* species (Demuyter et al., 2004; Masneuf-Pomarede et al., 2010; Tofalo et al., 2013; Tosi et al., 2008). Through wine yeast metabolism, the fermentable sugars from the grape must are converted into ethanol and carbon dioxide, which forms the basis of wine. Winemaking countries are universally classified into “Old World” and “New World”; “Old World” indicates traditional winemaking countries around the Mediterranean region (e.g. France, Germany, Italy, and Spain) while “New World” denotes winemaking countries that were established after Europe’s colonial expansion (e.g. United States, Canada, and Australia) (Johnson and Robinson, 2013). With a significant history in winemaking, “Old World” countries emphasize the origin of their grapes and the *terroir* of specified winemaking regions in their

wine marketing strategy, through legally identified geographical indications called “appellations” (Remaud and Couderc, 2006). The “Old World” winemaking establishment is usually comprised of family-owned business and features specialized blends (e.g. Bordeaux, Burgundy, Champagne) (Faith and Robinson, 2015; Remaud and Couderc, 2006; Wilson et al., 2015). By contrast, “New World” countries emphasize individual grape varietals and proprietary brand in their advertisement strategy.

According to Agriculture and Agri-Food Canada, the Canadian winemaking industry is comprised of businesses that participate principally in wine manufacturing, grape growing, wine blending, cider production and brandy distillation (Agriculture and Agri-Food Canada, 2016). Even though Canada represents only 0.3% of the global wine production, Canada is the world’s biggest manufacturers of ice wine (Canadian Vintners Association, 2017). Furthermore, the Canadian wine industry provides 9 billion dollars to the Canadian economy annually and 37,000 jobs are generated in Canada to support the wine industry (Canadian Vintners Association, 2018).

In Canada, the two largest wine and grape producing provinces are British Columbia (BC) and Ontario, with small contributions from Quebec and Nova Scotia (Canadian Vintners Association, 2018). The BC wine industry alone contributes 2.8 billion dollars to the provincial economy annually, which is a noteworthy impact to the BC economy. Also, around 12,000 jobs are created in BC to support the wine industry. The major wine region in BC is the OV; smaller regions include the Similkameen Valley, Fraser Valley, and Vancouver Island. (BC Wine Institute, 2018).

According to the BC Wine Institute, the OV holds 84% of the vineyard acreage (8,619 acres) in BC, which represents the dominant grape growing region in the province (BC Wine Institute, 2018). To date, the OV spans over 250 kilometers in length. The common red wine grape varietals planted in the OV include Merlot, Pinot Noir, and Cabernet Sauvignon, while the common white wine grape varietals planted in the OV include Pinot Gris, Chardonnay, and Gewürztraminer.

Table 1-2 Examples of Okanagan Valley sub-regions and grapevine-related information[1]

Sub-region	Average GDD	General Soil Composition	Acres planted	Number of Wineries
Kelowna-Lake country	1200	• Thick soils with sandy loam, clay and limestone	904	44
Naramata-Penticton	1319	• Glacial Lake sediments in the form of silt, sand and gravels.	898	41
Oliver-Osoyoos	1520	• Sloping hills with granite bedrock, covered by deep-glacial river sandy sediments. • Golden Mile Bench- River sediments are composed of gravelly, stony and sandy loams that have good drainage	5844	57

[1]The information are sourced from BC Wine Institute, 2018.

As shown in Table 1-2, there are sub-regions in the OV with significantly different average GDD, soil composition and acres planted with grapevines (BC Wine Institute, 2018).

Kelowna-Lake country has the oldest wine making history among OV sub-regions and is home to many of the first families of the BC wine industry. For instance, the first grapevine of the province was planted at Kelowna in 1859 and the surviving oldest operating winery of the province (Calona Vineyards) has been located in Kelowna since 1932. By contrast, NP and OO are more recently developed wine-making regions with around thirty and forty years of history, respectively. Almost half of the total BC grapevines are located in OO; this sub-region has a distinctive climate at the west and the east side, with the west soaking up the morning sun that is ideal for the cultivation of white grapes, and the east busking under the afternoon sun that is great for the cultivation of red grapes. In particular, Osoyoos is the hottest place in Canada and thus more suitable for red wine production. Furthermore, the term sub-geographical indication (sub-GI) is given to officially recognize a sub-region with a unique *terroir*. In particular, the first official sub-GI in the OV was credited to the Golden mile bench in 2015. Located at the southwest of Oliver, the Golden mile bench has a warm climate subject to the morning sun, along with a distinguishing soil composition (Table 1-2).

1.4 Pinot Noir

Nowadays, most of the wine in the world is made with an European species of grapevine, called *Vitis vinifera* (Harding, 2015). Within this species, there are around 5,000 – 10,000 grape varieties but only a few dominate the wine market (This et al., 2006). These grape varieties may demonstrate different and recognizable styles of wine (Vouillamoz et al., 2015). Among all *Vitis vinifera* varieties, Pinot Noir is one most well-known and widely planted wine grape varieties. According to *The Oxford Companion to Wine*, "Pinot" is a word created to name the French vine variety and it describes the pinecone shape of a Pinot grape brunch (Oxford University Press, 2015). Pinot is assumed to have been selected from wild vines and Pinot Noir is considered as the oldest and most important form of Pinot. Pinot Noir is an early ripening vine and therefore cool climate regions provide the best viticulture site to prevent the pre-maturation of grapes which results in loss of acidity and aroma. Pinot Noir is currently being transplanted to nearly every wine producing region (excluding the very hot regions). In general, Pinot Noir has a sweet fruitiness and relatively low levels of tannins and anthocyanins as compared to other French red varieties. Yet, Pinot Noir is a very variable vine and has little consistency in its characteristics all around the world. Specifically, the Burgundians in Eastern France use Pinot Noir as a way to examine the *terroir* of each vineyard (Oxford University Press, 2015). While Pinot Noir grows best in a slim temperature range of 14^{o}C – 16^{o}C after fruit set, it is generally suitable to grow in viticultural region with less than 2500 GDDs (Baldy, 2009; Tattersall and Wynne, 2015).

1.5 Wine Yeast – Saccharomyces cerevisiae and Saccharomyces uvarum

Wine is a fermented beverage with over thousands of years of history, as the DNA materials of wine yeasts have been discovered from ancient wine jars (3150 BC) in Egypt (Tofalo et al., 2013).

Among all yeast species involved in the wine fermentation process, *S. cerevisiae* plays a dominant role in most fermentations, while some winemakers have identified *S. uvarum* as the major wine yeast species in their fermentations (Demuyter et al., 2004; Masneuf-Pomarede et al., 2010; Tosi et al., 2008). With their high ethanol tolerance and the Crabtree effect (see 1.5.2), these two species can outcompete most other yeast species at the latter stage of the fermentation.

1.5.1 Genetic Information of Wine Yeast

Under the influence of environmental stress, wine yeast genomes are susceptible to chromosome and gene rearrangements, duplications and deletions that may result in genetic diversity, phenotypic variation and the development of regional-specific strains (Almeida et al., 2015).

1.5.1.1 Genome of *S. cerevisiae*

In 1996, the *S. cerevisiae* genome, approximately 12.07 megabases, was completely sequenced for the first time using the S288c laboratory strain (Goffeau et al., 1996). The genome is grouped into sixteen chromosomes with 5885 protein-encoding genes. At this time, it was discovered that *S. cerevisiae* contains a remarkably compact genome compared to other yeasts and fungi species. In particular, nearly 70% of the whole *S. cerevisiae* genome contains open reading frames (ORFs), which correspond to a density of protein-encoding genes of one gene every two thousand bases of the yeast genome.

1.5.1.1.1 Differences of *S. cerevisiae* Genomes between Laboratory Strains and Wine Strains

The genomes of *S. cerevisiae* wild and industrial strains may not be best represented by the genome of a laboratory strain because S288c is a haploid strain while most of the wild and wine strains are in a diploid state that implies better growth fitness traits (Peter et al., 2018). Furthermore, geography, ecology and human activity have together shaped the global population structure of *S. cerevisiae* (Almeida et al., 2015). Through human intervention, *S. cerevisiae* has had opportunity for multiple rounds of domestication, artificial selection and out-crossing, to select for desirable functions. The impact of humans using yeast for industrial processes has resulted in a large number of *S. cerevisiae* strains that contain distinctive phenotypes characteristic to specific industrial applications (Borneman et al., 2011). Specifically, many essential genes identified in S288c are present in other industrial strains, while many accessory genes present in industrial strains, which are associated with various metabolic or cellular potential, are absent in S288c (Liti et al., 2009; Borneman et al., 2011). Also, there are at least 102 ORFs identified in industrial *S. cerevisiae* strains but not S288c (Borneman et al., 2011). With these genetic differences, industrial strains have a better survival rate in certain

environments (i.e. high ethanol concentration and poor nutrient availability), while lab strains have a faster growth in nutrient rich laboratory media (Liti et al., 2009; Borneman et al., 2011).

1.5.1.1.2 The Population Structure of the *S. cerevisiae* Genome among Global Wine Strains

The *S. cerevisiae* genomes of wine strains around the globe, including selected commercial strains and strains isolated from grape must, have demonstrated close genetic relatedness. In a study by Liti et al., the researchers recognized five *S. cerevisiae* lineages based on their usage or isolated geographical regions, namely the Malaysian, West African, sake, North American and wine/European lineages (Liti et al., 2009). The wine/European lineage contains many *S. cerevisiae* strains isolated from Europe and during wine fermentation. Owing to frequent human interaction, worldwide *S. cerevisiae* wine strains cannot be satisfactorily differentiated simply based on geographic boundaries (Liti et al., 2009). With an intimate genetic association between global *S. cerevisiae* wine strains and the *S. cerevisiae* wine strains isolated in Europe, European wine strains are considered to have migrated around the globe and maintained as a separate population through phenotypic selection (Borneman et al., 2011). Furthermore, in a study by Borneman et al., wine strains isolated from various geographical regions genetically fall into one sub-population, which is separate from other industrial strains including the sake strains and the ale strains (Borneman et al., 2011). Studies have shown that there is an obvious genetic separation of *S. cerevisiae* genomes based on their industrial usage (Borneman et al., 2011; Schacherer et al., 2009). This finding is also supported by another study, in which the genomes of wine strains, including 106 commercial wine strains from 9 suppliers and other non-commercial wine strains (i.e. wild strains), were discovered to be extremely similar genetically compared to strains isolated from industries or environments not related to wine (Borneman et al., 2016). In particular, some commercial strains from multiple suppliers are genetically identical despite having different names (Bradbury et al., 2006). Thus, evidence supports the theory that there is a single domestication event associated with *S. cerevisiae* yeast for winemaking from the native yeast population (see 1.5.1.1.3 for more details) (Almeida et al., 2015, Borneman et al., 2016; Peter et al., 2018; Libkind et al., 2011; Schacherer et al., 2009).

In addition, the genetic diversity of *S. cerevisiae* wine strains should be higher with commercial strains than wild strains (Peter et al., 2018). This is to be expected because most genome modification of wild strains is associated with the accumulation of SNPs in a natural

environment, while wine strains selected for commercial usage may develop desirable features through experiencing rapid ORF gain or loss under selective pressure in an artificial environment (e.g. wineries).

1.5.1.1.3 *S. cerevisiae* Genome Ancestor of Wine Strains

In recent years, as hinted by the intimate genetic association between wine strains and European isolated strains, *S. cerevisiae* strains identified from Mediterranean oak tree were proposed to be the wild genetic ancestor of domesticated *S. cerevisiae* wine strains (Almeida et al., 2015). Oak-associated strains were resolved by their genomes into two groups; a group containing Mediterranean-oak isolates and another group containing a mixture of oak-isolates from North America and Japan (Almeida et al., 2015). Furthermore, based on the wine strains sharing the highest number of polymorphisms with the Mediterranean oak isolates, the genetic relatedness of wine strains is the highest with Mediterranean oak isolates, as compared to sake strains, beer strains and *S. cerevisiae* isolates from North American oak and Japanese oak (Almeida et al., 2015). This finding is supported by another study, in which *S. cerevisiae* strains from the wine/European group have the closest genetic relatedness with Mediterranean oak isolates, as compared to the sake strains and other wild strains isolated from North America and Malaysia (Peter et al., 2018). In addition, the population migration rate of Mediterranean oak *S. cerevisiae* isolates into the wine strain population is much higher than the reverse direction (Almeida et al., 2015). This information suggests the Mediterranean oak isolates are ancestors, instead of descendants, of wine strains. By assuming Mediterranean oak *S. cerevisiae* isolates are the ancestors of wine strains, the domestication of *S. cerevisiae* is estimated to have occured around 1,300 years to 10,300 years ago, which matches with the first biochemical evidence of wine in 5400 – 5000 BC.

1.5.1.1.4 Specialization of Wine Strain Genomes

Wine strains are characterized by a cluster of five ORFs that are integrated into different sites of the wine yeast genomes (Borneman, 2011). For these five ORFs, the sequences are highly conserved between wine strains, yet the genomic location and copy number can be varied. This five-gene cluster may be associated with a high ethanol production efficiency because it is

also found in a bioethanol strain. Aside from this five-gene cluster, a yeast stress response gene *MPR1* was identified in over 92% of examined wine strains but is not present in ale strains, demonstrating another specialization of wine strain genomes (Borneman et al., 2016). In addition, the *S. cerevisiae* Mediterranean oak isolates and wine strains can be separated by three domestication fingerprints (Almeida et al., 2015). These domestication fingerprints were obtained from horizontal gene transfer, including a 17-kilobases insertion from the *Zygosaccharomyces baillii* genome. The three horizontal gene transfer events are believed to improve the sugar and nitrogen metabolism of wine strains, which increases fitness in grape must. In addition, at least one of these three genome regions are observed in 78% of examined wine strains, but do not exist in the Mediterranean oak isolates (Almeida et al., 2015).

Among wine strains, there are differences in accessory loci (i.e. genes not identified in S288c) resulting from horizontal gene transfer, gene loss, chromosomal translocations, and recombination events among the parental populations (Borneman, 2016). In addition, these commercial wine strains might have various accessory genes that are associated with phenotypic differences in their fermentative performance. Please refer to section 1.5.3.3 for an example.

1.5.1.2 Genome of *S. uvarum*

In general, the size of the *S. uvarum* genome is slightly smaller than *S. cerevisiae* (11.54 megabases), but also contains sixteen chromosomes (Ngyuen and Boekhout, 2017). Unlike *S. cerevisiae*, there are limited studies focusing on the global phylogeography and domestication events of *S. uvarum* (Almeida *et al.*, 2014). Except for *S. cerevisiae, S. uvarum* is one of the few natural and non-hybrid *Saccharomyces* species that has been identified from industrial wine fermentations. *S. uvarum* has been frequently isolated from European fermentations, while rarely being identified in the natural environment in temperate Europe and North America. Yet, in South America, there are a considerable number of *S. uvarum* isolates discovered in Patagonia and Australasia from *Nothodagus* (southern beech) (Almeida *et al.*, 2014). This is an interesting observation because these two geographical regions are the only places in the world where *Nothodagus* is native. According to the Almeida *et al.* study, three *S. uvarum* clades were identified from sixty-one strains evaluated based on geographical isolation – a group of South American (SA-A) strains, a group of Australasian strains, and a group composed of Holarctic strains and South America (SA-B) strains. The South American *S. uvarum* strains have all been

isolated from Patagonia where there are cooler climactic conditions. As stated above, one group of the Patagonian *S. uvarum* strains (SA-B) are phylogenetically more closely related to the Northern Hemisphere (Holarctic) than another group of Patagonian strains (SA-A) strains. Also, there is low genetic diversity in *S. uvarum* strains isolated from the Northern Hemisphere compared to a significantly high genetic diversity amongst *S. uvarum* strains from the Southern Hemisphere. Therefore, *Nothodagus* in Patagonia is suggested to be the primal niche of *S. uvarum*, with migration of *S. uvarum* from Patagonian to the Northern Hemisphere.

The *S. uvarum* wine strains are suspected to have been domesticated in Europe after the migration of *S. uvarum* from the Southern Hemisphere (Almeida *et al.*, 2014). Particularly, certain Holarctic *S. uvarum* strains have introgressions from other *Saccharomyces spp.* across the genome. The number and sizes of genome introgressions differ in each strain. For instance, there are prevalent and extensive introgressions of *S. uvarum* from *S. eubayanus,* and this introgression is exclusive to European *S. uvarum* strains found in cider and wine fermentation (Almeida *et al.*, 2014). In addition, several wine fermentation-related genes including *ASP1* (cytosolic L-asparaginase used to degrade asparagine and utilized as nitrogen source) are believed to have been transmitted from the *S. cerevisiae* genome to the *S. uvarum* genome through horizontal gene transfer. With these genome modifications, possibly through selective pressures under anthropic environments, European *S. uvarum* wine strains have developed with genetically and phenotypically distinctive features compared to *S. uvarum* wild strains.

It is worth noting that there is low genetic diversity between *S. uvarum* strains isolated from the Northern Hemisphere when compared to the vast colonized geographical area (Almeida *et al.*, 2014). Therefore, the dispersal of Northern Hemisphere *S. uvarum* strain has happened rapidly to limit the accumulation of genome mutations from different geographical locations in the Northern Hemisphere.

1.5.2 Wine Yeast Metabolism

During wine fermentation, fermentable sugars from the grape must are converted into ethanol and carbon dioxide by the metabolic activity of wine yeast. The sugar content of grape must can be measured in ^{o}Brix, which is defined as the percentage of soluble solids by weight (i.e. gram) in a volume of liquid (i.e. litre) (Coombe, 2015). Since around 90% of soluble solutes in ripe grapes are sugars, ^{o}Brix is an adequate scale for approximating the sugar concentration in

grapes (Coombe, 2015). The fermentable sugars of grapes are dominated by glucose and fructose, which exist in a 1:1 ratio and range from 21 ^{o}Brix to 24 ^{o}Brix for red wine grapes (Fugelsang and Edwards, 2007). As shown from previous studies, the majority of wine yeasts are glucophilic, with preference of glucose utilization over fructose utilization (Fugelsang and Edwards, 2007). Thus, the rate of glucose uptake is around five times higher than the rate of fructose uptake by the wine yeasts during alcoholic fermentation (Fugelsang and Edwards, 2007). Furthermore, wine yeasts are able to produce extracellular invertases to hydrolyse the small amount of sucrose (0.2% – 1%) from the grape must into glucose and fructose for fermentation (Fugelsang and Edwards, 2007). Moreover, pentoses (five-carbon sugars) are minor soluble solids in grapes and wine yeasts cannot metabolize these compounds (Fugelsang and Edwards, 2007).

In a typical anaerobic environment during fermentation, fructose and glucose are utilized by yeast through glycolysis, resulting in two pyruvate molecules, two ATP and a NADH as the end product (Fugelsang and Edwards, 2007). Instead of entering the Krebs cycle, the metabolism of glucose enters a fermentative pathway and the pyruvate molecule is decarboxylated into an acetaldehyde molecule, which is further reduced into an ethanol molecule by NADH oxidation. NADH oxidation is important under anaerobic conditions because NAD+ is required to maintain the glycolytic pathway. Therefore, in addition to the fermentative pathway, a mid-product during glycolysis, namely dihydroxyacetone-phosphate, can also be reduced into glycerol to regenerate NAD+ when acetaldehyde is not available.

Furthermore, wine yeasts choose fermentation over respiration even under aerobic conditions to survive and outcompete other microorganisms (Fugelsang and Edwards, 2007). In the presence of a high amount of glucose (>9 g/L) and oxygen (such as the initial stages of wine fermentation), rather than entering the Krebs cycle, pyruvate is shunted into the fermentative pathway and ethanol production is continually executed by the wine yeast. The ability to carry out fermentation in the presence of oxygen when glucose is available is named the Crabtree effect. As the ethanol content quickly increases during the initial stage of fermentation, non-*Saccharomyces* yeasts and other microorganisms with low ethanol tolerance are killed. As well, glucose and fructose are rapidly metabolized by the wine yeasts, including both *S. cerevisiae and S. uvarum*. As a result, *S. cerevisiae*, or sometimes *S. uvarum*, is the dominant yeast population that survives in the wine fermentation. Moreover, in an anaerobic environment, certain enzymes

of the Krebs cycle of wine yeasts are still functional. Therefore, wine yeasts are able to synthesize important compounds and perform NAD+ regeneration for cellular function.

1.5.3 Aroma and Flavor Production

Yeasts have multiple ways to produce extraordinary aroma and flavor in wine during fermentation, including synthesis of metabolites, production of glycosides and autolysis (Fugelsang and Edwards, 2007).

1.5.3.1 Synthesis of Metabolites

Various types of volatile and nonvolatile compounds are metabolic by-products of yeast during fermentation. These compounds, collectively, are responsible for the sensory profile of wine (Fugelsang and Edwards, 2007). For instance, higher alcohols (i.e. alcohol with at least three carbons, also called fusel alcohols) are by-products of yeasts formed by the Ehrlich pathway. The first step in the Ehrlich pathway is a transamination reaction that occurs between an amino acid and α-ketoglutarate to generate an α-keto-acid. The α-keto-acids are further decarboxylated into aldehydes. These aldehydes are converted into higher alcohols, such as isoamyl alcohol, which influences the sensory profile of wine. Moreover, during wine fermentation, different types and quantities of esters are synthesized from alcohols and carboxylic acids by yeast metabolism, which contributes to the variety of wine aroma characteristics. The production of metabolic aroma by-products from yeast metabolism is subject to changes in environmental factors, the grape variety, ripeness, fermentation temperature and yeast strain.

1.5.3.2 Production of Glycosidases

Monoterpenes are volatile compounds which typically impart floral aromas. In grape must, however, most of these monoterpenes are bound with a glycoside and in a non-volatile form that is odorless (Fugelsang and Edwards, 2007). The glycoside compound can be cleaved off from the monoterpene by glycosidases, which are enzymes naturally found wine yeasts, such as *S. cerevisiae* and *S. uvarum*, along with some non-*Saccharomyces* species. As a result, monoterpenes are freed as volatiles and give rise to wine aroma and flavor characteristics.

1.5.3.3 Catalysis of Aromatic Alcohols

A novel ORF encoding for a unique aryl-alcohol dehydrogenase has been identified in multiple commercial wine strains (Borneman et al., 2011). Aryl-alcohol dehydrogenases are responsible for catalyzing the reduction of aldehyde and ketones into aromatic alcohols, which provides strain-specific aroma characteristics (Borneman et al., 2011).

1.5.3.4 Mannoproteins and Flavor Reduction

Mannoproteins are large and complexed hydrocolloids from the yeast cell walls that are released during yeast autolysis (Fugelsang and Edwards, 2007). Mannoproteins can bind with volatile compounds and tannins in wine, which contributes to the reduction in flavor and astringency. Furthermore, mannoproteins may bind with other molecules and result in an elevated protein and tartrate stability of wine.

1.5.4 Differences between *S. cerevisiae* and *S. uvarum*

Both *S. cerevisiae* and *S. uvarum* are able to initiate wine fermentation, however their physiological characteristics are slightly different. First, these two species have different temperature preferences. *S. cerevisiae* is a thermotolerant microorganism while *S. uvarum* is a cryotolerant microorganism (Masneuf-Pomarede et al., 2010). In particular, among Saccharomyces *spp.*, *S. cerevisiae* has the highest optimal growth temperature of 32 ºC and *S. uvarum* has the lowest optimal growth temperature of 26 ºC (Lo´pez-Malo et al, 2013; Salvadó et al., 2011). Yet, in the study of Masneuf-Pomarede et al., *S. uvarum* demonstrated low ethanol tolerance and low fermentation capacity at 24 ºC, which resulted in stuck fermentations (Masneuf-Pomarede et al., 2010). Instead, *S. uvarum* showed higher cell viability and better fermentation capacity at 13 ºC, as evidence of low-temperature adaption associated with wine fermentation. Second, *S. cerevisiae* has a stronger fermentative competitiveness as compared to *S. uvarum* (Lo´pez-Malo et al., 2013; Masneuf-Pomarede et al., 2010). In the Lo´pez-Malo et al. study, *S. cerevisiae* was shown to have quicker sugar uptake and higher ethanol tolerance and production in wine fermentations at both 12 ºC and 28 ºC compared to *S. uvarum* (Lo´pez-Malo et al., 2013). In addition, in the study of Masneuf-Pomarede et al., *S. cerevisiae* was able to

complete the wine fermentation at both 13 ºC and 24 ºC while *S. uvarum* was only able to complete the fermentation at 13 ºC (Masneuf-Pomarede et al., 2010). Third, these two species have metabolic differences. For instance, most *S. uvarum* strains are able to metabolize melibiose whereas the enzymes required for melibiose metabolism are absent in most *S. cerevisiae* strains (Masneuf-Pomarede et al., 2010; Rainieri et al., 1999). Fourth, the chemical profiles of wine fermented by these two species have significant differences. In a study by Tosi et al., *S. cerevisiae* and *S. uvarum* wine fermentations carried out at 16.5 ºC were compared (Tosi et al., 2009). Wine fermented by *S. cerevisiae* had a significantly higher concentration of acetic acid, which is a source of volatile acidity, than wine fermented by *S. uvarum* (Tosi et al., 2009). By contrast, wine fermented by *S. uvarum* had a significantly higher concentration of glycerol, than wine fermented by *S. cerevisiae* (Tosi et al., 2009). Glycerol is a source of sweetness and it is also the most abundant product in wine fermentation after ethanol and carbon dioxide. Also, 2-phenylethanol, a higher alcohol with a rose-like aroma, was found to be five times more abundant in *S. uvarum*-fermented wine as compared to *S. cerevisiae*-fermented wine (Tosi et al., 2009).

1.5.5 Differences between Spontaneous Fermentation and Inoculated Fermentation

Spontaneous fermentation is a traditional practice; the fermentation is carried out by wine yeasts solely present on grapes and winery equipment (Pretorius, 2000). In nature, wine yeasts are spread by birds and insects to various geographical locations (Tofalo *et al.*, 2013). Interestingly, among all yeast species presented on undamaged berries in the vineyards, *S. cerevisiae* comprises only 0.1% of the population (Tofalo *et al.*, 2013). In comparison, berries damaged by insects or birds (i.e. one in every thousand berries) have a significantly higher proportion of *S. cerevisiae* (24%). Therefore, at the beginning of spontaneous fermentation, alcoholic production is accomplished by the predominant non-*Saccharomyces* species (i.e. *Candida, Hanseniaspora*) (Fleet, 2008). Nevertheless, most of these yeast species have low ethanol tolerance and die off quickly during the early stages of the fermentation. Therefore, at the peak of spontaneous fermentation, *S. cerevisiae* takes over as the predominant microorganism and produces ethanol efficiently.

Owing to the metabolic activities of various yeast species, the resulting wine of a spontaneous fermentation may develop a complex and diverse sensory profile. Yet, there are

several risks associated with spontaneous fermentation. First, the vineyard and winery yeast populations are dynamic and varies by vintages and geographical location (Knight and Goddard, 2015, Martiniuk et al., 2016, Rantsiou et al., 2016, Schuller et al., 2005). Thus, the resulting wine quality is less predictable and a stuck fermentation may occur. As well, the presence of some non-*Saccharomyces* yeast species in the fermentation, such as *Brettanomyces* and *Kluyveromyces*, may create undesirable aroma characteristics in the wine (Pretorius, 2000). Furthermore, spontaneous fermentation is time-consuming because the accumulation of *S. cerevisiae* is necessary before the initiation of vigorous ethanol production (Pretorius, 2000).

Consequently, nowadays, many winemakers have embraced inoculated wine fermentations. Inoculated fermentations have a much shorter history and were introduced by Muller-Thurgau in 1980, who came up with the idea to inoculate wine fermentation by pure wine yeast starter culture (Pretorius, 2000). Commercial wine yeast starter cultures are usually in dried powder form for stability and transport-convenience (Donalies *et al*., 2008). They are selected to have high ethanol tolerance, efficient metabolism of nutrients and sugars in the grape must, and rapid growth during fermentation to limit the chance of microbial contamination. Owing to these designated traits, inoculated wine yeast strains can outcompete other yeast species, including native wine yeast strains, and quickly take over the wine fermentation process. Since inoculated fermentation is rapid and reliable, and also guarantees the uniformity, predictability and reproducibility of the wine sensory profile to a certain extent, it is widely used in many large-scale wine productions and in the New World (Pretorius, 2000). It should be noted that, however, many Old World winemakers still perform spontaneous fermentation to incorporate vintage variation and uniqueness to their resulting wine.

1.5.6 Indigenous Wine Yeast Populations

Multiple biogeographical studies have been conducted in the past five years to elucidate the association between *terroir* and native wine yeast strains, especially *S. cerevisiae*. Through environmental-microbial and microbial-microbial interactions, wine yeast can adapt to the surroundings with naturally occurring gene and genomic changes that creates regional-specific strains (Zhang et al., 2015). In Knight and Goddard's study, the researchers isolated 3,900 *S. cerevisiae* isolates from six major grape growing areas in New Zealand spanning over 1000 km (Knight and Goddard, 2015). Among these isolates, 295 genotypes were identified by

microsatellite analysis as regional-specific when comparing to their database with 246 genotypes, including 78 commercial strains (Knight and Goddard, 2015). Also, a biodiversity investigation of *S. cerevisiae* strains in Monferrato, Italy concluded that 37 regional-specific strains were identified by interdelta polymerase chain reaction (δ PCR) (Rantsiou et al., 2016). Furthermore, a three-year study conducted by Schuller et al. in Portugal used mitochondrial DNA restriction fragment length polymorphism (mtDNA RFLP) to identify 8 vineyard-specific indigenous *S. cerevisiae* strains that were persistently isolated from the same vineyard across vintages (Schuller et al., 2005). Therefore, evidence suggests that a variety of indigenous *S. cerevisiae* strains exist among different geographical locations.

In addition, there are studies focused on the significance of regional-specific *S. cerevisiae* strains with regards to wine production. In the study by Knight et al., wine fermentations were conducted with multiple regional-specific *S. cerevisiae* strains isolated from New Zealand (Knight et al., 2015). The researchers were able to recognize a significant relationship between regional-specific *S. cerevisiae* strains and the phenolic profiles of the resulting wine. Although 29 out of 39 examined volatile compounds in the finished wine differed in abundance based on the geographical origin of *S. cerevisiae* strains, none of these compounds was found to be exclusively responsible for the regional-specific flavor profiles of the wine (Knight et al., 2015). Therefore, a complicated association between regional-specific *S. cerevisiae* strains and volatile production during wine fermentation was identified in this study. Moreover, a group of researchers in Italy conducted single-strain inoculated fermentations for sixty-three indigenous *S. cerevisiae* strains isolated from two grape growing regions (Capece et al., 2016). Similarly, through chemical analysis, the sensory profiles of the resulting wine had significant differences with respect to the regions of yeast isolation. For instance, wine fermented by regional-specific *S. cerevisiae strains* from Aglianico had higher acetic acid content as compared to wine fermented by regional-specific *S. cerevisiae* strains from Sangiovese (Capece et al., 2016). Interestingly, the researchers discovered the possible adaption of *S. cerevisiae* strains to locally-grown berries; 5 of the regional-specific *S. cerevisiae* strains from Aglianico failed to complete the fermentation using grape must from Sangiovese (Capece et al., 2016). Therefore, there is good evidence of a correlation between regional-specific *S. cerevisiae* strains and sensory characteristics of the resulting wine.

