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Title: Enrichment Isolation of *Brettanomyces* Yeasts to Probe the Relationship Between Vineyard and Winery Populations

Abstract approved:

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Christopher D. Curtin

The budding yeast *Brettanomyces bruxellensis* has the potential to spoil fermented beverages and cause financial losses, but also contribute positively to certain products such as Lambic beer. It is most notorious for causing "Brett" spoilage of wine, which is characterized by undesirable aromas such as "bandaid" and "barnyard", therefore most research on this species has focused on understanding this impact. However, little is known about the ecology and physiological properties of this species outside of isolates obtained from within fermentation facilities. A limited number of previous studies have had mixed success enriching *B. bruxellensis* from vineyards, and there have been no comprehensive comparisons of populations from vineyards and wineries, to establish their connectivity.

In this work, enrichment culturing using a new, simplified enrichment media (*Brettanomyces* Enrichment Media - BEM) was successfully applied in the recovery of 12 isolates of *B. bruxellensis* from a vineyard in Oregon. While BEM did restrict the growth of common vineyard yeasts likely to out-compete *B. bruxellensis* in enrichment

cultures, several other yeast species infrequently described in vineyard ecology, such as *Nakazawea ishiwadae* and *Ogataea polymorpha* were recovered. Investigation into the competitive nature of these yeasts in BEM suggests that the relatively slow growth of *B. bruxellensis* relative to other vineyard yeast is the main factor hampering successful enrichment.

In parallel, whole genome sequencing was performed on 120 *B. bruxellensis* isolates from New Zealand wineries. The goal of this sequencing was to compare winery populations from different winemaking regions around the world and serve to generate baseline data against which Oregon winery and vineyard isolates could be compared. Analysis of the sequenced *B. bruxellensis* isolates revealed grouping into five distinct clades, consistent with results from other recent genome-sequencing studies. However, the relative distribution of isolates in these groups differed from previous studies, with New Zealand isolates exhibiting lower relative abundance of sulfite tolerant isolates compared to previous studies in Europe and Australia.

Future work will involve genome sequencing of the Oregon vineyard and winery isolates gathered in this study, and evaluation of their phylogenetic relatedness to one another and other winery populations of *B. bruxellensis* from other wine producing regions. Doing so may reveal the possible movement of *B. bruxellensis* from the vineyard to the winery, a relationship that has long been suspected. Such evidence could shed light upon the origins of *B. bruxellensis* infection in the winery.

Enrichment Isolation of *Brettanomyces* Yeasts to Probe the Relationship Between Vineyard and Winery Populations

by
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Kevin E. Pigao, Author

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CONTRIBUTION OF AUTHORS

Funding for work in Chapter 3 was obtained by Dr. Chris Curtin and Dr. James Osborne from the Oregon Wine Board. Dr. Osborne was also involved in the design of the work, coordination of vineyard and winery sampling. Ryan O'Donnell evaluated enrichment media formulations and performed the initial screening experiment of *Brettanomyces* species and non-*Saccharomyces* growth in BEM. Kevin Pigao performed enrichment culturing, isolation and identification of yeasts, evaluated fitness of isolates, interpreted data and prepared the manuscript.

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Dr. Chris Curtin contributed to development of bioinformatic pipelines necessary for work in Chapter 4, study design and data interpretation for both chapters, and provided assistance with manuscript and thesis editing.

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1 THESIS INTRODUCTION

Wine, made from fermented grape juice, has cultural and economic importance around the globe. Wine grapes are grown and fermented everywhere from California to France to Australia. Central to wine's success is its intoxicating effect and complex flavor and aromas. In order to minimize economic losses, winemakers must take every precaution in order to prevent spoilage, or the presence of off-flavors in wine. One of the most notorious causes of spoilage in red wines is the budding yeast *Brettanomyces bruxellensis*, often referred to colloquially as "Brett." *Brettanomyces* can produce volatile phenols, which produce an unpleasant aroma in wine. They are often associated with the descriptors "horsey," "barnyard," and "medicinal." The presence of "Brett character" almost universally leads to consumer rejection, resulting in damaged reputation and wasted wine.

A challenge of *B. bruxellensis* is that little is known about its ecology outside of fermentation environments. While it has been reliable isolated from spoiled beverages and associated production facilities it has only been isolated from outside of the facility, in the vineyard, on two occasions. It has never been isolated from an environment completely unrelated to the fermentation industry. Genomic analysis of the isolates that have been obtained from fermentation environments shows that they group into distinct clades, which tend to share oenologically relevant properties.

The most common countermeasure against *Brettanomyces* is the addition of sulfites (SO_2) to the wine. Sulfites have both antioxidant and antimicrobial properties, allowing them help prevent both chemical and biological spoilage. However, some strains of *B. bruxellensis* exhibit greater tolerance to sulfites than others. Compounding this problem is consumer

backlash to the use of sulfites, incentivizing some winemakers to minimize their use. This makes the characterization of *B. bruxellensis* isolates and their sulfite tolerance in a given winemaking region important, as it allows winemakers to make informed decisions about how much sulfites they should be using for the control *Brettanomyces*.

The following two studies address both of these questions. The first study sought to expand understanding of Brettanomyces ecology by enrichment isolation from a vineyard in Oregon over two harvest years. Twelve *B. bruxellensis* isolates were successfully enriched from Pinot Noir grape clusters. Also enriched were several uncommon fermentative yeasts which may have oenological relevance, including *Nakazawaea ishiwadae* and *Lodderomyces elongisporus*.

In the second study, whole-genome sequencing and sulfite tolerance assays were performed on a representative sampling of *B. bruxellensis* isolates from wineries in New Zealand, a region where the *B. bruxellensis* population had not previously been studied. The population structure and phenotypic properties observed were similar to those previously reported in Australia and France, with differences in relative proportions of globally-dispersed strains.

For future work, sequencing of the Oregon vineyard isolates as well as isolates from the co-located winery is planned. The data can then be compared against the results of the New Zealand sequencing data.

2 LITERATURE REVIEW

2.1 Introduction

Fermented beverages have been consumed by humans for thousands of years. The fermenting of foods can have many positive outcomes, including increased food safety (Waite & Daeschel, 2007), enhanced flavor(Swiegers & Pretorius, 2005), and intoxicating effects (Giacopassi & Stein, 1991). Wine, in particular, is one of the oldest fermented beverages (Cavalieri et al., 2003; Pretorius, 2000) and has held cultural significance from ancient times until today. Indeed, the ancient Greeks dedicated a god, Dionysus, to wine, and today wine is considered sacred in Catholicism. Wine has been produced and consumed long before humans understood the mechanism behind fermentation and that microbes were responsible for fermentation.

We now know that microbes are ingrained in almost every sensory aspect of wine, from alcohol content to flavor and mouthfeel (Cordente et al., 2012; Pretorius, 2000; Swiegers & Pretorius, 2005). Wines with pleasant sensory qualities are far more likely to be profitable than those with negative qualities, which may be rejected by the consumer (Prescott et al., 2005). A year or even a batch of poor-quality wine can spell economic ruin for a winemaker. Thus, great attention must be paid to the microbiology of wine in order to prevent good wine from becoming spoiled.

One of the most feared causes of wine spoilage is the yeast *Brettanomyces bruxellensis*. When allowed to grow in wine, *B. bruxellensis* imparts phenolic off-flavors, resulting in consumer rejection and tarnished reputations (Lattey et al., 2010). While the ecology of *B. bruxellensis* is not yet fully understood, it his frequently isolated from fermented beverages

and related production facilities. It has also been observed that different strains have different spoilage potential through different production profiles of volatile compounds (Cibrario et al., 2020). In order to control *Brettanomyces* in the winery, every aspect of *B. bruxellensis* must be understood, including its interactions with the wine, and the origins of this spoilage yeast outside of the winery.

2.2 Wine production

The typical winemaking process begins even before the grapes are harvested. Grapes are carefully monitored for sugar content, acidity, tannin content, and ripeness. When the desired level of ripeness has been reached, then harvest begins (Bindon et al., 2013). Harvested grapes are usually treated with the preservative, Sulfur dioxide (SO_2), transported to the cellar and crushed to release the juice. The exact steps following crushing vary based upon wine style (Figure 2.1). White wines are typically pressed to remove skins and seeds from grapes prior to primary fermentation. For red wines, pressing is not performed until after primary fermentation. This allows for extended contact time with the skins and greater extraction of compounds associated with color, flavor, and mouthfeel. The goal of primary fermentation is to use yeast to convert the sugars from the grape juice into ethanol. After primary ethanolic fermentation, malolactic fermentation is sometimes performed, in which malic acid is converted into lactic acid (Bauer & Dicks, 2004). This can serve to reduce the perceived sourness of a wine. It also has the added benefit of consuming some of the remaining nutrients leftover from primary fermentation, making subsequent spoilage less likely. Some wines are then aged in barrels, usually oak barrels, in order to impart flavor from the oak. For styles where this is not desirable, the wine may

be aged in stainless steel tanks. During the aging process wine is treated with SO_2 to minimize the risk of spoilage. Finally, wine is packaged before reaching the consumer, usually in bottles, but increasingly other forms of packaging, for reasons of sustainability (Barber, 2010).

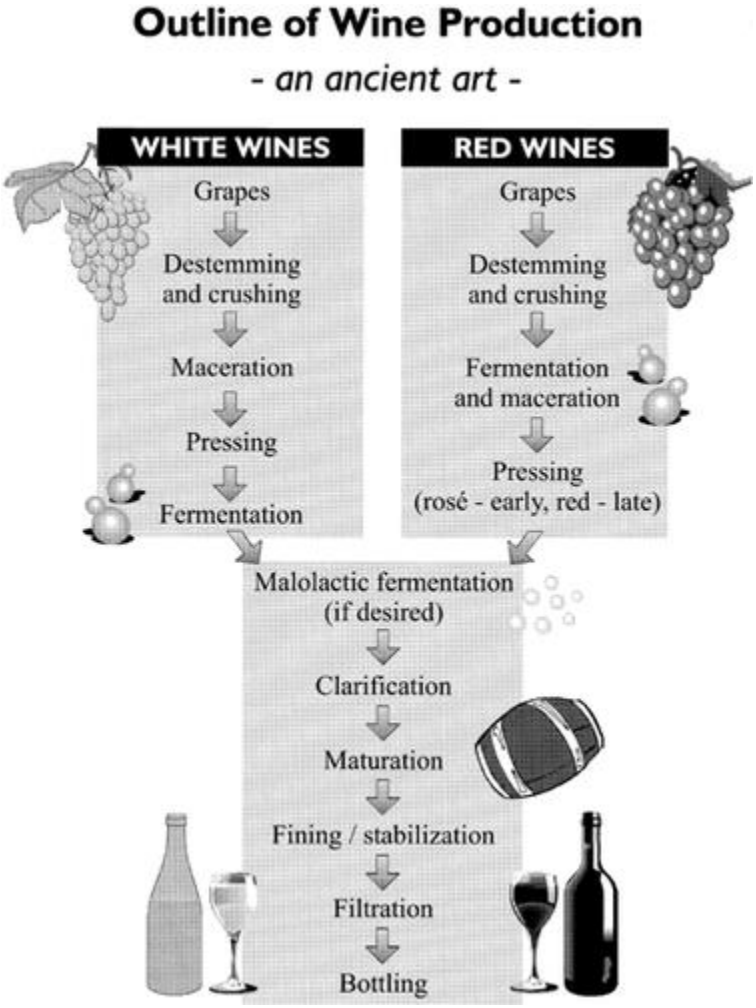

Figure 2.1. The general winemaking process (Pretorius, 2000).

A key consideration for wine packaging is controlling the ingress of oxygen, which affects the shelf-life of the wine through chemical reactions (Toit et al., 2006) and by stimulating

growth of aerobic spoilage bacteria (Bartowsky et al., 2003; Drysdale & Fleet, 1988; Toit et al., 2006).

2.3 Microbiology of winemaking

Winemaking is far more complex than previously thought. A great deal of time in modern winemaking is dedicating to manipulating microbes. Equipment is thoroughly sanitized between batches to avoid unintentional contamination of the product (Barata et al., 2013). Sulfur dioxide (SO_2), often in the form of potassium metabisulfite, is used as a chemical sterilizer and added directly to the wine both before and after fermentation to inhibit less tolerant microbe populations (Zuehlke & Edwards, 2013). Primary fermentation, driven by *Saccharomyces* converting sugar to ethanol is no longer left to chance. Winemakers now routinely inoculate grape must with pure cultures of *Saccharomyces cerevisiae* strains with desirable traits(Pretorius, 2000). However, there is a growing trend of some winemakers to return to traditional fermentation practices, where fermentation is allowed to begin spontaneously, begun by yeasts present in the vineyard and winery (Frezier & Dubourdieu, 1992; Medina et al., 2013).