1.6 Microsatellite Analysis

Microbiogeography is a field that focuses on the distribution of microorganism biodiversity among time and space (Tofalo *et al.*, 2013). Microsatellite analysis is one of the most widely-used molecular tools to study the microbiogeography of wine yeasts by a genetic approach owing to its reliability, simplicity and low price (Richards *et al.*, 2009). Microsatellites, also referred as short tandem repeats (STRs) or simple sequence repeats (SSRs), are DNA repeats consisting of one to six base pairs that have a high degree of polymorphism. SSRs are abundant and frequently found in the DNA non-coding regulatory region of wine yeasts. Particularly, some SSRs of *S. cerevisiae* and *S. uvarum* have higher allelic diversity for population differentiation and genetic relationship establishment among geographical regions than other SSRs (Schuller and Casal, 2007). In previous studies, as compared to other genotyping methods such as mtDNA RFLP and δ PCR, analysis of six microsatellite loci was recognized to have identical discriminatory power (Perez et al., 2001, Schuller et al., 2004). Thus, in order to enhance the discriminatory power of microsatellite analysis, it is essential to examine a greater number of STRs in the *Saccharomyces* DNA material.

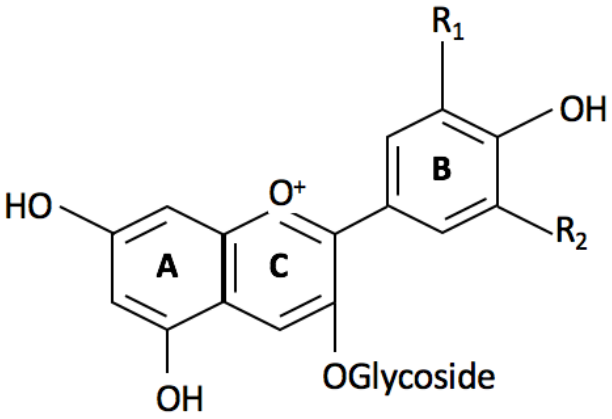
1.7 Flavonoid Compounds in Grapes

Flavonoid compounds in grapes are secondary metabolites that serve important roles in plant-insect interactions, plant defense mechanisms, and plant stress responses (Falcone Ferreyra et al., 2012).

1.7.1 Anthocyanins

Anthocyanins are water-soluble dyes and the primary contributor of the color of grape skins. As shown in Figure 1-2, each anthocyanin molecule is composed of an anthocyanidin aglycone glycosidically bonded to one or more sugar molecules (Flamini and Traldi, 2010). In particular, there are five different non-acylated anthocyanins found in Pinot Noir grapes, including the glucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin respectively (Lee and Skinkis, 2013). According to a study by Mattivi et al., these different types of anthocyanins account for 4.95%, 4.44%, 5.68%, 34.65% and 50.27% (based on the order above) of the total anthocyanin content in Pinot Noir grapes (Mattivi et al., 2006). The color of

anthocyanins varies with the number of hydroxyl groups on the B-ring; di-substituted anthocyanins generally have a magenta hue while tri-substituted anthocyanins typically have a purple hue (Bakowska-Barczak, 2005) (Figure 1-2). Anthocyanin accumulation in wine grapes begins at veraison and continues during fruit maturation (Braidot et al., 2008; Downey et al., 2006).



	R_1	R_2
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OH	OCH_3
Malvidin	OCH_3	OCH_3

Figure 1-2 Chemical structures of anthocyanins found in Pinot Noir grapes.
The bold letters indicate the rings in the flavonoid compound.

In grape, the vibrant color of anthocyanins plays an important role in attracting frugivores for seed dispersal and germination (Gould and Lister, 2005). Furthermore, similar to flavonols in chemical structure, with multiple hydroxyl groups on the B-ring, anthocyanins are effective antioxidants against DNA damage. Also, anthocyanins are proposed to have health benefits associated with antioxidant, anti-inflammatory and anti-carcinogenic properties (Mazza et al., 1999). In addition, anthocyanin accumulation can be highly varied by almost all biotic and abiotic stressors (e.g. temperature) (Gould and Lister, 2005).

1.7.1.1 Grapes Anthocyanin Content and *Terroir*

The importance of grape anthocyanin content for wine production and stress-response has resulted in multiple studies to determine if grape anthocyanin content changes according to variation in *terroir*.

In 2001, the anthocyanin content of Cabernet Sauvignon and Merlot grapes from four different Bordeaux *terroirs* were analyzed (Gaulejac et al., 2001). The researchers concluded that

terroir is a significant factor in determining the total and individual anthocyanin content of grapes. Particularly, the proportion of malvidin-3-glucoside in Merlot grape skin varied from 22% – 35% among the four different *terroirs*. Moreover, research conducted with Carignan noir grapes in Maule Valley, Chile, demonstrated that the total and individual anthocyanin content of Carignan noir grapes was significantly different in six different *terroirs* (Martinex-Gil et al., 2016). Furthermore, Pinot Noir grapes were used to examine the effect of *terroir* between two viticultural regions in Romania. Pinot Noir grapes grown in Dealu Mare had at least four times more extracted anthocyanin content compared to the Pinot Noir grapes from Murfatlar (Artem et al., 2016). In the same study of flavonol content where Pinot Noir grapes were sampled from eleven distinctive localities in southern Spain and central Germany, grape anthocyanin content of all five aglycones were also found to be varied (Del-Castillo-Alonso et al., 2016). For instance, malvidin-3-O-glucoside ranged from 12.3 to 54.6 mg g^{-1} dry weight among the eleven Pinot Noir sampling sites. Therefore, anthocyanin content is a good indicator of the association between *terroir* and grape quality components (e.g. pigmentation). Nevertheless, many of these studies have focused on identifying the differences in anthocyanin content among different *terroirs*, instead of pinpointing a particular climatological component to explain the results, owing to the complicated interactions between environmental constituents.

1.7.2 Tannins

Tannins, especially condensed tannins, are a group of astringent and bitter flavonoid compound found in both grape skin and seeds (Flamini and Traldi, 2010). When grape tannins are precipitated with salivary proteins, a puckering and drying response occurs in the mouth that is referred to as astringency (Villamor *et al.*, 2009). In grapes, tannins function to defend plants against herbivory, and to protect against bacteria and fungi infection (Furlan *et al.*, 2011). Typically, tannin accumulation happens from fruit set until veraison (Braidot et al., 2008). After veraison, the concentration of skin tannin may decline due to the association with other skin components (e.g. cell wall polysaccharide, proteins) and the concentration of seed tannin may decline due to oxidation (i.e. turn seeds from green to brown color). As well, the total tannin concentration is reduced owing to grape berry expansion and consequent dilution of the tannins accumulated before veraison (Downey et al., 2003; Kennedy, 2002).

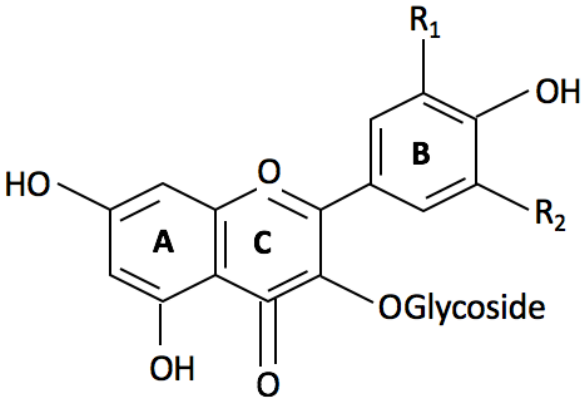
Condensed tannins are the most abundant flavonoid compounds in grapes and are polymers of flavan-3-ols linked by $C_4 – C_6$ and $C_4 – C_8$ interflavan bonds (Teixeira et al., 2013). The primary subunits are catechin, epicatechin, epigallocatechin and epicatechin gallate (Harbertson *et al.*, 2002). In the study by Harbertson et al., there is more than two times as much seed tannin per berry as skin tannin (Harbertson et al., 2002). Furthermore, when looking into the molecular structure, there is more epicatechin gallate present as terminal units in seed tannins than skin tannins, while epigallocatechin is absence from seed tannins. Despite these molecular differences, there is no significant difference in astringency between skin tannins and seed tannins (Brossaud *et al.*, 2001). Most research has focused on examining the total tannin content in grape skins and seeds instead of classifying the tannin content based on the various lengths and chemical compositions.

1.7.2.1 Grapes Tannin Content and *Terroir*

Interestingly, even though tannins contribute significantly to the astringency and bitterness of grapes and red wine, limited research studies have assessed the association of grapes tannin content with *terroir*, and the results are not consistent (Artem et al., 2016; Fernandez-Marin et al., 2013; Mateus et al., 2001). As suggested by the Mateus et al. study, tannin composition is influenced more by environmental fluctuation as compared to the tannin concentration (Mateus et al., 2001). In the Fernandez-Marin et al. study with four varieties of grapes (Syrah, Merlot, Cabernet Sauvignon, and Pinot Noir), there was no significant variation in skin, seed, and total tannin contents among grapes grown in four distinctive Andalusian terroirs (Fernandez-Marin et al., 2013). Similarly, in the study by Artem et al., Pinot Noir grapes from two different *terroirs* in Romania were examined and the difference in seed tannin content was not significant (Artem et al., 2016). Nevertheless, in the same study, the skin tannin content of Pinot Noir grapes from Dealu Mare was found to be two-fold greater compared to the Pinot Noir grapes in Murfatlar (Artem et al., 2016). Therefore, these results are not sufficient to explain the impact of *terroir* on grape tannin content and more research has to be completed to fully understand the relationship.

1.7.3 Flavonols

Flavonols are white and yellowish compounds synthesized exclusively in the form of 3-O-glycosides in grape skins; some examples of glycosides include glucoside, galactoside and glucuronide (Makris *et al.*, 2006). Interestingly, flavonols are considered to be one of the oldest polyphenolic compounds in the world, based on the fact that ancient land plants, including mosses and liverworts, all have the genes required for flavonol biosynthesis (Pollastri and Tattini, 2011). In a study by Mattivi et al., the researchers analyzed flavonol content in ninety-one grape varieties and attempted to classify grape species based on their flavonol profiles (Mattivi et al., 2006). Six flavonol aglycones were present at significant levels in red wine grapes, namely myricetin, quercetin, laricitrin, kaempferol, isorhamnetin and syringetin (Figure 1-3). On average, for Pinot Noir grapes, these flavonol aglycones account for 16.28%, 59.30%, 4.73%, 10.14%, 7.11% and 2.44% (according to the order above) of the total flavonol content respectively. Furthermore, myricetin, laricitrin and syringetin are missing from all white grape varieties. Interestingly, flavonol biosynthesis in wine grapes occurs in two discrete periods, the first one starting at near flowering (fruit set) and the second one beginning at one to two weeks after veraison until the harvest (Braidot et al., 2008; Downey et al., 2006).



	R_1	R_2
Kaempferol	H	H
Quercetin	OH	H
Myricetin	OH	OH
Isorhamnetin	OCH_3	H
Laricitrin	OCH_3	OH
Syringetin	OCH_3	OCH_3

Figure 1-3 Chemical structures of flavonols found in Pinot Noir grapes.
The bold letters indicate the rings in the flavonoid compound.

In grapes, flavonols are known as photo-protective compounds against ultraviolet B (UVB) radiation stress (Pollastri and Tattini, 2011). Reactive oxygen species (ROS) production increases in grape plants under UVB irradiation, which leads to light-induced oxidative damages. Flavonols, which are strong antioxidants, are able to protect the plant DNA material from oxidative damage by multiple mechanisms, including the donation of hydrogen atoms to scavenge free radicals (i.e. ROS) and screening out UV-B irradiation (Makris *et al.*, 2006;

Pollastri and Tattini, 2011). Therefore, flavonols are also important bioactive compounds for human health because these compounds can scavenge alkoxyl and peroxyl radicals, resulting in the termination of lipid peroxidation. Structural features of flavonols are important in determining the antioxidant efficiency. In particular, flavonols with an O-dihydroxy structure at the B-ring (e.g. quercetin, myricetin, laricitrin) have a greater antioxidant efficiency. The O-dihydroxy structure at the B-ring is a target site for free radicals and the structure maintains stability after donating an electron to stabilize the free radicals (i.e. electron dislocation). Also, for all flavonol aglycones, the 2,3-double bond in conjugation with the 4-keto functional group at the C-ring participate in electron delocalization from the B-ring.

Flavonols have high affinity to a wide range of proteins in plants, which produce signaling compounds that are important for cell growth and development regulation (Pollastri and Tattini, 2011). Furthermore, intermolecular co-pigmentation was identified with the noncovalent association between flavonols and anthocyanins, resulting in color intensity enhancement of grape skins (Trouillas *et al.*, 2016).

1.7.3.1 Grape Flavonol Content and *Terroir*

Studies looking at the association between grape flavonol content and *terroir* are scarce. In a study that compared Pinot Noir grapes from eleven distinctive localities among southern Spain and central Germany, grape flavonol content was shown to vary based on *terroir* (Del-Castillo-Alonso et al., 2016). For example, the content of quercetin-3-O-glucoside in Pinot Noir grapes ranged from 17.7 to 181 mg g^{-1} dry weight among the eleven sampling sites. Yet, more studies from other geographical locations are required before general conclusions can be reached.

1.7.4 Similarity between Flavonol and Anthocyanin Biosynthesis

Even though tannins, flavonols and anthocyanins are all sub-classes of flavonoids, only tannins are characterized by a high degree of polymerization in grapes. Thus, grape flavonols and anthocyanins are more similar in the sense that these molecules accumulate in red wine grapes in the form of 3-O-glycosides (Mattivi et al., 2006). In particular, some of the biosynthesis pathway between flavonols and anthocyanins are analogous (Mattivi et al., 2006). First, with the same

flavonoid precursor, 3'-hydroxylation at the B-ring would result in the production of anthocyanin cyanidin and flavonol quercetin, while 3'5'- hydroxylation at the B-ring would result in anthocyanin delphinidin and flavonol myricetin. (Mattivi et al., 2006). Second, the formation of the flavonol isorhamnetin (from quercetin) and the anthocyanin peonidin (from cyanidin) results from 3' O-methylation at the B ring. Third, 3' and 5' O-methylation occur for both flavonols and anthocyanins, determining the biosynthesis of the anthocyanins petunidin and malvidin (from delphinidin) and the flavonols laricitrin and syringetin (from myricetin). With similarities between the flavonol and anthocyanin biosynthetic pathways, it is rational to predict that the differences in anthocyanin and flavonol accumulation amongst different *terroirs* could correlate.

1.8 Research Objective

The objective of this study was to examine the distribution of wine yeast strains as well as to evaluate the flavonoid profile of Pinot Noir grapes among three sub-regions of the OV (KE, NP, OO) in the 2017 vintage. In particular, this thesis is comprised of three objectives: (i) to compare the distribution of vineyard-associated *S. cerevisiae* and *S. uvarum* strains among the three sub-regions; (ii) to identify regional-specific *S. cerevisiae* and *S. uvarum* strains; (iii) to describe the differences in the tannin, flavonol and anthocyanin content and profile of Pinot Noir grapes among the three sub-regions.

2 Chapter 2- Microbial Analysis

2.1 Introduction

Wine, a popular beverage that has historical significance, is a product of grape juice fermentation by wine yeasts, including *S. cerevisiae* and *S. uvarum* (Demuyter et al., 2004; Masneuf-Pomarede et al., 2010; Tofalo et al., 2013; Tosi et al., 2008). Through wine yeast metabolism, the fermentable sugars from the grape must are converted into ethanol and carbon dioxide, which forms the basis of wine. Nowadays, wines in Canada, especially those made in large scale production facilities, are mostly produced by inoculated fermentation, where the fermentation is initiated by the addition of a commercial wine yeast starter culture into the grape

must (Pretorius, 2000). This practice is beneficial because of its rapidity and reliability, which also guarantees the uniformity and reproducibility of the finished wine to a certain extent.

With globalization, the competition in the wine market has never been greater (Pretorius, 2000). As a result, many winemakers strive to produce wine of higher quality and uniqueness in order to stand out from other wine products. Therefore, spontaneous fermentation, a traditional way of wine production, where the fermentation is solely carried out by wine yeasts on harvested grapes and winery equipment, has regained its popularity (Pretorius, 2000). Even though the resulting wine product is hardly reproducible and stuck fermentations may occur owing to the unpredictability of the yeast communities in nature, various wine yeast strains are involved in the fermentation and thus, the resulting wine can develop a complex and diverse sensory profile. Moreover, in the past five years, multiple studies from New Zealand, Italy, Portugal and *etc.* have suggested that wine yeasts can adapt to their environment with naturally occurring gene and genomic changes that creates regional-specific strains (Knight and Goddard, 2015; Rantsiou et al., 2016; Schuller et al., 2005; Zhang et al., 2015). Furthermore, when a variety of strains are involved in wine fermentation, the metabolic interactions of the regional-specific wine yeast strains can result in wine with unique and distinctive sensory profiles (Knight et al., 2015).

The OV, which is the major winemaking region in BC, is a geographical location with great natural resources for grape growing and wine production (BC Wine Institute, 2018). Even so, no large-scale study that characterizes regional-specific wine yeast strains has been published. Therefore, the research objectives of this thesis were to analyze the distribution of vineyard-associated *S. cerevisiae* and *S. uvarum* strains as well as to identify regional-specific *S. cerevisiae* and *S. uvarum* strains among three distinctive winemaking sub-regions of the OV (KE, NP and OO) in the 2017 vintage. We hypothesized that the distribution of vineyard-associated wine yeasts would be different between the three sub-regions quantitatively and qualitatively, along with the identified regional-specific wine yeast strains.

2.2 Methods

2.2.1 Experimental Design and Grape Sampling

Between September 2017 and October 2017, Pinot Noir grape samples were collected from thirteen vineyards across three sub-regions (OO, NP, KE) of the OV spanning around 100 km

within a week prior to the winery harvesting date. The sampled Pinot Noir grapes have insignificant differences in total soluble solid level among the three sub-regions, between 21 to 23 ºBrix (Table 3.1). The locations of the vineyards are shown in Figure 2-1 and the names of the vineyards remain confidential upon request by the owners. The three sub-regions, KE, NP and OO, have distinctive terroir differences (1.3).

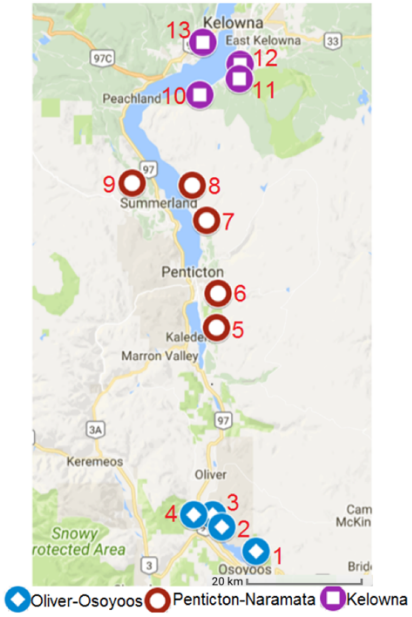


Figure 2-1 Vineyard Locations for Pinot Noir Grape Sample Collection.
The map shows a section of the OV that spans around 100 km. Each circle with a number represents a vineyard; blue represents vineyards in OO, red represents vineyards in NP and purple represents vineyards in KE. Courtesy of Jonah Hamilton.

At each Pinot Noir vineyard, the sampling site was a ~0.25 hectare section of the vineyard. The section was sub-divided into thirty-two portions; each portion had two rows with three posts or panels on each row. The outermost rows of the section and the first two posts or panels of each row were excluded from the sampling. For vineyard 13, since the posts were very long, five grapevines were counted as one sampling post.

For microbial analysis, berry clusters were sampled from six regions of each vineyard that were randomly selected (six replicates). Each sampled region of the vineyard consisted of five to six fence posts. Within these six regions, five healthy grape clusters were randomly and aseptically harvested per post or panel, resulting in a sample size of thirty grape clusters from each portion. Grape samples from each region were placed into sterile stomacher bags and

transported to the Wine Research Center (UBC, Vancouver) on ice. The grapes were then crushed and fermented aseptically in the lab within twenty-four hours after sampling.

2.2.2 Processing of Pinot Noir Grape Samples

Erlenmeyer flasks were autoclaved and pre-weighed prior to the processing of grape samples. Also, bungs and airlocks were sterilized by immersing in 70% ethanol solution. At the Wine Research Center, bags of grape samples were manually crushed for fifteen minutes. From each bag, around 500 ml of crushed grape juice was aseptically transferred to a pre-weighed and autoclaved Erlenmeyer flask; each flask was further sealed with a sterile bung and airlock. In order to dissolve the carbon dioxide gas that is created during fermentation, sterile distilled-deionized water (ddH_2O) was pipetted into the airlock. The flasks were incubated at 25 ºC, weighed right after processing and everyday up to forty days of fermentation.

2.2.3 Isolation of Wine Yeasts

The weight loss of the fermenting grape juice was tracked to estimate the degree of sugar depletion. When the fermentation reached around two-thirds sugar depletion, a small volume of fermented grape juice was serially diluted (10^{-4} to 10^{-6}) and plated on Yeast extract-peptone-dextrose agar (YPD) plates with 0.015% biphenyl (BPH) and 0.01% chloramphenicol (CAN) in duplicates. CAN is a bacteriostatic agent that binds to the bacterial ribosome (50S subunit) and inhibits protein elongation through peptidyl transferase inactivity (Singleton and Sainbury, 2006). In addition, mold growth is reduced by BPH through inhibition of carotin synthesis (Luck and Jager, 1997). For fermentation that did not reached two-thirds sugar depletion, no yeast colony was examined in this study. The plates were incubated at 25 ºC for three days. After forty days of fermentation, no further fermented grape juice samples were taken.

2.2.4 Purification of Wine Yeasts

For each fermented grape juice sample, after incubation on YPD plates for three days at 25 ºC, the lowest plate dilution with 30 – 300 colonies was selected for purification of wine

yeasts. Forty-eight randomly selected single colonies were struck onto the YPD plates and incubated at 25 ºC for two days.

2.2.5 Identification of Wine Yeasts

Wallerstein nutrient agar (WNA) was purchased from Sigma-Aldrich and the agar plates were made accordance to the manufacturer's instructions. The yeast colonies on the YPD plates were patched on the WNA plates for *Saccharomyces* yeast identification (Spedding, 2000). A commercial *S. cerevisiae* wine strain (M2) and a *Hanseniaspora uvarum* (*H. uvarum)* strain were included as the positive and negative controls, respectively. These plates were incubated at 25 ºC for two days.

After incubation, colonies with light green or creamy shades on the WNA plates were identified as *Saccharomyces* yeast colonies. Colonies with dark green shades were further patched onto a lysine plate and incubated at 25 ºC for two days. A commercial *S. cerevisiae* colony (M2) and a *H. uvarum* colony were included as the positive and negative control respectively. After incubation, colonies that did not grow on the lysine plates were also identified as wine yeast colonies.

For all *Saccharomyces* yeast colonies, they were once again patched from the WNA plates to YPD plates and incubated at 25 ºC for two days to remove the color dye. Next, the *Saccharomyces* yeast were arrayed with 96 colonies per plate.

2.2.6 Extraction of DNA Materials

The DNA of the *Saccharomyces* yeast colonies were extracted with Zymolyase 20T. Zymolase buffer (1.2M sorbitol, 0.1M KH_2PO_4 in KOH at pH 7.2) was mixed with Zymolyase 20T solution (5 mg/ml) at a 1: 4 ratio. A ninety-six pin-head pinner was used to lift a small volume of the yeast colony followed by mixing with 20 µl of the Zymolase mixture in a ninety-six well plate. In order to achieve cell wall disruption and DNA extraction, the mixture was warmed at 37 ºC for thirty minutes, then heated to 95 ºC for ten minutes. Finally, the DNA material was separated from the cell debris by centrifugation at 4000 g for ten minutes. After determining the concentration of the DNA by the nanodrop spectrophotometer, the DNA was diluted to 25 µg/µl in sterile 10 mM Tris at pH 8.0.

2.2.7 Differentiation of *S. cerevisiae* and *S. uvarum*

S. cerevisiae and *S. uvarum* were differentiated based on polymorphism at the *MET2* locus using RFLP-PCR. The M2 strain and the CBS 7001 strain were used as positive control for *S. cerevisiae* and *S. uvarum* respectively. The diluted DNA (1 µl) was added into 19 µl of *MET2* PCR master mix [20% 5X dNTPs 10% 10X BioBasic Taq Buffer, 10% $MgSO_4$ (20 mM), 0.6% MET2 forward primer (100 µM), 0.6% MET2 reverse primer (100 µM), 0.5% BioBasic Taq Polymerase]. Then, the *MET2* fragment was amplified with the following conditions: initial denaturation step (five minutes at 94 ºC), thirty-five cycles of PCR (thirty seconds at 94 ºC, forty-five seconds at 50 ºC and one minute at 72 ºC) and a final elongation step (ten minutes at 72 ºC). Two endonucleases, *EcoRI* and *PstI*, were used to digest the amplified *MET2* DNA. To identify *S. cerevisiae* DNA, the *MET2* PCR product (3.3 µl) was added to 6.7 µl of *EcoRI* master mix (15% NEB CutSmart buffer, 1.5% 100X BSA, 1.5% *EcoRI* High Fidelity restriction enzymes). Similarly, to identify *S. uvarum* DNA, the same volume of *MET2* PCR product was added into 6.7 µl of *PstI* master mix (15% NEB CutSmart buffer, 1.5% 100X BSA, 1.5% *PstI* High Fidelity restriction enzyme). Both mixtures were warmed at 37 ºC for thirty minutes. Afterwards, *EcoRI*-digested and *PstI*-digested products (8 µl) were ran on a 1.2% agarose gel at 110V for forty-five minutes, and read by UVP gel reader at 365 nm transillumination. *MET2* amplified from *S. cerevisiae* DNA produces two *EcoRI*-digested products that migrate at ~250 and 400 base pairs (bp) whereas *MET2* amplified from *S. uvarum* DNA produces two equally sized *PstI*-digested products that migrate at ~250 and 400 bp.

2.2.8 Microsatellite Analysis of *S. cerevisiae*

Microsatellite fragments from diluted DNA previously extracted from *S. cerevisiae* colonies (2.2.5) were amplified using the Qiagen multiplex PCR kit (#206143) with ten *S. cerevisiae* SSR primers (Table 2-1) (Richards *et al.*, 2009; Tantikachornkiat, 2017). As previously established by Jay Martiniuk, the *S. cerevisiae* SSR primer sequences and the composition of primer mix are described in Table A-1 under the Appendix A. For each reaction, there were 5 µl of PCR Kit, 2 µl of primer mix, 2 µl of milli-Q ddwater and 1 µl of diluted DNA. The PCR reactions were carried out by a thermocycler program with an initial denaturation step

(five minutes at 95 ºC), thirty-four cycles of PCR (thirty seconds at 94 ºC, ninety seconds at 54 ºC and one minute at 72 ºC) and a final elongation step (forty-five minutes at 68 ºC). The size of the amplified fragments was determined by an AB3730 DNA analyzer coupled with capillary electrophoresis at the UBC Sequencing + Bioinformatics Consortium. The microsatellite amplicons were analyzed using Genemapper software. Allele-binning was performed on the microsatellite data by MsatAllele (a R package) to generate a multi-locus genotype (MLGs) for each sample and each distinctive MLG was considered as an individual strain.

Table 2-1 SSR Primers used in Microsatellite Analysis of *S. cerevisiae*

Locus	Chromosome	Tag	SSR	Alleles[a]	Size range
YGL139W (C3)*	VII	FAM	CAA	18	94 – 158
YLR177W (YLR)	XII	PET	CAG	13	80 – 160
YFR028C (C5)	VI	VIC	GT	34	119 – 228
YGL014W (C8)	VII	NED	TAA	10	122 – 160
YOL109W (C4)	XV	VIC	TAA, TAG	26	230 – 373
YML091C (O91C)	XIII	NED	AAT	31	218 – 331
YOR267c (SCY)	XV	PET	CAA	27	258 – 390
YLL049W (AT4)	XII	FAM	TA	15	270 – 305
YDR160W (SCAAT3)	IV	NED	AAT	21	345 – 499
YPL009C (009C)	XVI	FAM	CTT	21	385 – 456

*Sign indicated in the parentheses is the common name of the loci. [a]The total number of unique alleles in the database.

2.2.9 Microsatellite Analysis of *S. uvarum*

Microsatellite analysis of *S. uvarum* was performed in a similar manner to *S. cerevisiae,* except eleven different SSR primers were used (Table 2-2) (Masneuf-Pomarede *et al.*, 2016; Zhang *et al.*, 2015). As previously established by Garrett McCarthy, the *S. uvarum* SSR primer sequences and the composition of primer mix are described in Table A-2 under the Appendix A. For each reaction, there were 5 µl of PCR Kit, 4 µl of primer mix, and 1 µl of diluted DNA. The PCR reactions was carried out by a thermocycler program with an initial denaturation step (five minutes at 97 ºC), thirty-four cycles of PCR (thirty seconds at 95 ºC, one minute at 54 ºC and two minutes at 72 ºC) and a final elongation step (ten minutes at 72 ºC). Afterwards, the

amplified fragments were measured and analyzed using Genemapper as described for *S. cerevisiae*.

Table 2-2 SSR Primers used in Microsatellite Analysis of *S. uvarum*

Locus	Chromosome	Tag	SSR	Alleles[a]	Size range
NB1	X	FAM	ATG	5	193 – 212
NB4	X	FAM	TGT	4	334 – 350
NB8	XVI	FAM	TGT	5	415 – 450
NB9	XV	FAM	AT	6	111 – 121
SuARS409 (L1)*	X	FAM	GT	3	163 – 167
SuYBR049c (L2)	II	FAM	ATT	8	285 – 306
SuYKL017c (L3)	XI	FAM	TA	4	219 – 229
SuYKR045c (L4)	XI	VIC	CTG	9	293 – 334
SuHTZ1PLB3 (L7)	XII	NED	TC	4	261 – 271
SuYHR102W (L8)	VIII	NED	GTT	11	202 – 254
SuYIL130W (L9)	IX	VIC	ATT	19	173 – 290

*Sign indicated in the bracket is the common name of the loci. [a]Number of unique alleles in the database and generated from this project.

2.2.10 Microbial Statistical Treatment

As *S. cerevisiae* and *S. uvarum* were amplified by different SSR primers, statistical treatment was performed separately on these two species. The microsatellite data were processed by R (version 3.3.3) with the Poppr package (version 2.6.0) (Kamvar *et al.*, 2014; Kamvar *et al.*, 2015).