Winemaking is not a sterile process, and equipment, winemakers, and the grapes themselves all harbor communities of microorganisms that can interact with wine(Barata et al., 2012; Morrison-Whittle & Goddard, 2018). The low pH and high ethanol content of wine prevent most of these organisms from growing in wine, instead favoring a select few yeasts and bacteria. Chief amongst these are yeasts of the genus *Saccharomyces*, which are the main contributors to ethanol formation. However, several other organisms play key roles in changing the flavor and composition of the wine(Cordente et al., 2012). Early in

fermentation, several non-*Saccharomyces* yeasts present, usually originating from the surface of the grapes. These are often the first yeasts to begin fermentation. *S. cerevisiae* follows in succession as higher ethanol content starts to prevent the growth of the majority of these yeasts(Gayevskiy & Goddard, 2012a). Once ethanolic fermentation has neared completion, lactic acid bacteria can convert malic acid to lactic acid and consume some of the remaining nutrients in a process known as malolactic fermentation(Bauer & Dicks, 2004). If exposed to oxygen post fermentation, oxidative yeasts or bacteria can convert ethanol to acetic acid (Bartowsky et al., 2003). In small amounts this can be acceptable. In large amounts, this can be undesirable, unless vinegar is the desired end product, as acetic acid is responsible for the distinct vinegar aroma. When stored in barrel, growth of spoilage yeast can occur (Loureiro & Malfeito-Ferreira, 2003). This can lead to various problems formation of films on the surface of the wine, production of off flavors or restarting of fermentation.

2.3.1 *Saccharomyces*

Ancient humans understood the concept of "backslopping," or adding a portion of a previous batch of wine to the current batch, in order to promote a more vigorous and successful fermentation (Gallone et al., 2016). Today we understand that yeasts are responsible for alcoholic fermentation. (Pretorius, 2000). The primary yeast genus responsible for ethanol fermentation is *Saccharomyces*. There are seven species in the genus, but not all are typically associated with the fermentation industry (Boynton & Greig, 2014). *S. cerevisiae* is the predominant yeast used in the fermentation industry and is used to make most types of fermented beverages including wines and ales (Pretorius, 2000). A

notable exception is *S. pastorianus*, a hybrid of *S. cerevisiae* and *S. eubayanus*, which is used in the production of lager beers (Hebly et al., 2015; Peris et al., 2014). *S. eubayanus*, comprising the other half of the lager lineage, has been isolated from beech forests in Patagonia, and only relatively recently. (Libkind et al., 2011). *S. uvarum* is occasionally isolated from fermented beverages, especially those that are kept at low temperature (Pulvirenti et al., 2000). *S. paradoxus*, the closest relative to S. cerevisiae, is commonly isolated from the bark of oak trees (Sniegowski et al., 2002; Tsai et al., 2008) but is not a feature in industrial processes. *S. kudriavzevii* has been isolated from soil and decaying leaves (González et al., 2006) but is also not in industrial processes.

Several interspecies hybrids of *Saccharomyces* have been identified. While the most well-known and commonly used is *S. pastorianus*, hybrids of *S. kudriavzevii* x *S. cerevisiae* and *S. kudriavzevii* x *S. cerevisiae* x *S. bayanus* have been isolated from wine, particularly in Switzerland (González et al., 2006). *S. kudriavzevii* is cryotolerant (González et al., 2006, 2007), raising the possibility that these hybrids flourished due to greater tolerance for conditions unfavorable for S. cerevisiae. Experimental evidence has shown that these hybrids do indeed ferment better than *S. cerevisiae* at temperatures below that of typical wine fermentation (González et al., 2007). Interspecies hybrids therefore present an interesting opportunity for carrying out fermentations with varied methods, possibly resulting in unique finished products.

In addition to producing ethanol, yeasts produce aroma compounds during fermentation. Many of these aroma compounds can contribute positively to the flavor of wine. *S. cerevisiae* can produce esters, which often have a fruity aroma, such as isoamyl acetate,

which has a banana-like aroma, and ethyl hexanoate which has an anise-like aroma (Saerens et al., 2008). Compounds with negative sensory impact can also be produced during fermentation Fusel alcohols, or alcohols with longer carbon chains than ethanol, are also produced by catabolism of amino acids in a pathway known as the Ehrlich pathway (Figure 2.2) (Hazelwood et al., 2008).

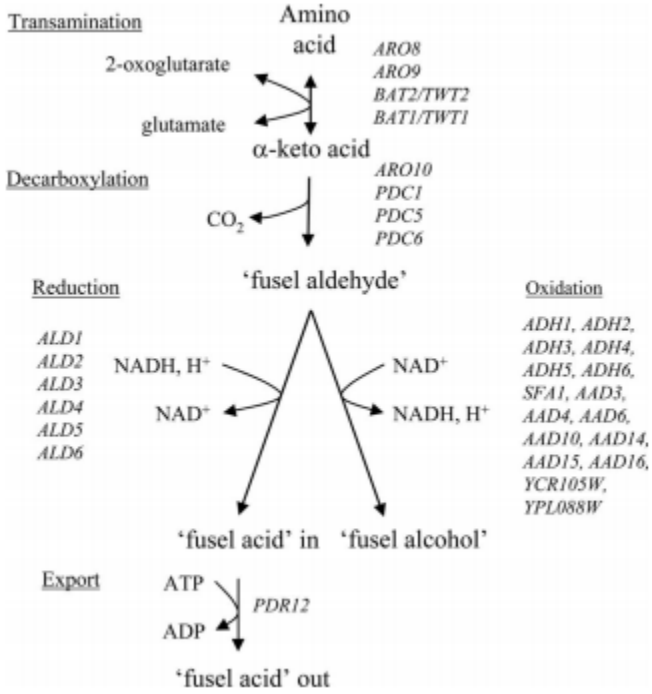

Figure 2.2. The Ehrlich pathway for production of fusel acids and fusel alcohols from amino acids in Saccharomyces and other fermentative yeasts(Hazelwood et al., 2008).

Saccharomyces can produce hydrogen sulfide (H_2S) in reductive environments (Jiranek et al., 1995), which can smell of rotten eggs (Pretorius, 2000). The balance of these compounds leads to distinct "flavor profiles" of different *S. cerevisiae* strains (Swiegers & Pretorius, 2005), sometimes referred to as "yeast bouquet" (Cordente et al., 2012). The differing flavor profiles of *S. cerevisiae* strains can lead to winemakers choosing to

inoculate with different strains depending on desired outcome. For example, only a select few *S. cerevisiae* commercial strains do not produce H_2S in Syrah juice (Kumar et al., 2010). A winemaker desiring to minimize H_2S in the flavor profile of their wine may choose one of these strains in order to achieve this effect.

Population genetics studies of *S. cerevisiae* have revealed that industrial isolates cluster according to their substrate of origin, rather than by geographical delimitation (Liti et al., 2009; Sicard & Legras, 2011), and that vineyard populations are not distinct from those isolated from wine itself (Fay & Benavides, 2005).

2.3.2 Lactic Acid and Acetic Acid Bacteria

In addition to yeasts, bacteria can also play a role in the winemaking process. Two common categories of bacteria involved in winemaking are lactic acid bacteria (LAB) and acetic acid bacteria (AAB).

After primary ethanolic fermentation, malolactic fermentation (MLF) can be carried out in wine if desired. This fermentation converts malic acid to lactic acid and is carried out by lactic acid bacteria (Sumby et al., 2019). This conversion can reduce the perceived sourness of the wine (Bartowsky & Borneman, 2011). MLF can also have the added benefit of consuming nutrients and producing antimicrobial compounds therefore creating a more hostile environment for spoilage organisms (Bauer & Dicks, 2004). The most common bacteria associated with malolactic fermentation is *Oenococcus oeni,* (Campbell-Sills et al., 2017), formerly known as *Leuconostoc oenos*. Several other bacterial species can be implicated to a lesser degree, such as *Pediococcus damnosus, Leuconostoc meseteroides* and *Lactobacillus* spp. (Bauer & Dicks, 2004).

Post fermentation, acetic acid production by acetic acid bacteria (AAB) can occur. This is almost always unintentional, unless vinegar is the desired end product. Two bacterial genera, *Acetobacter* and *Gluconoacetobacter*, are the most common AAB implicated in wine spoilage (Bartowsky et al., 2003; Drysdale & Fleet, 1988), while a third genus, *Gluconobacter*, is most commonly isolated from grapes and unfermented grape must (Bartowsky et al., 2003). Acetic acid production requires the presence of oxygens, reinforcing the importance of controlling oxygen concentration and contact in wine.

2.3.3 Non-*Saccharomyces* yeasts

Several non-Saccharomyces yeasts can be associate with wine production, particularly prior to ethanolic fermentation. The yeast-like basidiomycete fungus *Aureobasidium pullulans* is the dominant fungus on grapes prior to ripening of the grapes (Pinto et al., 2014; Vincent Renouf et al., 2005). However, as harvest approaches, fermentative ascomycete yeasts such as *Hanseniaspora uvarum*, *Metchnikowia* spp., *Pichia* spp., and *Candida* spp. become dominant on the skin of grapes (Barata et al., 2012; Gayevskiy & Goddard, 2012a).

These yeasts can participate in the early stages of fermentation and have noticeable impacts on the quality of the finished product. Well documented is the ability of *H. uvarum* to produce ethyl esters which have the ability to both enhance (Hu et al., 2018) and detract (Johnson et al., 2020) from the finished product depending on the quantity and variety of esters produced. Production of ethyl acetate, which has a solvent-like aroma, is of chief concern (Cordente et al., 2012). The majority of these non-*Saccharomyces* yeasts begin to decline in relative abundance once *S. cerevisiae* fermentation begins and ethanol content

begins to rise (Barata et al., 2012; Morrison-Whittle & Goddard, 2018). However, a select few have been found to persist well into fermentation, such as *Lachancea thermotolerans* and *Starmerella bacillaris* (Bagheri et al., 2020).

Post-fermentation, other yeasts can infect the wine and contribute to spoilage. One of the simplest forms of spoilage is the restarting of fermentation. This can lead to elevated ethanol content, decreased sugar content, and CO_2 production. The production of CO_2 at this stage can be particularly hazardous if the wine has already been packaged, leading to explosion of bottles(Loureiro & Malfeito-Ferreira, 2003). So-called film yeasts can also cause spoilage. These yeasts, mostly *Candida* spp., and *Pichia membranifaciens* get their name because they form a visible film on the surface of the wine where there is contact with oxygen(Rankine, 1966; Volleková et al., 1996). The primary fault caused by these yeasts is production of high levels of ethyl acetate, causing a harsh, solvent like aroma in wine. While tolerable at low levels, high concentrations can lead to consumer rejection.

One of the most notorious non-*Saccharomyces* yeasts responsible for wine spoilage is *Brettanomyces bruxellensis*. The potential economic damage that *B. bruxellensis* can cause has led to interest in understanding all aspects of this organism in order to better control spoilage.

2.4 Brettanomyces yeasts

The genus *Brettanomyces* contains several species that can act as spoilage organisms in a variety of foods. Yeast of this genera can grow have sensory impact in a variety of foods and beverages, including wine, beer, fruit juice, kombucha, soda, and olives (Custers, 1940; Esch, 1992; Greenwalt et al., 2000; Roach & Borneman, 2020; Rodrigues et al., 2001). The

various species of *Brettanomyces*, have been shown to have different preferences for different foods. *B. anomalus*, *B. custersianus*, and *B. nanus* have been found to primarily be located from beer (Roach & Borneman, 2020; Rodrigues et al., 2001). *B. naardensis* has primarily been isolated from soft drinks and fruit juice beverages (Esch, 1992; Roach & Borneman, 2020). Most recently, *Brettanomyces acidodurans* has been found to be a cause of spoilage in olive brine (Péter et al., 2017). In wine, the *Brettanomyces* species that causes spoilage is *B. bruxellensis*.

Brettanomyces bruxellensis is important to many areas of the fermentation industry as a source of both spoilage and flavor. *B. bruxellensis* has been observed in diverse substrates in addition to wine, such as beer, cider, kombucha, soft drinks, and fruit juice. *B. bruxellensis* has also been observed to contaminate bioethanol production facilities (Abbott & Ingledew, 2005). In wine, it is almost universally regarded as spoilage (Chatonnet et al., 1995). In some styles of beer, particularly Belgian beers such as Lambics, it can be a positive contributor to flavor (Thompson Witrick et al., 2017). It is also been shown to associate with and impact sensory qualities kombucha and soft drinks (Greenwalt et al., 2000). The primary reason for this is the ability of *Brettanomyces* to produce volatile phenols such as 4-ethylphenol (Heresztyn, 1986). These compounds create unpleasant flavor and aroma, that can often be described as “barnyard”, “cloves” and “medicinal” (A. Romano et al., 2008) . For this reason, *Brettanomyces* has the potential to cause both economic losses through spoilage and economic gain through production of desirable compounds depending on the beverage being fermented.

2.4.1 History and Taxonomy

Brettanomyces was first isolated in 1904 from beer by N.H. Claussen in Brussels (Gilliland, 1961). It was over a decade later in 1921 when *Brettanomyces* was isolated from Lambic beers and classified as B. bruxellensis, leading to the realization that it played a major role in the flavor profile of these styles (Custers, 1940). *Brettanomyces* was only isolated from wine as *Mycotorula intermedia*. (Krumbholz & Tauschanoff, 1933) . It was observed to produce ascospores in leading to the creation of the teleomorph genus *Dekkera* in 1964 (van der Walt, 1964). Based upon current conventions regarding yeast species with teleomorphic and anamorphic forms, *Brettanomyces* is the only genus name now in use. Today, six species are recognized in the *Brettanomyces* genus; *B. bruxellensis*, *B. naardensis*, *B. nanus*, *B. custersianus*, *B. anomalus*, and *B. acidodurans* (Péter et al., 2017; Shen et al., 2018).

2.4.2 Ecology

Though *B. bruxellensis* was first isolated from beer, we now realize that it can be found wherever alcohol is produced. Indeed, it has been isolated form beer, wine, cider, kombucha, and bioethanol, as well as the facilities and equipment associated with these substrates, such as vineyards, barrels, fermentation vessels and even the air circulating within the facilities (Connell et al., 2002). *B. bruxellensis* has earned a reputation for being hardy, stubborn and difficult to eradicate from any environment that it inhabits. The physiology of *B. bruxellensis* seems to suggest a transient yeast, that is ubiquitous in these environments in low numbers, only proliferating when favorable conditions are found. This is evidenced by numerous adaptations (described in section 2.4.3), that suggest that *B.*

bruxellensis has evolved to opportunistically grow after ethanolic fermentation by *Saccharomyces* has occurred.

2.4.3 Physiology

Brettanomyces bruxellensis is an ascomycetous budding yeast. It is capable of fermenting arabinose and cellobiose to ethanol, which *S. cerevisiae* is incapable of (Reis et al., 2014). It has a tolerance of low pH, being able to grow in pH 3.5 and above, with some instance reported of as low as pH 3.0 (Conterno et al., 2006). Wine strains of *B. bruxellensis* have a maximum ethanol tolerance of 14-14.5% ethanol v/v in red wine (Wedral et al., 2010). Compared to yeast that inhabit similar substrates, *B. bruxellensis* grows remarkably slowly. Colonies take an average of 3-5 days to appear on rich media. This causes it to be present in low number at the start of fermentation, and only increase in numbers once S. cerevisiae starts to decline(Wedral et al., 2010) .

2.4.3.1 Carbon Metabolism

B. bruxellensis, like *S. cerevisiae*, is able to utilize carbon sources both in aerobic and anaerobic conditions. It prefers to grow on monosaccharides such as glucose, fructose, and galactose, but is also capable of growing on the disaccharides sucrose, maltose, cellobiose and trehalose (Conterno et al., 2006), albeit with some strain-level variation. For example, beer isolates are more commonly able to utilize maltose effectively (Colomer et al., 2020). This suggests that beer isolates have adapted to the maltose rich environment of the brewery. Also unlike *S. cerevisiae*, *B. bruxellensis* exhibits ß-glucosidase activity, which allows it to utilize cellobiose (Colomer et al., 2020; Crauwels et al., 2015; Crauwels et al., 2014). Cellobiose is common in wood, suggesting that *B. bruxellensis* may have adapted

to growth in wooden barrels post-fermentation, where other fermentative carbon sources become scarce.

Similar to *Saccharomyces, Brettanomyces* is able to ferment sugars to ethanol and carbon dioxide to obtain energy, though at a slower rate than what is typically observed in *Saccharomyces* fermentations (Ciani et al., 2003)). Also similar to *S. cerevisiae*, *B. bruxellensis* exhibits the "Crabtree effect", where fermentation of sugars is preferred over respiration in the presence of oxygen (Rozpędowska et al., 2011). While anaerobic fermentation is less efficient than aerobic respiration, it has the added benefit of ethanol production, which can help to inhibit competing microbes (De Deken, 1966; Pfeiffer and Morley, 2014). A distinctive feature of *B. bruxellensis* is the "Custer's effect", where ethanol production is inhibited during transition from aerobic to anaerobic conditions (Agnolucci et al., 2017; Wijsman et al., 1984).

Brettanomyces is also able to convert ethanol to acetic acid in aerobic conditions through an oxidative pathway to obtain additional energy (Uscanga et al., 2003; Freer, 2002; Wijsman et al., 1984). The ability to use ethanol as a carbon source may also be an adaptation to grow in carbohydrate poor beverages post *S. cerevisiae* fermentation (Conterno et al., 2006).

2.4.3.2 Nitrogen Utilization

An aspect of *B. bruxellensis* metabolism that sets it apart from *S. cerevisiae* is its nitrogen utilization. Like *S. cerevisiae*, *B. bruxellensis* is able to use ammonium as a nitrogen source (Conterno et al., 2006). However, unlike *S. cerevisiae*, *B. bruxellensis* is able to use nitrate as a sole nitrogen source (Borneman et al., 2014; Conterno et al., 2006; Crauwels et al.,

2014). Interestingly, it has been observed that isolates from beer are less able to utilize nitrates. (Colomer et al., 2020; Crauwels et al., 2014; de Barros Pita et al., 2011)

B. bruxellensis has also been shown to have efficient utilization of amino acids (Parente et al., 2018), suggesting that it has adapted to use nitrogen sources left over after fermentation by *S. cerevisiae* (Oelofse et al., 2016) . This provides further evidence of *B. bruxellensis* being specialized to use nutrients present after *S. cerevisiae* has completed its life cycle, as autolysis of *S. cerevisiae* releases amino acids into the wine (Burattini et al., 2008).

2.4.3.3 Stress Tolerances

While slow-growing, B. bruxellensis displays stress-tolerance attributes that enable it to out-compete most other yeast species found in the ecology of winemaking. In particular, its tolerance to low pH and high alcohol is notable. This allows *B. bruxellensis* to survive in wine, where pH is commonly 3.0-3.5 and ethanol content can approach 15%. *B. bruxellensis* has been shown to grow in laboratory media with up to 14.5-15% ethanol v/v (Cibrario et al., 2019; Dias et al., 2003). Some isolates even show improved growth in the presence of some (5-10% v/v) ethanol (Cibrario et al., 2019). This extreme tolerance allows it to grow in the majority of wines even when *S. cerevisiae* fermentation has fully completed. This ethanol tolerance is greater than most other fermentative yeasts which may be encountered in wineries or the vineyard (Fleet, 1990; Renouf et al., 2006). Interestingly, recent studies of *B. bruxellensis* isolated from various substrates show that there is no significant difference in ethanol or pH tolerance between groups (Cibrario et al., 2019). However, there are some outliers among the wine strains which are more tolerant to pH than isolates from other substrates (Cibrario et al., 2019).

B. bruxellensis is also considered to be tolerant to sulfites, the common antioxidant and antimicrobial agent used in the wine industry. This attribute, however, is highly variable (Conterno et al., 2006) Recent studies have demonstrated sulfite-tolerance to be genotype-dependent (Curtin et al., 2012). Curtin et al. (2012) found that the most tolerant clades could withstand 0.5 mg/l of free SO_2. The less tolerant clades could only withstand SO_2 levels of 0.25 mg/l. Later investigation by Avramova et al (2018) concurred with this difference by clade (Avramova, Vallet-Courbin, et al., 2018).

The mechanism of sulfite tolerance is understood in *S. cerevisiae*, whereby a sulfite efflux pump encoded by the gene *SSU1* pump sulfites out of the cell (Avram & Bakalinsky, 1997; Park & Bakalinsky, 2000). While not fully elucidated in *B. bruxellensis*, there is evidence that different isoforms of *B. bruxellensis* Ssu1p impart differing degrees of sulfite-tolerance when expressed in a *S. cerevisiae* wine strain (Varela et al., 2019).

2.4.4 Metabolic by-products and flavor impacts

2.4.4.1 Volatile phenols

B. bruxellensis is able to produce volatile phenols, namely 4-ethylphenol and 4-ethylguiacol (Suárez et al., 2007). It does this by converting hydroxycinnamic acids such as ferulic acid and p-coumaric acid to the 4-vinyl form via hydroxycinnamate decarboxylase and then to the 4-ethyl form via vinylphenol reductase (Figure 2.3). (Heresztyn, 1986; Suárez et al., 2007). Because hydroxycinnamates are toxic to some microorganisms, it is thought to do this in order to detoxify the surrounding environment (Edlin et al., 1998; Valdetara et al., 2017). Hydroxycinnamate decarboxylase and vinylphenol reductase (VPR) are thought to be encoded in the genes DbCD and DbVPR

respectively (Curtin et al., 2012; Valdetara et al., 2017). Granato et al. (2015) isolated a protein from *B. bruxellensis* which appeared to have VPR activity (Granato et al., 2015) and further experiments cloning and expressing the gene responsible for this protein into *S. cerevisiae* suggested that it does indeed have vinylphenol reductase activity (D. Romano et al., 2017).

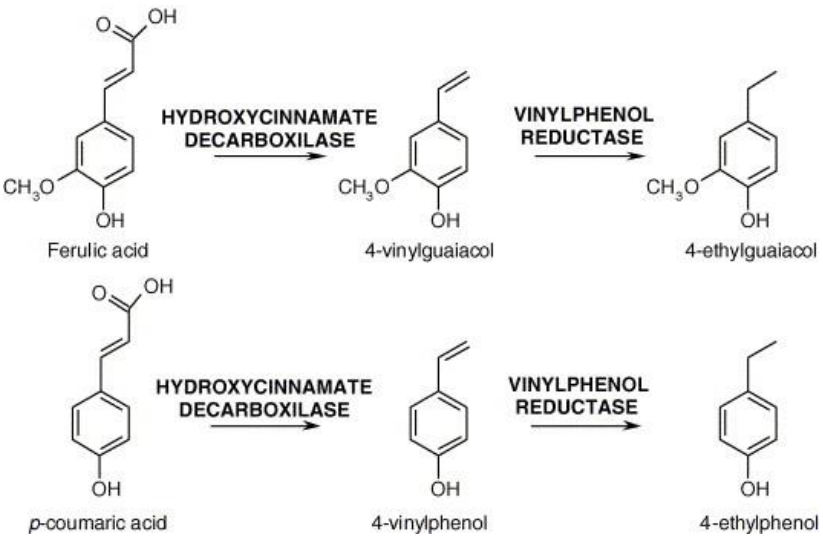

Figure 2.3. Production pathway of volatile phenols by *B. bruxellensis* (Suárez et al., 2007)

The effect of volatile phenols is often referred to as Phenolic Off Flavor (POF), and yeast that produce them can be referred to as "POF positive". Descriptors associated with POF include "barnyard", "cloves" and "medicinal" (Heresztyn, 1986; Holt et al., 2018; Romano et al., 2017; Suárez et al., 2007; Wedral et al., 2010). In wine and most beer styles, these flavors are almost universally considered spoilage. In some beers, particularly Lambic beers and some barrel age beers, these flavors can be considered a positive (Crauwels et al., 2015; Colomer et al., 2019).

While vinylphenols and ethylphenols are both produced, ethylphenols have a lower sensory threshold and therefore greater impact on wine than vinylphenols. In addition to vinylphenols being reduced to ethylphenols, in red wine they can bind to pigmented compounds, leaving them sensorially inert (Morata et al., 2007). Consequently, there is little vinylphenol content in red wine, and phenolic off-flavors are wholly due to presence of ethylphenols.

Some strains of *S. cerevisiae* are capable of producing volatile phenols, and are even used for this reason in certain styles of beer, such as German Hefeweizen beers (Holt et al., 2018). These yeasts can become a problem if present in primary fermentation. However, *B. bruxellensis* poses a unique threat compared to POF positive *S. cerevisiae*, as *B. bruxellensis* can produce volatile phenols long after primary fermentation, during the aging of the wine (Wedral et al., 2010). This constant threat of volatile phenol production even after successful primary fermentation makes *Brettanomyces* the most dangerous threat in terms of volatile phenol production in wine. While some bacteria and the yeast *Meyerozyma guillermndii* have been shown to produce ethylphenols in laboratory settings, this production has not been shown to be relevant to wine (Romano et al., 2009) and is why volatile phenols are often considered evidence of clear *B. bruxellensis* infection in wine. (Kheir et al., 2013).

2.4.4.2 Volatile acids

B. bruxellensis can produce a host of volatile acids. While acetic acid is the main concern during wine making, isovaleric acid can also have sensory impacts. Acetic acid results in

a distinctive vinegar aroma. Isovaleric acid has been described as “rancid” (Romano et al., 2008).

Acetic acid is produced by oxidation of acetaldehyde, which is itself produced by oxidation of ethanol or (Freer, 2002), producing additional energy for the cell. Because of this, wine is at greatest risk of acetic acid spoilage when exposed to high levels of oxygen. Acetic acid begins to be detrimental to the flavor profile of wine once concentration rises above 1.2-1.4g/l and may lead to consumer rejection (Drysdale & Fleet, 1988). Acetic Acid production by *Brettanomyces* has been well documented (Uscanga et al., 2003; Freer, 2002; Romano et al., 2008; Wedral et al., 2010). *B. bruxellensis* does have the ability to produce acetic acid at concentrations greater than the sensory detection threshold (Uscanga et al., 2003; Freer, 2002). However, it requires large amounts of oxygen to produce appreciable amounts of acetic acid, much more oxygen than is usually found in wine (Freer, 2002; Freer et al., 2003). There is some strain level variability in B. bruxellensis acetic acid production (Freer, 2002). However, all strains produce less acetic acid than AAB and AAB can produce acetic acid in the presence of much smaller quantities of oxygen, such as the small amount that enters a bottle through the cork (Bartowsky et al., 2003). This slow ingress of oxygen and slow but sustained production of acetic acid is more likely to result in acetic acid spoilage than B. bruxellensis.