To identify the potentially indigenous strains from commercial strains, Bruvo's distance, which takes into account stepwise mutations in microsatellites, was calculated. All strains with an average Bruvo's distance < 0.25 from a commercial strain were considered to be commercial-related (i.e. commercial strains) and otherwise, the strain was considered as a potentially indigenous yeast strain. The Bruvo's distance is set at a distance threshold of 0.25 because this distance is suggested in the Poppr package for defining multilocus genotypes (Kamvar *et al.*, 2014; Kamvar *et al.*, 2015; Martiniuk et al., 2016). For *S. cerevisiae*, the Bruvo's genetic distance of each strain isolated from this study was compared to the commercial *S. cerevisiae* database in the Measday's lab, that contains 174 *S. cerevisiae* strains collected or donated from wineries or yeast companies, to visualize the stepwise mutations in SSRs (Bruvo et al., 2004). The Bruvo's genetic distance between strains was calculated as $= 1 - 2^{-|x|}$; where x is the number

of repeat units differed between two alleles, specifically the isolated strain and a strain in the database (Kamvar, n.d.). The identity of the isolated *S. cerevisiae* strains was determined by the average Bruvo's genetic distance of the ten loci.

For *S. uvarum*, the data was processed in the same fashion, except the strains isolated in this study were compared to 20 *S. uvarum* strains previously isolated around the world, 10 *S. uvarum* strains previously isolated in OV wineries and 1 *S. uvarum* commercial strain (BMV 58) (Almeida et al., 2014). With an average Bruvo's distance of the eleven loci < 0.25, the isolated *S. uvarum* strain was considered as a previously isolated yeast strain; otherwise, the isolated *S. uvarum* strain was considered as a newly discovered yeast strain.

The Venn diagram was created in Jvenn to visualize the distribution of wine yeast strains among three sub-regions of the OV. Analysis of molecular variance (AMOVA) was calculated to measure the population differentiation via the microsatellite markers, in order to understand the variation of yeast strains at the level of sub-regions, vineyards and sample level (Kamvar *et al.*, n.d.). Also, a significance test was conducted to examine the population differentiation at a significance level of 0.05. In addition, Bruvo's genetic distance matrixes were constructed by Adonis and the matrixes were further visualized by a minimum spanning network. The minimum spanning network was created to understand the genetic relationships among potentially indigenous *S. cerevisiae* strains and all isolated *S. uvarum* genotypes from this study. Furthermore, phylogenetic networks were produced by SplitTree 4.14.6 via the Neighbor-Net algorithm (distance-based) to investigate the genetic relationships between potentially indigenous *S. cerevisiae* strains and commercial *S. cerevisiae* strains, as well as between newly discovered *S. uvarum* strains and previously isolated *S. uvarum* strains (Bryant and Moulton, 2004).

2.3 Results

2.3.1 Fermentation Results

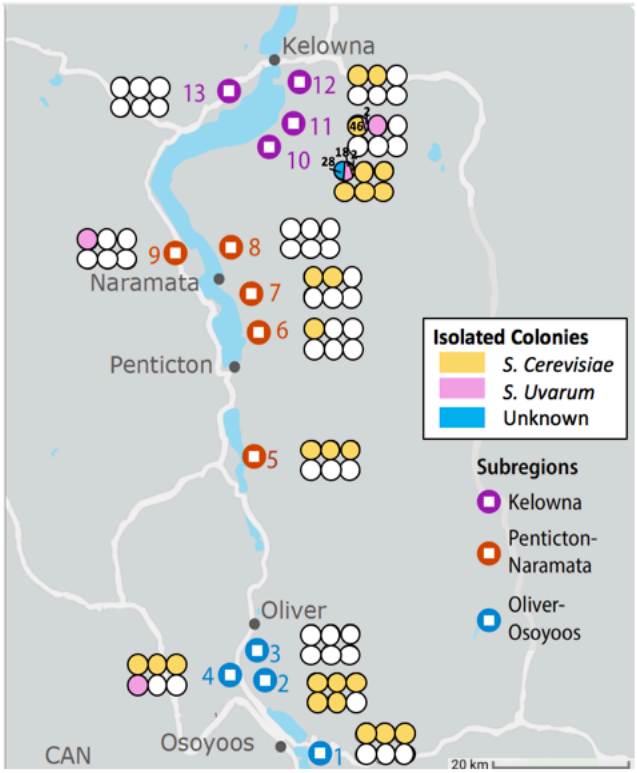


Figure 2-2 Map of Vineyards with Successful Fermentations (Two-thirds Sugar Depletion) and the Wine Yeast Species Isolated.
Each circled square with a number represents a vineyard. The associated sub-region to each vineyard is indicated (purple indicates KE, orange indicates NP and dark blue indicates OO). Each circle represents a spontaneous fermentation. The colored circles (e.g. yellow, pink and sky blue) are fermentations that have reached two-thirds sugar depletion whereas the white circles are fermentations that did not reach two-thirds sugar depletion. Isolation of *S. cerevisiae* is indicated by yellow, isolation of *S. uvarum* is indicated by pink and unknown *Saccharomyces spp.* are indicated by sky blue. Each colored circle represents isolation of 48 wine yeast colonies corresponding to the color of the circles, unless otherwise indicated (vineyard 10 and 11).

Table 2-3 Number of Fermentations with Two-thirds Sugar Depletion and Wine Yeast Colonies Isolated from Each Vineyard and Sub-region

Sub-region	Vineyard	Fermentations with two-thirds sugar depletion[a]	Number of Isolated *S. cerevisiae* colonies	Number of Isolated *S. uvarum* colonies
Oliver-Osoyoos	1	3/6[b]	144	0
	2	5/6	240	0
	3	0/6	0	0
	4	4/6	144	48
Naramata-Penticton	5	3/6	144	0

	6	1/6	48	0
	7	2/6	96	0
	8	0/6	0	0
	9	1/6	0	48
Kelowna	10	6/6	242 [c]	18 [c]
	11	2/6	46	50
	12	2/6	96	0
	13	0/6	0	0

[a] Sugar depletion was calculated by weight loss due to CO_2 generation. [b] The fraction indicates the number of fermentation that reached two-thirds sugar deletion out of the six replicates. [c] For vineyard 10, 28 colonies were categorized as unknown *Saccharomyces spp.* (2 %) and not depicted in the Table.

Separate spontaneous fermentations were carried out for each vineyard replicate (13 vineyards, 6 replicates each). Of the 78 spontaneous fermentations conducted in the 2017 vintage, 29 (37%) reached two-thirds sugar depletion, which was the criteria for wine yeast isolation (Figure 2-2 and Table 2-3). In total, 1,392 *Saccharomyces* colonies were identified via WNA and lysine agar. WNA is a differential agar for differentiating yeasts and bacteria in wine fermentations (Spedding, 2000). Bromocresol green is the differential indicator in WNA and this color dye is utilized by yeasts in various degrees. For instance, *S. cerevisiae* colonies are frequently coloured white or pale green, whereas *H. uvarum* colonies are commonly dark green due to their inability to reduce bromocresol green. In addition, lysine agar is a selective agar because *Saccharomyces sp.* cannot grow with lysine as the major source of nitrogen, whereas non-*Saccharomyces* yeasts can use lysine as a nitrogen source (Spedding, 2000; Morris and Eddy, 1957). Therefore, *Saccharomyces* yeast do not grow on lysine plates whereas non-Saccharomyces yeast do.

Of the 1,392 *Saccharomyces* colonies isolated from spontaneous fermentation, 1,200 colonies were identified as *S. cerevisiae* (86%), 164 colonies were identified as *S. uvarum* (12%) and 28 colonies were categorized as unknown *Saccharomyces spp.* (2%) through microsatellite analysis (Figure 2-2). In particular, the unknown colonies were recognized by WNA and lysine agar as *Saccharomyces spp.*, but DNA extracted from these colonies was not amplified by either the *S. cerevisiae* nor *S. uvarum* microsatellite multiplex PCR mix. Preliminary data suggests that the unknown colonies may be *Saccharomyces paradoxus* but this remains to be confirmed through Sanger sequencing. In all three OV sub-regions, both *S. cerevisiae* and *S. uvarum* were identified, while the unknown *Saccharomyces spp.* were only isolated from vineyard 10. Each of the three sub-regions contained 1 vineyard where none of the 6 spontaneous fermentation

reached two-thirds sugar depletion (vineyard 3, 8 and 13). Both *S. cerevisiae* and *S. uvarum* were isolated in vineyard 4 (OO), 10 and 11 (KE), whereas *S. uvarum* was identified as the only wine yeast species from vineyard 9 (NP). Generally, there were more vineyards with *S. cerevisiae* colonies isolated (9 vineyards) compared to *S. uvarum* colonies isolated (4 vineyards).

Of the three examined sub-regions, NP had the least number of spontaneous fermentations reaching two-thirds sugar depletion (i.e. 7 spontaneous fermentations) and the smallest number of isolated wine yeast colonies (i.e. 288 *S. cerevisiae* colonies and 48 *S. uvarum* colonies). In comparison, with 4 sampled vineyards, OO had the highest number of spontaneous fermentations reaching two-thirds sugar depletion (i.e. 12 spontaneous fermentations), along with the largest number of isolated wine yeast colonies (i.e. 528 *S. cerevisiae* colonies and 48 *S. uvarum* colonies). Also, for KE with 4 sampled vineyards, there were 10 spontaneous fermentation that reached two-thirds sugar depletion, which resulted in the isolation of 384 *S. cerevisiae* colonies, 64 *S. uvarum* colonies and 28 unknown *Saccharomyces spp.* colonies.

2.3.2 *Saccharomyces cerevisiae*

2.3.2.1 Distribution of *Saccharomyces cerevisiae* Strains among the Three OV Sub-regions

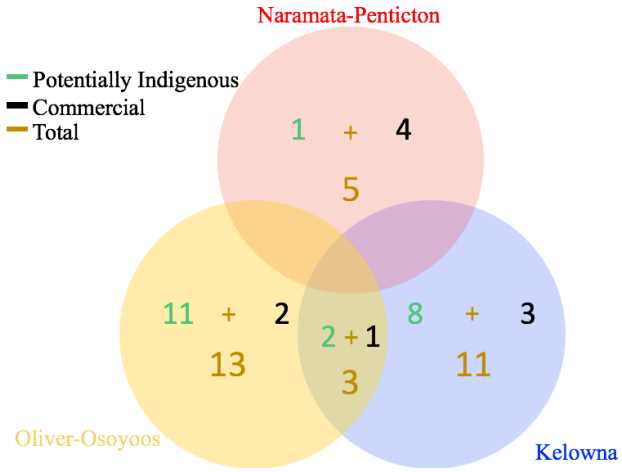


Figure 2-3 Venn Diagram of *S. cerevisiae* Strain Distribution by Sub-regions
Brown numbers represent the total number of isolated *S. cerevisiae* strains; green numbers represent the potentially indigenous strains (>0.25 Bruvo's distance) whereas black numbers represent the commercial strains (<0.25 Bruvo's distance) by comparison with the lab commercial strain reference database.

In total, based on Bruvo's distance, the 32 *S. cerevisiae* strains were classified into 10 commercial and 22 potentially indigenous strains in this study (Figure 2-3). As shown in the Venn diagram, the majority of isolated *S. cerevisiae* strains did not overlap between sub-regions. In fact, only 3 *S. cerevisiae* strains overlapped between OO and KE, while *S. cerevisiae* strains found in NP were not isolated in other sub-regions. In addition, the number of total *S. cerevisiae* strains and potentially indigenous *S. cerevisiae* strains that were found exclusively in each sub-region was positively correlated with the number of *S. cerevisiae* colonies isolated from each sub-regions, showing that our sampling method was sufficient to capture genetic variation of *S. cerevisiae* strains among sub-regions. Specifically, there were 11 potentially indigenous *S. cerevisiae* strains isolated exclusively from OO, 8 potentially indigenous *S. cerevisiae* strains recognized uniquely from KE, and only 1 potentially indigenous *S. cerevisiae* strain discovered from NP.

Table 2-4 Analysis of Molecular Variance (AMOVA) of *Saccharomyces cerevisiae* Strains

	Component of variance (%)		Significance (p)	
	w C	w/o C	w C	w/o C
Variations between sub-region	11.49	19.57	0.001	0.001
Variations between vineyards within sub-region	54.58	55.49	0.001	0.001
Variations within vineyards	33.92	24.95	0.001	0.001

Significance is calculated by 999 permutations. w C= with Commercial strains isolated from this study; w/o C= without commercial strains

According to the AMOVA table, for potentially indigenous *S. cerevisiae* strains (w/o C on Table 2-4), the molecular variance was highest between strains within sub-regions (55.49%), which accounted for more than half of the variance, followed by variations within vineyards (24.95%) and variations between sub-region (19.57%) (Table 2-4). In comparison, when including commercial *S. cerevisiae* strains isolated from this study in AMOVA (w C), the molecular variance within vineyard was observably increased (33.92%), while the variations between sub-region was noticeably decreased (11.49%). Also, even though the molecular variance was still the highest between strains within a sub-region, the absolute value was slightly lowered (54.58%). Either with or without commercial *S. cerevisiae* strains, the population strata at all examined levels has a p-value equal to 0.01. Therefore, the results of this study suggest that there is a significant *S. cerevisiae* population structure among the three OV sub-regions.

2.3.2.2 Genetic Relatedness of the Isolated *S. cerevisiae* Strains

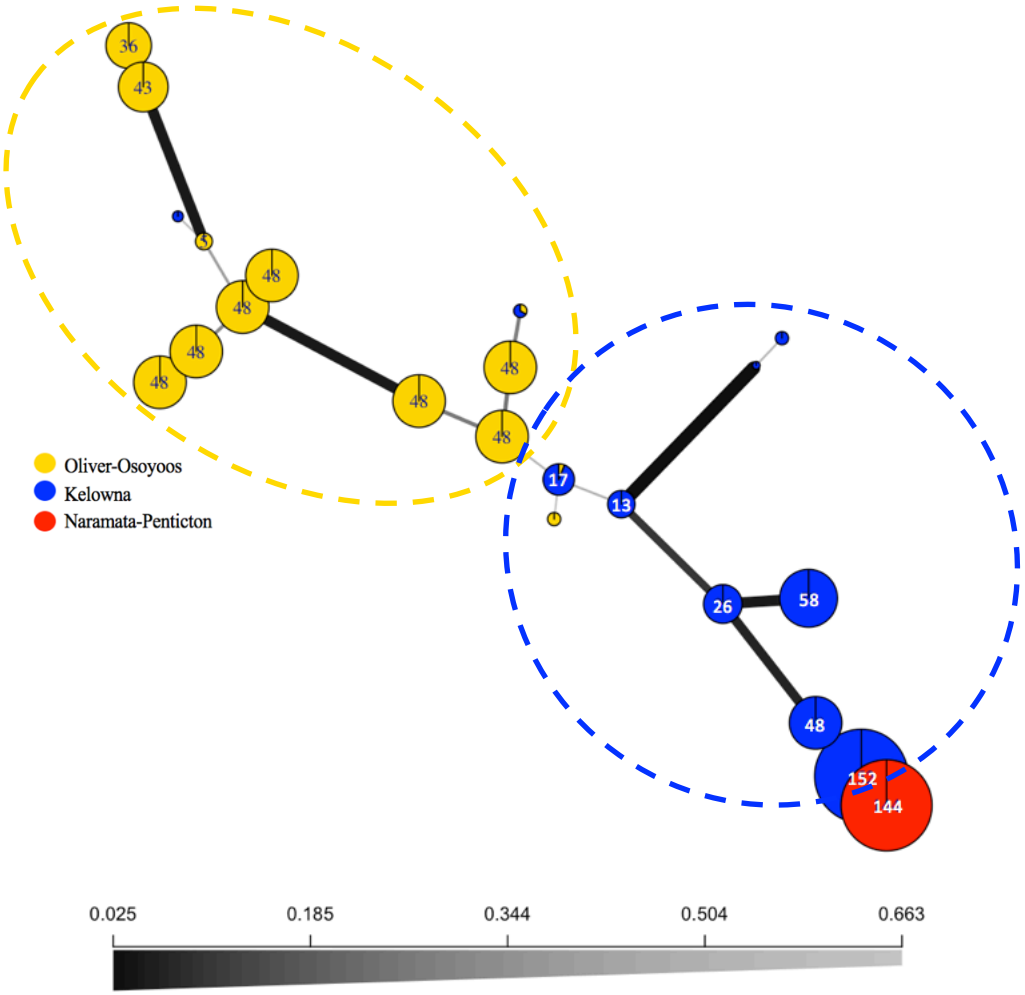


Figure 2-4 Minimum Spanning Network of Potentially Indigenous *S. cerevisiae* Strains
Each node represents one potentially indigenous *S. cerevisiae* strain; the color of the node indicates the sub-region(s) the strain was isolated from; the node size is proportional to the number of isolated colonies and the line thickness is proportional to the level of genetic relatedness between two potentially indigenous *S. cerevisiae* strains. Nodes with two colors indicate a potentially indigenous strain that was isolated from two sub-regions. Circles were used to denote obvious clusters of potentially indigenous strains.

The minimum spanning network is constructed based on the Bruvo's distance to visualize the genetic relatedness and the sampling abundance among potentially indigenous *S. cerevisiae* strains (Figure 2-4). Within the minimum spanning network, each potentially indigenous *S. cerevisiae* strain is forced to connect with at least one potentially indigenous *S. cerevisiae* strain (s), in order to construct a network that has no cycle and showing the possible way to connect all strains with the minimum possible genetic distance (Salipante and Hall, 2011).

From the minimum spanning network, two clusters of potentially indigenous *S. cerevisiae* strains, one dominated with strains isolated from OO (yellow circle) and one dominated with strains isolated from KE (blue circle), were identified (Figure 2-4). As the network is established based on Bruvo's distance, these two clusters demonstrate that *S. cerevisiae* strains within each sub-region were more genetically related than between sub-regions. Also, there are variations in the number of colonies for each potentially indigenous *S. cerevisiae* strains identified in this study. In particular, potentially indigenous *S. cerevisiae* strains solely isolated in OO has less variation in the number of isolated colonies (i.e. 7 out of the 11 identified strains were isolated with 48 colonies) as compared to potentially indigenous *S. cerevisiae* strains solely isolated in KE (i.e. varied blue node sizes). For the two potentially indigenous *S. cerevisiae* strains isolated from both KE and OO, there were higher sampling abundance from KE as compared to OO. For the single potentially indigenous *S. cerevisiae* strain isolated from NP, it was genetically more closely related to strains isolated from KE. Also, the node size demonstrates that the NP strain was isolated from more than one spontaneous fermentation, since forty-eight colonies were isolated from each fermentation. Moreover, when comparing strains within each cluster, there is a higher amount of genetic relatedness between strains found in KE than strains isolated in OO, as presented by the thicker connecting line.

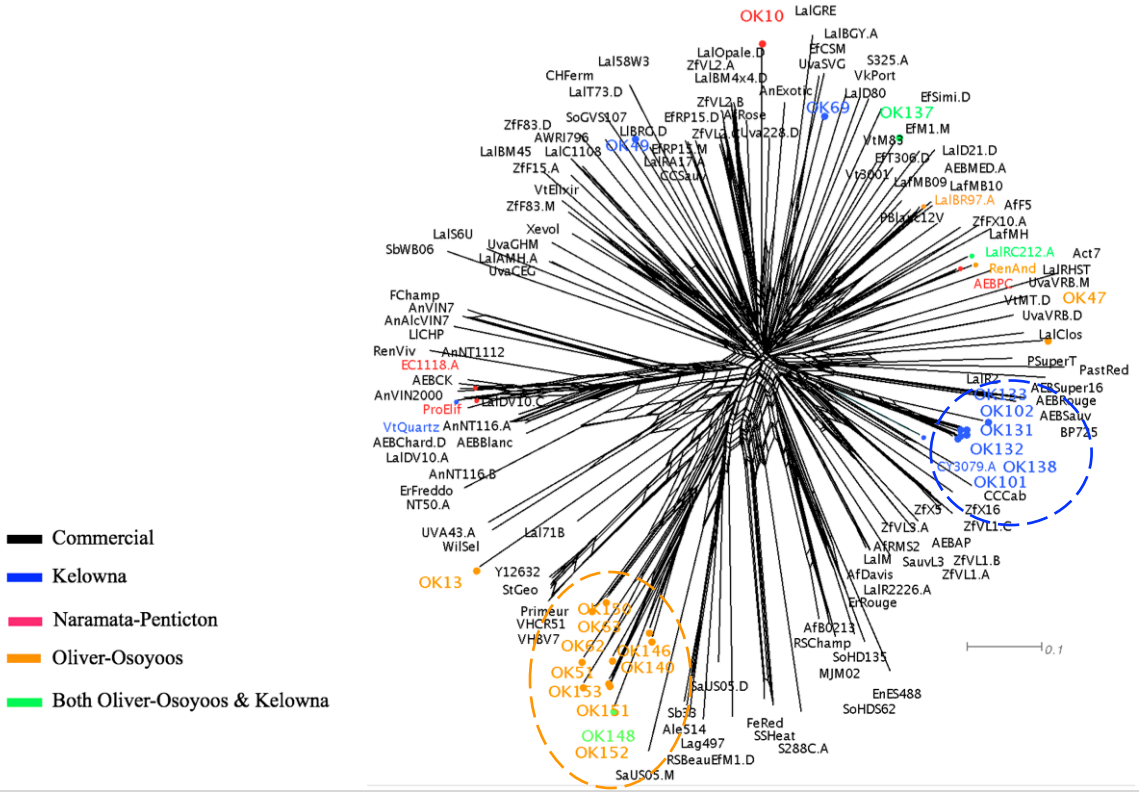


Figure 2-5 Phylogenetic Network of *S. cerevisiae* Strains Isolated from the OV and Commercial *S. cerevisiae* Strains from the Lab Reference Database.
The phylogenetic relationship was established using the Neighbor-Net algorithm based on Bruvo's distance. Potentially indigenous strains are labelled as "OK"; the color of the letters indicates the region where the commercial strain and the potentially indigenous strain was isolated from according to the legend. Black letters represent commercial strains from the lab reference database but not isolated from this study. Circles were used to denote obvious clusters of potentially indigenous strains.

Even though the minimum spanning network is adequate to visualize the genetic relatedness among potentially indigenous *S. cerevisiae* strains, the network tends to over-connect the strains in order to create a network with strains linked by the minimum possible genetic distance (Salipante and Hall, 2011). With a large dataset, a minimum spanning network may demonstrate too many connections between strains which provides an overwhelming and meaningless network (Salipante and Hall, 2011). Therefore, a phylogenetic network is constructed based on the Neighbor-Net method (i.e. distance-based) with a Bruvo's distance matrix to visualize the genetic relatedness among potentially indigenous *S. cerevisiae* strains and commercial *S. cerevisiae* strains (Figure 2-5). A phylogenetic network can be rapidly produced by Neighbor-Net method with good scaling, which is useful to provide an informative and detailed overview structure of a large dataset (Bryant & Moulton, 2003).

In the phylogenetic network, two clusters of potentially indigenous *S. cerevisiae* strains, isolated from OO and KE respectively, were clearly recognized (shown by the circles in Figure 2-5). Thus, most of the potentially indigenous strains isolated from OO and KE were more genetically related to strains isolated from the same sub-region as compared to strains between sub-regions or other commercial strains. Also, based on the distances in the network, none of the commercial *S. cerevisiae* strains isolated from this study are genetically closely related to any potentially indigenous *S. cerevisiae* strains, except CY3079.A that was identified in the cluster of strains isolated from KE. For the only potentially indigenous *S. cerevisiae* strain identified in NP (i.e. OK10), it is not closely genetically related to the commercial strains isolated from NP in the 2017 vintage, including EC1118.A, ProElif and AEBPC (Figure 2-5). For potentially indigenous *S. cerevisiae* strains isolated from both OO and KE, one was found in the cluster of strains isolated from OO (OK148), demonstrating close genetic relatedness with the strains in the cluster, while another strain (OK137) is not closely related genetically to any isolated strain from this study. Instead, OK137 is similar to commercial strains such as VtM83 and EfM1.M (Figure 2-5).

2.3.3 *Saccharomyces uvarum*

2.3.3.1 Distribution and Genetic Relatedness of *Saccharomyces uvarum* Genotypes among the Three OV Sub-regions

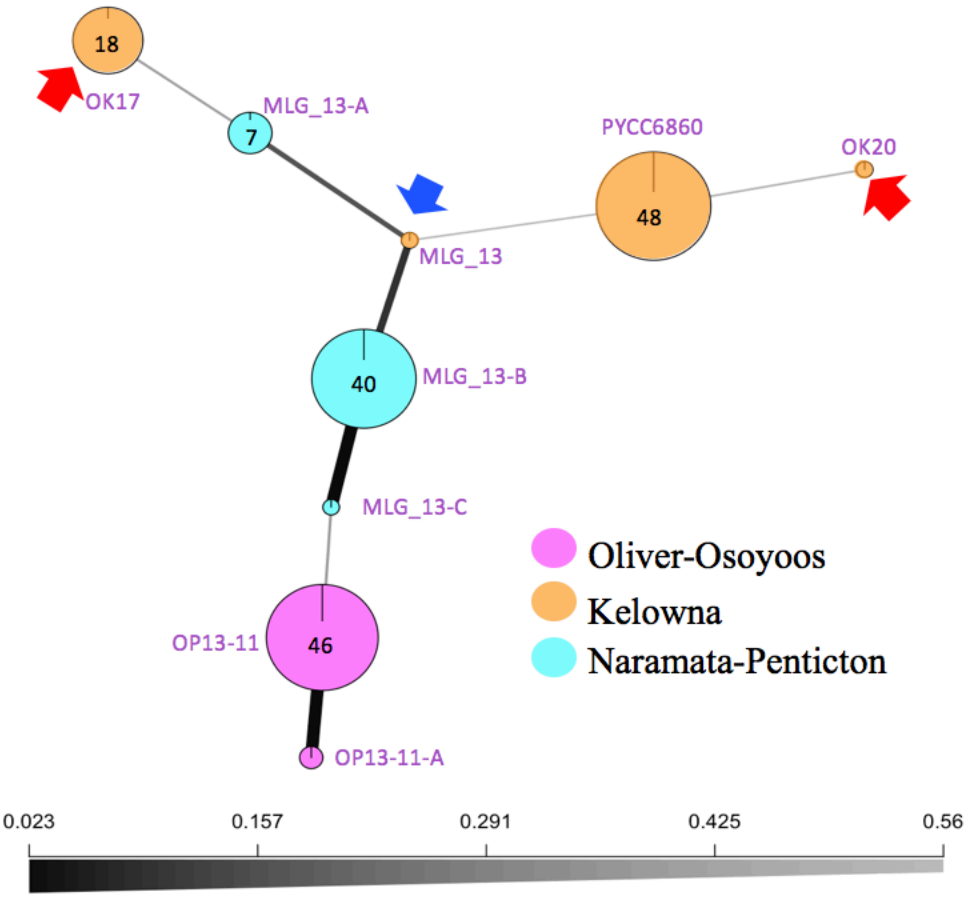


Figure 2-6 Minimum Spanning Network of all Isolated *S. uvarum* Genotypes in this study. Each node represents one isolated *S. uvarum* genotype; the color of the node indicates the sub-region where the genotype was isolated from; the node size is proportional to the number of isolated colonies and the line thickness is proportional to the level of genetic relatedness between two *S. uvarum* genotypes. Since there is only one known commercial *S. uvarum* strain and it was not isolated from this study, all isolated *S. uvarum* colonies and identified *S. uvarum* genotypes are included in the minimum spanning network. The two red arrows indicate the newly discovered *S. uvarum* genotypes from this study. The blue arrow indicate the previously isolated *S. uvarum* genotype that was identifed from KE but closely related genetically to strains isolated from NP. The purple labels are the corresponding strains of the identified genotypes. Four genotypes are the equivalent of MLG_13 and two genotypes are the equipvalent of OP13-11, when Bruvo's distance (< 0.25) is applied and therefore labelled with a hyphen and a letter of the alphabet to indicate a highly similar genotype to MLG_13 and OP13-11, respectively.

Microsatellite profiles across the eleven selected loci were completed for all 164 isolated *S. uvarum* colonies and 9 different multi-locus genotypes were identified. Because there is only one known commercial *S. uvarum* strain (BMV 58), the isolated genotypes were compared to a list of strains previously isolated from OV wineries and around the world (Table 2-5). Therefore, all genotypes with an average Bruvo's distance < 0.25 from a previously isolated strain are considered as previously isolated and otherwise, the genotypes were considered to represent newly discovered strains. In total, the 9 isolated *S. uvarum* genotypes were classified into 7 previously isolated genotypes and 2 newly discovered genotypes in this study.

Table 2-5 List of Previously Isolated *S. uvarum* Strains

Strain	Locality/Country of origin
CBS7001	Spain [a]
CBS8690	Moldova [a]
CBS8696	USA [a]
CBS8711	France [a]
A1	New Zealand [b]
A4	New Zealand [b]
A9	New Zealand [b]
7A6	New Zealand [b]
7D4	New Zealand [b]
8B11	New Zealand [b]
CBS 395	Netherlands [b]
PYCC6330	South America [c]
PYCC6860	Hornby Island, British Columbia, Canada [c]
PYCC6861	Hornby Island, British Columbia, Canada [c]
PYCC6862	Japan [c]
PYCC6871	Portugal [c]
PYCC6895	Moldova [c]
PYCC6901	USA [c]
PYCC6902	USA[c]
PYCC6994	France [c]
BMV 58	Spain (Commercial strain) [d]
OP13-07	OV wineries, British Columbia, Canada [e]
OP13-34	OV wineries, British Columbia, Canada [e]
OP13-02	OV wineries, British Columbia, Canada [e]
OP13-04	OV wineries, British Columbia, Canada [e]
OP13-11	OV wineries, British Columbia, Canada [e]
MLG160	OV wineries, British Columbia, Canada [e]
MLG 13	OV wineries, British Columbia, Canada [e]

MLG 79	OV wineries, British Columbia, Canada [e]
MLG 103	OV wineries, British Columbia, Canada [e]
Su01	OV wineries, British Columbia, Canada [e]

Collection acronyms: CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; PYCC: Portuguese Yeast Culture Collection. [a] Ngyuen and Boekhout, 2017. [b] Zhang et al., 2015. [c] Almeida et al., 2014. [d] Pérez-Torrado et al., 2016. [e] Measday and Durall lab, unpublished data.

The minimum spanning network was used to visualize the genetic relatedness of all isolated *S. uvarum* genotypes (Figure 2-6). The two newly discovered genotypes, both isolated from KE, are indicated by the red arrows in the figure (Figure 2-6).

None of the *S. uvarum* genotypes overlapped between sub-regions. The highest number of *S. uvarum* genotypes was isolated from KE (i.e. 4 genotypes), followed by 3 genotypes isolated from NP and 2 genotypes isolated from OO. Although a small number of *S. uvarum* genotypes were identified, most of the *S. uvarum* genotypes were linked within each sub-region in the network. In particular, *S. uvarum* genotype isolated from OO and NP respectively were more closely related genetically within sub-regions than between sub-regions, which is represented by a thick connection line in Figure 2-6. Yet, *S. uvarum* genotypes isolated from KE were distantly related within sub-region, as they are linked by thin connecting line. It is also noteworthy that a *S. uvarum* genotype isolated from KE more closely related genetically to genotypes isolated from NP than genotypes in the same sub-region, as indicated by the blue arrow in the figure (Figure 2-6). Moreover, based on the node size, each sub-region was quantitatively dominated by one *S. uvarum* genotype in our isolation (Figure 2-6).