Another volatile acid produced by *B. bruxellensis* with sensory significance is Isovaleric acid. Isovaleric acid, also referred to as 3-Methylbutanoic acid, produces a “rancid” or “cheesy” aroma (Cordente et al., 2012; Henick-Kling et al., 2000) which can be perceived negatively in wine. Isovaleric acid is produced via the Ehrlic pathway during metabolism

of leucine (Hazelwood et al., 2008). Leucine is transaminated to α-ketoisocaproate, which is then decarboxylated to the fusel aldehyde isoamyladehyde. Isoamlyaldehyde can then be oxidized to isoamyl alcohol or reduced to isovaleric acid (Hazelwood et al., 2008).

Isovaleric acid may be quite important to the perception of "Brett Character". Studies have shown that ethylphenols without the presence of isovaleric acid, even when the ethylphenols were well above the sensory threshold, did not always lead to consumers identifying the aroma as "Brett Character" (Licker et al., 1998; Romano et al., 2009). This can be important, especially in beer, when Brett character is desirable (Thompson Witrick et al., 2017)

2.4.4.3 Mousy Off-Flavor

"Mousy" off flavor is another fault in wine associated with *Brettanomyces* as well as lactic acid bacteria (LAB) (Costello & Henschke, 2002; Grbin & Henschke, 2000). The flavor has been described as mouse-urine or mouse like. mousiness is almost always considered a detriment to the beverage, hence the term mouse-taint, and no technique currently exists to remove this flavor from wine once present (Snowdon et al., 2006). Mousiness can be attributed to the production of N-heterocyclic compounds from the metabolism of ornithine and lysine (Bartowsky, 2009), including 2-acetyltetrahydropyridine (ATHP), 2-acetylpyrroline (APY), and 2-ethyltetrahydropyridine (ETHP). (Grbin & Henschke, 2000; Snowdon et al., 2006)

While there is evidence that *B. bruxellensis* can produce the compounds associated with mousiness, some argue that LAB such as *Oenococcus oeni* and *Lactobacillus* spp. are primarily responsible for mouse taint in wine. ATHP has been detected in previously

sterilized wines inoculated with B. bruxellensis, confirming that this yeast can produce the compounds associated with mouse-taint (Romano et al., 2008). While *Brettanomyces* has been shown to produce APY as well, the levels that it produces are not of sensory significance in wine (Snowdon et al., 2006). LAB on the other hand, can produce detectable quantities of APY and produce ATHP and ETHP at far greater quantities (Costello & Henschke, 2002; Snowdon et al., 2006). The evidence suggests that *B. bruxellensis* is only a minor source of mouse taint when compared to LAB.

2.4.4.4 Esters

B. bruxellensis can produce esters during its growth and metabolism. While ester production of *B. bruxellensis* is much lower than that of *S. cerevisiae* (Romano et al., 2009), it can still have a noticeable impact on fermented products (Thompson Witrick et al., 2017). Since esters are often pleasant and fruity or floral, they are desirable compounds when brewing with *B. bruxellensis.* (Colomer et al., 2019; Thompson Witrick et al., 2017) *B. bruxellensis* can also produce the ester ethyl acetate (Curtin et al., 2013), which in large quantities can be perceived negatively as a harsh, solvent aroma (Cordente et al., 2012). Notably, it has been shown that the ester production profile of *B. bruxellensis* can differ significantly between strains (Curtin et al., 2013), and some have argued that positive contributions can be made by *B. bruxellensis* through ester production in infected wine if the concentration of volatile phenols is not above the sensory threshold (Joseph et al., 2017). However, 4-ethylphenol can overshadow these differences, meaning there is little practical value of *Brettanomyces* derived esters in wines (Curtin et al., 2013).

In addition to producing esters, *B. bruxellensis* can also degrade esters previously produced by *S. cerevisiae* in primary fermentation (Curtin et al., 2013; Spaepen & Verachtert, 1982). In particular, *B. bruxellensis* has been shown to contain three homologs of isoamyl acetate hydrolase *IAH1* (Curtin et al., 2012), which encodes a protein that degrades isoamyl acetate, an ester with a distinctive banana aroma, and phenylethyl acetate which imparts "honey" and "rose" aromas (Cordente et al., 2012). *B. bruxellensis* was shown to decrease concentrations of both acetate esters in model wine (Curtin et al., 2013) The ability to degrade desirable esters in its substrate demonstrates another mechanism by which *B. bruxellensis* may be detrimental to the flavor and aroma of beverages..

2.4.5 Genetic diversity and population structure

The most comprehensive population genetics study performed to date on *B. bruxellensis* utilized microsatellite PCR markers and profiled 1488 isolates from around the world and from different substrates (Avramova et al., 2018). Phylogenetic analysis suggested that *B. bruxellensis* can be divided into six distinct clades (Figure 2.4), which appear to group by substrate of isolation (Avramova et al., 2018). There are three distinct wine groups, of which two are triploid and one is diploid. The beer isolates tend to group together and present as triploid or aneuploid based upon allelic variation of markers. Kombucha isolates tend to group together and present as diploid, and tequila/ethanol isolates tend to group together as diploid or triploid strains (Avramova et al., 2018).

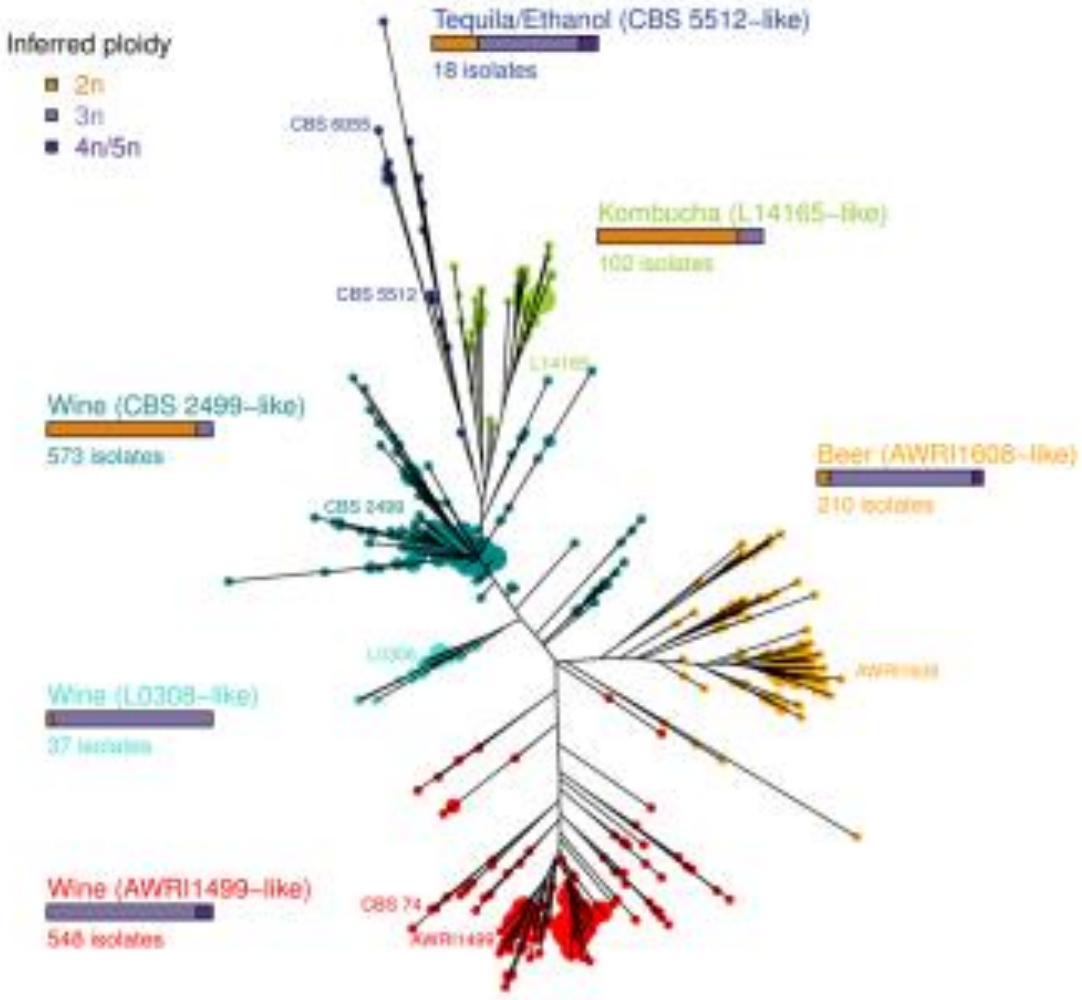

Figure 2.4, Dendrogram of *B. bruxellensis* isolates using microsatellite markers. (Avramova et al., 2018)

Whole Genome Sequencing of *B. bruxellensis* provides further context, and deeper understanding of *B. bruxellensis* population structure. The first whole genome assembly of *B. bruxellensis* strain AWRI1499 was published in 2012 (Curtin et al., 2012). Assembled in its native triploid state, the AWRI1499 haploid-equivalent genome was 12.7 Mb long. Subsequent assemblies of diploid (Piškur et al., 2012; Tiukova et al., 2019) and haploid (Roach & Borneman, 2020) isolates were similar in length and gene content, and the Roach et al. (2020) and Tiukova et al. (2019) resolved into similar numbers of scaffolds. Tiukova

made use of optical mapping to resolve the assembly of CBS11270 into 4 chromosomes. Interestingly, substantial karyotype variation within the species was observed by Piskur et al. (2009), with individual *B. bruxellensis* strains apparently harboring between 3 and 9 chromosomes. This may be explained by variation in ploidy noted amongst sequenced strains, and evident in the microsatellite-PCR based phylogeny. This difference in ploidy helps to explain some of the strain grouping. Some phenotypes do correlate with ploidy, with triploid state being associated with greater SO_2 tolerance (Avramova, Vallet-Courbin, et al., 2018; Curtin & Pretorius, 2014). There is some speculation that the extra copies of chromosomes resulted from hybridization with an as of yet undiscovered species in the *Brettanomyces* genus (Curtin et al., 2012).

Studies of the Brettanomyces population has shown that these strain groups appear in different proportions, with the AWRI1499-like group representing the largest proportion of winery isolates in studies conducted in both Europe and Australia (Avramova et al., 2018; Curtin et al., 2007). However, isolates from beer appear to show low occurrence of AWRI1499-like strains, suggesting that these populations are distinct. Further analysis of beer isolates suggest that they do cluster into a distinct group (Avramova et al., 2018; Colomer et al., 2020), with some exceptions where wine-like isolates appear in beer. (Colomer et al., 2020). This could be because wine barrels discarded by wineries are sometimes used by breweries to house barrel aged beers.

2.5 Environmental Isolation and Enrichment of Yeasts

When attempting to isolate a microbe from the environment two factors need to be taken into consideration. First is culturability, meaning is it known under what conditions the

microbe can be grown in the laboratory. B. bruxellensis has been well characterized and is able to be cultured routinely in the laboratory. Second is the prevalence of the target microbe relative to other microbes in the environment. If the CFU count of the target is lower in relative abundance than other organisms, then direct plating may not be capable of detecting the target organism, even if culturable. In this case enrichment techniques must be used. Enrichment is the process of using selective media to increase the number of cells of a target organism in order to bring it up beyond the threshold of detection. However, the drawback of this technique is that it does not allow for quantification of initial microbe levels

2.5.1 *Saccharomyces* isolation

For a long time, the origins of *S. cerevisiae* were unclear. While S. cerevisiae has been proven to be associated with fermented beverages since 1863 (Barnett, 1998), its habitat outside of industrial fermentation was only discovered relatively recently. It has since been found to associate with trees, oak galls, and rotting fruit (Bowles & Lachance, 1983). Prior to 1996 there was debate as to whether *S. cerevisiae* was indeed present in the vineyard (Török et al., 1996). Further studies revealed that while present in low numbers on intact grapes, *S. cerevisiae* in much more common on damaged grapes where the juice and sugar have been exposed (Mortimer & Polsinelli, 1999). More recent metagenomic surveys have detected *S. cerevisiae* in the vineyard, albeit rarely (Bokulich et al., 2014; Morrison-Whittle & Goddard, 2018; Taylor et al., 2014), further suggesting that *S. cerevisiae* is only present in low numbers outside of production environments.

Because of these low numbers, enrichment methods were needed. Much of the work done regarding yeast enrichment has been inspired by previous work on *S. cerevisiae* enrichment. *S. cerevisiae* has been successfully enriched in high sugar media from natural environments including tree sap, soil near oak trees, oak galls, and rotting fruit (Bowles & Lachance, 1983). However, these oak isolates are genetically distinct from wine isolates (Zhang et al., 2010) in fact, wine isolates cluster narrowly into their own group separate from isolates from beer and other beverages, suggesting that human wine fermentation has resulted in adaptation of *S. cerevisiae* for this manmade condition (Fay & Benavides, 2005).

2.5.2 Brettanomyces Isolation

Brettanomyces has been consistently isolated from wineries, breweries, and other fermentation environments in addition to the beverages. However, it has not been consistently isolated from non-fermentation environments. It is commonly believed that this is due to low cell counts in these environments, necessitating enrichment. Initial attempts to enrich the yeasts from within the winery resulted in the development of a specialized media, *Dekkera/Brettanomyces* Differential Medium (DBDM), which sought to isolate Brettanomyces from within the winery (Rodrigues et al., 2001). This media relied upon the addition of p-coumaric acid, which *Brettanomyces* would convert into 4ethylphenol, producing a distinctive smell, and bromescol green, which would turn yellow due to acid production by *B. bruxellensis*. The medium also contains cycloheximide to inhibit the growth of *S. cerevisiae* and other cycloheximide sensitive yeast. DBDM media has been successfully used to isolate *B. bruxellensis* from wine barrels (Barata et al., 2013).

In order to enrich from the vineyard, another media, Enrichment *Brettanomyces bruxellensis* (EBB), was developed (Vincent Renouf & Lonvaud-Funel, 2007). This media was specially developed for use with grapes and contains grape juice to serve as the primary carbon source. It also contains Tween 80 to serve as a surfactant to wash the yeast cells from the surface of the grapes

Using EBB, *Brettanomyces* has been enriched from vineyards in Italy and France from the skins of red wine grapes (Oro et al., 2019; Vincent Renouf & Lonvaud-Funel, 2007). In the French study, *B. bruxellensis* was detected using culture independent PCR methods (Vincent Renouf & Lonvaud-Funel, 2007). The later Italian study successfully isolated pure cultures of B. bruxellensis (Oro et al., 2019). In this instance, fifteen *B. bruxellensis* isolates were isolated from vineyards within the same region of Italy. *B. bruxellensis* was also isolated from various locations within associated wineries. These isolates were then compared via the use of RAPD-PCR. This comparison found isolates from the winery and vineyard contained the same strain groupings, suggesting that vineyard isolates and winery isolates were not distinct and separate populations (Oro et al., 2019). Similar results were found for a small sample set using microsatellite-PCR fingerprinting (Albertin et al., 2014).

2.6 Objectives

The research objectives of this thesis are:

1. To isolate *Brettanomyces* yeasts from vineyards in Oregon,

2. To investigate the properties of other yeasts enriched alongside *B. bruxellensis* in order to guide future improvement of enrichment methodology, and

3. To evaluate population structure of *B. bruxellensis* from New Zealand wineries and use this dataset for comparison to Oregon populations.

Chapter 3 focuses on the enrichment of *B. bruxellensis* from grape clusters and evaluation of competitiveness of other yeasts which may be present in the vineyard.

Chapter 4 describes the whole genome sequencing and sulfite tolerance assays of *B. bruxellensis* isolates from New Zealand wineries, and the use of these datasets to compare the New Zealand *B. bruxellensis* population to those previously described in Australian and French studies.

3. ENRICHMENT OF *BRETTANOMYCES* AND OTHER NON-*SACCHAROMYCES* FERMENTATIVE YEASTS FROM VINEYARD SAMPLES IN OREGON

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3.1 Introduction

Brettanomyces bruxellensis is a species of yeast that has long been associated with fermented foods. While almost universally considered a spoilage organism in wine (Curtin et al., 2015; Loureiro & Malfeito-Ferreira, 2003), it can also make positive contributions to some specialty beer styles (Thompson Witrick et al., 2017; Vanderhaegen et al., 2003) and kombucha (Greenwalt et al., 2000). This ability to help and hinder production of various beverages has made *B. bruxellensis* an organism of great interest to the fermentation industry. The primary significance of *Brettanomyces* yeasts in fermented beverage production is their production of volatile phenols (Heresztyn, 1986), such as 4-ethylphenol and 4-ethylguiacol, often described as "barnyard", "cloves" and "medicinal" (A. Romano et al., 2008) which are considered spoilage aromas in wine (Chatonnet et al., 1995) and most beer styles (Shimotsu et al., 2015). These off flavors can lead to consumer rejection of the products and result in economic losses.

With regards to wine production, *B. bruxellensis* is not a major contributor during alcoholic fermentation. It is frequently encountered in wine undergoing maturation (Fleet, 2003) and on winemaking equipment (Curtin et al., 2015; Oro et al., 2019; Rodrigues et al., 2001), and has been even been detected in air circulating within a winery (Connell et al., 2002). The combination of relatively high tolerance for ethanol (Wedral et al., 2010) and acidity (Cibrario et al., 2020), along with slow growth rates (Henick-Kling et al., 2000; Wedral et al., 2010), help to explain the association of *B. bruxellensis* with finished wine.

Previous attempts to isolate *B. bruxellensis* from the vineyard have taken some of these traits into consideration, in order to develop enrichment media (Vincent Renouf &

Lonvaud-Funel, 2007). The relatively few studies that describe attempts at enrichment of *Brettanomyces* from vineyard samples met with mixed success (Garijo et al., 2015; Oro et al., 2019). Efforts to isolate *Brettanomyces* without an enrichment step have failed, even when using semi-selective solid media (Garijo et al., 2015). These studies have suggested that *B. bruxellensis* recovery from the vineyard is possible but difficult, likely because it is present in low numbers relative to other yeasts (Oro et al., 2019; Vincent Renouf & Lonvaud-Funel, 2007).

Grape berries harbor complex communities of microbes. Early in berry development, basidiomycete yeasts and fungi are dominant, but as harvest approaches, fermentative Ascomycete yeast begin to dominate (Barata et al., 2012; Gayevskiy & Goddard, 2012b). *Saccharomyces* is the most notable alcoholic fermenter, but other non-*Saccharomyces* yeasts can be present in large quantities and persist into fermentation (Morrison-Whittle & Goddard, 2018). After ethanol concentration increases, these yeasts are typically replaced by *Saccharomyces* (Fleet, 2003). Renouf & Lonvaud-Funel (2007) explored whether the Basidiomycetes *Aureobasidium pullulan* and *Cryptococcus*, the most dominant fungi on berries during the early stages of their development, could interfere with *Brettanomyces* enrichment. They did not consider the impact of ascomycetes, despite detecting DNA of several species in enrichments of ripe grape berries. The results of Garijo et al. (2015) underscored that genera such as *Hanseniaspora* and *Candida*, prevalent on mature grape berries, are frequently recovered in vineyard sample enrichment cultures targeting *Brettanomyces*.

In this study we used enrichment culturing to isolate *Brettanomyces bruxellensis* from vineyards located in Oregon, USA. In addition, we assessed the capacity of non-*Saccharomyces* yeasts to interfere with recovery of *B. bruxellensis* during enrichment. Our results emphasize the difficulty in isolating this slow-growing yeast from vineyard samples, while also highlighting the potential for enrichment to facilitate isolation of other rare vineyard genera such as *Nakazawea, Kazachstania, Ogataea* and *Lodderomyces*.

3.2 Materials and Methods

3.2.1 Chemicals

Unless specified, all reagents were obtained from Sigma-Aldrich (St. Louis, USA).

3.2.2 Yeast isolates and propagation

Reference yeast strains and yeasts isolated in this study are listed in Supplementary Table S3.1. All isolates were cryopreserved in Yeast Extract Peptone Dextrose (YPD) broth (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose) containing 15% glycerol. Isolates retrieved from cryogenic storage were grown on YPD agar (15g/l agar, Bioplus, Altamonte Springs, USA) incubated at 30ºC, then single colonies were picked and transferred into YPD broth in 15ml ventilated centrifuge tubes (Techno Plastic Products, Trasadingen, Switzerland) incubated at 30ºC on orbital shaking platform at 150rpm until stationary phase was achieved.

3.2.3 Vineyard sampling

Grapes clusters were sampled in 2018 and 2019, from a single vineyard in the central Willamette Valley, Oregon, USA, on the same day fruit was to be commercially harvested.

A sterile 710ml sample bag (Whirl-pak WPB01020WA, Nasco Sampling, Fort Atkinson, USA) was placed around each cluster to be sampled. Shears sanitized with 70% ethanol were used to cut the vine as close as possible to the top of the cluster without touching the grape berries. Care was taken not to damage grape berries during sampling or transport. Harvested clusters were immediately transported (<2hrs) to Oregon State University and stored at 4ºC until processed (within 24hrs).

3.2.4 Grape washing

In order to determine the optimal method for recovering yeast from grape clusters, berries were removed from a subset of ripe Pinot Noir clusters aseptically and randomized. They were then placed into sterile sample bags (Whirl-pak WPB01020WA, Nasco Sampling, Fort Atkinson, USA) at a rate of 15g and 45g per bag, with an equal volume of wash solution comprising sterile peptone water (0.1% v/v) with or without Tween 80 (0.2% v/v). Triplicate bags were sonicated in a bath sonicator (Kendal model HB-S-36DHT) for varying times (0, 30, 60, 120 sec). After processing, 100 µl of wash solution was plated onto YPD agar media and incubated at 30°C. Based upon results of these tests, subsequent grape cluster samples were washed using an equal volume of sterile peptone water (0.1% v/v) with Tween 80 (0.2% v/v) by sonication for 30 sec.

3.2.5 Enrichment and isolation of yeasts from grape clusters

Brettanomyces enrichment media (BEM) was based on Enrichment *Brettanomyces bruxellensis* (EBB) broth (Vincent Renouf & Lonvaud-Funel, 2007) with modifications. BEM contained 4% (v/v) ethanol, 40 g/l glucose, 1.5 g/l malt extract, 1.5 g/l yeast extract, 0.5 g/l Ammonium sulfate, 0.2 g/l Magnesium sulfate, and 0.1% (v/v) Tween 80, pH was

adjusted to 3.5 with orthophosphoric acid. Ethanol and antibiotics were added after autoclaving at 121ºC for 15 minutes.

Following sonication of grape clusters (or glass beads inoculated with varying population sizes of *B. bruxellensis* Y78), wash fluid was aseptically transferred into a sterile 50ml centrifuge tube (VWR, Radnor, USA) and centrifuged for 5 minutes at 3900 RPM. The resultant pellet was re-suspended in 15ml of BEM media and transferred into a 15ml culture tube, which was then sealed and placed into an incubator at 27°C. At 14 days and also at 2-months, 100 µl of enrichment culture was spread plated onto YPD agar media containing 10 mg/l cycloheximide, 10 mg/l chloramphenicol, and 150 mg/l biphenyl. Plates were then incubated at 30°C for seven days and checked daily for growth. Representatives of all observed colony and cell morphologies were streaked for isolation on YPD and incubated again at 30°C until isolated colonies were visible. Isolates were then prepared for cryogenic storage.

Glass beads were inoculated with *B. bruxellensis* Y78 by pipetting 100 µl of culture diluted to the relevant concentration in 0.1% peptone water. Bags were then sealed and shaken by hand. Samples were incubated at room temperature for 15 minutes before being processed identically to grape samples. Calculations were made as to equivalent grape mass based upon relative density of marbles and grapes.

3.2.6 Yeast isolate identification

YPD broth cultures of all isolated yeasts were utilized for DNA extraction. Briefly, 1 ml of culture in 1.5ml microcentrifuge tubes (Argos Technologies EW-22999-00, Vernon Hills, USA) was centrifuged at 13,000 g (Eppendorf 5424), and the resultant pellet

extracted using the Gentra Puregene YeastBact kit (Qiagen, Hilden, Germany). The internal transcribed spacer (ITS) regions flanking the 5.8S rDNA gene were amplified by polymerase chain reaction (PCR) as described by (Guillamón et al., 1998), using 2x Econotaq plus green (Lucigen, Middleton, USA) and products run on 2% TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) agarose gels, using 6x Gel-red loading dye (supplier). Agarose gels were visualized under UV light and photographed Biorad Gel Doc XR+ gel imager (Biorad, Hercules, USA) and PCR product size compared with a known control, Y78 (AWRI1499).

For PCR products consistent in size with *B. bruxellensis*, RFLP was performed by digestion with HaeIII, CfoI and HinFI restriction enzymes (ThermoFisher, Waltham, USA), and all products were electrophoresed and visualized as described above. Positive identification of isolates as *B. bruxellensis* was performed by comparison of fragment sizes against a known control Y78, and those described in Guillamón *et al.* (1998).

All other PCR products werer purified using the EZ-10 Spin Column PCR Products Purification Kit (Bio Basic, Markham, Canada) and submitted to the Oregon State University Center for Genome Research and Biocomputing (OSU CGRB) for Sanger sequencing. A BLAST search of the resulting sequences was performed against the NCBI non-redundant nucleic acid database and the species top-hit used to assign identity for each isolate.

3.2.7 Relative growth rates of *Brettanomyces* and other yeasts in BEM

Stationary phase YPD starter cultures were adjusted to the same cell concentration based upon haemocytometer counts to determine cell concentration. Once cell concentration was

determined, cultures were diluted to $1x10^7$ cells/ml in 0.1% peptone water, and then diluted 1:100 to $1x10^5$ cells/ml in BEM to a final volume of 200µl. Plates were sealed with permeable Breathe-Easy membranes (BEM-1, Diversified Biotech, Boston, USA) then incubated at 30°C. During the first growth rate experiment plates were manually loaded into a Spectramax M2 spectrophotometer (Molecular Devices, San Jose, USA) and optical density at 600nm (OD_{600}) was measured periodically over 4 days. In subsequent experiments, a single plate was incubated at 30°C in the spectrophotometer and OD_{600} recorded hourly for 130 hours. OD_{600} readings were analyzed in R Studio (RStudio Team 2020) using the Growthcurver package (Sprouffske & Wagner, 2016) to estimate maximum specific growth rate and growth delay, defined as the time at which the population density reaches half of the maximum concentration.