Table 2-6 Analysis of Molecular Variance (AMOVA) of *Saccharomyces uvarum* Genotypes

	Component of variance (%)	P
Variations between sub-region	30.62	0.001
Variations between vineyard within sub-region	54.84	0.001
Variations within vineyard	14.54	0.013

Significance calculated by 999 permutations. All *S. uvarum* strains isolated from this study were included in the AMOVA table.

Similar to results of the isolated *S. cerevisiae* strains, the molecular variance of isolated *S. uvarum* genotypes was highest between strains within a sub-region (54.84 %), while there was a higher variation between sub-region (30.62%) as compared to within vineyard (14.54%) (Table 2-6). Also, the population strata were significant at all examined levels, while

the significance of variation within vineyard was lower (p= 0.013) as compared to another two population strata (p= 0.001). Nevertheless, it should be noted that this AMOVA table has low statistical power owing to the small number of vineyards that *S. uvarum* were isolated from.

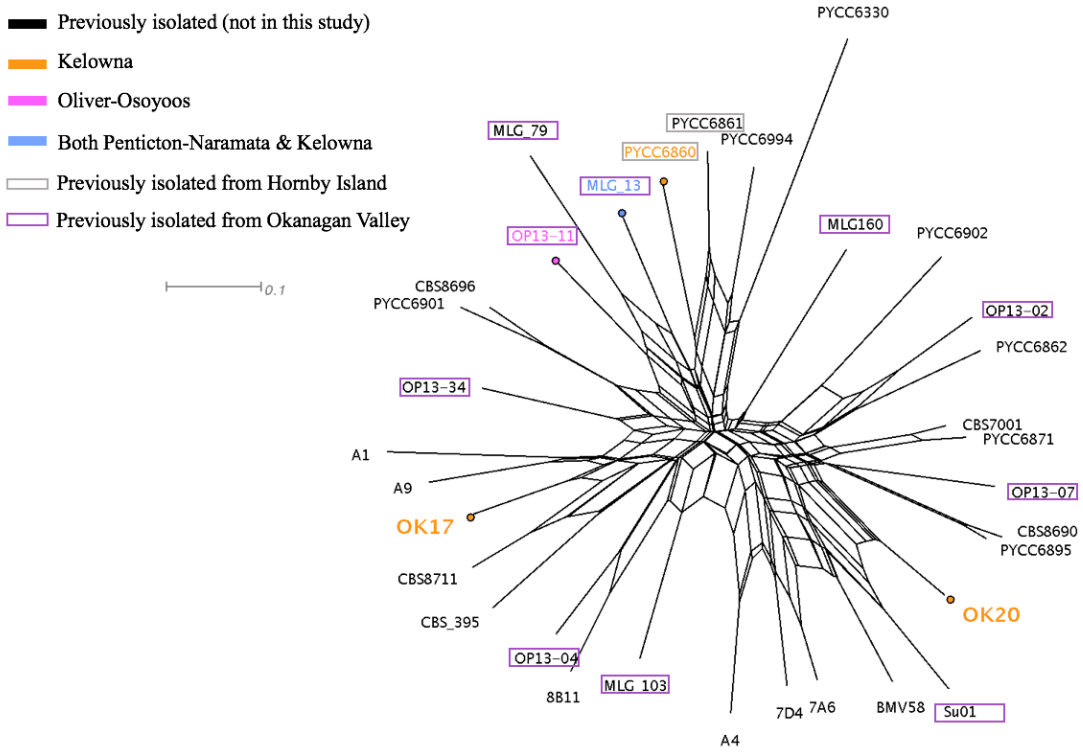


Figure 2-7 Phylogenetic Network composed of the *Saccharomyces uvarum* Strains Isolated from This Study and Previously Isolated Strains.
The phylogenetic relationship was established by the Neighbor-Net algorithm based on Bruvo's distance. Newly discovered strains are labelled as "OK"; the color of the word indicates the location of strain isolation while a black color represents strains that were only identified from the lab reference database (not in this study). The color of the boxes corresponds to the locations of strains previously isolated in British Columbia, Canada (Table 2-5).

Different from the minimum spanning network, this phylogenetic network not only includes the previously isolated *S. uvarum* strains from the lab database, but also shows the previously isolated *S. uvarum* genotypes identified from this study only by the name of the most closely associated previously isolated *S. uvarum* strains from the lab reference database (i.e. Bruvo's distance < 0.25) (Figure 2-7). Therefore, although 3 different *S. uvarum* genotypes were identified from NP in the minimum spanning network (i.e. MLG_13-A, MLG_13-B, MLG_13-C), they were all recognized as strain MLG_13 in this phylogenetic network, which is a strain previously isolated from OV wineries and a strain isolated from KE in this study (Measday and Durall lab, unpublished data). Similarly, the 2 different *S. uvarum* genotypes identified from OO

were both recognized as strain OP13-11 in this phylogenetic network, which was a strain previously isolated from OV wineries (Measday and Durall lab, unpublished data). Also, aside from the 2 newly discovered strains, a *S. uvarum* genotype isolated from KE was identified as strain PYCC6860 in this phylogenetic network (Almeida et al., 2014). Particularly, the 4 strains (i.e. 2 previously isolated, 2 newly discovered) isolated from KE were genetically distantly related. Even though one of the newly discovered *S. uvarum* strains (OK 20) were genetically similar to a strain previously isolated in OV wineries (Su01), Su01 was not identified in this study (Table 2-4).

2.4 Discussion

2.4.1 Spontaneous Fermentation

In this study, only 37% of the spontaneous fermentations performed achieved two-thirds sugar depletion within forty days of fermentation. In part, this may be due to our method of grape sampling whereby thirty clusters were picked from six discrete areas of the vineyard. If a particular region of the vineyards has lower levels of *S. cerevisiae* or higher levels of a competing organism, the spontaneous fermentation will not proceed. Previous studies have demonstrated that there is an unpredictability associated with spontaneous fermentation, including a dynamic vineyard yeast population between vintages and geographical locations (Knight and Goddard, 2015, Martiniuk et al., 2016, Rantsiou et al., 2016, Schuller et al., 2005). In particular, as *S. cerevisiae* was found to comprise only 0.1% of the yeast population on undamaged berries, spontaneous fermentation can become stuck or not even start due to the insignificant presence of *S. cerevisiae* and *S. uvarum* colonies in the grape must (Tofalo *et al.*, 2013). In fact, a vigorous mold growth was visualized in some of the stuck spontaneous fermentation in this study, which likely outcompeted the growth of wine yeast strains.

A general observation is that both *S. cerevisiae* and *S. uvarum* were identified in each of the three OV sub-regions (Table 2-3). In particular, in some vineyards none of the spontaneous fermentations reached two-thirds sugar depletion (vineyard 3,8,12) while all of the spontaneous fermentations from vineyard 10 proceeded (Figure 2-2). Also, the isolation of either *S. cerevisiae*, *S. uvarum*, or both species from each vineyard had no clear pattern. In particular, vineyard 10 was the only vineyard with isolation of an unknown *Saccharomyces spp*. This result

may correspond to the dynamic composition of vineyard-associated wine yeasts found on sampled Pinot Noir grapes in nature, which vary due to the presence of wild biological vectors in a particular vintage or geographical locations (i.e. birds, insects).

Moreover, the number of isolated *S. cerevisiae* colonies (i.e. 1200 colonies) is more than seven-fold higher than the number of isolated *S. uvarum* colonies (i.e. 164 colonies), and *S. cerevisiae* colonies were isolated from more vineyards (9 vineyards) than *S. uvarum* colonies (4 vineyards) in the 2017 vintage. Based on previous studies, *S. uvarum* has poor ethanol tolerance that results in stuck fermentation at 24 ^{o}C (Masneuf-Pomarede et al., 2010). Furthermore, due to its crytolerant traits, including a good wine fermentation capacity at low temperature, *S. uvarum* has typically been identified in white wine fermentations (~15 ^{o}C) (Demuyter et al., 2003; Lo´pez-Malo et al, 2013; Masneuf-Pomarede et al., 2010). Therefore, it was surprising to isolate *S. uvarum* from this study, because the experimental red grape fermentation had an incubation temperature (25 ^{o}C) that should promote low ethanol tolerance of *S. uvarum* (Masneuf-Pomarede et al., 2010). Moreover, although *S. uvarum* was shown to out-compete *S. cerevisiae* in mixed culture fermentations (i.e. fermentation with both *S. uvarum* and *S. cerevisiae* presence) at 12 ^{o}C, our results have demonstrated that *S. uvarum* was isolated as a minority species in fermentations at 25 ^{o}C (Su et al., 2019). As *S. cerevisiae* has a better fermentative competitiveness and ethanol tolerance at 25 ^{o}C, *S. cerevisiae* should be preferentially enriched by our fermentation method as compared to *S. uvarum*.

Even so, it is worth mentioning that in the study of Knight and Goddard, the relative abundance of *S. cerevisiae* and *S. uvarum* were 56% and 40%, respectively, from 3,780 colonies isolated from the spontaneous fermentation at 15 ^{o}C of Sauvignon Blanc grapes in New Zealand (Knight and Goddard, 2015). As the relative abundance of *S. uvarum* is only 12% in our sampled colonies, the result implies that differences in fermentation temperature, variety of fermented grapes as well as the geographical location can influence the population structure of vineyard-associated wine yeast populations.

In addition, the number of spontaneous fermentations that reached two-thirds sugar depletion were unbalanced among the three OV sub-regions, with the greatest occurrence in OO (i.e. 12 fermentation), followed by KE (i.e. 10 fermentation), and the least in NP (i.e. 7 fermentation). The remarkably lower isolation of wine yeasts from NP may be explained by the available weather information – NP has the highest cumulative precipitation among the three OV

sub-regions in the 2017 vintage (Figure B-1 in Appendix B and further discussion in Chapter 3). Therefore, one hypothesis is that the high volume of rainwater washed off wine yeast colonies resident on the berry surfaces, which resulted in a negligible presence of wine yeast colonies at the beginning of spontaneous fermentation. A second hypothesis is because NP has the lowest calculated GDD among the three OV sub-regions and this cool climate may not be desirable for the inhabitation of *S. cerevisiae*, nor the residency of wild biological vectors (birds and insects) that are involved in the dispersal of wine yeasts (Tofalo *et al.*, 2013). In fact, OO with the highest calculated GDD had the greatest percentage (42%) of spontaneous fermentations reaching two-thirds sugar depletion in this study.

2.4.2 *Saccharomyces cerevisiae*

2.4.2.1 Isolation of *S. cerevisiae* Strains

In this study, the quantity of isolated *S. cerevisiae* colonies was positively correlated to the number of total and potentially indigenous strains identified in each sub-region. A higher number of isolated colonies increased the chance of identifying *S. cerevisiae* strains with a distinctive microsatellite profile. In addition, as shown by the node size of the minimum spanning network in Figure 2-4, there is higher variation in the number of sampled colonies for each potentially indigenous *S. cerevisiae* strains isolated from KE as compared to OO. In particular, some potentially indigenous strains were identified from more than one fermentation in KE, which implies that these strains were wide-spread geographically, through biological vectors or human activities, and therefore enriched in multiple spontaneous fermentations. Also, some strains isolated with less than 48 colonies from KE and OO and thus some fermentations were facilitated by more than one type of *S. cerevisiae* strain. This is correlated to a general benefit of spontaneous fermentation, which is the possibility to have various wine yeast strains involved in the fermentation and resulted in wine with complex sensory profile (Pretorius, 2000).

As there is a higher number of colonies classified as potentially indigenous *S. cerevisiae* strains (i.e. 22 strains) than the commercial strains (i.e. 10 strains) in this study based on Bruvo's distance, it is unlikely that the majority of vineyard-associated *S. cerevisiae* strains are directly related to the use of commercial starter cultures in wineries. Instead, these vineyard-associated *S. cerevisiae* strains possibly represent a native yeast population in OV, or commercial strains that

have undergone genetic rearrangement under the influence of local *terroir* conditions (Peter et al., 2018). Among the 32 identified *S. cerevisiae* strains, there were only 2 potentially indigenous *S. cerevisiae* strains and 1 commercial *S. cerevisiae* strain that overlapped between OO and KE and no strains that were identified in all three sub-regions (Figure 2-3). As OO and KE are located at either end of the OV, separated by NP, the overlapped *S. cerevisiae* strains found in these two sub-regions suggest yeast dispersal related to human activity. For instance, the same group of viticulturalists may work at vineyards located within OO and KE. If the viticulturalists are transported by a vehicle directly between these two sub-regions, the vehicle can prevent the viticulturists with wine yeast-contaminated clothing from having direct contact with the NP sub-region. Also, the two potentially indigenous *S. cerevisiae* strains isolated from both KE and OO had a higher sampling abundance for KE as compared to OO, which provides the basis for a very interesting observation that will be discussed in 2.4.2.2. Otherwise, most of the strains were exclusive to only one sub-region, showing regional specificity. Since our results have identified regional specificity of *S. cerevisiae* populations, we have discovered a unique microbial *terroir* in each of the three OV sub-regions. The finding of regional-specific and potentially indigenous strains suggests the possibly of utilizing these strains in spontaneous fermentation for the production of regional aroma and flavor in wine during fermentation (Fugelsang and Edwards, 2007).

Unlike the study of wine yeast population structure conducted in New Zealand, in which over 90% of the isolated *S. cerevisiae* strains were potentially indigenous (i.e. regional-specific), nearly one-third of the isolated *S. cerevisiae* strains (10 out of 32) in this study were identified as commercial strains (Knight and Goddard, 2015). The discrepancy in these two studies can be explained by the differences in sampling types and enrichment methods, particularly because soil samples was also examined in New Zealand. A spontaneous fermentation study of Pinot Noir and Chardonnay, conducted in OV wineries found that commercial *S. cerevisiae* strains were dominant from early to late stages of fermentation (Scholl et al., 2016 and Hall et al., 2011). However, our results show that vineyard-associated spontaneous fermentations contain a higher relative abundance of *S. cerevisiae* strains that are potentially indigenous (Scholl et al., 2016). Thus, even though commercial strains may survive industrial-scale fermentations better than potentially indigenous strains, our data suggests that potentially indigenous strains have the

potential to survive in the vineyards and withstand outdoor influences such as temperature fluctuation.

2.4.2.2 Genetic Relatedness of *S. cerevisiae* Strains

In both the minimum spanning network and the phylogenetic network, there were two clusters of *S. cerevisiae* strains, one dominated by potentially indigenous strains isolated from OO and another dominated by potentially indigenous strains isolated from KE (Figure 2-4 and Figure 2-5). Thus, these clusters indicate that most of the strains from OO and KE have strong genetic relatedness within sub-regions and weak genetic relatedness between sub-regions, demonstrating regional-specificity of the isolated potentially indigenous *S. cerevisiae* strains.

Based on conversations with viticulturists and winemakers, commercial *S. cerevisiae* strains have been used in inoculated wine fermentation for all wineries near the examined vineyards. Consequently, it is very likely that commercial *S. cerevisiae* strains identified in this study have been used in inoculated wine fermentation by the wineries within the isolated sub-regions. Aside from CY3079.A, none of the commercial strains isolated from this study was genetically close to any isolated potentially indigenous *S. cerevisiae* strain.

Based on previous studies, most genome modifications of wild *S. cerevisiae* strains are associated with the accumulation of SNPs (Peter et al., 2018). By contrast, under selective pressure, commercial wine *S. cerevisiae* strains develop through rapid ORF gain or loss, in order to acquire desirable phenotypic characteristics. Even though microsatellite analysis cannot detect ORF gain/loss or the accumulation of SNPs, our data suggests that commercial *S. cerevisiae* strains isolated within a sub-region are less related genetically than the wild *S. cerevisiae* strains identified from the same sub-region. As mentioned in the study of Borneman et al., there is an intimate genetic association between global *S. cerevisiae* wine strains and the *S. cerevisiae* wine strains isolated in Europe, which implies that European wine strains have migrated around the globe and maintained as a separate population through phenotypic selection (Borneman et al., 2011). As *Vitis vinifera* has been transplanted to the OV, the occurrence of potentially indigenous strains identified in this study may be explained by at least two theories associated with the genetic modification of wine strains found on European *Vitis vinifera* plant materials. In the first theory, the same European wine strains may have localized among these sub-regions when *Vitis vinifera* was first brought to OV. To adapt to specific *terroirs* in these sub-regions,

the vineyard-associated strains may evolve by acquiring mutations from environmental pressures, such as the survival in a much cooler climate of the OV as compared to some European vineyards. These vineyard-associated strains may have additional genetic modifications through genome rearrangement, mutation or breeding (Borneman et al., 2016; Peter et al., 2018; Libkind et al., 2011). In the second theory, different European wine strains may have localized among these sub-regions when first brought to the OV. Thus, the significant genetic differences of potentially indigenous strains identified from the OV may have developed in Europe, and further genetic differences within sub-regions may have accumulated through genome rearrangement, mutation or breeding. To identify the most likely theory, further studies characterizing the genomic similarity between potentially indigenous OV strains and European wine strains using whole genome sequencing will be necessary to understand the origin of the *S. cerevisiae* strains identified in the OV.

From the minimum spanning network, there is higher genetic relatedness (thicker connecting lines) between *S. cerevisiae* strains isolated from the KE cluster as compared to *S. cerevisiae* strains isolated from the OO cluster (Figure 2-4). Furthermore, the Lalvin commercial strain CY3079.A was identified within the KE cluster of *S. cerevisiae* strains in the phylogenetic network (Figure 2-5). With a commercial strain identified within the KE cluster of *S. cerevisiae* strains that has high genetic relatedness, this may imply that some of the potentially indigenous *S. cerevisiae* strains isolated from KE resulted from mutations that altered the length of the microsatellite repeats in CY3079.A (Peter et al., 2018). This can happen under the influence of *terroir* in a particular sub-region and result in several strains that have higher genetic relatedness within the cluster. By contrast, there was no commercial *S. cerevisiae* strains identified in the OO cluster. Therefore, most of the potentially indigenous *S. cerevisiae* strains in the OO cluster were connected by relatively thin connecting lines, which shows a weaker genetic relatedness in the minimum spanning network than strains in the KE cluster (Figure 2-4). The OO strains may possibly be vineyard-associated to the particular OV sub-region since the transplantation of *Vitis vinifera*, and have evolved continuously under the selective pressure of *terroir*, resulting in low genetic relatedness between strains within the cluster. Furthermore, for potentially indigenous *S. cerevisiae* strains that are not within any clusters, including the only strain from NP (OK10), they have possible evolved under the influence of *terroir* associated to a particular vineyard that separates them from the regional cluster of strains.

For the two potentially indigenous *S. cerevisiae* strains isolated in both OO and KE, one strain (OK 148) clusters with strains isolated from OO while the other strain (OK 137) is more similar to commercial strains than either the OO or KE strain clusters. These two strains might have been dispersed between the two sub-regions through human activity. Yet, obtaining a larger sample size between KE and OO would be necessary to identify the locality of OK 148 because of its close genetic relatedness with other potentially indigenous *S. cerevisiae* strains in the OO cluster while having a higher isolation frequency from KE in this study. Furthermore, as OK 137 was not included in any regional clusters, this potentially indigenous *S. cerevisiae* strain may have evolved based on the unique *terroir* of a particular vineyard, or it is a genetically different strain that was transplanted with the *Vitis vinifera* to OV decades ago.

One limitation of this study is that the commercial *S. cerevisiae* strains utilized by the wineries associated with each individual vineyard were not well-documented. Therefore, it is not known which commercial *S. cerevisiae* strains used in these wineries may have been transmitted to the vineyards. Also, the origin of *Vitis vinifera* transplanted to each vineyard was not investigated. Thus, it is not possible to identify the closest ancestor of *S. cerevisiae* strains identified in this study.

2.4.2.3 Geographical Distribution of *S. cerevisiae* strain

As shown by the component of variance, more than half of the variation was due to *S. cerevisiae* strains between vineyards within a sub-region (Table 2-4). Therefore, this result may suggest that vineyards within a sub-region have significant environmental and viticultural differences, leading to the discovery of unique potentially indigenous *S. cerevisiae* strains. For instance, each vineyard may be altered by manual viticulture practices, such as various fertilization methods and the usage of netting, which create distinctive microclimates for vineyards within the same sub-region. Also, based on the dynamic composition of vineyard-associated *S. cerevisiae* colonies, it is reasonable to have a higher component of variance for variations within vineyards than variations between sub-region, as the isolated *S. cerevisiae* strains can vary significantly between the six spontaneous fermentations that were performed with randomly sampled Pinot Noir grapes within one vineyard. Furthermore, the variation between sub-regions was decreased and the variation within vineyards was increased with the consideration of commercial *S. cerevisiae* strains in the component of variance analysis. This

result can be rationalized because multiple different commercial *S. cerevisiae* strains were isolated in the spontaneous fermentation performed from the same vineyard, while the same commercial strain (Lalvin RC212) was identified by both OO and KE as mentioned above.

For the significance test, variations at all population strata (between sub-region, between vineyards within sub-region, within vineyards) were significant at p= 0.001. Therefore, the occurrence of different *S. cerevisiae* strains among the three OV sub-regions was not a random event and there is *S. cerevisiae* population structure at all examined levels, suggesting the discovery of regional specific *S. cerevisiae* strains.

2.4.3 *Saccharomyces uvarum*

2.4.3.1 Isolation of *S. uvarum* Strains

Most of *S. uvarum* colonies sampled were isolated from KE (41%) and 4 out of the 9 *S. uvarum* genotypes were recognized from KE in this study (Figure 2-6). With a larger number of isolated colonies in a particular sub-region, it might provide a higher chance of identifying more distinctive *S. uvarum* genotypes.

The uneven node size of the minimum spanning network (Figure 2-6) indicates that each *S. uvarum* genotype was identified in different quantities from the isolated colonies, with one genotype being dominant in each sub-region. As we have discussed previously, because *S. uvarum* has a low ethanol tolerance at the incubation temperature (25 ^{o}C) of the spontaneous fermentation, some *S. uvarum* genotypes might have been isolated in a lower proportion due to a weakened competitiveness at this incubation temperature (25 ^{o}C). Moreover, since different proportions of *S. uvarum* genotypes were identified in this study, the data demonstrates that multiple genotypes and species of wine yeasts can be isolated from the same fermentation, which is a distinctive feature of spontaneous fermentation, demonstrating that grape must contains a dynamic wine yeast population (Fleet, 2008).

Unlike *S. cerevisiae*, none of the identified *S. uvarum* genotypes overlapped between sub-regions, which may indicate that *S. uvarum* genotypes were exclusively associated to a sub-region. However, one possibility is that these *S. uvarum* genotypes have weakened fermentative ability at 25 ^{o}C and thus our conditions were not ideal to isolate *S. uvarum* from *S. cerevisiae*-dominated fermentations. In addition, among the 9 *S. uvarum* genotypes identified in this study,

only 2 genotypes isolated from KE are newly discovered. As demonstrated by the phylogenetic network (Figure 2-7), the remainder of the *S. uvarum* genotypes were previously isolated in the OV wineries, with the exception of PYCC 6860 which was previous isolated from Hornby island, BC (Almeida et al., 2014; Measday and Durall lab, unpublished data). Because there is only one known commercial *S. uvarum* strain (BMV 58), this result reveals that the previously isolated *S. uvarum* strains (except PYCC 6860) are prevalent to the OV. The discovery of PYCC6860 in this study, which was isolated from an oak tree on Hornby island, may reveal the possibility of wine yeast dispersal through biological and environmental vectors (e.g. humans, birds, insects, wind) within the province. Yet, because PYCC6860 was only identified in KE, this may also suggest a better adaption of this strain to the *terroir* of KE (i.e. cooler, wetter climate).

2.4.3.2 Genetic Relatedness of *S. uvarum* Strains

With only 9 identified *S. uvarum* genotypes, it is impossible to identify regional-specific clusters of *S. uvarum* from the minimum spanning network (Figure 2-6). Still, genotypes isolated from OO and NP are genetically more closely associated within sub-regions than genotypes between sub-regions. In particular, for genotypes identified in OO and NP respectively, they were linked by thick connecting lines, showing higher genetic relatedness of genotypes within sub-regions as compared to strains isolated from KE, which are connected with thin connecting lines. It should be noted that, however, in the phylogenetic network which identified *S. uvarum* strains based on Bruvo's distance (< 0.25), the 2 *S. uvarum* genotypes isolated from OO were recognized as one strain (OP13-11). Also, all *S. uvarum* genotypes identified from NP are genetic modifications of MLG_13, which was a strain also identified from KE in this study. Thus, the different *S. uvarum* genotypes present in OO and NP are very likely genetic rearrangement products that may correspond to the *terroir* of the sub-regions. Consequently, it is rational for these genotypes to demonstrate high genetic relatedness in the minimum spanning network within sub-regions (Figure 2-6). Considering the 4 genotypes isolated from KE, they are identified as 4 distinctive strains. In fact, similar to what has shown in the minimum spanning network, these 4 strains are genetically distantly related, as they are located far from one another in the phylogenetic network (Figure 2-7). In addition, a strain isolated from KE has the same genotype as a previously identified strain (PYCC 6860 from Hornby Island, BC). Interestingly,

in the 2017 vintage, both PYCC 6860 and MLG_13 were also identified in white grape spontaneous fermentations conducted at the Mission Hill Family Estate Winery, which is located in West Kelowna (McCarthy, 2019). Therefore, the discovery of these two previously isolated *S. uvarum* strains as vineyard-associated strains in Kelowna suggests that PYCC 6860 and MLG_13 are prevalence in Kelowna, and these two strains may have contaminated the sampled Pinot Noir grapes in Kelowna through human activities or biological vectors (e.g. wind).

Also, as shown by the phylogenetic network, the newly discovered strains from this study, OK17 and OK 20, are not genetically closely associated (Figure 2-7). Instead, OK 20 is genetically more closely related to Su01, which was a previously isolated strain from the OV wineries (Measday and Durall lab, unpublished data). Thus, these strains from KE may have developed under the influence of *terroir* associated to the particular vineyard that separates them genetically within the sub-region. As a limitation of this study, OK 20 may be genetically rearranged versions of the previously isolated strain (Su01), that was not identified in this study owing to the undesirable fermentation temperature and the dynamic wine yeast population of Pinot Noir grapes. OK 17 is possibly a vineyard-specific *S. uvarum* strains since it is not genetically closely related to any previously isolated strains from OV wineries and it was only isolated from one vineyard.

S. uvarum strains have been identified from Hornby Island, BC in nature and from industrial white wine fermentations in the OV wineries. As mentioned in the Almeida et al. study, *S. uvarum* strains associated with European wine fermentation have prevalent and extensive introgressions from *S. eubayanus* whereas strains isolated from the environment, such as PYCC6860 do not (Almeida et al. 2014). Therefore, further studies of *S. uvarum*, that target these *S. eubayanus* domestication fingerprints, may help elucidate whether the strains isolated from this study are genetic modifications of a native strain from Hornby Island, or if they are genetically modified European *S. uvarum* wine strains. It should be noted that, *S. uvarum* genotypes tend to have more genetic relatedness within sub-regions as compared to between sub-regions. Similar to *S. cerevisiae* strains, the slight genetic differences within sub-regions can be accumulated through genome rearrangement, mutation or breeding, which may reflect on the altered length of microsatellite repeats (Peter et al., 2018). Yet, owing to a low sample size, isolation of additional *S. uvarum* strains from the OV will be necessary to identify the regional-specific genomic differences in *S. uvarum*.

2.4.3.3 Geographical Distribution of *S. uvarum* strain

As shown by the component of variance, over 50% of the variation was due to *S. uvarum* strains isolated between vineyards within a sub-region (Table 2-6). Therefore, in agreement with the genetic relatedness results of *S. uvarum* strains isolated from KE, there may be distinctive *terroir* features for vineyards within each sub-region, in addition to the general *terroir* of the sub-region. Also, since no *S. uvarum* genotypes overlapped between sub-regions, it is reasonable to have a higher variation of *S. uvarum* strains between sub-regions than within vineyards, as *S. uvarum* strains were only isolated from one vineyard, and one spontaneous fermentation, for each of the OO and NP sub-regions.

For the significance test, variations at all population strata (i.e. between sub-region, between vineyards within sub-region, within vineyards) were significant (Table 2-5). Therefore, there is *S. uvarum* population structures at all examined levels, suggesting the discovery of regional-specific *S. uvarum* strains. It should be noted that, the variations within vineyard have lower significance ($p=0.013$) as compared to the other population strata ($p=0.001$). Once again, this result is correlated with the fact that *S. uvarum* strains were only identified from one spontaneous fermentation in each of the OO and NP sub-regions. Since *S. uvarum* strains were isolated from a limited number of fermentation replicates and vineyards, the AMOVA table in Table 2-6 has low statistical power.

2.5 Conclusions

In this study, 37% of the lab-scale spontaneous fermentation of Pinot Noir grapes reached two-thirds sugar depletion, which demonstrates the unpredictability and the dynamic nature of vineyard-associated yeast populations, as shown in other studies (Knight and Goddard, 2015, Martiniuk et al., 2016, Rantsiou et al., 2016, Schuller et al., 2005). The low number of isolated *S. cerevisiae* colonies from the NP sub-region suggests that a cool climate with a high level of cumulative precipitation may limit the occurrence of *S. cerevisiae* on wine grapes, as compared to OO and KE where a warmer climate with a lower level of cumulative precipitation was associated with a higher number of *S. cerevisiae* colony isolation.

Based on the results from the microsatellite analysis, there were 10 commercial strains and 22 potentially indigenous *S. cerevisiae* strains identified. Only 3 *S. cerevisiae* strains were identified in more than one sub-region, showing the geographical exclusiveness of *S. cerevisiae* strains identified in this study. Moreover, potentially indigenous *S. cerevisiae* strains with close genetic relatedness within sub-regions may have arisen from a wild *S. cerevisiae* progenitor strain that has evolved into different genotypes with variations in the lengths of some of the microsatellite repeats loci. In our study, geographical clusters of potentially indigenous *S. cerevisiae* strains were identified from OO and KE.