3.2.8 Head-to-head fitness in BEM

For a subset of isolates, relative fitness in BEM media was assessed using head-to-head competition assays. Briefly, triplicate 15ml centrifuge tubes containing BEM were co-inoculated with stationary phase YPD cultures of *B. bruxellensis* Y78 and one of; *Kazachstania aerobia* Y429, *K. marxianus* Y2, *Nakazawaea ishiwadae* Y446, or *P. membranifacens* Y69. These experiments were designed to simulate low numbers of yeast recovered from grape clusters, therefore *B. bruxellensis, K. aerobia*, and *N. ishiwadae* were inoculated at $1x10^3$ cells/ml. Controls were triplicate BEM tubes containing each yeast alone at $1x10^3$ cells/ml. All BEM tubes were incubated at 27µC., and enumerated at 0, 7, 14, days by spread-plating 100 µl of relevant dilutions (in 0.1% peptone water) onto Wallerstein Laboratory (WL) media, which allowed for distinction of *B. bruxellensis*

colonies from those of other yeasts based upon morphology, pigmentation, and colony size. Plates were incubated at 30°C and checked daily for growth for seven days.

3.2.9 Competitive growth spot plates

To evaluate negative growth interactions between *B. bruxellensis* and other yeasts recovered from BEM enrichments, a solid-media co-culture experiment was performed. Briefly, 1 ml of stationary phase YPD *B. bruxellensis* Y78 culture was diluted to span the range from 10^2 to 10^6 CFU/ml and spread on separate WL plates to create a lawn. Three spots of the assayed yeasts, *N. ishiwadae* Y446 and *K. aerobia* Y429 were dropped on each plate by pipetting 1µl of yeast culture at dilutions varying from 10^2 to 10^6 CFU/ml. After inoculation, plates were incubated at 30ºC for 5 days and checked for growth. Once a clear lawn of *Brettanomyces* was evident, zones of inhibition around "spots" of assayed yeast were evaluated.

3.2.10 Statistical analyses

Statistical analyses were performed in R using R studio and R studio cloud. Growth curves were calculated and fitted using growthcurver and analyzed through ANOVA. Pairwise comparisons were analyzed using Tukey HSD. Figures were created using ggplot2.

3.3 Results

3.3.1 Evaluation of yeast growth characteristics in BEM

Representative isolates from the *Brettanomyces* genus, along with selected vineyard/fermentation-relevant yeast species, were inoculated into YPD and BEM, and their relative growth characteristics quantified (Figure 3.1). As expected, all yeast isolates assayed were able to grow in YPD broth (Figure 3.1A and 3.1B). BEM was selective against some species, as evidenced by the failure of *S. cerevisiae*, *T. delbrueckii* and *H. uvarum* to grow (Figure 3.1C and 3.1D). Importantly, all *Brettanomyces* isolates grew in BEM, however they did exhibit some variation in growth rate and growth delay between them. *B. bruxellensis* Y1, a commercial beer isolate, grew significantly faster in BEM than *B. bruxellensis* Y16, Y78 and Y8, all wine isolates ($p<0.0043$). On the other hand, *B. bruxellensis* Y1 displayed a longer growth delay than both Y78 and Y8 ($p<0.0182$). Of the non-*Saccharomyces* yeasts able to grow in BEM, the most likely to outcompete *Brettanomyces* species appeared to be *K. marxianus*. It was able to grow significantly faster than three of the *B. bruxellensis* isolates (Y16, Y78, and Y8) and the *B. custersianus* isolate Y52 ($p< 0.0041$). Indeed, no isolate of any species assayed exhibited a growth rate that was significantly greater than *K. marxianus* ($P=0.1068$). Additionally, *K. marxianus* displayed a significantly shorter growth delay than all other isolates ($p<0.0001$).

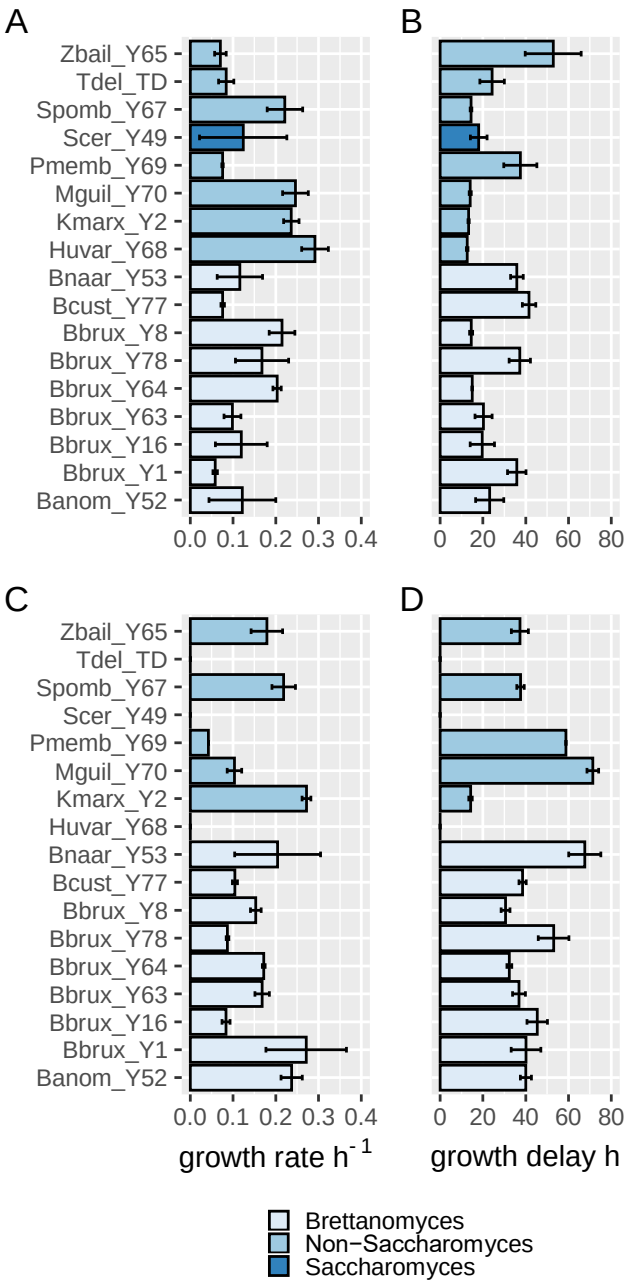

Figure 3.1. Growth rate and growth delay of *Brettanomyces* and oenologically-relevant non-*Saccharomyces* species in BEM and YPD media. Fitted growth curves generated from triplicate wells of 96-well microtiter plates were used to estimate parameters, and error bars represent standard deviation of these estimates. Growth rate of isolates in YPD (A), Growth delay of isolates in YPD (B), Growth rate of isolates in BEM (C), and Growth delay of isolates in BEM (D).

3.3.2 Head-to-head fitness of select non-*Saccharomyces* yeasts and *B. bruxellensis* in BEM enrichments

Based upon relative growth characteristics in BEM, the potential risk of non-*Saccharomyces* yeasts outcompeting *B. bruxellensis* during BEM enrichment was evaluated by performing head-to-head growth experiments in mixed culture. The relatively fast-growing *K. marxianus* was chosen to represent potential high-risk, and *P. membranifaciens* was used to confirm that it was of low risk to interfere with *Brettanomyces* enrichment. Results depicted in Figure 3.2 support these assessments. When inoculated by itself in BEM, *P. membranifaciens* culturable population size decreased at 7- and 14-days, below the limit of detection. Consequently, when *P. membranifaciens* was co-inoculated with *B. bruxellensis*, at 7- and 14-days *B. bruxellensis* was the only yeast recovered.

Despite apparent differences in growth rates estimated from 96-well plate growth curves, the population sizes of *B. bruxellensis* and *K. marxianus* did not differ at 7-days ($p=0.37$) or 14-days ($p=0.59$) in this experiment during BEM monoculture. A competitive advantage for *K. marxianus* was, nevertheless, revealed when both *K. marxianus* and *B. bruxellensis* were co-inoculated. *B. bruxellensis* colonies were only observable for a single replicate at day 7. *K. marxianus* was also dominant at day 14 ($p=0.039$), although *B. bruxellensis* colonies were recovered from all three replicates, and for one replicate *B. bruxellensis* and *K. marxianus* were recovered in nearly equal numbers.

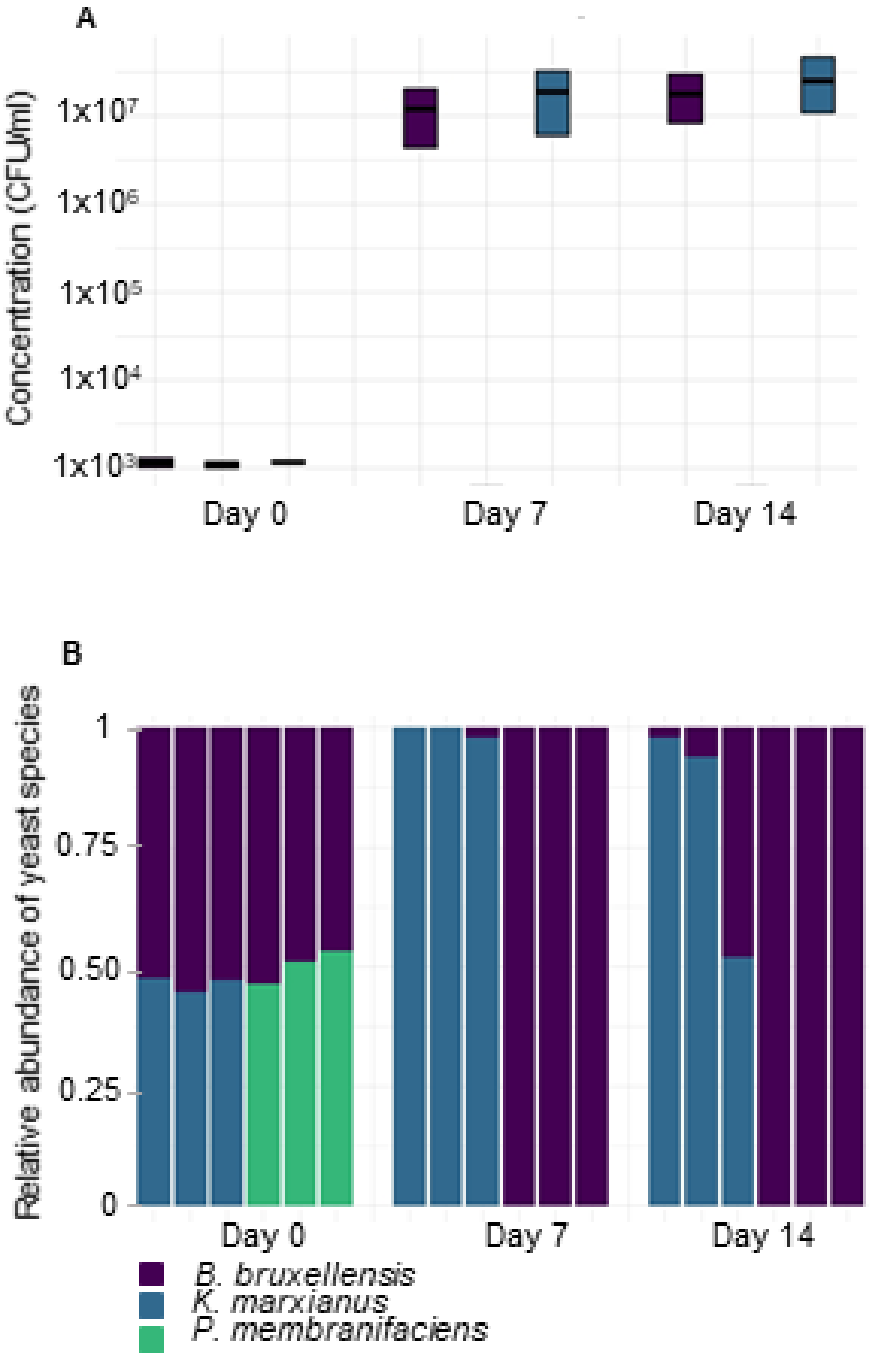

Figure 3.2. Head-to-head fitness in BEM media. *B. bruxellensis* was co-inoculated with *K. marxianus* or P. *membranifaciens* and incubated alongside single-culture controls. CFU/ml and relative abundance were determined by plating on WL media. Cell concentration of control single cultures at each sampling point (A), Relative abundance of each yeast in mixed culture (B).