Furthermore, *S. uvarum* was unexpectedly isolated from Pinot Noir spontaneous fermentations conducted in the 2017 vintage. *S. uvarum* has lower fermentative competitiveness as compared to *S. cerevisiae* and low ethanol tolerance at the experimental fermentation temperature (i.e. 25 ^{o}C) (Kennedy and He, 2005; Lo´pez-Malo et al, 2013; Masneuf-Pomarede et al., 2010). Thus, *S. uvarum* is typically identified in white wine fermentations (Demuyter et al., 2003; Lo´pez-Malo et al, 2013; Masneuf-Pomarede et al., 2010). While *S. uvarum* has been identified from red grape spontaneous fermentation in other countries, this is the first time that *S. uvarum* has been identified in a spontaneous fermentation study with red wine grapes in the OV (Raymond Eder et al., 2018). Furthermore, 9 *S. uvarum* genotypes were identified with no overlap between OV sub-regions, showing geographical exclusiveness of the identified genotypes. Also, there were 3 previously isolated *S. uvarum* strains and 2 newly discovered *S. uvarum* strains identified in this study. In fact, aside from 2 of the newly discovered *S. uvarum* strains, 1 strain was identified as a strain previously isolated from Hornby Island, BC and 2 strains were identified as strains previously isolated from the OV wineries.

Overall, the population structure of both *S. cerevisiae* and *S. uvarum* isolated from Pinot Noir vineyards was found to be significant at the regional-specific level. Thus, regional-specific *S. cerevisiae* strains were identified in this study, which implies a significant impact of *terroir* upon genomic rearrangement of wine strains. Nevertheless, the limited number of *S. uvarum strains* isolated from this study prevent the possibility of making conclusions about *S. uvarum* population structure. With the identification of potentially indigenous strains, future investigation is required to elucidate the differences in phenotypic characteristics of these regional-specific strains. Also, regional-specific starter culture for wine fermentation may be developed based on these preliminary studies.

3 Chapter 3- Flavonoid Analysis

3.1 Introduction

Pinot Noir, as one of the most popular red grape varieties, is cultivated in nearly every wine producing regions (excluding the very hot regions) all around the world (Oxford University Press, 2015). Pinot Noir grapes carry a range of flavonoid compounds, which serve important biological functions in grape plants and these compounds are considered as quality determinants for red wine production.

Anthocyanins determine the color of the red wine and they are also the primary contributors of the color of grape skins in attracting frugivores for seed dispersal (Gould and Lister, 2005). Interestingly, in Pinot Noir grapes, anthocyanins are accumulated solely in five different non-acylated form, including the glucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin (Lee and Skinkis, 2013).

Tannins in grape skins and seeds play a key role in protecting the grape plants against herbivory and bacterial infection (Furlan *et al.*, 2011) Tannins confer astringency and bitterness sensorial notes to grapes and wines, and provide texture to red wines (Flamini and Traldi, 2010).

Flavonols influence wine quality by forming non-covalent interactions with anthocyanin molecules. The interaction of flavonols with anthocyanins results in color intensity enhancement of grape skins (Pollastri and Tattini, 2011; Trouillas *et al.*, 2016). Furthermore, flavonols are recognized as photo-protective compounds that limit light-induced oxidative DNA damages in grape plants (Pollastri and Tattini, 2011; Trouillas *et al.*, 2016). There are six flavonol aglycones significantly presented in Pinot Noir grapes: myricetin, quercetin, laricitrin, kaempferol, isorhamnetin and syringetin, which are synthesized and accumulated in the form of 3-O-glycosides in the berry skins (Makris *et al.*, 2006).

Grape varieties can display distinctive phenotypes with different flavonoid profiles accordingly to the region (geographical locations) where they are grown. Thus, the *terroir* – which is a term used to describe environmental effects on the crop phenotype – can affect the flavonoid composition of grapes (Knight et al., 2015). In fact, several European studies on Pinot Noir grapes have shown that there are variations in grape anthocyanin content among geographical regions (i.e. *terroir*) (Artem et al., 2016; Del-Castillo-Alonso et al., 2016). Nevertheless, there were limited studies assessing the association between grape tannins and

flavonols with *terroir* and the results from these studies were not consistent (Artem et al., 2016; Del-Castillo-Alonso et al., 2016; Fernandez-Marin et al., 2013). Furthermore, even though *terroir* can be vintage-specific, there was no known study conducted on the influence of Canadian *terroir* on the flavonoid composition of Pinot Noir.

Consequently, in order to fill in the knowledge gap, the research objective was to denote the differences in tannins, flavonols, and anthocyanins profiles of Pinot Noir grapes among three distinctive winemaking sub-regions of the Okanagan Valley (Kelowna, Naramata-Penticton, and Oliver-Osoyoos) in the 2017 vintage.

3.2 Methods

3.2.1 Experimental Design and Sample Collection for Flavonoid Analysis

Four of the six portions (i.e. four replicates) selected for the microbial analysis were sampled (2.2.1). Forty berries per replicate were randomly picked by hand for total soluble solids (i.e. Brix) measurement. Another forty berries per replicate were randomly harvested with scissors – by cutting off the berry at the pedicel level, in order to avoid any damage that could create oxidation to the berry – for flavonoid analysis. The grape samples were frozen in the field using dry ice and transported to the Wine Research Center (UBC, Vancouver) where they were stored in a -80 ºC freezer until processing.

3.2.2 Processing of Pinot Noir Grapes

For total soluble solids measurement, berry juice was analyzed with the Sper Scientific 300017 digital refractometer after the berries were weighed and squeezed. For flavonoid analysis, the Pinot Noir grapes were kept frozen by liquid nitrogen to prevent polyphenols degradation. Each replicate of Pinot Noir grapes was weighted and pedicels were removed. The berries were carefully dissected by a scalpel to separate the skin and seed materials. The skin materials were weighted while the seeds were both counted and weighted. Then, the skin and seed materials were grinded into a fine powder separately using cleaned mortars and pestles under liquid nitrogen. The fine powder of skins and seeds was stored in a -80 ºC freezer before flavonoid analysis.

3.2.3 Anthocyanin and Flavonol Analyses

Anthocyanins and flavonols were analyzed only from skin since they are presented only in trace amounts in other berry tissues.

3.2.3.1 Anthocyanin and Flavonol Extraction

An aliquot of 0.18 g of skin powder was mixed with the extraction solvent (50% methanol (v/v), 1% formic acid (v/v) in MilliQ ddwater) in a 1:10 ratio; the mixture was gently shaken and sonicated for twenty minutes under room temperature. Then, the mixture was centrifuged at 14000 g for ten minutes; 1 ml of the supernatant was filtered by a 3-ml Luer-Lok Tip syringe coupled with a 0.22 um X 13 mm PVDF filter and collected into an amber vial. The remaining pellet was extracted again by the 1.8 ml of extraction solvent, while the supernatant (1 ml) was filtered and added to an amber vial. The two extracts were mixed and diluted ten-fold with the extraction solvent. The diluted extracts were stored at -20 ºC until analysis.

3.2.3.2 Anthocyanin and Flavonol Measurement

Each diluted extract (5 µl) was injected into an Agilent 1100 Series LC/MSD Trap XCT Plus System equipped with an Agilent ZORBAX SB-C18 Column (1.8 µm, 4.6 X 50 mm), which consisted of an electrospray ionization mass analyzer and a spectrophotometric diode-array detector. The mobile phases were composed of a solvent A (water with 2% formic acid) and a solvent B (acetonitrile with 2% formic acid). The binary solvent gradient for the LC chromatographic separation was achieved as followed: 0 min, 5% solvent B; 6 mins, 20% solvent B; 9 mins, 80% solvent B; 10 mins, 90% solvent B; 11 mins, 5% solvent B. The analysis was run at a flow rate of 1.20 mL/min in a constant temperature of 67 ºC. The mass spectrum peaks of anthocyanins and flavonols were recognized accordingly to the elution order reported in previously published manuscripts and confirmed by comparing the spectrum of each compound to the ones reported for grapes anthocyanins and flavonols (Del-Castillo-Alonso et al., 2016; Flamini and Traldi, 2010). The peaks of anthocyanins were examined by the spectrophotometric diode-array detector (520 nm) while the peaks of flavonols were examined by the electrospray

ionization mass analyzer, since co-elution occurred and an accurate quantification of peaks at 353 nm was impossible.

3.2.3.3 Anthocyanin and Flavonol Standard Curves

An anthocyanin standard curve was prepared with Malvidin-3-O-glucoside (M-3-G) in the range of 0 to 25 µg/ml through diluting a stock solution of M-3-G in the extraction solvent. Similarly, a flavonol standard curve was prepared with quercetin-3-O-glucoside (Qu-3-Glu) in the range of 0 to 10 µg/ml via diluting a stock solution of Qu-3-Glu in the extraction solvent. Every dilution was made in triplicates to account for manual error. The anthocyanin and flavonol concentrations of skin samples were calculated from the M-3-G and Qu-3-Glu standard curves, respectively, and reported in M-3-G and Qu-3-Glu equivalent, respectively.

3.2.4 Tannin Analysis

This analysis is based on the work of Harbertson *et al.* (2002) and changes were made to complement with our experiments. This protocol works the same for seed and skin samples unless otherwise specified. Analysis was performed in technical duplicates to account for manual error.

3.2.4.1 Tannin Extraction

The powdery sample of 0.18 g was mixed with 70% acetone in a 1:10 ratio; the mixture was shaken at 150 rpm for twenty-four hours at room temperature. Then, the mixture was centrifuged at 14000 g for ten minutes; 1 ml of the supernatant was collected and weighted. The supernatant was condensed by the rotary evaporator for one hour. Afterwards, the ddH_2O was added to the remaining supernatant to make up the volume loss. The extracts were diluted in a 1:4 ratio by a dilution buffer that consisted in: 12% ethanol (v/v), 5 g/L potassium tartrate, and adjusted to pH 3.3 with HCl.

3.2.4.2 Tannin Measurement

An aliquot of diluted extracts (500 µl) was mixed with 1 ml of protein buffer consisting in 1 mg/ml Bovine Serum Albumin in washing buffer (200 mM acetic acid, 170 mM NaCl and adjusted to pH 4.9 with NaOH) and shaken at 150 rpm for one hour at room temperature. Then, the mixture was centrifuged at 14000 g for ten minutes and the supernatant was discarded. An aliquot of 250 µl of washing buffer (200 mM acetic acid, 170 mM NaCl, and adjusted to pH 4.9 with NaOH) was added to the remaining precipitate; the mixture was vigorously vortexed, followed by centrifugation at 14000 g for five minutes. After discarding the supernatant, the previous step was repeated once to ensure the BSA protein content in the precipitate was negligible. The remaining precipitate was gently agitated with 1.75 ml of dissolving buffer (5% TEA (v/v) and 5% SDS (w/v)) for fifteen minutes to completely dissolve the tannins.

For seed samples, the dissolved tannin solution (875 µl) was immediately mixed with 125 µl of ferric chloride reagent (10 nM FeCl3 in 0.01 M HCl) and left at room temperature for ten minutes before measuring the absorbance by the Shimadzu UV-160 spectrophotometer at 510 nm.

For skin samples, the dissolved tannin solution was left at room temperature for ten minutes before measuring the background absorbance by the Shimadzu UV-160 spectrophotometer at 510 nm. Then, the dissolved tannin solution (875 µl) was mixed with the ferric chloride reagent (125 µl) and left at room temperature for ten minutes before measuring the total absorbance by the Shimadzu UV-160 spectrophotometer at 510 nm. The tannin absorbance was calculated as= [total absorbance – (background absorbance*0.875)].

3.2.4.3 Tannin Standard Curve

A standard curve was made with (+)-catechin in the range of 0 to 300 µg/ml. A catechin stock solution (1 mg/ml) in 10% ethanol was diluted with dissolving buffer to a final volume of 875 µl. Then, the diluted catechin mixture (875 µl) was combined to the ferric chloride reagent (125 µl) and left at room temperature for ten minutes before measuring the absorbance by the Shimadzu UV-160 spectrophotometer at 510 nm. Every dilution was made in triplicates to account for manual error. The tannin concentrations of skin and seed samples were calculated from the (+)-catechin standard curve. The tannin concentration was reported in (+)-catechin equivalents.

3.2.4.4 Statistics on Flavonoid Data

The flavonoid data were presented as mean ± standard error and analyzed by a one-way ANOVA coupled with a Tukey's test. The goal of the statistics was to compare the berry flavonoid content and compositions among the three sub-regions considered ($p < 0.05$). Also, to identify the factors that have higher impact on the berry flavonoid composition in each sub-region, a principal component analysis (PCA) was conducted on the tannin, anthocyanin, and flavonol data. Statistical analyses were performed using JMP 14 (Statistical Discovery™ from SAS).

3.3 Results

3.3.1 Weather Information

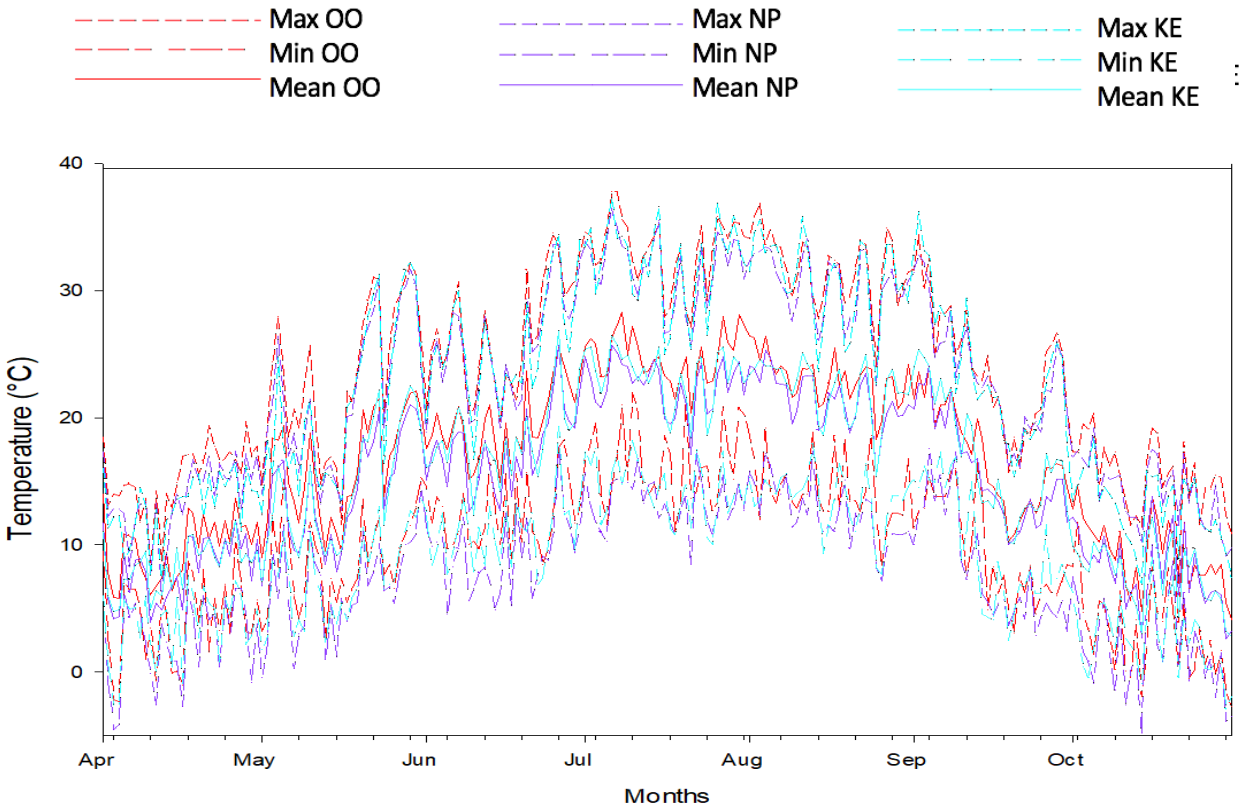


Figure 3-1 Daily Maximum, Minimum and Mean Temperature of the Three OV Sub-regions from April 1st to October 31st, 2017.
The color of the line indicates the associated sub-region while the pattern of the line presents the different type of temperature information from each sub-region
OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; Max= Maximum; Min= Minimum.

The weather information of the growing season (from April 1st to October 31st, 2017), including the maximum (max), minimum (min) and mean daily air temperatures, as well as the mean daily precipitation (mm), was measured by a nearby weather station in each of the three sampled OV sub-regions.

The presented OO, NP and KE weather information was provided by Environmental Canada through their weather stations in Oliver, Penticton, and the UBC Okanagan (Kelowna), respectively (Environmental Canada, 2019a). Among these three sub-regions, active heat summation (i.e. GDD) calculated as the sum of the daily average temperature minus 10 ºC from

1st April to 31 October 2017 was highest in OO, with 1513 DD, followed by KE, with 1263 DD, and lowest in NP, with 1157 DD. In addition, cumulative precipitation was highest in NP (198 mm), followed by OO (140 mm), and lowest in KE (121 mm). Furthermore, the number of days with max daily temperature reaching 35 °C was greatest in OO with 12 days, followed by 7 days in KE and 2 days in NP, which correlated with the GDD results. Even so, none of the sub-region had mean daily temperature reaching 35 °C.

As shown in Figure 3-1, these three OV sub-regions had comparable max, min, and mean daily temperature throughout the growing season, except the min and mean daily temperatures of OO that were notably higher in July and August, which again determined the considerably higher GDD of OO.

3.3.2 General Berry Parameters

Table 3-1 General Berry Parameters of Pinot Noir Grapes among Three OV Sub-regions

	OO	NP	KE
Berry weight (g)	1.24±0.03	1.36±0.04	1.31±0.06
Skin Weight/Berry (μg)	108.27±3.74	113.91±3.91	108.43±3.46
Skin/Berry (%)	8.62±0.15	8.56±0.38	8.38±0.31
Seed Weight/Berry (μg)	36.61±0.66	37.03±0.35	38.36±1.68
Seed/Berry (%)	6.41±0.17	7.06±0.18	6.69±0.25
Seed number	2.17±0.05^{B}	2.58±0.08^{A}	2.31±0.11^{AB}
Total Soluble Solids (°Brix)	22.19±0.36	21.82±0.33	22.54±0.29

The values are averages among all the vineyards of each sub-region. Abbreviations indicated: OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna

The berry, skin, and seed weight, the skin and seed to berry ratio (expressed as percentage), and the total soluble solids of Pinot Noir grape samples did not vary among the three OV sub-regions (Table 3-1). In particular, the average total soluble solids of Pinot Noir grapes in these sub-regions ranged from 21.82±0.33 °Brix to 22.54±0.29 °Brix. Also, there is higher seed number in grapes collected from NP as compared to the ones from OO.

3.3.3 Anthocyanin Content

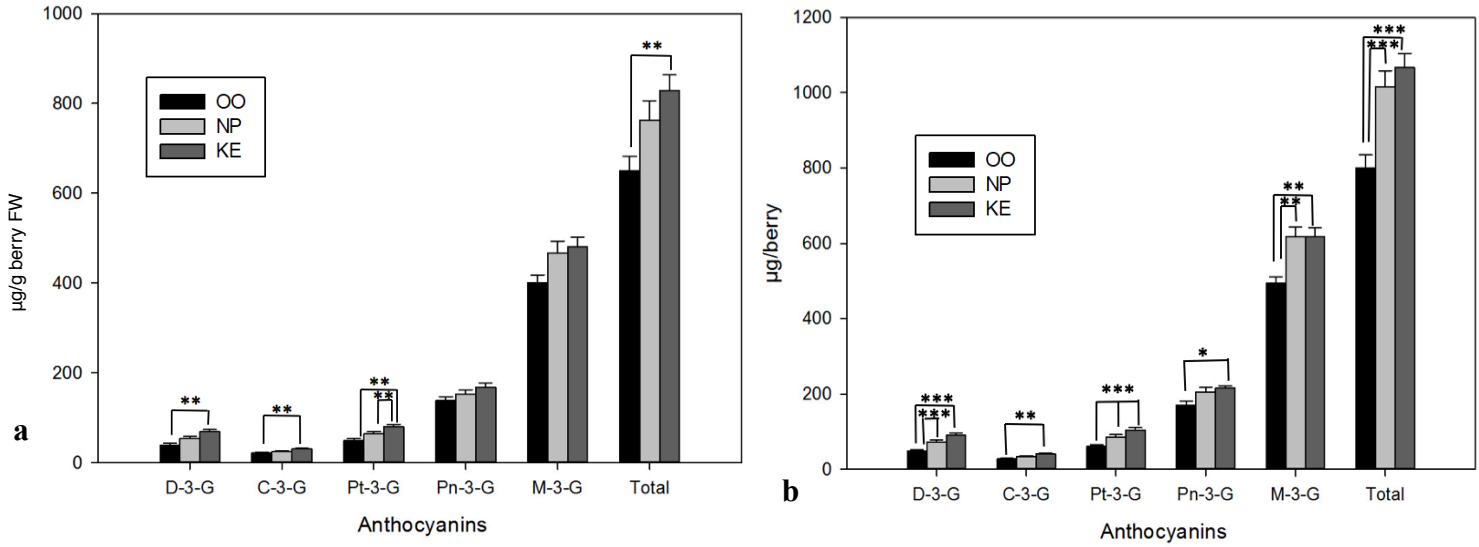
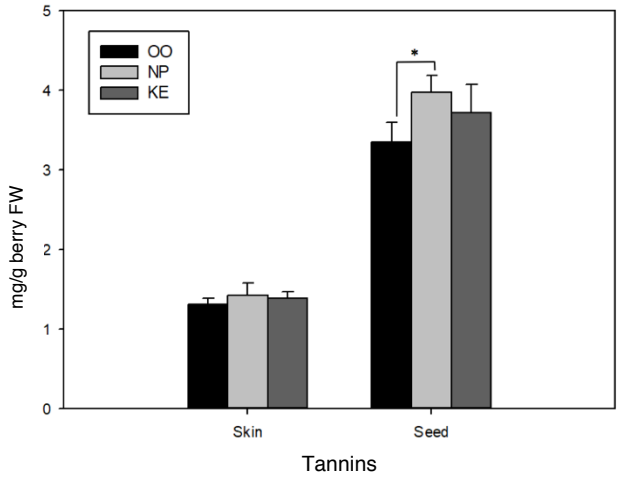


Figure 3-2 Anthocyanin Content of Pinot Noir Grapes expressed as (a) µg/g berry FW and (b) µg/berry among Three OV Sub-regions.
Abbreviations indicated: ***= p<0.0001; **= p<0.01; *=p<0.05; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; D-3-G= Delphinidin-3-glucoside; C-3-G= Cyanidin-3-glucoside; Pt-3-G= Petunidin-3-glucoside; Pn-3-G= Peonidin-3-glucoside; M-3-G= Malvidin-3-glucoside.
Vertical bars indicate standard error of bioreplicates within each sub-region.

In this study, about 40 berries were sampled for each replicate, each vineyard has 4 replicates and 4 vineyards were sampled for each sub-region (except NP which has 5 sampled vineyards). In total, we have examined 2,081 Pinot Noir berries and this corresponds to 16 replicates from OO, 20 replicates from NP and 16 replicates from KE. Expressed in both µg/g and µg/berry, malvidin-3-glucoside (M-3-G) was the most abundant anthocyanin while cyanidin-3-glucoside (C-3-G) was the least abundant anthocyanin identified in this study (Figure 3-2). When anthocyanins were expressed as µg/g berry FW (i.e. concentration in the berry), the delphinidin-3-glucoside (D-3-G), cyanidin-3-glucoside (C-3-G), petunidin-3-glucoside (Pt-3-G), and total anthocyanin content were higher in Pinot Noir grapes from KE as compared to the ones from OO, with a p-value less than 0.01 (Figure 3-2a). When anthocyanins were expressed as µg/berry (i.e. total amount accumulated in a berry), all classes of anthocyanin were greater in samples from KE than the ones from OO (Figure 3-2b). In addition, in µg/berry, D-3-G, Pt-3-G, and M-3-G were higher in berries collected from NP than berries collected from OO. These three anthocyanins are all tri-substituted anthocyanins.

3.3.4 Tannin Content

a



b

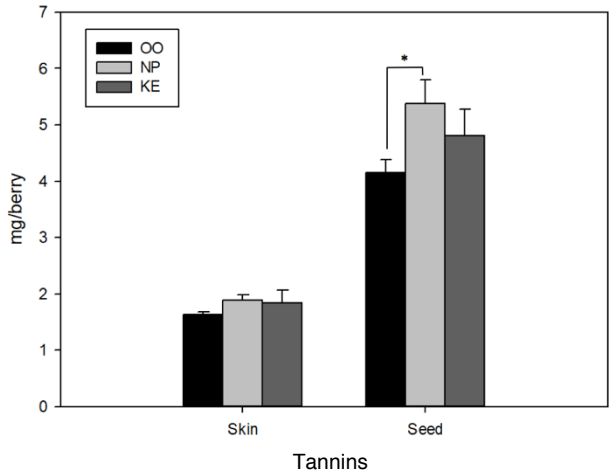
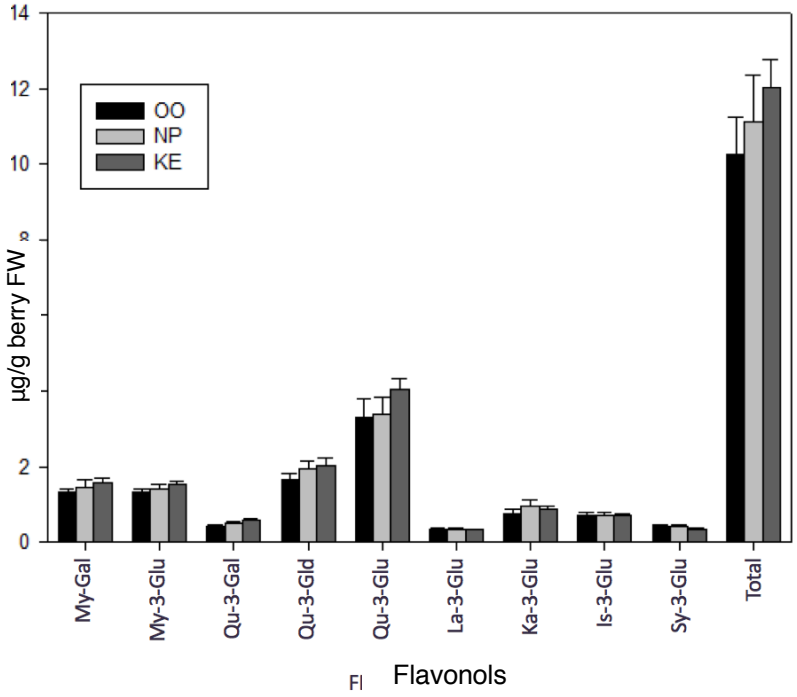


Figure 3-3 Skin and Seed Tannin Content of Pinot Noir Grapes Expressed as (a) mg/g berry FW and (b) mg/berry among Three OV Sub-regions.
Abbreviations indicate: *=p<0.05; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna. Vertical bars indicate standard error of the bioreplicates within each sub-region.

The tannin content of Pinot Noir grape skin (mg/g berry FW and mg/berry) did not vary among the three OV sub-regions, ranged from 1.63±0.05 mg/berry to 1.88±0.1 mg/berry (Figure 3-3a). By contrast, Pinot Noir grape seeds from NP had significantly higher tannin content (5.37±0.42 mg/berry) as compared to the ones from OO (4.15±0.22 mg/berry) (Figure 3-3b).

3.3.5 Flavonol Content

a



b

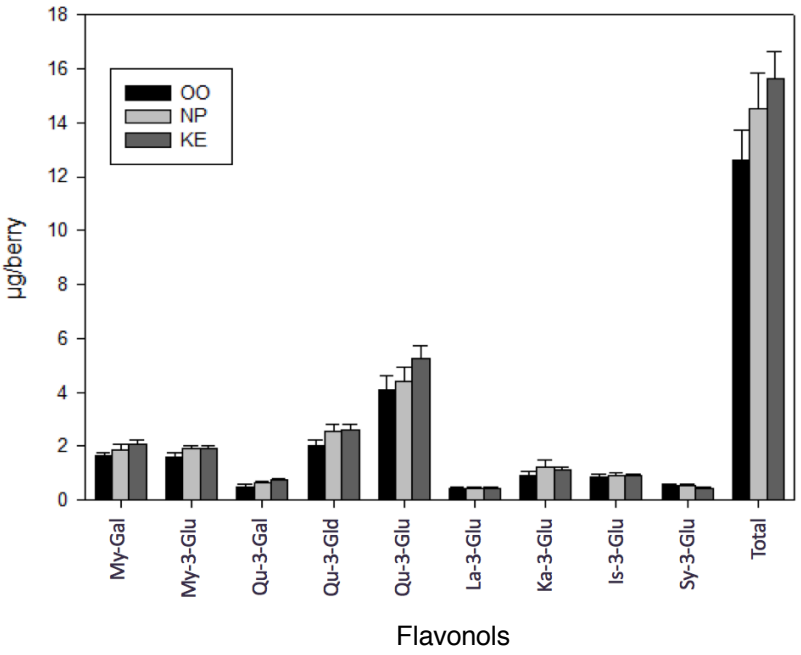


Figure 3-4 Flavonol Content of Pinot Noir Grapes expressed as (a) µg/g berry FW and (b) µg/berry among Three OV Sub-regions.
Abbreviations indicated: OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; My-3-Gal= Myricetin-3-galactoside; My-3-Glu= Myricetin-3-glucoside; Qu-3-Gal= Quercetin-3-

galactoside; Qu-3-Gld= Quercetin-3-glucuronide; Qu-3-Glu= Quercetin-3-glucoside; La-3-Glu= Laricitrin-3-glucoside; Ka-3-Glu= Kaempferol-3-glucoside; Is-3-Glu= Isorahamnetin-3-glucoside; Sy-3-Glu= Syringetin-3-glucoside. Vertical bars indicate standard error of bioreplicates within each sub-region.

The flavonol content of Pinot Noir grape skins, either calculated in µg/g berry FW or µg/berry, did not vary among the three OV sub-regions (Figure 3-4). Quercetin-3-glucoside (Q-3-Glu) was the most abundant flavonol compound in the examined Pinot Noir berries while laricitrin-3-glucoside (La-3-Glu) and syringetin-3-glucose (Sy-3-Glu) were the least abundant flavonols identified in this study.

3.3.6 Composition (Relative Abundances) of Anthocyanins and Flavonols

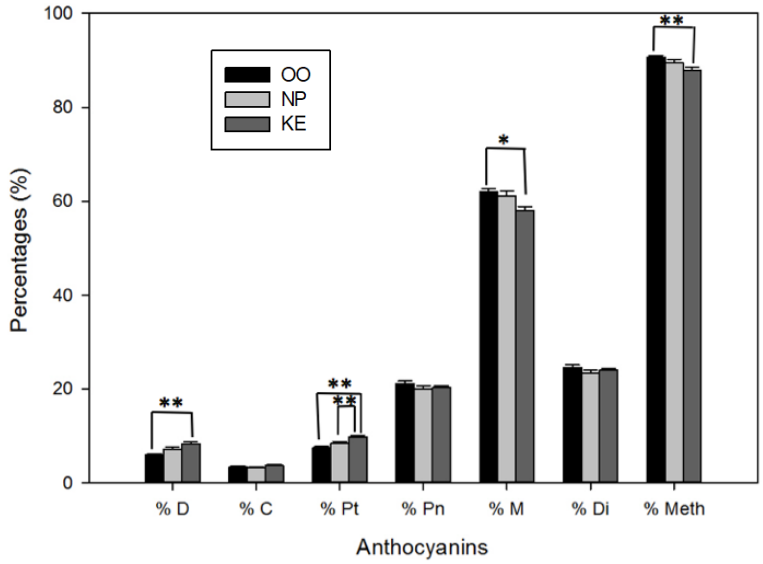


Figure 3-5 Relative Abundance of Individual Anthocyanins and Anthocyanin Fractions based on Level of Substitution and Methoxylation in Pinot Noir Grape Skins among Three OV Sub-regions.
Abbreviations indicated: **= $p<0.01$; *=$p<0.05$; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; C= Cyanidin; Pn= Peonidin; D= Delphinidin; Pt= Petunidin; M= Malvidin; Di= Di-substituted; Meth= Methoxylated.
Vertical bars indicate standard error of the bioreplicates within each sub-region.

The malvidin-3-glucoside (M-3-G) was the most dominant anthocyanin, accounting for over 55% of the total anthocyanin content, and contributing partially to the high relative abundance of methylated anthocyanin in Pinot Noir grapes (Figure 3-5). This was observed across the three sub-regions. In particular, the relative abundance of methoxylated anthocyanin in

Pinot Noir grapes from OO, NP and KE were 90.65±0.39%, 89.55±0.65% and 87.95±0.56%, respectively. Also, while the relative abundance of M-3-G and methoxylated anthocyanin were significantly lower in KE than from OO grapes, the relative abundances of Pt-3-G and D-3-G in KE grapes were significantly higher than in OO grapes. Remarkably, the individual anthocyanins that changed in the relative abundance among the three OV sub-regions (M-3-G, Pt-3-G and D-3-G) were all tri-substituted anthocyanins in Pinot Noir grapes. Even so, the relative abundance of total di-substituted anthocyanins, and therefore total tri-substituted anthocyanins, did not vary among the three OV sub-regions.

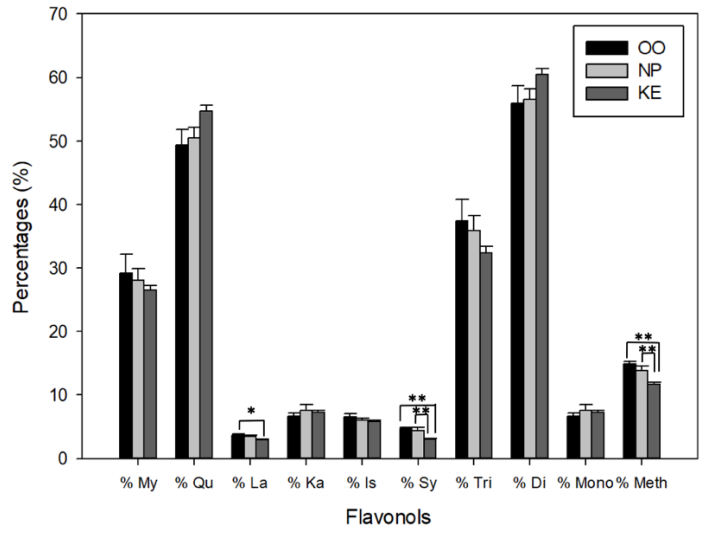


Figure 3-6 Relative Abundance of Individual Flavonols and Flavonol Fractions based on Level of Substitution and Methoxylation in Pinot Noir Grape Skins among Three OV Sub-regions. Abbreviations indicate: **= p<0.01; *=p<0.05; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; My= Myricetin; Qu= Quercetin; La= Laricitrin; Ka= Kaempferol; Is= Isorhamnetin; Sy= Syringetin; Tri= Tri-substituted; Di= Di-substituted; Mono= Mono-substituted; Meth= Methoxylated. Vertical bars indicate standard error of the bioreplicates within each sub-region.

Among flavonols, quercetin (Qu) was the most dominant aglycone, accounted for around 50% of the total flavonol content, and was the major contributor to the high relative abundance of di-substituted flavonols in Pinot Noir grapes. The relative abundance of tri-, di-, and mono-substituted flavonols did not vary among the three OV sub-regions (Figure 3-6).

In comparison, the relative abundance of laricitrin (La) and syringetin (Sy) were found to be higher in Pinot Noir grapes from OO as compared to the ones from KE (Figure 3-6). In addition, the relative abundance of Sy in Pinot Noir grapes from NP was higher than the ones

from KE. Interestingly, both of these two flavonol aglycones are methoxylated and tri-substituted. Consequently, the total methoxylated aglycones (i.e. La, Sy, and Is) followed the same trend, with a higher relative abundance in grapes from OO (14.86±0.46%) and from NP (13.86±0.71%) than in grapes from KE (11.66±0.34%).

There were several commonalities in the relative abundance of anthocyanins and flavonols from Pinot Noir grapes among the three OV sub-regions. For instance, the individual anthocyanin and flavonol compounds that have shown significant differences among Pinot Noir grapes from the three OV sub-regions were all tri-substituted molecules. Furthermore, the relative abundance of both total methoxylated anthocyanins and flavonols was higher in grapes from OO as compared to KE.

3.3.7 Principal Component Analysis of Flavonoid Compounds in Pinot Noir Grapes

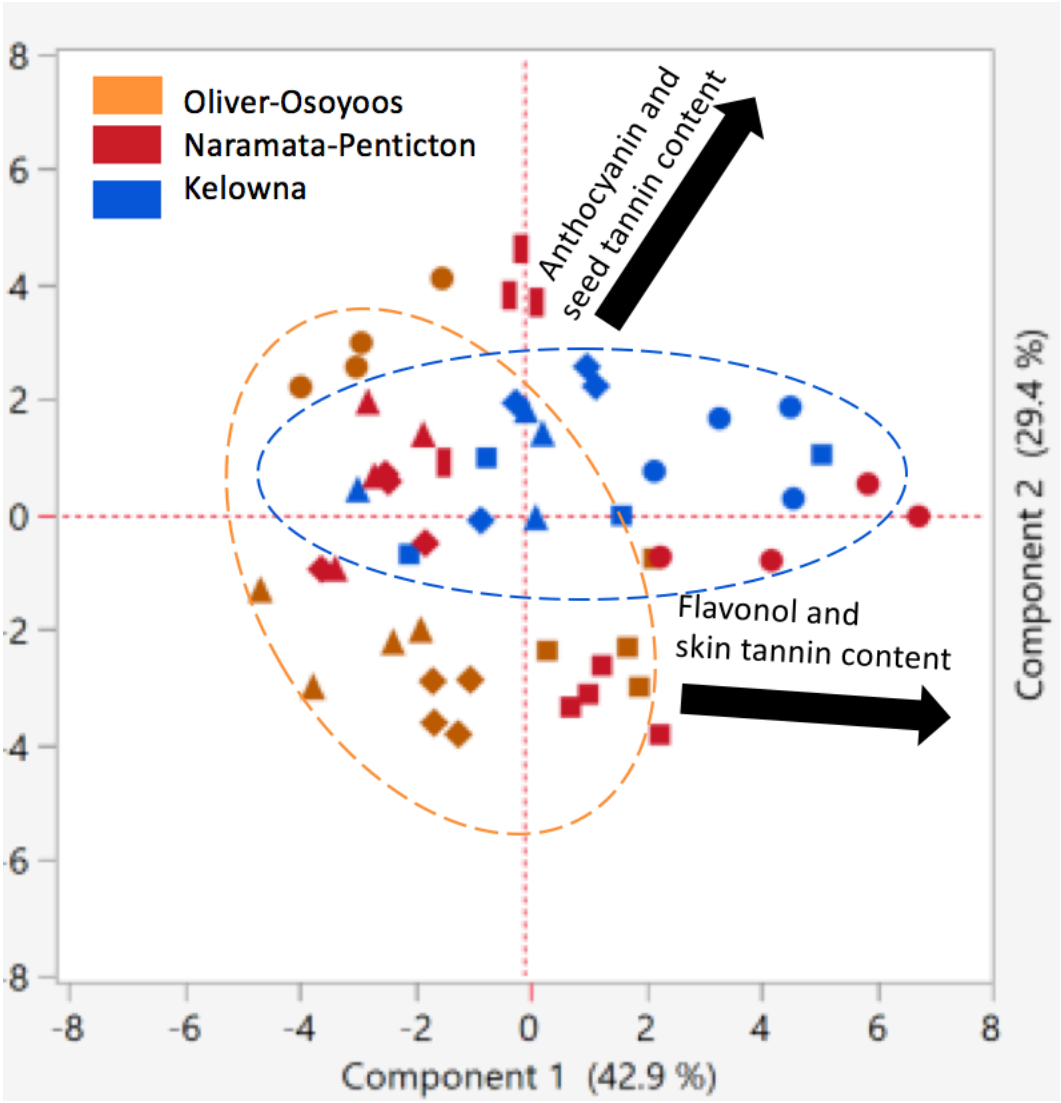


Figure 3-7 Score Plot of PCA grouping Pinot Noir Grape Samples based on the Anthocyanin, Tannin and Flavonol Content.
The label color represents sub-regions and the label shape identifies bioreplicates from the same vineyard.

According to Figure 3-7, PC1 and PC2 were accounted for 42.9% and 29.4% of the variation respectively, which was 72.3% in total. Interestingly, PC1 was dominated by factors that were not differed among sub-regions (i.e. flavonol and skin tannin content) while PC2 was mostly governed by factors that were varied among sub-regions (i.e. anthocyanins and seed tannin content).

Most of the replicates within each vineyard were grouped together on the PCA plot (Figure 3-7). Even though some samples did overlap in the PCA plot, two clusters can be

roughly identified for Pinot Noir grapes collected from OO (circled in yellow) and from KE (circled in blue), respectively. In particular, most of the variations between replicates collected from OO is attributed to PC2, while most of the variations between replicates collected from KE is attributed to PC1. By contrast, replicates collected from NP were segregated by both PC1 and PC2 in an ambiguous fashion, with no recognizable cluster.

3.4 Discussion

3.4.1 Weather Information

The calculated GDDs of OO (1513 DD) and KE (1263 DD) from the 2017 vintage have similar values compared to the average (from the past three decades) GDDs for OO (1520 DD) and KE (1200 DD), indicated in the British Columbia Wine Institute website (British Columbia Wine Institute, 2018; Environmental Canada, 2019a). Nevertheless, there is a high discrepancy between the average (from the past three decades) GDD of NP (1319 DD) and the calculated GDD of NP from the 2017 vintage (1157 DD) (British Columbia Wine Institute, 2018; Environmental Canada, 2019a). In addition, according to the line graph of Figure 3-1, the mean daily temperatures of KE and NP are similar throughout the 2017 growing season while the mean daily temperatures of OO were distinctively higher during July and August than the other two OV sub-regions. Therefore, in general, the calculated GDDs were positively correlated to the mean daily temperatures measured from the three OV sub-regions in the 2017 growing season.

Furthermore, based on previous research, 35 ^{o}C is known to inhibit the biosynthesis and initiate the degradation of anthocyanins (Artem et al., 2016). In this study, none of the recorded mean daily temperature has reached 35 ^{o}C in the 2017 vintage; however, maximum temperatures reached and exceeded 35 ^{o}C (Environmental Canada, 2019a).

The discrepancy between the average (from the past three decades) GDD value of NP with the calculated GDD value of NP from the 2017 vintage can be explained by the level of cumulative precipitation in the 2017 growing season. The southern region of the Okanagan (i.e. OO) has a dessert-like climate (drier, warmer) compared to the hemi-boreal climate (more humid and cooler) in the northern region of the Okanagan (i.e. KE) (Experience Wine Tours, 2019; Climatemps, 2014). According to Environmental Canada, from 1981 to 2010, the average annual rainfall (i.e. precipitation) in KE, NP and OO during the growing season (i.e. April to October)

are 243.5 mm, 218 mm and 189.2 mm respectively (Environmental Canada, 2019b). In other words, OO is expected to receive the lowest cumulative precipitation and KE is expected to obtain the highest cumulative precipitation among the three examined sub-regions. Yet, interestingly, the cumulative precipitation of OO, NP and KE in the 2017 growing season were 140 mm, 198 mm and 121 mm respectively (Environmental Canada, 2019a). As water has a high specific heat capacity, precipitation is an effective tool for removing the latent heat of evaporation, which in turn reduces the air temperature of a certain geographical region (Warf, 2010). Despite the fact that the recorded cumulative precipitations from the 2017 vintage were lower than the average from the past three decades, KE and OO have comparable GDDs in the 2017 growing season as compared to the average (from the past three decades) GDDs. Therefore, the distinctively high cumulative precipitation of NP among the three sub-regions may be directly associated with the lower calculated GDD value of NP in the 2017 vintage (1157 DD) than the average (from the past three decades) GDD (1319 DD), explaining the abnormality for this particular vintage.

Aside from temperature and precipitation, other important environmental factors influencing the *terroir* of a geographical region may have played a key role, including sunlight intensity and soil structure. Still, the relevance of these factors can be affected by specific viticulture practices adopted in a vineyard (e.g. canopy training, netting and fertilization), and we had less documented information than for temperature and precipitation about those practices.

3.4.2 General Berry Information

Since no differences were identified among the three OV sub-regions for the total soluble solids and berry weights of Pinot Noir grapes, we assume that the grape samples were generally collected at similar developmental stages and that the ripeness of the sampled grapes was similar among sub-regions. Therefore, *terroir* is assumed to be a major factor contributing to the variation in flavonoid composition and content of the grapes. Also, there was a higher seed number in grapes collected form NP as compared to the ones from OO. Although previous studies have shown that grape seed number is positively correlated to the berry weight, we observed no variation of berry weight among the three sub-regions in this study (Ollat et al., 2002; Prudent et al., 2014). Also, our results indicate that there was no difference in the seed

weight per berry, implying that that each grape seed collected from NP has a smaller weight as compared to the ones from OO.

3.4.3 Anthocyanin Content

In this study, five non-acylated anthocyanins were identified in Pinot Noir grapes collected from all three OV sub-regions, which were glucosides of delphinidin (D-3-G), cyanidin (C-3-G), petunidin (Pt-3-G), peonidin (Pn-3-G), and malvidin (M-3-G). As shown in Figure 3-2b, all classes of individual anthocyanins and the total anthocyanin content were higher in Pinot Noir grapes sampled from KE as compared to the ones from OO when the amount was expressed as μg/berry. Also, when the anthocyanin concentration was calculated in μg/g berry FW, all the anthocyanins except M-3-G and Pn-3-G changed in concentration among different sub-regions. Thus, larger differences in the anthocyanin content (μg/berry) than concentration (μg/g berry FW, that takes into consideration of the berry size effect) were identified. In other words, changes in anthocyanin accumulation were very likely associated to a stimulation of biosynthesis or degradation in specific regions rather than to variation in berry size among regions that can lead to increase in concentration of the metabolites accumulated in the skins (Roby et al., 2005). The stimulation of biosynthesis or degradation may be attributed to the changes in *terroir*s among the three OV sub-regions (discussed in later section).

In addition, individual anthocyanin compounds in Pinot Noir grapes have different degree of responses towards change of *terroir*s in OV. As mentioned above, the content of D-3-G, Pt-3-G, and M-3-G (μg/berry) were higher in Pinot Noir grapes collected from NP and from KE than the ones from OO. These compounds were all tri-substituted anthocyanin molecules. Thus, tri-substituted anthocyanin molecules have shown higher sensitivity to changes in *terroir*, as compared to the di-substituted anthocyanins (C-3-G and Pn-3-G).

In general, both individual anthocyanin and total anthocyanin content were highest in Pinot Noir grapes collected from KE, followed by NP, and were lowest in OO. Temperature is known to have a significant effect on the anthocyanin accumulation in grape berries (Spayd et al., 2002; Yamane et al., 2006; Nicholas et al., 2011; Mori et al., 2007). Particularly, anthocyanins accumulation and biosynthesis are promoted by moderate growing temperatures at 15 – 25 ^{o}C (Artem et al., 2016). At over 35 ^{o}C, anthocyanin accumulation is reduced by both anthocyanin degradation and inhibition of anthocyanin biosynthetic gene expression (Mori et al.,

2007). In the study of Mori et al., Cabernet Sauvignon grapes that grew at 35 ºC have less than half of the anthocyanin accumulation as compared to samples grew at 25 ºC (Mori et al., 2007). Similarly, in the study of Yamane et al., anthocyanins content was higher in potted grapevines exposed to 20 ºC as compared to 30 ºC (Yamane et al., 2006). In comparison, our result has correlated with these previous studies. Even though none of the average daily temperature achieved 35 ºC among the three OV sub-regions in the 2017 vintage, the number of days with maximum daily temperature reaching 35 ºC was positively correlated with the GDD in each sub-region. As the highest GDD (1513 DD) was recorded in the OO region, both individual anthocyanin compounds and the total anthocyanin content (μg/berry) were significantly lower in Pinot Noir grapes collected from OO as compared to grapes collected from KE, that has a remarkably lower GDD (1263 DD). In addition, tri-substituted anthocyanin compounds and total anthocyanin content (μg/berry) were significantly higher in Pinot Noir grapes collected from NP, with the lowest GDD (1157 DD) among the three sub-regions, as compared to grapes collected from OO. As stated above, OO had a warmer climate with more days reaching a maximum daily temperature at 35 ºC, a significantly lower total anthocyanin content from grapes collected from OO as compared to grapes from NP and KE may be associated with a long duration of anthocyanin degradation and inhibition of anthocyanin biosynthesis.

Even though KE has a recorded higher GDD as compared to NP, total anthocyanin content of Pinot Noir grapes collected from NP were generally, but not significantly, lower than samples from KE. In particular, a study of Castellarin et al. illustrated that water deficit either before veraison or after veraison has beneficial impact on anthocyanin accumulation by increasing the expression of genes responsible for anthocyanin biosynthesis (Castellarin et al., 2007). A higher cumulative precipitation may result in water availability for the plants in NP. Therefore, with a slight discrepancy of GDD between these two locations, a lower anthocyanin accumulation of grapes collected from NP than the ones from KE may have resulted from the high cumulative precipitation of NP (Figure B-1 in Appendix B).

In general, anthocyanin biosynthesis is known to greatly vary when the vine is exposed to biotic and abiotic stressors (Gould and Lister, 2005, Gaulejac et al. 2001, Martinex-Gil et al., 2016, Artem et al., 2016, Del-Castillo-Alonso et al., 2016). In the study of Del-Castillo-Alonso et al., M-3-G content of Pinot Noir grapes was found to vary by four-fold among eleven sampling sites spanning from southern Spain to central Germany (Del-Castillo-Alonso et al.,

2016). By contrast, we identified less than one-fold difference of M-3-G content in Pinot Noir grapes among the three OV sub-regions. Thus, because the three sampled OV sub-regions only spanned approximately 100 km (from north to south), we speculate that it is reasonable to observe a subtler yet significant difference in anthocyanin content of Pinot Noir grapes among sub-regions in this study than in the study of Del-Castillo-Alonso et al. (Del-Castillo-Alonso et al., 2016).

3.4.4 Tannin Content

The skin tannin content of Pinot Noir grapes was not significantly affected by the three OV sub-regions, while the seed tannin content (expressed both in mg/g berry FW and in mg/berry) was significantly higher in Pinot Noir grapes collected from NP than from OO.

Previous studies on the association of grapes tannin content to *terroir* have yielded inconsistent results. In a study of Pinot Noir grapes grown in four Andalusian *terroirs*, there was no significant difference identified for skin, seed, and total tannin content in grapes (Fernandez-Marin et al. 2013). In another study performed in Romanian wine regions, there was a significant difference in skin tannin content but not in seed tannin content in Pinot Noir grapes collected from two distinctive *terroirs* (Artem et al. 2016). Furthermore, although studies have suggested that tannin content reduction during ripening is accelerated under high growing temperatures (i.e. 35 ^{o}C), controversial results have been obtained by other researchers (Cohen et al., 2008; Nicholas et al., 2011). Also, studies of the effect of precipitation (i.e. water availability) on tannin accumulation are not consistent and have shown either positive, negative or no impact (Genebra et al., 2014; Kyraleou et al., 2017; Zarrouk et al., 2012).

Differences in grape growing conditions among the various geographical regions can explain the inconsistencies among the studies. Tannin production is known to halt at grape veraison and the tannin concentration is subsequently reduced owing to grape cell expansion and consequent dilution of the compounds (Downey et al., 2003; Kennedy et al., 2000). Moreover, seed tannins may decline due to oxidation (i.e. when seeds turn from green to brown color) after veraison (Downey et al., 2003; Kennedy et al., 2000). Thus, the changes in tannin content examined from this study may have resulted from the complex interactions between different constituents of *terroir,* which contribute to an unique regional climate (macroclimate), vineyard mesoclimate, and a vine microclimate. For instance, based on our study, a high temperature

coupled with low precipitation (i.e. OO) is likely to reduce seed tannin accumulation while a low temperature coupled with high precipitation (i.e. NP) is likely to enhance seed tannin accumulation.

In addition, a higher seed number was discovered from grapes collected from NP as compared to the ones from OO. Based on the study of Harbertson et al., seed tannin content varies with the seed number but not the tannin content per seed (Harbertson et al., 2002). Also, seed number is known to be negatively influenced by heat stress and water deficit in field pea (Wery and Lecoeur, 2003). Therefore, a lower seed number in Pinot Noir grapes from OO than in grapes from NP may be associated with the climate discrepancies, which consequently lead to lower seed tannin content in grapes collected from OO than from NP. Further studies should be conducted in these three OV sub-regions in order to elucidate the most influential factor(s) of *terroir* towards tannin content accumulation in these specific geographical regions, including whether exposure to high growing temperature (i.e. > 35 ^{o}C) and low precipitation level would affect the number of seeds developed by the berries.

In this study, the average skin and seed tannin content of all sampled Pinot Noir grapes were 1.79 and 4.78 mg/berry respectively. In the study of Harbertson et al., that considered the same tannin measurement method, the skin and seed tannin content of Pinot Noir grapes were measured as 0.74 – 1.22 and 1.1 – 2.1 mg/berry respectively (Harbertson et al., 2002). In both studies, the skin tannin content was identified to be lower than the seed tannin content, which is consistent with multiple grape tannin studies (Mateus et al., 2001, Fernandez-Marin et al., 2013, Artem et al., 2016). However, a slightly higher skin tannin content and a double seed tannin content were observed from our sampled grapes as compared to the study of Harbertson et al. (Harbertson et al., 2002). This may be associated with the differences in the vintages and *terroirs* between these two studies. In particular, vintages have shown a strong effect on the flavonoid content (e.g. tannin and anthocyanin) of grapes at harvest (Bucchetti et al., 2011).

The change in tannin composition is known to be more obvious than tannin content in response to *terroir* variation (Mateus et al., 2001). As the total skin tannin content was shown to be similar in grapes among the three OV sub-regions, tannin content may not be sufficient to reflect all of the *terroir* effects from these geographical locations. In future studies, tannin composition should be measured together with tannin content to obtain a better understanding on the association between *terroirs* of OV and grape tannins (Downey et al., 2003; Kyraleou et al.,

2017). Moreover, tannin accumulation of Pinot Noir grapes was enhanced with increased heat summation between fruit set and veraison, as well as between budburst and flowering (Nicholas et al., 2011). Future studies might focus on OV vineyards with distinct heat summations during these phenological stages in order to assess if that component of *terroir* affects skin and seed tannins in Pinot Noir grapes grown in OV.

3.4.5 Flavonol Content

In this study, nine flavonol compounds were identified in Pinot Noir grapes collected from the three OV sub-regions, which were glucosides of myricetin (My-3-Glu), quercetin (Qu-3-Glu), laricitrin (La-3-Glu), kaempferol (Ka-3-Glu), isorhamnetin (Is-3-Glu), and syringetin (Sy-3-Glu), galactosides of quercetin (Qu-3-Gal) and myricetin (My-3-Gal), as well as a glucuronide of quercetin (Qu-3-Gld). None of the individual flavonol compounds and total flavonol content of Pinot Noir grapes differed among the three OV sub-regions.

Temperature variation has little effect on flavonol accumulation (Azuma et al., 2012; Downey et al., 2004; Price et al. 1995, Spayd et al. 2002). Furthermore, water availability (i.e. precipitation) did not affect total flavonol accumulation in grapes and has an inconsistent impact on individual flavonol compounds (Martinez-Luscher et al., 2014; Torres et al., 2017). In our study, there were no significant differences identified for total flavonol content between sub-regions (Figure 3-4). This result suggests that, other factors of *terroir* that did not vary significantly among sub-regions in our study, may have a stronger impact on flavonol accumulation. In particular, sunlight intensity is positively correlated to flavonol biosynthesis in grape berries (Azuma et al., 2012; Price et al. 1995; Spayd et al. 2002). Specifically, the effect of light intensity has a much greater impact on the expression of flavonol genes than the effect of temperature (Azuma et al., 2012). In order to explain our observation on flavonol content of Pinot Noir grapes, additional measurements are required that include a variety of *terroir* factors, including the light intensity received from the grapes among the three OV sub-regions.

3.4.6 Relative Abundance of Anthocyanins and Flavonols

3.4.6.1 Relative Abundance of Anthocyanins

Five anthocyanidins were identified in Pinot Noir grapes collected from the three OV sub-regions, which were delphinidin, cyanidin, petunidin, peonidin and malvidin. The average relative abundances of anthocyanidin in Pinot Noir grapes identified from this study were 7.24%, 3.46%, 8.66%, 20.5% and 60.1%, based on the compound order above. Comparably, in the study of Mattivi et al. on the metabolite profile of Pinot Noir grapes, relative abundances of anthocyanidin were identified as 4.95%, 4.44%, 5.68%, 34.65% and 50.27%, same order as the compound order above (Mattivi et al., 2006). In both studies, cyanidin and malvidin were identified as the anthocyanins with the lowest and highest relative abundance in Pinot Noir grapes, respectively. Also, variations in the anthocyanidin proportion values can be attributed to the differences of experimental settings in these two studies, including the viticultural locations. Specifically, all samples in this study were sampled from OV commercial vineyards while the samples in the study of Mattivi et al. were sampled from a research station in Northern Italy (Mattivi et al., 2006).

3.4.6.2 Relative Abundance of Flavonols

Six aglycones of flavonols were identified in Pinot Noir grapes collected from all three OV sub-regions, which were myricetin, quercetin, laricitrin, kaempferol, isorhamnetin, and syringetin. The average relative abundances of flavonol aglycones in Pinot Noir grapes identified from this study were 27.87%, 51.53%, 3.33%, 7.14%, 6.12% and 4.01%, based on the compound order above. In the study of Mattivi et al. on the metabolite profile of Pinot Noir grapes, relative abundances of flavonol aglycones were recognized as 16.28%, 59.30%, 4.73%, 10.14%, 7.11% and 2.44%, same order as the compound order above (Mattivi et al., 2006). Quercetin was identified as the major flavonol aglycone in both studies. And yet, with a distinctive difference in experimental settings and viticultural locations, there was some variation in the flavonol aglycone proportions.

Among studies focused on the flavonol composition in Pinot Noir grapes, researchers have published a different list of flavonol compounds depending on their research purposes (Jin et al., 2009; Del-Castillo-Alonso et al., 2016; Mazza et al., 1999; Price et al. 1995). For instance, 12 flavonol compounds were measured to elucidate the impact of environmental factors on Pinot Noir grapes grown in European vineyards (Del-Castillo-Alonso et al., 2016). In comparison, only 7 flavonol compounds were reported to elucidate the variation of Pinot Noir grapes grown in

Northwest China between two vintages (Jin et al., 2009). In our study, we have only identified the major flavonol compounds in order to examine the effect of *terroir* on Pinot Noir grapes collected from three OV sub-regions. The flavonol compounds present in very low or trace concentrations were omitted from this study. These minor compounds are foreseen to have a very minor impact on the relative abundance of flavonol aglycones in the Pinot Noir grapes.

3.4.6.3 Comparing the Relative Abundance of Anthocyanins and Flavonols among the Three OV Sub-Regions

The individual flavonoids with significant differences in Pinot Noir grapes collected from OO and KE were all tri-substituted flavonoids. For anthocyanins, they are the relative abundance of delphinidin (%D), petunidin (%Pt) and malvidin (%M). For flavonols, they are the relative abundance of laricitrin (%La) and syringetin (%Sy). Thus, tri-substituted flavonoids have a higher response than di-substituted flavonoids to *terroir* variation. Yet, there was no difference in the relative abundance of total tri-substituted anthocyanins and flavonols in Pinot Noir grapes collected from these three OV sub-regions. As shown in Figure 3-6, the relative abundance of each tri-substituted flavonol (%La, %Sy and %My) tend to be higher in grapes collected from OO as compared to the grapes from KE. Also, for tri-substituted anthocyanins, there was higher %M in Pinot Noir grapes collected from OO as compared to the ones from KE, while there was lower %D and %Pt in grapes collected from OO than the ones from KE.

The relative abundances of both total methoxylated anthocyanins and flavonols were higher in grapes collected from OO as compared to the ones from KE (Figure 3-5). Furthermore, three out of the four flavonoid compounds that are both tri-substituted and methoxylated (laricitrin, syringetin and malvidin) were higher in relative abundance in grapes collected from OO as compared to the ones from KE (Figure 3-5 and 3-6). To our knowledge, this study is the first research to identify an impact of *terroir* on the relative abundance of methoxylated flavonoid compounds in grapes.

In the 2017 vintage, OO had a remarkably higher GDD and a longer period of high growing temperature (i.e. > 35 ^{o}C) compared to KE, with a similar cumulative precipitation in these two regions. For anthocyanins, compounds with a higher number of hydroxylated groups present in the B-ring are lower in stability, while compounds with a higher number of methoxylated groups present in the B-ring are higher in stability (Yang et al., 2018). Moreover, a

warmer temperature (but < 35 ºC) was shown to enhance the relative abundance of tri-hydroxylated and methoxylated anthocyanins and flavonols in grape skins (Zhu et al., 2017). Thus, our results can be explained by two hypotheses. In the first hypothesis, the biosynthesis of tri-hydroxylated and methoxylated anthocyanins and flavonols was stimulated by the overall warmer temperature of the OO climate. For example, with the same mono-hydroxylated flavonoid precursor (dihydrokaempferol), either the 3'5'-hydroxylation enzyme activity (or gene expression level) could have been promoted or the 3'-hydroxylation enzyme activity (or gene expression level) could have been impaired in grapes collected from OO, resulting in a higher % of tri-substituted compounds, such as %La, and %Sy, than grapes collected from KE. Also, O-methoxylation enzyme activity (or gene expression level) of flavonol myricetin and the anthocyanin delphinidin could have been stimulated in grapes collected from OO, resulting in a higher %La, %Sy and %Mv than grapes collected from KE. In the second hypothesis, the high growing temperature (i.e. > 35 ºC) of OO may have stimulated the degradation of anthocyanin compounds with lower stability. In particular, because of a lower molecular stability associated with an higher degree of hydroxylation, petunidin and delphinidin were more vulnerable to degradation in grapes collected from OO as compared to grapes collected from KE. Also, malvidin has a higher degree of methoxylation than delphinidin and petunidin, and it has shown a better tolerance to degradation under high growing temperature (i.e. > 35 ºC) (Mori et al., 2017). By contrast to anthocyanins, a high growing temperature (i.e. > 35 ºC) has a lower impact on flavonol degradation, which is consistent with our results (Gouot et al., 2019). A further study looking at the anthocyanin and flavonol biosynthetic gene expression would be helpful to elucidate the association between *terroir* and the relative abundance of flavonoid compounds.