3.3.3 Optimization of method to recover yeast from grape berries

A preliminary experiment was performed to evaluate the use of sonication and surfactant (Tween 80) to wash epiphytic yeast off of grape clusters. Compared to gentle orbital shaking for long periods (Vincent Renouf & Lonvaud-Funel, 2007), short-term sonication and collection of washed-off microbes in supernatant is more scalable for processing of many samples . However, direct enumeration of wash fluid on YPD agar showed no significant difference in mean number of colonies recovered for variations in grape-washing method (ANOVA p=0.99, data not shown). The two replicates from which highest colony counts were recovered had been sonicated for 30 seconds in peptone water that contained surfactant. While recovery was not significantly greater than other methods, as our aim was to recover *Brettanomyces* potentially present on grapes at very low numbers, this method was utilized for the remainder of the study.

3.3.4 Enrichment of vineyard grape cluster samples

Prior to processing grape samples, we evaluated the sensitivity of our enrichment protocol using glass marbles as a proxy for grapes. Bags of marbles that had been inoculated with *B. bruxellensis* (mock samples) were subjected to the same washing and enrichment protocol as used for grape clusters. As shown in Table 3.1, recovery was achieved from all replicates at $5x10^2$ CFU/100g.

All uninoculated samples and those inoculated at $5x10^1$ CFU/100g and below showed no recovery. A single sample out of three replicates showed recovery at $1x10^2$ CFU/100g. Mock samples were also processed contemporaneously with vineyard samples, yielding similar results (data not shown). Recovery of *B. bruxellensis* colonies on YPD plates was only possible from mock samples inoculated with $1x10^3$ CFU/100g.

Table 3.1. Recovery of *B. bruxellensis* from mock samples (inoculated glass marbles)

Inoculation Rate[a]	**Recovery from enriched samples**[b]
Uninoculated	0 of 3 (0%)
$1x10^1$CFU/100g	0 of 3 (0%)
$5x10^1$CFU/100g	0 of 3 (0%)
$1x10^2$CFU/100g	1 of 3 (33%)
$5x10^2$CFU/100g	3 of 3 (100%)
$1x10^3$CFU/100g	3 of 3 (100%)

[a]100g of glass marbles were inoculated with *B. bruxellensis* strain OSCL-Y0078 to simulate varied levels of bunch contamination

[b]Confirmed *B. bruxellensis* colonies observed on YPD plates following 14-day enrichment in modified EBB.

Sampling in 2018 resulted in isolation of *B. bruxellensis* from 19% of the grape clusters enriched (Table 3.2.). Several other yeast species were also recovered. In each case, only a single colony morphotype was observed on spread-plates of BEM enrichments, suggesting the 14-day enrichment protocol selected for a single dominant yeast species from the communities present on each cluster. Notably, *N. holstii* was recovered from a large number of samples, while *K. aerobia* and *Ogataea* species were also prominent.

During the 2019 harvest season, enrichments did not result in isolation of *B. bruxellensis* (0/83 samples) and yeast were recovered from fewer samples. While there was some overlap in the yeast species recovered through enrichment, in 2019 the predominant yeast species was *K. aerobia*.

Repeat plating of the enrichments from both years after 2-months of incubation did not result in any additional samples from which *Brettanomyces* could be recovered (data not shown).

Table 3.2. Recovery of yeast from vineyard enrichment.

Yeast	**Number Isolated 2018**	**Number Isolated 2019[a]**
Brettanomyces bruxellensis	12	0
Rhodotorula mucilaginosa	1	0
Kazachstania aerobia	3	13
Ogataea polymorpha	3	0
Ogataea parapolymorpha	1	0
Lodderomyces elongisporus	1	1
Nakazawaea holstii	9	1
Nakazawaea ishiwadae	0	1
Hanseniaspora uvarum	0	1
Hanseniaspora valbynenesis	0	1
Unidentified[b]	2	1
Total Number of Samples	64	85
% samples yeast recovered	50%	20.5%
% samples *B. bruxellensis* recovered	18.8%	0%

[a]Multiple isolates of the same species from same enrichment tube omitted
[b]Unidentified yeasts did not produce conclusive ITS sequences

3.3.5 Evaluation of vineyard yeast isolate growth characteristics in BEM

Representative isolates from species recovered during vineyard enrichments were inoculated alongside control yeasts into BEM in a 96-well microtiter plate, and their relative growth characteristics were quantified (Figure 3.3). Control yeasts *T. delbrueckii* and *P. membranifaciens* were unable to grow within the time period of the experiment, while *M. guilliermondii* displayed an extended growth delay. These results were largely consistent with previous experiments. Amongst the vineyard *B. bruxellensis* isolates there was no significant difference in growth rate ($p=0.94$), however, there were significant differences in growth delay. *B. bruxellensis* Y344 showed a significantly greater growth delay than the other vineyard isolates Y350, and Y333 ($p<0.0001$). *B. bruxellensis* Y344

and Y343 also displayed a significantly greater growth delay than *O. polymorpha* ($p=0.018$) and *N. ishiwadae*, ($p=0.010$) with Y344 further showing a greater growth delay than *K. aerobia* ($p=0.003$), *N. holstii* ($p=0.0002$), and *L. elongisporus* ($p=0.003$). Results also confirmed that some vineyard isolates of *Hanseniaspora* are capable of growing in BEM media, despite earlier results showing an inability of *H. uvarum* to grow. In fact, the *H. valbynenesis* isolate showed a significantly greater growth rate than all four vineyard *B. bruxellensis* isolates assayed ($p=0.03$), and a shorter growth delay than two of the isolates, Y344 ($p<0.00001$) and Y343 ($p=.0002$). When considered as a group, vineyard *B. bruxellensis* exhibited a slower growth rate ($p=0.003$) and greater growth delay ($p=0.018$) than the other vineyard isolates. Overall, these results suggest that when other non-*Saccharomyces* yeasts are present in enriched vineyard samples, they may pose a significant threat of outcompeting *B. bruxellensis* and hampering recovery.

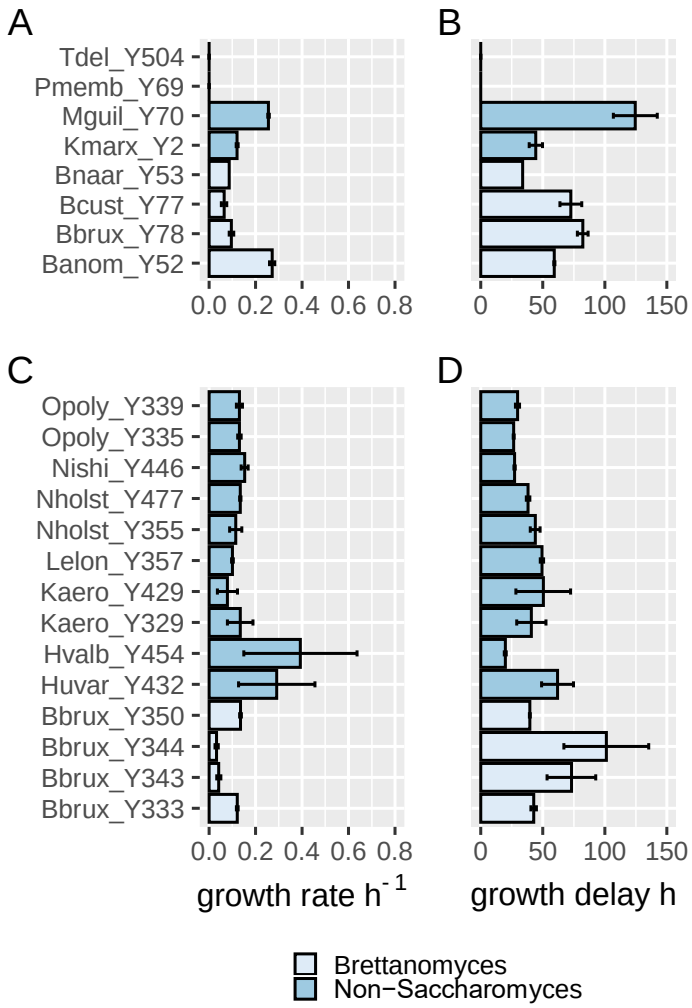

Figure 3.3. Growth rate and delay of vineyard yeast isolates and representative control yeasts in BEM media. Each isolate grown in triplicate wells of 96-well microtiter plates. Growth monitored by measuring optical density. Parameters estimated from fitted growth curves. Growth rate of control isolates in BEM (A). Growth delay of control isolates in BEM (B). Growth rate of vineyard isolates in BEM (C). Growth delay of vineyard isolates in BEM(D).

3.3.6 Head-to-head fitness of select vineyard non-*Saccharomyces* yeasts and *B. bruxellensis* in BEM enrichments

In order to assess the ability of vineyard non-Saccharomyces yeast isolates to interfere with *B. bruxellensis* recovery from BEM enrichments, head-to-head growth experiments in mixed culture were carried out (Figure 3.4). *N. ishiwadae* was chosen to represent the genus *Nakazawaea*, as it exhibited shorter growth delay than *N. holstii* and has recently been shown to persist into fermentation and possibly have wine quality impacts (Ruiz et al., 2019). *K. aerobia* was chosen due to its prevalence in the second year of sampling when we failed to recover *B. bruxellensis* from enrichments. When inoculated in mixed culture, *N. ishiwadae* was dominant over *B. bruxellensis* at day seven ($p<0.0001$), with recovery only observed in a single sample, and also at day-14 ($p=0.005$), therefore severely inhibited the recovery of *B. bruxellensis* compared to the monoculture control. *K. aerobia* dominated initially at day 7 ($p= 0.002$), despite having a lower CFU in the control culture relative to the *B. bruxellensis* control. However, at day 14, *K. aerobia* was below the threshold of detection in mixed culture. The *K. aerobia* control culture showed a drop in population size at this time point, with one control culture showing less than $1x10^4$ CFU/ml. This suggests that there may be some interaction between *B. bruxellensis* and *K. aerobia* beyond nutrient competition.

As a first step to explore the nature of possible interactions, *N. ishiwadae* and *K. aerobia* were spot-inoculated in the presence of *B. bruxellensis* lawns, and incubated for 7 days. No zones of inhibition were observed around the *N. ishiwadae* or *K. aerobia* spots (Appendix 1), suggesting an absence of direct antagonism against *B. bruxellensis*.

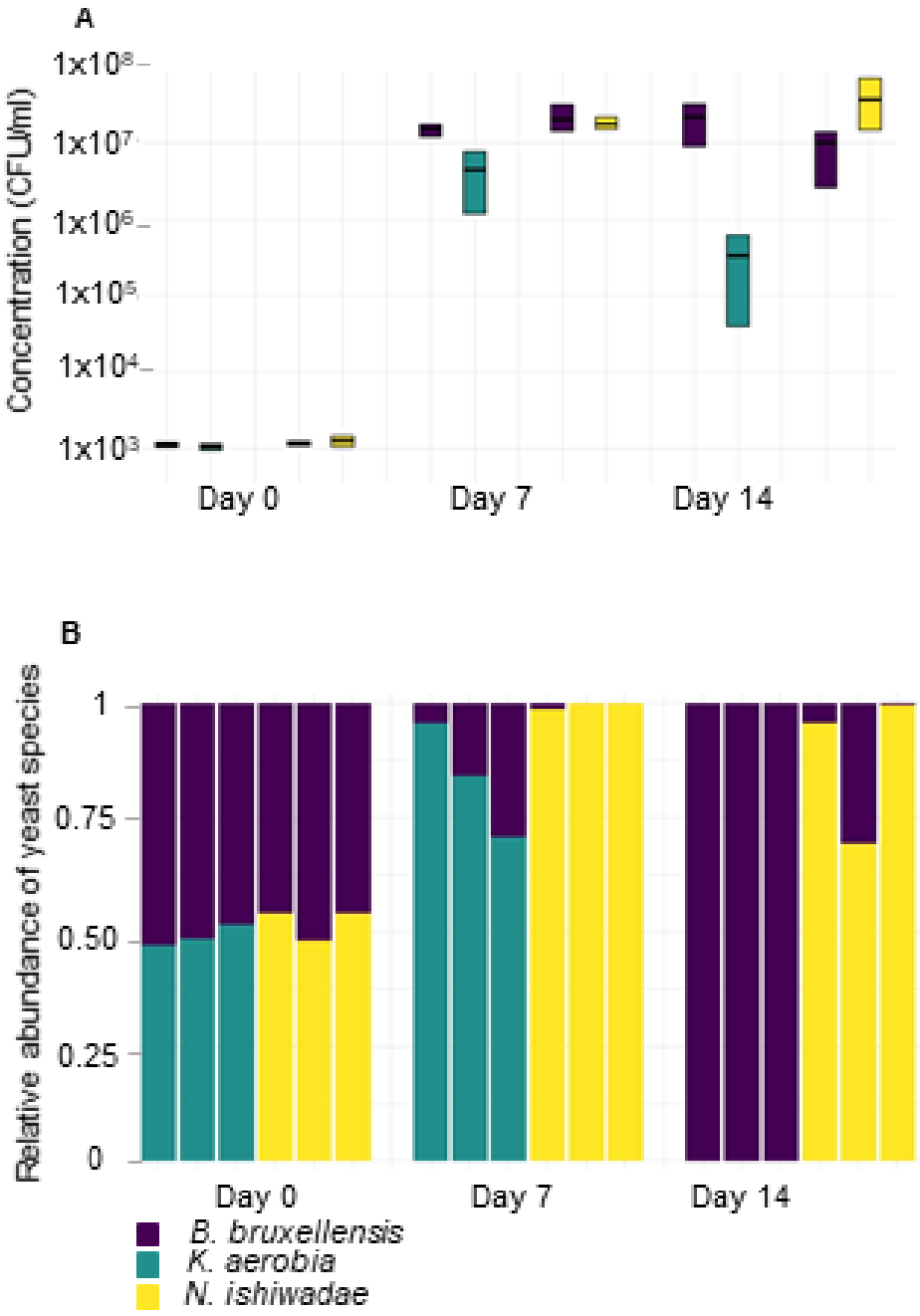

Figure 3.4. Head-to-Head fitness in BEM media. *B. bruxellensis* co inoculated with *K. aerobia* and *N. ishiwadae* cultures and controls in BEM media**.** and incubated alongside single-culture controls. CFU/ml and relative abundance were determined by plating on WL media. Cell concentration of control single cultures at each sampling point (A), Relative abundance of each yeast in mixed culture (B).