Even though NP has the lowest GDDs among the three OV sub-regions in the 2017 vintage, the most changes in relative abundance of flavonols and anthocyanins are recorded between OO and KE. Thus, aside from air temperature, other factors of terroir may play a role in the relative abundance of anthocyanins and flavonols in grapes. For instance, the relative abundance of methoxylated flavonols were higher in grapes collected from NP as compared to the ones KE. With similar GDDs between these two sub-regions, a higher cumulative precipitation level in NP may justify the changes of methoxylated flavonols. Yet, water availability was shown to have no impact of the relative abundance of methoxylated flavonols (Martinez-Luscher et al., 2014). Therefore, studies looking at association between specific

environmental factors and methoxylated flavonoid compounds would be helpful to explain our observations.

3.4.7 Principal Component Analysis of Flavonoid Compounds in Pinot Noir Grapes

To compare and assess the differentiability of Pinot Noir grapes collected from three OV sub-regions, Principal Component Analysis (PCA) was generated based on the flavonoid (anthocyanins, tannins, flavonols) profiles of the grape samples. The PCA result demonstrates a limited differentiation of the Pinot Noir grapes based on the geographical sub-region of origin. As shown in Figure 3-7, within each sub-region, there was a considerable vineyard variation. Particularly, most of the replicates (i.e. dots in Figure 3-7) within each vineyard (i.e. a distinct shape of the dots in Figure 3-7) were close together on the PCA plot but some vineyards within each sub-region segregated from other vineyards. Even though Pinot Noir grapes collected from OO and KE could be grouped into clusters, portions of the clusters overlap among sub-regions, showing that some of the vineyards cannot be differentiated as part of a specific sub-region based on the flavonoid profiles. In addition, the grape samples collected from NP have dispersed among the PCA plot, which overlapped with both the OO and KE clusters and showed no specific geographical clustering.

Also, unexpectedly, PC1 is governed by factors that were not significantly affected among the three OV sub-regions (i.e. flavonol and skin tannin content), while PC2 is governed by factors that were different among the three OV sub-regions (i.e. anthocyanins and seed tannin content). Since PC1 did not reflect the flavonoid variation at the sub-region level, we speculate that there is more flavonoid variation in grapes among vineyards within a sub-region than among sub-regions.

As a limitation of this study, the clones and rootstocks of Pinot Noir grapes were not purposefully selected among commercial vineyards, and only clone 115 was identified in all three sub-regions (Table C-2 in Appendix C). Thus, even though the ripeness and maturity of Pinot Noir grapes were not significantly affected by the sub-regions, flavonoid variation may exist among the distinctive Pinot Noir clones. Also, the viticultural practices of each vineyard was kept confidential by some growers. Therefore, the discussion of *terroir* influence against flavonoid composition is limited to the GDDs and cumulative precipitation of the three *terroirs* considered, which may not completely reflect the unique regional climate conditions formed by

the interaction among different constituents of *terroir,* including sunlight intensity, soil structure, *etc.* (Gladstones *et al.*, 2015a).

3.5 Conclusion

In this study, anthocyanins were significantly lower in Pinot Noir grapes collected from OO as compared to from KE. Temperature is known to have a significant effect on the anthocyanin accumulation of grape berries (Spayd et al., 2002; Yamane et al., 2006; Nicholas et al., 2011; Mori et al., 2007). Thus, with a high frequency of extreme maximum daily temperatures observed in OO (> 35 ºC), the low anthocyanin content of Pinot Noir grapes from OO may be due to anthocyanin degradation and/or inhibition of anthocyanin biosynthesis. Also, there was a slightly higher anthocyanin accumulation in grapes collected from KE than the grapes from NP, suggesting that a lower cumulative precipitation in KE may have upregulated the gene responsible for anthocyanin biosynthesis (Castellarin et al., 2007).

Seed tannin content was higher in grapes collected from NP as compared to OO, while skin tannin content was not affected by OV sub-regions. Although previous studies on the association between *terroir* and tannin content have inconsistent results, our data suggests a negative correlation between GDD and seed tannin accumulation, in which a warmer climate may associate with acceleration of tannin content reduction after veraison (Artem et al. 2016; Fernandez-Marin et al. 2013; Nicholas et al., 2011). Furthermore, no significant difference was identified in flavonol content of Pinot Noir grapes among the three OV sub-regions, which may indicate GDD and cumulative precipitation are not the major driving forces for flavonol accumulation.

In terms of the relative abundance of individual anthocyanin and flavonol compounds in Pinot Noir grapes, our study suggests that the general warmer climate of OO may promote the biosynthesis of tri-substituted/methoxylated anthocyanins and flavonols, while the degradation of anthocyanins with lower stability can be stimulated by high growing temperature (i.e. > 35 ºC) (i.e. tri-hydroxylated compounds).

Finally, Pinot Noir grapes collected from the same sub-region can only be partially separated based on the flavonoid profile in the PCA plot, with higher vineyard-associated variation as compared to sub-regional-associated variation. A future study is required to elucidate the interaction of the vineyard-specific *terroirs* and viticultural practices that might

influence the flavonoid amount and profile of Pinot Noir grapes. The identification of the impact of regional *terroirs* on the flavonoid profile of Pinot Noir grapes will help local wineries to build up product differentiation and become more competitive in the globalized market.

4 Conclusion and Future Directions

4.1 Conclusion

The present study characterized the population structure of vineyard-associated wine yeast strains and examined the flavonoid profile of Pinot Noir grapes among the three OV sub-regions (KE, NP, and OO) in the 2017 vintage. For objective 1, both *S. cerevisiae* and *S. uvarum* strains were identified from all three examined OV sub-regions. In total, 10 commercial *S. cerevisiae* strains, 22 potentially indigenous *S. cerevisiae* strains, 2 previously isolated *S. uvarum* strains from OV, 1 previously isolated *S. uvarum* strain from Hornby Island (BC, Canada) and 2 newly discovered *S. uvarum* strains were isolated and identified in this study (Figure 2-3, 2-7). An unexpectedly low number of wine yeast colonies were isolated from NP, suggesting that a cool climate with high cumulative precipitation may impede the inhabitation of wine yeast on Pinot Noir grapes. In fact, based on our knowledge, this is the first time that *S. uvarum* has been identified in a spontaneous fermentation study from fermenting red grapes from OV.

For objective 2, the genotypes of isolated *S. cerevisiae* and *S. uvarum* colonies were grouped based on Bruvo's distances. For both *S. cerevisiae* and *S. uvarum*, the genetic relatedness of isolates identified within a sub-region was higher than among sub-regions (Figure 2-4, 2-6). Based on geographical isolation, a cluster of *S. cerevisiae* strains was identified for OO and KE respectively (Figure 2-5). Thus, distinctive *terroir* in each OV sub-region may contribute to genetic modifications of wine yeasts strains under selective environmental pressure. From AMOVA, as the population structure was shown to be significant at the sub-region level, it can be concluded that regional-specific *S. cerevisiae* strains were identified in this study (Table 203). Yet, with limited number of *S. uvarum strains* isolated, we are unable to make conclusions about *S. uvarum* population structure (Table 2-5).

Lastly, for objective 3, the tannin, flavonol, and anthocyanin profiles of Pinot Noir grapes collected from the three OV sub-regions were examined. The concentration of anthocyanin compounds identified was lower in grapes collected from OO as compared to from KE (Figure 3-2b). The lower anthocyanin contents in Pinot Noir berries from OO may be due to anthocyanin degradation and/or inhibition of biosynthesis in the warmer climate of OO (Mori et al., 2007). Similarly, seed tannin content was found to be lower in grapes collected from OO as compared to from NP, demonstrating that the *terroir* in OO is likely to reduce seed tannin accumulation

more than the *terroir* in NP. Interestingly, grapes collected from OO had a higher relative abundance of methoxylated and tri-substituted flavonoid compounds than the other two sub-regions. Thus, the flavonoid profile of Pinot Noir grapes was varied by the sub-regional *terroir* of the OV.

In conclusion, vineyard-associated *S. cerevisiae* and *S. uvarum* strains were genetically examined in this study and *S. cerevisiae* was found to have regional specificity. Also, the flavonoid profiles of Pinot Noir grapes vary among the three OV sub-regions. These findings represent the first characterization of a regional-specific population of wine yeast strains and the flavonoid profile of Pinot Noir grapes in the three sub-regions of the OV.

4.2 Future Directions

In this study, vineyard-associated wine yeast population and flavonoid profiles of Pinot Noir grapes were only examined for one vintage. Yet, *terroir* is defined as all environmental factors and interactions at a specific geographical site, which may vary in each vintage. Particularly, a remarkably high cumulative precipitation was identified from NP in the 2017 vintage. Also, as mentioned before, vineyard-associated yeast population is dynamic and vary with vintages and geographical locations. Therefore, at least another year of study is necessary to compare with the results obtained from this study, in order to identify persistent vineyard-associated *S. cerevisiae* strains from the OV and reveal consistent impacts of *terroir* towards the flavonoid profile of Pinot Noir grapes.

Moreover, in order to select regional-specific wine yeast strains that have enological potential and may be useful as a starter culture, the phenotypic characteristics of the *S. cerevisiae* and *S. uvarum* strains should be examined during wine fermentation. For instance, the ethanol production efficiency and acetic acid production rate are important considerations for the utilization of starter cultures.

Also, each wine yeast genome was genotyped by microsatellite analysis, which is only a small portion of the whole yeast genome. As a result, mutations that have occurred due to environmental conditions may be underrepresented by this non-coding repetitive region of the yeast DNA. Thus, a whole genome sequencing study is necessary to obtain a better understanding of the environmental impact against the wine yeast genomes by comparison to

strains isolated from other environments and to identify the ancestor of vineyard-associated wine yeast strains.

In addition, while the flavonoid profile of Pinot Noir grapes was shown to vary significantly among the three OV sub-regions, it is still unclear whether these differences are reflected to the resulting wine products. Thus, a follow-up fermentation study with Pinot Noir grapes collected from these three OV sub-regions will help to elucidate the *terroir* impact on the resulting wine.

Lastly, even though Pinot Noir is one of the most cultivated varieties in the OV, there were a few limitations to its flavonoid profile. In particular, Pinot Noir contains only non-acylated anthocyanin compounds. Therefore, the impact of *terroir* against acylated anthocyanin compounds cannot be elucidated from this study. A future study should focus on another grape variety, such as Merlot, that is planted in the three OV sub-regions, in order to understand the impact of *terroir* towards other flavonoid compounds absented in Pinot Noir grapes.

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Appendices

Appendix A – Primer Mix Composition and Primer Sequences for Microsatellite Analysis

Table A-1 *S. cerevisiae* Primer Mix Composition and Primer Sequences

Locus	Volume[a] (µl)	Primer Sequence
C3 F[b]	9.5	GTGTCTCTTTTTATTTACGAGCGGGCCAT
C3 R[c]	9.5	AAATCTCATGCCTGTGAGGGGTAT
YLR F	5.4	CTTAAACAACAGCTCCCAAA
YLR R	5.4	ATGAATCAGCGCATCAGAAAT
C5 F	9.5	GTGTCTTGACACAATAGCAATGGCCTTCA
C5 R	9.5	GCAAGCGACTAGAACAACAATCACA
C8 F	18	GTGTCTCAGGTCGTTCTAACGTTGGTAAAATG
C8 R	18	GCTGTTGCTGTTGGTAGCATTACTGT
C11 F	9	TTCCATCATAACCGTCTGGGATT
C11 R	9	TGCCTTTTTCTTAGATGGGCTTTC
C4 F	48.4	GTGTCTAGGAGAAAAATGCTGTTTATTCTGACC
C4 R	48.4	TTTTCCTCCGGGACGTGAAATA
O91 F	0	GTGTCTAAGCCTCTTCAAGCATGAC
O91 R	30	GTGTCTGGACAATTTTGCCACCTTA
SCY F	8	TACTAACGTCAACACTGCTGCCAA
SCY R	8	GGATCTACTTGCAGTATACGGG
AT4 F	20	GCAACATAATGATTTTGAGGT
AT4 R	20	GTGTCTTGTGTGAGCATAGTGGAGAA
SCAAT3 F	90	GTGTCTGAGGAGGGAAATGGACAG
SCAAT R	90	GCCTGAAGATGCTTTTAG
009C F	20	GTGTCTGGGTTTTGGATTTTTATGGA
009C R	20	GTGTCTTTCAATTTTCCTCTTTTACCAC
Milli-Q water	1464.4	

[a]The volume is for 2000 reactions and concentrations of the primers were 100 µM. [b]F= forward primers; [c]R= reverse primers

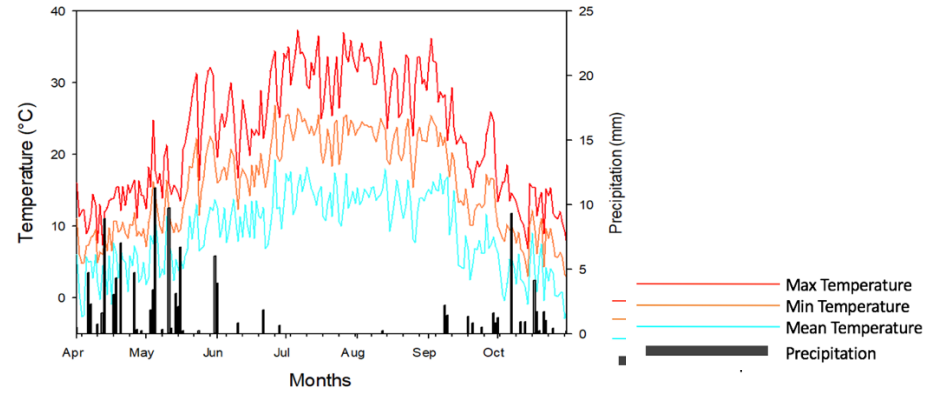
Table A-2 *S. uvarum* Primer Mix Composition and Primer Sequences

Locus	Volume[a] (µl)	Primer Sequence
NB1 F[b]	20	GTGCTCCATGGACTTGTATGAAGCAA
NB1 R[c]	20	GTTCGTTACCTTCAGTGCTC
NB4 F	60	GTGCTCGACATTGTAAAAGCACAGCA
NB4 R	60	ACGGGGCTTCTCTAGATATT
NB8 F	80	GTGCTCTGCATGAAAGATTGTAAAGG
NB8 R	80	TCCACAACGATATCAAGACA
NB9 F	80	GTGCTCAAACAAGAAACTGTGGTCGT
NB9 R	80	TGCTTTAATTTCAAGAAACA
L1 F	60	CGTGTTGAAGACATAATTG
L1 R	60	AATCTGAACGACAGGAAT
L2 F	160	TGCCCTTCTTATTCTTGT
L2 R	160	GAAAATATCAACGCATTAAA
L3 F	130	GTATGCATCACTATTTTTCG
L3 R	130	AATTTGGTAATTTGAATGTG
L4 F	60	GGACACTAGAGTTCGTCTCG
L4 R	60	GCCACCACTATCAGTTCG
L7 F	50	GTAGAATTCACCACAGGTC
L7 R	50	CCGTATATAAAACAGCACTT
L8 F	50	CACGGCAATCAGCACATTT
L8 R	50	TGAAGTTTCATCATCGGCAA
L9 F	160	AAAAAGCAACCTTAAAAGCAACA
L9 R	160	CTTTACGTAGGCTCATGGCA
Milli-Q water	180	

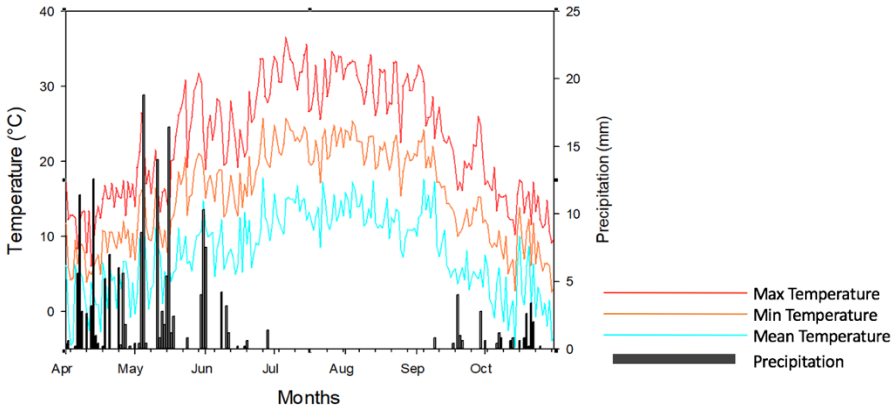
[a]The volume is for 2000 reactions. The concentrations of the primers were 10 µM for all primers except L3. L4, L7, L8 and L9, which were 15 uM. [b]F= forward primers; [c]R= reverse primers

Appendix B – Weather Information

a



b



c

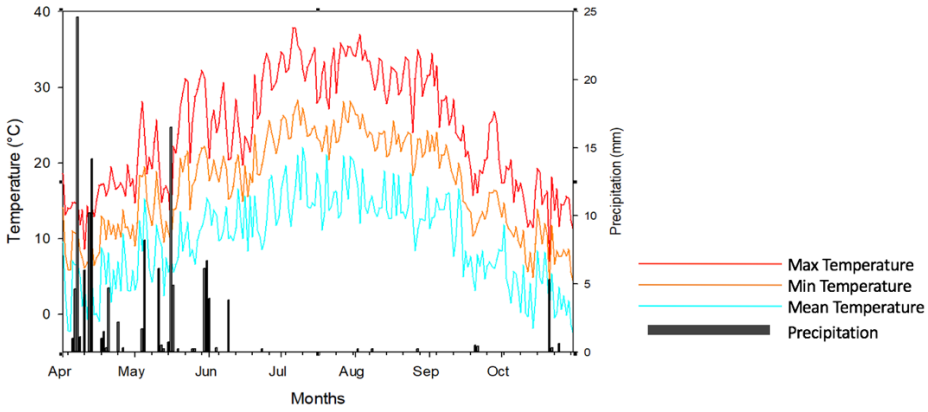


Figure B-1 Daily Temperature and Precipitation data of (a) Kelowna (b) Naramata-Penticton (c) Oliver-Osoyoos from April 1st to October 31st, 2017.
The line graphs provide the fluctuation of daily air temperature (°C) while the bar charts present the daily precipitation data. Abbreviation indicates: Max= Maximum; Min= Minimum.

Appendix C – General Berry Parameters

Table C-1 General Berry Parameters of Pinot Noir Grapes among sampled OV Vineyards

	Vineyard 1 (OO)	Vineyard 2 (OO)	Vineyard 3 (OO)	Vineyard 4 (OO)	Vineyard 5 (NP)	Vineyard 6 (NP)	Vineyard 7 (NP)	Vineyard 8 (NP)	Vineyard 9 (NP)	Vineyard 10 (KE)	Vineyard 11 (KE)	Vineyard 12 (KE)	Vineyard 13 (KE)
Berry weight (g)***	1.17±0.03^{BC}	1.35±0.02^{AB}	1.16±0.02^{BC}	1.29±0.09^{AB}	1.4±0.08^{AB}	1.35±0.07^{AB}	1.13±0.05^{BC}	1.38±0.05^{AB}	1.52±0.05^{A}	1.4±0.04^{AB}	0.96±0.07^{C}	1.42±0.05^{AB}	1.47±0.04^{A}
Skin Weight/Berry (ug)**	101.06±1.47 ABC	127.69±3.17 A	96.05±2.13^{B} C	108.3±8.33^{A} BC	116.26±2.63 ABC	95.5±5.14^{C}	114.17±13.87^{ABC}	123.58±5.3^{A} B	120.05±8.42 ABC	103.45±2.95 ABC	99.08±10.01 BC	112.6±3.97^{A} BC	118.61±6.12 ABC
Skin/Berry (%)**	8.63±0.18^{AB} C	9.45±0.17 ABC	8.00±0.08 ABC	8.39±0.14 ABC	8.62±0.22 ABC	7.13±0.24^{C}	10.37±1.51 A	8.88±0.22 ABC	7.81±0.36^{BC}	7.38±0.17^{C}	10.18±0.15 AB	7.98±0.36^{BC}	7.98±0.40 ABC
Seed Weight/Berry (ug)***	38.32±0.95^{B}	37.07±1.05^{B}	35.98±0.28^{B}	35.08±2.17^{B}	37.57±1.01^{B}	36.87±1.02^{B}	36.23±0.76^{B}	37.4±0.84^{B}	37.06±0.41^{B}	35.18±0.6^{B}	33.23±2.01^{B}	37.11±1.14^{B}	47.93±2.81^{A}
Seed/Berry (%)***	6.70±0.04 ABCD	6.40±0.23 CD	7.05±0.17 ABC	5.47±0.04^{D}	6.32±0.13 CD	6.47±0.49 BCD	7.52±0.20 ABC	7.23±0.33 ABC	7.73±0.35 AB	6.31±0.14 CD	7.90±0.19^{A}	7.03±0.11 ABC	5.52±0.41^{D}
Seed Number***	2.05±0.03 CD	2.34±0.11^{BC}	2.28±0.09 BCD	2.01±0.04 CD	2.35±0.09^{BC}	2.36±0.16^{BC}	2.33±0.02^{BC}	2.66±0.03 AB	3.17±0.12^{A}	2.51±0.05^{BC}	2.28±0.07 BCD	2.70±0.03 AB	1.74±0.28^{D}
Total Soluble Solids (^{o}Brix)***	22.4±0.08^{AB} CD	20.28±0.61^{D}	22.3±0.11^{AB} CD	23.78±0.26^{A}	21.28±0.32^{B} CD	20.65±0.22^{C} D	23.1±0.35^{AB} CD	22.05±0.29^{A} BCD	22±1.4^{ABCD}	23±0.12^{ABC}	20.95±0.57^{C} D	23.45±0.22^{A} B	22.75±0.26^{A} BCD

Abbreviations indicated: ***= p<0.0001; **= p<0.01; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna.
Numerical values followed by no letter or the same letter are not significantly different.

Table C-2 Rootstock and Clone information of the sampled Pinot Noir Grapes among sampled OV Vineyards

Vineyards	Rootstock/Clone
1 (OO)	ND/115
2 (OO)	ND/114
3 (OO)	S04/115
4 (OO)	3309/115
5 (NP)	ND/828
6 (NP)	3309/777
7 (NP)	ND/115
8 (NP)	101-14/115
9 (NP)	ND/828&667
10 (KE)	ND/828
11 (KE)	S04/115
12 (KE)	101-14/667
13 (KE)	101-14/943

The information was verbally provided by viticulturists from these sampled vineyards

Appendix D – Supplementary Berry Flavonoid Information

Table D-1 Anthocyanin Concentration of Pinot Noir Grapes in µg/g berry FW among Three OV Sub-regions

	OO	NP	KE
D-3-G*	40.78±9.77^{B}	53.76±11.39^{AB}	70.52±10.48^{A}
C-3-G	26.05±5.54	29.86±4.81	37.22±5.52
Pt-3-G*	77.93±11.76^{B}	94.96±19.88^{AB}	118.90±10.86^{A}
Pn-3-G	215.45±26.02	234.81±23.20	257.12±43.23
M-3-G	444.52±26.47	477.39±55.17	473.84±47.60
Total	804.74±65.00	890.77±110.97	957.61±112.71

Abbreviations indicated: *=p<0.05; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; D-3-G= Delphinidin-3-glucoside; C-3-G= Cyanidin-3-glucoside; Pt-3-G= Petunidin-3-glucoside; Pn-3-G= Peonidin-3-glucoside; M-3-G= Malvidin-3-glucoside. Numerical values followed by no letter or the same letter are not significantly different.

Table D-2 Anthocyanin Content of Pinot Noir Grapes in µg/berry among Three OV Sub-regions

	OO	NP	KE
D-3-G**	49.85±10.43^{B}	70.38±11.48^{AB}	89.99±13.70^{A}
C-3-G	32.08±5.78	40.08±4.99	48.78±8.76
Pt-3-G**	95.90±11.12^{B}	123.82±18.63^{AB}	152.98±12.78^{A}
Pn-3-G	268.59±29.47	311.45±21.15	326.23±43.43
M-3-G	552.31±34.94	634.00±39.05	613.06±68.22
Total*	998.74±64.78^{B}	1179.74±87.78^{AB}	1231.03±137.60^{A}

Abbreviations indicated: ***= p<0.0001; **= p<0.01; *=p<0.05; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; D-3-G= Delphinidin-3-glucoside; C-3-G= Cyanidin-3-glucoside; Pt-3-G= Petunidin-3-glucoside; Pn-3-G= Peonidin-3-glucoside; M-3-G= Malvidin-3-glucoside. Numerical values followed by no letter or the same letter are not significantly different.

Table D-3 Skin and Seed Tannin Content of Pinot Noir Grapes Expressed as mg/g berry FW and mg/berry among Three OV Sub-regions

	OO	NP	KE
mg/g berry FW for skin	1.31±0.07	1.42±0.16	1.39±0.07
mg/g berry FW for seed*	3.35±0.24^{B}	3.97±0.22^{A}	3.71±0.37^{AB}
mg/berry for skin	1.63±0.05	1.88±0.10	1.84±0.23
mg/berry for seed**	4.15±0.22^{B}	5.37±0.42^{A}	4.81±0.47^{AB}
Skin tannins/total (%)	22.47±1.04	22.40±1.09	23.43±2.43

Abbreviations indicate: *=p<0.05; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna. Numerical values followed by no letter or the same letter are not significantly different.

Table D-4 Flavonol Concentration of Pinot Noir grapes in µg/g berry FW among Three OV Sub-regions

	OO	NP	KE
My-3-Gal	1.32±0.1	1.44±0.2	1.6±0.09
My-3-Glu	1.32±0.11	1.42±0.1	1.52±0.08
Qu-3-Gal	0.41±0.07	0.48±0.08	0.57±0.06
Qu-3-Gld	1.65±0.18	1.95±0.2	2.05±0.17
Qu-3-Glu	3.31±0.48	3.38±0.48	4.05±0.29
La-3-Glu	0.36±0.02	0.35±0.03	0.34±0.02
Ka-3-Glu	0.74±0.11	0.96±0.17	0.87±0.07
Is-3-Glu	0.7±0.09	0.7±0.09	0.7±0.06
Sy-3-Glu	0.44±0.03	0.43±0.04	0.35±0.02
Total	10.26±0.99	11.13±1.23	12.05±0.75

Abbreviations indicated: OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; My-3-Gal= Myricetin-3-galactoside; My-3-Glu= Myricetin-3-glucoside; Qu-3-Gal= Quercetin-3-galactoside; Qu-3-Gld= Quercetin-3-glucuronide; Qu-3-Glu= Quercetin-3-glucoside; La-3-Glu= Laricitrin-3-glucoside; Ka-3-Glu= Kaempferol-3-glucoside; Is-3-Glu= Isorahamnetin-3-glucoside; Sy-3-Glu= Syringetin-3-glucoside.
Numerical values followed by no letter or the same letter are not significantly different.

Table D-5 Flavonol Content of Pinot Noir Grapes in µg/berry among Three OV Sub-regions

	OO	NP	KE
My-3-Gal	1.63±0.11	1.86±0.2	2.09±0.14
My-3-Glu	1.62±0.11	1.91±0.13	1.94±0.07
Qu-3-Gal	0.51±0.08	0.62±0.09	0.74±0.07
Qu-3-Gld	2.03±0.21	2.57±0.23	2.62±0.21
Qu-3-Glu	4.07±0.56	4.38±0.54	5.27±0.43
La-3-Glu	0.44±0.03	0.46±0.03	0.44±0.03
Ka-3-Glu	0.91±0.13	1.25±0.22	1.14±0.11
Is-3-Glu	0.86±0.1	0.91±0.11	0.91±0.07
Sy-3-Glu	0.54±0.03	0.56±0.04	0.45±0.02
Total	12.62±1.12	14.54±1.3	15.61±1.04

Abbreviations indicated: OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; My-3-Gal= Myricetin-3-galactoside; My-3-Glu= Myricetin-3-glucoside; Qu-3-Gal= Quercetin-3-galactoside; Qu-3-Gld= Quercetin-3-glucuronide; Qu-3-Glu= Quercetin-3-glucoside; La-3-Glu= Laricitrin-3-glucoside; Ka-3-Glu= Kaempferol-3-glucoside; Is-3-Glu= Isorahamnetin-3-glucoside; Sy-3-Glu= Syringetin-3-glucoside.
Numerical values followed by no letter or the same letter are not significantly different.

Table D-6 Relative Abundance of Individual Anthocyanins and Anthocyanin Fractions based on Level of Substitution and Methoxylation in Pinot Noir Grape Skins among Three OV Sub-regions

	OO	NP	KE
%D**	4.90±0.78^{B}	5.74±0.59^{AB}	7.22±0.42^{A}
%C	3.19±0.59	3.32±0.22	3.84±0.24
%Pt*	9.54±0.91^{B}	10.13±0.87^{AB}	12.61±0.95^{A}
%Pn	26.58±1.62	26.62±1.16	26.44±1.56
%M*	55.79±2.36^{A}	54.19±1.45^{AB}	49.89±1.13^{B}
%Di	29.78±1.25	29.93±1.11	30.27±1.56
%Meth**	91.91±1.13^{A}	90.94±0.80^{AB}	88.94±0.62^{B}

Abbreviations indicated: **= p<0.01; *=p<0.05; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; C= Cyanidin; Pn= Peonidin; D= Delphinidin; Pt= Petunidin; M= Malvidin; Di= Di-substituted; Meth= Methoxylated. Numerical values followed by no letter or the same letter are not significantly different.

Table D-7 Relative Abundance of Individual Flavonols and Flavonol Fractions based on Level of Substitution and Methoxylation in Pinot Noir Grape Skins among Three OV Sub-regions.

	OO	NP	KE
%My	29.13±3.00	28.02±1.85	26.45±0.80
%Qu	49.40±2.43	50.51±1.58	54.68±0.91
%La*	3.69±0.18^{A}	3.41±0.25^{AB}	2.89±0.19^{B}
%Ka	6.61±0.58	7.60±0.84	7.22±0.31
%Is	6.54±0.46	6.05±0.27	5.78±0.22
%Sy**	4.64±0.28^{A}	4.40±0.47^{A}	3.00±0.16^{B}
%Tri	37.46±3.31	35.84±2.38	32.33±1.06
%Di	55.93±2.76	56.56±1.67	60.46±0.95
%Mono	6.61±0.58	7.60±0.84	7.22±0.31
%Meth**	14.86±0.46^{A}	13.86±0.71^{A}	11.66±0.34^{B}

Abbreviations indicate: **= p<0.01; *=p<0.05; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; My= Myricetin; Qu= Quercetin; La= Laricitrin; Ka= Kaempferol; Is= Isorhamnetin; Sy= Syringetin; Tri= Tri-substituted; Di= Di-substituted; Mono= Mono-substituted; Meth= Methoxylated. Numerical values followed by no letter or the same letter are not significantly different.

Table D-8 Anthocyanin Concentration of Pinot Noir Grapes in µg/g berry FW among sampled OV Vineyards

	Vineyard 1 (OO)	Vineyard 2 (OO)	Vineyard 3 (OO)	Vineyard 4 (OO)	Vineyard 5 (NP)	Vineyard 6 (NP)	Vineyard 7 (NP)	Vineyard 8 (NP)	Vineyard 9 (NP)	Vineyard 10 (KE)	Vineyard 11 (KE)	Vineyard 12 (KE)	Vineyard 13 (KE)
D-3-G***	45.45±4.64^{BC}	29.56±3.85^{C}	54.09±6.74^{A} BC	28.88±1.19^{C}	57.97±3.05^{A} BC	39.9±9.9^{BC}	84.29±9.98^{A}	35.9±2.16^{BC}	52.96±7.96^{A} BC	60.42±3.74^{A} BC	69.81±11.25 AB	84.16±12.42 A	61.09±4.1^{AB} C
C-3-G**	28.49±2.72^{ABC}	18.78±2.34^{BC}	24.04±1.65^{A} BC	16.73±1.65^{C}	22.88±1.32^{A} BC	18.38±3.51^{B} C	34.7±4.71^{A}	22.07±2.85^{A} BC	27.32±2.9^{AB} C	25.51±2^{ABC}	31.49±3.92^{A} B	33.52±2.37^{A}	31.64±3.44^{A} B
Pt-3-G***	55.01±4.19^{CD}	38.11±3.33^{D}	68.6±6.94^{AB} CD	36.02±1.54^{D}	60.25±3.59^{B} CD	49.95±7.92^{D}	97.36±11.29 A	45.61±2.03^{D}	68.48±8.05^{A} BCD	70.14±5.12^{A} BCD	88.12±12.98 ABC	91.97±11.41 AB	72.22±4.32^{A} BCD
Pn-3-G***	131.55±5.48^{DE}	123.16±11.88^{DE}	187.58±12.41^{ABC}	110.45±4.81 DE	102.49±6.03 E	122.7±10.44 DE	192.5±17.87 AB	156.53±11.8 BCD	190.48±9.34 AB	136.63±11.57^{CDE}	214.52±11.46^{A}	160.12±6.82 BCD	161.7±6.3^{BC} D
M-3-G***	422.18±6.11^{BC} D	364.96±2.62^{CD}	495.73±12.67$_{ABC}$	319.64±7.7^{D}	346.52±14.15^{CD}	434.13±59.83^{BCD}	641.47±44.14^{A}	434.48±34.77^{BCD}	474.37±29.71^{BC}	402.18±32.92^{CD}	573.32±21.76^{AB}	450.26±22.3 BCD	497.46±46.11^{ABC}
Total***	682.68±21.99^{C} DE	574.57±19.16^{E}	830.04±32.86^{ABC}	511.73±13.16^{E}	590.1±25.28 DE	665.07±67.41^{CDE}	1050.33±80.58^{A}	694.59±43.31^{CDE}	813.61±36.12^{BCD}	694.89±54.92^{CDE}	977.25±45.76^{AB}	820.04±54.32^{BC}	824.11±50.08^{ABC}

Abbreviations indicated: ***= p<0.0001; **= p<0.01; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; D-3-G= Delphinidin-3-glucoside; C-3-G= Cyanidin-3-glucoside; Pt-3-G= Petunidin-3-glucoside; Pn-3-G= Peonidin-3-glucoside; M-3-G= Malvidin-3-glucoside. Numerical values followed by no letter or the same letter are not significantly different

Table D-9 Anthocyanin Content of Pinot Noir Grapes in µg/berry among sampled OV Vineyards

	Vineyard 1 (OO)	Vineyard 2 (OO)	Vineyard 3 (OO)	Vineyard 4 (OO)	Vineyard 5 (NP)	Vineyard 6 (NP)	Vineyard 7 (NP)	Vineyard 8 (NP)	Vineyard 9 (NP)	Vineyard 10 (KE)	Vineyard 11 (KE)	Vineyard 12 (KE)	Vineyard 13 (KE)
D-3-G***	53.44±6.2^{BC} DE	40.06±5.51^{D} E	62.92±7.73^{B} CDE	37.43±3.63^{E}	80.74±3.64^{A} BCDE	53.99±13.91 BCDE	94.58±10.27 AB	49.7±3.6^{CDE}	81.62±14.65 ABCDE	84.57±4.6^{AB} CD	67±10.82^{BC} DE	118.15±13.65^{A}	89.53±5.63^{A} BC
C-3-G**	33.53±3.74^{A} B	25.46±3.33^{B}	28.02±2.15^{A} B	21.98±3.58^{B}	31.77±0.93^{A} B	24.87±5.01^{B}	38.95±4.94^{A} B	30.83±4.95^{A} B	41.95±5.65^{A} B	35.75±2.77^{A} B	30.35±4.28^{A} B	47.45±2.02^{A}	46.58±5.44^{A}
Pt-3-G***	64.62±5.8^{CD}	51.59±4.9^{D}	79.83±8.06^{B} CD	46.67±4.49^{D}	83.64±2.41^{B} CD	67.11±10.85 BCD	109.15±11.32^{AB}	63.03±3.13^{C} D	105.29±15.46^{ABC}	98.05±5.79^{A} BC	84.22±11.9^{B} CD	129.42±11.77^{A}	105.81±5.64 ABC
Pn-3-G***	154.29±8.27 DE	166.67±16.98^{CDE}	218.7±16.52 BC	142.75±12.83^{E}	142.16±2.34 E	163.6±6.39^{C} DE	216.25±19.11^{BCD}	216.14±16.52^{BCD}	290.12±16.22^{A}	191.08±14.2 BCDE	204.74±10.91^{BCDE}	227.21±2.57 ABC	237.11±8.3^{A} B
M-3-G***	494.66±12.41^{CDE}	493.09±10.21^{DE}	576.78±15.2 BCD	411.06±24.8 E	482.05±9.86 DE	574.5±46.27 CD	719.04±40.78^{AB}	596.26±25.72^{ABCD}	720.09±34.13^{AB}	562.1±38.38 CD	546.53±15.81^{CDE}	638.46±11.71^{ABC}	725.49±46.47^{A}
Total***	800.54±36.1 EF	776.87±33.88^{EF}	966.26±42.7 CDE	659.88±48.62^{F}	820.36±10.71^{EF}	884.07±40.04^{EF}	1177.98±78.35^{ABC}	955.95±35.05^{CDE}	1239.08±63.31^{A}	971.55±65.01^{BCDE}	932.84±44.92^{DE}	1160.68±37.79^{ABCD}	1204.52±39.5^{AB}

Abbreviations indicated: ***= p<0.0001; **= p<0.01; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; D-3-G= Delphinidin-3-glucoside; C-3-G= Cyanidin-3-glucoside; Pt-3-G= Petunidin-3-glucoside; Pn-3-G= Peonidin-3-glucoside; M-3-G= Malvidin-3-glucoside. Numerical values followed by no letter or the same letter are not significantly different.

Table D-10 Skin and Seed Tannin Content of Pinot Noir Grapes Expressed as mg/g berry FW and mg/berry among sampled OV Vineyards

	Vineyard 1 (OO)	Vineyard 2 (OO)	Vineyard 3 (OO)	Vineyard 4 (OO)	Vineyard 5 (NP)	Vineyard 6 (NP)	Vineyard 7 (NP)	Vineyard 8 (NP)	Vineyard 9 (NP)	Vineyard 10 (KE)	Vineyard 11 (KE)	Vineyard 12 (KE)	Vineyard 13 (KE)
mg/g berry FW for skin	1.39±0.05	1.26±0.06	1.46±0.11	1.13±0.16	1.4±0.06	1.33±0.09	2.02±0.57	1.17±0.12	1.18±0.15	1.58±0.09	1.23±0.08	1.33±0.08	1.41±0.06
mg/g berry FW for seed***	$3.63\pm0.07^{CD\ EF}$	3.29 ± 0.1^{EFG}	$3.8\pm0.09^{BCD\ E}$	2.69 ± 0.09^{G}	$3.41\pm0.07^{DE\ FG}$	$3.63\pm0.24^{BC\ DEF}$	$4.29\pm0.18^{AB\ CD}$	$3.9\pm0.17A^{B\ CDE}$	4.62 ± 0.29^{A}	$3.25\pm0.06^{EF\ G}$	$4.32\pm0.09^{AB\ C}$	4.35 ± 0.06^{AB}	2.93 ± 0.21^{FG}
mg/berry for skin**	1.63 ± 0.09^{AB}	1.71 ± 0.11^{AB}	1.69 ± 0.12^{AB}	1.5 ± 0.28^{AB}	1.97 ± 0.15^{AB}	1.78 ± 0.11^{AB}	2.23 ± 0.55^{AB}	1.63 ± 0.19^{AB}	1.82 ± 0.28^{AB}	2.21 ± 0.12^{A}	1.18 ± 0.1^{B}	1.89 ± 0.08^{AB}	2.08 ± 0.15^{A}
mg/berry for seed***	$4.25\pm0.13^{CD\ E}$	$4.45\pm0.12^{CD\ E}$	$4.42\pm0.11^{CD\ E}$	3.49 ± 0.35^{E}	4.77 ± 0.19^{CD}	4.87 ± 0.25^{CD}	4.82 ± 0.2^{CD}	5.38 ± 0.19^{BC}	7 ± 0.24^{A}	$4.55\pm0.12^{CD\ E}$	4.15 ± 0.32^{DE}	6.2 ± 0.16^{AB}	$4.32\pm0.39^{CD\ E}$
% skin tannin/total***	$22.97\pm1.05^{A\ BC}$	$20.56\pm0.86^{B\ C}$	$25.2\pm1.45^{AB\ C}$	$21.15\pm2.02^{B\ C}$	$23.23\pm1.21^{A\ BC}$	$24.8\pm1.23^{AB\ C}$	$24.34\pm1.64^{A\ BC}$	$19.53\pm1.18^{B\ C}$	$20.11\pm2.05^{B\ C}$	29.34 ± 1.55^{A}	18.08 ± 0.96^{C}	$21.23\pm0.93^{B\ C}$	$25.09\pm1.74^{A\ B}$

Abbreviations indicate: ***= p<0.0001; **= p<0.01; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna. Numerical values followed by no letter or the same letter are not significantly different.

Table D-11 Flavonol Concentration of Pinot Noir grapes in µg/g berry FW among sampled OV Vineyards

	Vineyard 1 (OO)	Vineyard 2 (OO)	Vineyard 3 (OO)	Vineyard 4 (OO)	Vineyard 5 (NP)	Vineyard 6 (NP)	Vineyard 7 (NP)	Vineyard 8 (NP)	Vineyard 9 (NP)	Vineyard 10 (KE)	Vineyard 11 (KE)	Vineyard 12 (KE)	Vineyard 13 (KE)
My-3-Gal***	1.82±0.16^{B}	0.99±0.13^{C}	1.12±0.14^{BC}	1.35±0.08^{BC}	1.14±0.05^{BC}	0.91±0.06^{C}	3.08±0.36^{A}	0.97±0.07^{C}	1.12±0.08^{BC}	1.54±0.29^{BC}	1.60±0.15^{BC}	1.83±0.09^{B}	1.41±0.15^{BC}
My-3-Glu***	1.26±0.07^{CD}	1.08±0.09^{D}	1.97±0.09^{A}	0.98±0.08^{D}	0.96±0.05^{D}	1.12±0.08^{D}	1.90±0.21^{AB}	1.38±0.11^{BC} D	1.76±0.14^{AB} C	1.24±0.12^{CD}	1.96±0.04^{A}	1.49±0.05^{AB} CD	1.40±0.13^{BC} D
Qu-3-Gal***	0.76±0.06^{AB}	0.25±0.05^{DE} F	0.14±0.02^{F}	0.51±0.05^{BC} D	0.74±0.03^{AB}	0.19±0.01^{EF}	0.99±0.07^{A}	0.26±0.04^{DE} F	0.24±0.02^{DE} F	0.51±0.13^{BC} DE	0.62±0.10^{BC}	0.76±0.08^{AB}	0.40±0.04^{CD} EF
Qu-Gld***	2.77±0.11^{A}	1.38±0.17^{BC} D	0.99±0.11^{D}	1.47±0.14^{BC} D	2.77±0.21^{A}	1.36±0.16^{BC} D	3.02±0.22^{A}	1.55±0.23^{BC} D	1.07±0.15^{D}	2.15±0.42^{AB} C	2.40±0.23^{AB}	2.38±0.27^{AB}	1.26±0.07^{CD}
Qu-3-Glu***	5.96±0.20^{AB}	2.18±0.37^{EF} G	1.21±0.19^{G}	3.88±0.29^{CD} E	4.70±0.26^{BC} D	1.49±0.10^{FG}	6.78±0.47^{A}	1.97±0.14^{FG}	1.95±0.09^{FG}	3.90±0.86^{CD} E	4.13±0.39^{CD}	5.07±0.34^{AB} C	3.09±0.24^{DE} F
La-3-Glu***	0.46±0.04^{AB}	0.32±0.01^{BC}	0.25±0.00^{C}	0.40±0.04^{BC}	0.28±0.02^{BC}	0.28±0.05^{C}	0.61±0.03^{A}	0.26±0.03^{C}	0.31±0.03^{BC}	0.30±0.05^{BC}	0.40±0.03^{BC}	0.33±0.01^{BC}	0.32±0.06^{BC}
Ka-3-Glu***	1.37±0.05^{B}	0.54±0.09^{DE} F	0.20±0.03^{F}	0.86±0.07^{CD} E	1.87±0.10^{A}	0.36±0.04^{F}	1.87±0.16^{A}	0.29±0.04^{F}	0.44±0.06^{EF}	0.90±0.16^{CD}	0.86±0.04^{CD} E	1.14±0.09^{BC}	0.58±0.04^{DE} F
Is-3-Glu***	1.04±0.04^{AB}	0.48±0.07^{EF}	0.28±0.02^{F}	0.99±0.10^{AB} C	0.94±0.11^{AB} CD	0.34±0.05^{F}	1.35±0.11^{A}	0.45±0.06^{EF}	0.44±0.07^{EF}	0.62±0.14^{CD} EF	0.80±0.12^{BC} DE	0.85±0.07^{BC} DE	0.54±0.05^{DE} F
Sy-3-Glu***	0.56±0.05^{AB}	0.39±0.03^{BC}	0.38±0.02^{BC}	0.45±0.06^{BC}	0.23±0.02^{C}	0.39±0.09^{BC}	0.71±0.07^{A}	0.40±0.03^{BC}	0.42±0.05^{BC}	0.32±0.05^{BC}	0.45±0.04^{BC}	0.34±0.02^{BC}	0.31±0.04^{C}
Total***	15.99±0.23^{A} B	7.61±0.80^{EF}	6.54±0.50^{F}	10.90±0.84^{C} DEF	13.61±0.58^{B} CD	6.44±0.19^{F}	20.30±1.17^{A}	7.54±0.60^{EF}	7.74±0.31^{EF}	11.48±2.18^{C} DE	13.21±0.91^{B} CD	14.19±0.82^{B} C	9.31±0.54^{DE} F

Abbreviations indicated: ***= p<0.0001; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; My-3-Gal= Myricetin-3-galactoside; My-3-Glu= Myricetin-3-glucoside; Qu-3-Gal= Quercetin-3-galactoside; Qu-3-Gld= Quercetin-3-glucuronide; Qu-3-Glu= Quercetin-3-glucoside; La-3-Glu= Laricitrin-3-glucoside; Ka-3-Glu= Kaempferol-3-glucoside; Is-3-Glu= Isorahamnetin-3-glucoside; Sy-3-Glu= Syringetin-3-glucoside.
Numerical values followed by no letter or the same letter are not significantly different.

Table D-12 Flavonol Content of Pinot Noir grapes in µg/berry among sampled OV Vineyards

	Vineyard 1 (OO)	Vineyard 2 (OO)	Vineyard 3 (OO)	Vineyard 4 (OO)	Vineyard 5 (NP)	Vineyard 6 (NP)	Vineyard 7 (NP)	Vineyard 8 (NP)	Vineyard 9 (NP)	Vineyard 10 (KE)	Vineyard 11 (KE)	Vineyard 12 (KE)	Vineyard 13 (KE)
My-3-Gal***	2.14±0.20^{BC}	1.35±0.19^{C}	1.31±0.17^{C}	1.72±0.04^{BC}	1.59±0.11^{C}	1.23±0.10^{C}	3.45±0.36^{A}	1.33±0.08^{C}	1.71±0.16^{BC}	2.15±0.36^{BC}	1.54±0.20^{C}	2.60±0.05^{AB}	2.05±0.16^{BC}
My-3-Glu***	1.47±0.07^{CD}	1.47±0.14^{CD}	2.29±0.11^{AB}	1.25±0.07^{D}	1.33±0.09^{D}	1.49±0.04^{CD}	2.13±0.19^{AB} C	1.91±0.15^{BC} D	2.68±0.23^{A}	1.73±0.14^{BC} D	1.87±0.09^{BC} D	2.12±0.07^{AB} C	2.06±0.21^{AB} C
Qu-3-Gal***	0.89±0.08^{AB} CD	0.33±0.07^{EF} G	0.16±0.03^{G}	0.65±0.03^{CD} E	1.03±0.02^{AB} C	0.26±0.01^{FG}	1.11±0.09^{A}	0.36±0.05^{EF} G	0.36±0.03^{EF} G	0.71±0.17^{BC} DE	0.59±0.09^{DE} F	1.08±0.12^{AB}	0.58±0.05^{DE} F
Qu-Gld***	3.25±0.18^{AB}	1.87±0.24^{CD}	1.16±0.13^{D}	1.86±0.08^{CD}	3.87±0.36^{A}	1.85±0.26^{CD}	3.39±0.22^{AB}	2.13±0.28^{BC} D	1.62±0.23^{D}	2.98±0.53^{AB} C	2.27±0.15^{BC} D	3.38±0.35^{AB}	1.85±0.15^{CD}
Qu-3-Glu***	6.98±0.26^{AB}	2.95±0.51^{EF} G	1.41±0.24^{G}	4.93±0.17^{BC} DE	6.55±0.37^{AB} C	2.03±0.20^{FG}	7.66±0.73^{A}	2.72±0.18^{EF} G	2.96±0.10^{EF} G	5.44±1.12^{AB} CD	3.95±0.40^{DE} F	7.20±0.43^{AB}	4.51±0.27^{CD} E
La-3-Glu***	0.54±0.05^{AB}	0.43±0.02^{BC}	0.29±0.00^{C}	0.51±0.03^{AB} C	0.39±0.02^{BC}	0.37±0.05^{BC}	0.68±0.05^{A}	0.36±0.03^{BC}	0.48±0.04^{AB} C	0.42±0.06^{BC}	0.39±0.06^{BC}	0.46±0.03^{BC}	0.47±0.07^{AB} C
Ka-3-Glu***	1.60±0.07^{BC}	0.73±0.12^{DE} FG	0.24±0.03^{G}	1.09±0.04^{CD} E	2.59±0.09^{A}	0.49±0.07^{EF} G	2.12±0.26^{AB}	0.40±0.05^{FG}	0.66±0.07^{DE} FG	1.26±0.21^{CD}	0.82±0.07^{DE} FG	1.64±0.18^{BC}	0.85±0.05^{DE} F
Is-3-Glu***	1.22±0.07^{AB} C	0.65±0.09^{DE}	0.33±0.03^{E}	1.25±0.05^{AB} C	1.29±0.10^{AB}	0.45±0.05^{DE}	1.53±0.18^{A}	0.62±0.07^{DE}	0.66±0.10^{DE}	0.86±0.17^{BC} D	0.77±0.12^{CD} E	1.21±0.11^{AB} C	0.79±0.07^{BC} DE
Sy-3-Glu***	0.65±0.05^{AB}	0.52±0.05^{AB} C	0.44±0.03^{BC}	0.56±0.04^{AB} C	0.32±0.03^{C}	0.52±0.10^{BC}	0.80±0.08^{A}	0.56±0.04^{AB} C	0.63±0.07^{AB}	0.44±0.06^{BC}	0.44±0.07^{BC}	0.48±0.01^{BC}	0.45±0.04^{BC}
Total***	18.74±0.52^{A} BC	10.30±1.13^{E} F	7.63±0.64^{F}	13.83±0.28^{C} DE	18.97±0.87^{A} BC	8.69±0.44^{EF}	22.88±1.68^{A}	10.38±0.69^{D} EF	11.77±0.28^{D} EF	15.99±2.78^{B} CD	12.66±1.06^{D} EF	20.17±1.11^{A} B	13.62±0.53^{C} DE

Abbreviations indicated: ***= p<0.0001; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; My-3-Gal= Myricetin-3-galactoside; My-3-Glu= Myricetin-3-glucoside; Qu-3-Gal= Quercetin-3-galactoside; Qu-3-Gld= Quercetin-3-glucuronide; Qu-3-Glu= Quercetin-3-glucoside; La-3-Glu= Laricitrin-3-glucoside; Ka-3-Glu= Kaempferol-3-glucoside; Is-3-Glu= Isorahamnetin-3-glucoside; Sy-3-Glu= Syringetin-3-glucoside.
Numerical values followed by no letter or the same letter are not significantly different.

Table D-13 Relative Abundance of Individual Anthocyanins and Anthocyanin Fractions based on Level of Substitution and Methoxylation in Pinot Noir Grape Skins among sampled OV Vineyards

	Vineyard 1 (OO)	Vineyard 2 (OO)	Vineyard 3 (OO)	Vineyard 4 (OO)	Vineyard 5 (NP)	Vineyard 6 (NP)	Vineyard 7 (NP)	Vineyard 8 (NP)	Vineyard 9 (NP)	Vineyard 10 (KE)	Vineyard 11 (KE)	Vineyard 12 (KE)	Vineyard 13 (KE)
%D***	6.61±0.46$^{AB}_{CD}$	5.11±0.55^{D}	6.46±0.63$^{BC}_{D}$	5.64±0.14^{CD}	9.84±0.37^{AB}	6.12±1.53^{CD}	7.97±0.43$^{AB}_{CD}$	5.21±0.36^{CD}	6.53±0.94$^{BC}_{D}$	8.73±0.22$^{AB}_{C}$	7.07±0.93$^{AB}_{CD}$	10.1±0.86^{A}	7.45±0.48$^{AB}_{CD}$
%C	4.15±0.28	3.24±0.31	2.89±0.1	3.27±0.33	3.87±0.1	2.83±0.56	3.28±0.32	3.22±0.48	3.37±0.35	3.67±0.11	3.21±0.34	4.09±0.14	3.89±0.48
%Pt***	8.02±0.35$^{AB}_{CD}$	6.61±0.42^{D}	8.22±0.61$^{AB}_{CD}$	7.03±0.18^{CD}	10.2±0.27^{AB}	7.6±1.18^{BCD}	9.2±0.39$^{ABC}_{D}$	6.6±0.28^{D}	8.44±0.94$^{AB}_{CD}$	10.11±0.1$^{AB}_{C}$	8.92±1.01$^{AB}_{CD}$	11.09±0.65^{A}	8.8±0.45$^{ABC}_{D}$
%Pn***	19.25±0.22$^{B}_{CD}$	21.34±1.55$^{A}_{BC}$	22.6±1.13^{AB}	21.56±0.45$^{A}_{BC}$	17.33±0.32^{D}	18.53±0.43$^{C}_{D}$	18.31±0.93$^{C}_{D}$	22.55±1.21$^{A}_{B}$	23.4±0.22^{A}	19.64±0.19$^{A}_{BCD}$	21.94±0.39$^{A}_{BC}$	19.62±0.52$^{A}_{BCD}$	19.75±0.94$^{A}_{BCD}$
%M*	61.96±1.24$^{A}_{B}$	63.7±1.81^{AB}	59.83±1.11$^{A}_{B}$	62.49±0.98$^{A}_{B}$	58.76±0.95$^{A}_{B}$	64.91±3.66^{A}	61.25±1.94$^{A}_{B}$	62.42±1.9^{AB}	58.26±2.16$^{A}_{B}$	57.85±0.3^{AB}	58.86±2.29$^{A}_{B}$	55.1±1.09^{B}	60.11±2.28$^{A}_{B}$
%Di**	23.4±0.45^{AB}	24.58±1.64$^{A}_{B}$	25.49±1.17$^{A}_{B}$	24.83±0.68$^{A}_{B}$	21.21±0.4^{B}	21.36±0.97^{B}	21.59±1.24^{B}	25.77±1.54$^{A}_{B}$	26.77±0.33^{A}	23.31±0.29$^{A}_{B}$	25.15±0.49$^{A}_{B}$	23.71±0.54$^{A}_{B}$	23.64±1.4^{AB}
%Meth**	89.23±0.72$^{A}_{BC}$	91.64±0.84^{A}	90.65±0.69$^{A}_{BC}$	91.09±0.46$^{A}_{B}$	86.29±0.47$^{B}_{C}$	91.05±2.08$^{A}_{B}$	88.75±0.73$^{A}_{BC}$	91.57±0.82^{A}	90.1±1.29$^{AB}_{C}$	87.59±0.26$^{A}_{BC}$	89.72±1.26$^{A}_{BC}$	85.81±0.9^{C}	88.66±0.95$^{A}_{BC}$

Abbreviations indicated: ***= p<0.0001; **= p<0.01; *=p<0.05; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; C= Cyanidin; Pn= Peonidin; D= Delphinidin; Pt= Petunidin; M= Malvidin; Di= Di-substituted; Meth= Methoxylated. Numerical values followed by no letter or the same letter are not significantly different.

Table D-14 Relative Abundance of Individual Flavonols and Flavonol Fractions based on Level of Substitution and Methoxylation in Pinot Noir Grape Skins among sampled OV Vineyards

	Vineyard 1 (OO)	Vineyard 2 (OO)	Vineyard 3 (OO)	Vineyard 4 (OO)	Vineyard 5 (NP)	Vineyard 6 (NP)	Vineyard 7 (NP)	Vineyard 8 (NP)	Vineyard 9 (NP)	Vineyard 10 (KE)	Vineyard 11 (KE)	Vineyard 12 (KE)	Vineyard 13 (KE)
%My***	19.31±1.22^{E} F	28.21±3.72^{C} D	47.42±1.00^{A}	21.58±1.10^{D} EF	15.45±0.61^{F}	31.43±1.01^{B} C	24.45±1.64^{C} DE	31.44±2.31^{B} C	37.36±2.37^{B}	24.96±1.52^{C} DE	27.08±0.86^{C} DE	23.52±0.90^{C} DEF	30.23±0.65^{B} C
%Qu***	59.29±1.12^{A} B	49.11±3.29^{C} DE	35.47±1.34^{F}	53.71±0.83^{A} BCD	60.26±0.82^{A}	47.18±3.10^{D} E	53.11±0.79^{A} BCD	49.97±2.17^{C} DE	42.04±1.44^{E} F	56.33±1.71^{A} BC	53.87±1.39^{A} BCD	57.61±1.56^{A} BC	50.91±0.83^{B} CD
%La**	2.86±0.22^{AB} C	4.30±0.37^{A}	3.88±0.26^{AB}	3.69±0.20^{AB} C	2.08±0.15^{C}	4.40±0.78^{A}	3.05±0.35^{AB} C	3.50±0.26^{AB} C	4.03±0.32^{AB}	2.66±0.18^{AB} C	3.12±0.38^{AB} C	2.31±0.12^{BC}	3.45±0.50^{AB} C
%Ka***	8.53±0.18^{BC}	6.94±0.77^{BC} D	3.07±0.25^{F}	7.91±0.19^{BC} D	13.71±0.43^{A}	5.55±0.61^{DE} F	9.26±0.78^{B}	3.84±0.32^{EF}	5.63±0.50^{DE}	7.90±0.40^{BC} D	6.54±0.29^{CD}	8.17±0.87^{BC}	6.26±0.15^{CD} E
%Is***	6.53±0.33^{BC}	6.27±0.41^{BC}	4.30±0.23^{C}	9.05±0.36^{A}	6.83±0.57^{AB}	5.29±0.75^{BC}	6.63±0.40^{BC}	5.90±0.35^{BC}	5.58±0.72^{BC}	5.33±0.35^{BC}	5.94±0.53^{BC}	5.99±0.27^{BC}	5.84±0.60^{BC}
%Sy***	3.47±0.26^{BC} DE	5.18±0.45^{AB} CD	5.85±0.24^{AB}	4.07±0.26^{AB} CDE	1.67±0.14^{E}	6.15±1.48^{A}	3.50±0.21^{BC} DE	5.35±0.20^{AB} C	5.35±0.45^{AB} C	2.82±0.25^{DE}	3.45±0.37^{BC} DE	2.40±0.11^{E}	3.30±0.27^{CD} E
%Tri ***	25.64±1.00^{F} G	37.69±4.24^{B} CDE	57.16±1.40^{A}	29.34±0.86^{E} F	19.20±0.66^{G}	41.99±2.79^{B} C	31.00±1.74^{D} EF	40.29±2.41^{B} CD	46.74±1.76^{B}	30.44±1.81^{E} F	33.65±1.45^{C} DEF	28.23±1.04^{E} FG	36.98±1.18^{C} DE
%Di ***	65.82±0.83^{A}	55.38±3.64^{B} CD	39.77±1.43^{E}	62.76±0.80^{A} B	67.09±0.72^{A}	52.47±2.44^{C} D	59.74±1.06^{A} BC	55.87±2.49^{B} CD	47.63±1.47^{D} E	61.66±1.78^{A} B	59.81±1.70^{A} BC	63.60±1.55^{A} B	56.75±1.07^{B} C
%Mono ***	8.53±0.18^{BC}	6.94±0.77^{BC} D	3.07±0.25^{F}	7.91±0.19^{BC} D	13.71±0.43^{A}	5.55±0.61^{DE} F	9.26±0.78^{B}	3.84±0.32^{EF}	5.63±0.50^{DE}	7.9±0.40^{BCD}	6.54±0.29^{CD}	8.17±0.87^{BC}	6.26±0.15^{CD} E
%Meth**	12.86±0.44^{A} B	15.75±0.74^{A}	14.03±0.47^{A} B	16.81±0.45^{A}	10.58±0.54^{B}	15.84±2.77^{A}	13.18±0.48^{A} B	14.75±0.27^{A} B	14.97±1.41^{A} B	10.81±0.33^{B}	12.52±0.69^{A} B	10.70±0.33^{B}	12.60±0.70^{A} B

Abbreviations indicate: ***= p<0.0001**= p<0.01; *=p<0.05; OO= Oliver-Osoyoos; NP= Naramata-Penticton; KE= Kelowna; My= Myricetin; Qu= Quercetin; La= Laricitrin; Ka= Kaempferol; Is= Isorhamnetin; Sy= Syringetin; Tri= Tri-substituted; Di= Di-substituted; Mono= Mono-substituted; Meth= Methoxylated. Numerical values followed by no letter or the same letter are not significantly different.