3.4 Discussion

Brettanomyces yeasts are a ubiquitous problem in wineries across the world, and there is growing evidence that *B. bruxellensis* wine strains are globally dispersed (Avramova, Cibrario, et al., 2018; Curtin et al., 2007)similar to *Saccharomyces cerevisiae* wine/European strains (Liti et al., 2009). However, there has been little success isolating *Brettanomyces* from the vineyard, despite this being one of the obvious ecological niches that connects wine producing regions. Is this simply a matter of inadequate sampling? Or does it reflect a relatively low prevalence of *Brettanomyces* in the vineyard, where large populations of faster-growing non-Saccharomyces yeasts are frequently encountered? Here, we used an enrichment protocol to isolate *B. bruxellensis* from a vineyard in Oregon and evaluated their relative fitness alongside oenologically-relevant/vineyard-isolated non-*Saccharomyces* yeasts in enrichment media. Our results provide a basis for development of improved methods for environmental recovery of *Brettanomyces* yeasts, while also showing that other rare vineyard yeast genera of potential interest to the wine industry can be recovered.

3.4.1 BEM is suitable for *Brettanomyces* enrichment culturing

All previously successful attempts to detect *Brettanomyces* from French (Vincent Renouf & Lonvaud-Funel, 2007) and isolate *Brettanomyces* from Italian (Oro et al., 2019) vineyards have required an enrichment step, using media that incorporates diluted grape-juice, despite a lack of evidence that this complex and compositionally variable ingredient is necessary. Indeed, other researchers previously utilized a minimal media for enrichment culturing (Rodrigues et al., 2001). We adapted elements of both in a simplified medium, BEM, that provides selectivity through low pH (3.5), moderate ethanol content (4% v/v),

and the inclusion of cycloheximide. Preliminary experiments confirmed that all *Brettanomyces* species grow in BEM, while *Saccharomyces* and some common non-*Saccharomyces* vineyard yeasts could not. Initial screening included several yeasts which were thought to be relevant to fermentation and potentially interferants in enrichment. Other species of *Brettanomyces, B. custersianus*, *B naardensis*, and *B. anomalus*, were included in order to capture the breadth of behavior of the genus *Brettanomyces*. *H. uvarum*, *M. guillermondii*, and *P. membranifaciens* were chosen as they are commonly found in vineyard settings (Hall et al., 2019; Lin et al., 2020). *S. cerevisiae*, *T. delbrueckii*, *S. pombe*, and *Z. bailii* were included due to their relevance to wine fermentation (Gallander, 1977; Schuller et al., 2000; Patricia Taillandier et al., 2014). *K. marxianus* was chosen to represent the *Kluyveromyces*/*Lachanthea* genus, as several members of this genus have been detected in wine (Kapsopoulou et al., 2005; Xufre et al., 2006).

All assayed species and isolates of *B. bruxellensis* were capable of growth in BEM media. In fact, some isolates showed faster growth rate in BEM than YPD. This may be because *B. bruxellensis* has adapted to grow in the presence of other fermentative yeasts, and therefore grows more optimally in the presence of some ethanol (Cibrario et al., 2020). The most successful yeast in BEM was *K. marxianus*, showing the shortest growth delay of any isolate. While *K. marxianus* is capable of growth in wine (Xufre et al., 2006) and may have properties beneficial to winemakers (Kourkoutas et al., 2004; Rollero et al., 2018), it is not commonly found on the skins of grapes (Fonseca et al., 2008)and is therefore unlikely to pose a significant problem during grape-berry enrichments. BEM showed the ability to exclude *S. cerevisiae*, *T. delbrueckii*, and *H. uvarum*. The exclusion of *S. cerevisiae* is most likely due to its intolerance of cycloheximide (Coursen & Sisler, 1960). Renouf et al.

(2007) did, however, detect *S. cerevisiae* through the use of PCR in enrichment (Vincent Renouf & Lonvaud-Funel, 2007). Notably, their EBB formulation did not include cycloheximide.

P. membranifaciens and *K. marxianus* were chosen to represent slow-growing and fast-growing isolates in head-to-head fitness assays with *B. bruxellensis* in BEM. While *P. membranifaciens* grew poorly in BEM compared to *B. bruxellensis,* some strains of *P. membranifaciens* can produce PMKT and PMKT2, killer toxins which are inhibitory towards *B. bruxellensis* (Belda et al., 2017; Santos et al., 2009), potentially affecting recovery. The results indicate that growth rate in BEM was the more important factor in determining whether a yeast could inhibit recovery of *B. bruxellensis*.

3.4.2 Recovery of *Brettanomyces* from vineyard grape cluster samples

Overall, we were able to isolate *B. bruxellensis* from enrichment cultures of 12 of 149 grape clusters (18.8% of year one samples, 8% of total samples), sampled over two consecutive harvest seasons. Studies of French vineyards did not report the percentage of grapes from which *B. bruxellensis* was recovered (Vincent Renouf & Lonvaud-Funel, 2007). Enrichment from Italian vineyards resulted in recovery from 8 of 26 samples (30.8%) (Oro et al., 2019).There are several differences between this study and previous studies which could possible account for our lower recovery rate. This study processed grapes by first washing them and then pelletizing and resuspending in enrichment medium. The previous studies directly incubated grapes in the enrichment medium (Oro et al., 2019; Vincent Renouf & Lonvaud-Funel, 2007), which has been suggested to increase recovery rate (Vincent Renouf & Lonvaud-Funel, 2007), although at a cost in terms of materials and physical space required for enrichments. Moreover, both previous studies investigated

wine grape varieties other than Pinot Noir (Oro et al., 2019; Vincent Renouf & Lonvaud-Funel, 2007). The possibility exists that the prevalence of *B. bruxellensis* may differ between grape varieties or geographic location. More attempts at enrichment from more varieties of grapes and locations would be necessary to elucidate this.

When *S. cerevisiae* has been recovered from vineyards, the estimated abundance on grapes has varied, estimated to only be present rarely (Martini et al., 1996) and in low numbers, 10-100 CFU/g on intact grapes (Fleet, 2003) but present in greater numbers, 100 000 to 1 000 000 CFU per berry on damaged grapes (Mortimer & Polsinelli, 1999). Our method requiring an initial *B. bruxellensis* population of 5-6 CFU/g of berries (based upon mock sample recovery rates) for successful recovery, and we harvested undamaged clusters. Thus the size of *Brettanomyces* populations (when present) in the vineyard may be similar to that of *Saccharomyces*, but we can speculate they may be less prevalent overall. Indeed other data supports this assertion. Genomics-based studies of vineyard fungal communities have detected *S. cerevisiae* on grape skins, albeit rarely (Bokulich et al., 2014; Morrison-Whittle & Goddard, 2018; Taylor et al., 2014), but the same studies do not describe presence of *B. bruxellensis* DNA. Further work must be done to determine whether *B. bruxellensis* is more common on damaged grapes, which may enhance the success of efforts to isolate this species from the vineyard.

3.4.3 Recovery of non-*Saccharomyces* yeasts from vineyard grape cluster samples

During enrichment culturing for *Brettanomyces*, several yeast species were isolated. These mostly belonged to non-*Saccharomyces* genera that are infrequently described as belonging to the grape berry community, with the exception *H. uvarum* and *H. valbynenesis*. Both were evidently capable of growth in BEM, despite previous

experiments suggesting that it could exclude *H. uvarum* at the very least. It is notable that, however, that while *Hansenispora* is amongst the most prevalent yeasts on grape skins (Barata et al., 2012; Gayevskiy & Goddard, 2012b; Jolly et al., 2003), we only recovered this genera from 2/149 samples. More investigation would be necessary to determine whether or not the two vineyard *Hanseniaspora* isolates behave differently the *H. uvarum* strain used during preliminary experiments. Their recovery may simply reflect their presence in positive samples at very high population sizes, despite the media being unfavorable for the growth of this species.

Amongst the rare vineyard yeasts, *N. ishiwadae, L. elongisporus*, and *K. aerobia* have all been shown to have high tolerance to ethanol and the pH tolerance necessary to ferment and grow in wine (Jood et al., 2017; Ruiz et al., 2019), which may explain their recovery from BEM enrichments. Interestingly, *Ogataea* spp. were isolated from the vineyard. In whole-genome phylogenies, *Ogataea* is the amongst the genera related to *Brettanomyces* (Shen et al., 2018), but does not display several genomic adaptations found in niche partners *B. bruxellensis* and *S. cerevisiae* (Curtin et al., 2012)

Because we isolated other yeasts more frequently than *B. bruxellensis* and did not isolate any *Brettanomyces* yeasts in the second year of sampling, we wondered to what extent this might reflect their relative fitness in BEM. Relative growth experiments in BEM suggest that *B. bruxellensis* is slower growing in this medium compared to the other yeast isolated. This is consistent with a long history of *B. bruxellensis* being described as a slow growing yeast (Custers, 1940). Nevertheless, head-to-head fitness experiments suggested that even when *B. bruxellensis* is in the presence of some faster growing yeasts, it may eventually become dominant if given enough time in a favorable environment. Consistent with this,

enrichment culturing by Oro et al. (2019) yielded only a single *B. bruxellensis* positive sample at 10 days, and 8 positive samples after 80 days (Oro et al., 2019). While our repeat-plating did not enhance recovery, further optimization of enrichment methods could focus on duration of incubation as a factor.

3.4.4 Winemaking relevance of recovered non-Saccharomyces yeasts

The role of non-*Saccharomyces* yeasts during wine fermentation has been well documented (Johnson et al., 2020; Jood et al., 2017; Ruiz et al., 2019; van Wyk et al., 2020), at least for the species that participate in uninoculated fermentations at population sizes likely to have an impact. The species we recovered have not received as much attention, nevertheless some have been shown in other studies to have potential impact on fermentation, *N. ishiwadae* and *L. elongisporus* in particular (Ruiz et al., 2019; van Wyk et al., 2020). Both of these species have been shown to have relatively high tolerance to ethanol and SO_2 (Ruiz et al., 2019). Both have the ability to persist well into fermentation and potentially produce favorable compounds such as (Ruiz et al., 2019). *K. aerobia*, the most common vineyard isolate in year two, has been shown to have sensory impacts when used in mixed culture fermentation with *S. cerevisiae*, with one isolate shown to produce fruity and floral aromas, and another associated with rancid and harsh aroma (Jood et al., 2017). On the other hand, winemaking relevance of *N. holstii*, the most common vineyard isolate in year one, remains to be investigated.

3.5 Conclusion

Using BEM media we were able to enrich and isolate *B. bruxellensis* from a vineyard in Oregon. While able to limit growth of several common vineyard yeasts, BEM enrichment did result in isolation of other non-Saccharomyces yeasts, albeit relatively rare genera in

the context of vineyard/wine ecology. Notably, genera such as *Nakazawea* and *Loderomyces* have potential oenological relevance and may, therefore, be targets of interest for enrichment themselves. This study supports the existence of *Brettanomyces* as a low-prevalence component of vineyard microbiota and provides a basis to further refine enrichment protocols.

Supplementary Table S3.1. Table of vineyard and reference isolates.

Y#	Species	Origin	Other Identifiers
1	Brettanomyces bruxellensis	Reference	White Labs WLP648
2	Kluveromyces marxianus	Reference	NRRL Y-1109
8	Brettanomyces bruxellensis	Reference	AWRI1613
16	Brettanomyces bruxellensis	Reference	AWRI1608
65	Zygosaccharomyces bailii	Reference	MUCL27812
504	Torulaspora delbrueckii	Reference	Biodiva TD 291
67	Schizosaccharomyces pombe	Reference	MUCL28824
49	Saccharomyces cerevisiae	Reference	Lalvin EC1118
69	Pichia membranifaciens	Reference	MUCL27734
70	Meyerozyma guillermondii	Reference	MUCL29837
68	Hanseniaspora uvarum	Reference	MUCL31704
53	Brettanomyces naardensis	Reference	ARS Y-5740
77	Brettanomyces custersianus	Reference	ARS Y-6653
78	Brettanomyces bruxellensis	Reference	AWRI1499
64	Brettanomyces bruxellensis	Reference	MUCL27705
63	Brettanomyces bruxellensis	Reference	MUCL27701
52	Brettanomyces anomalus	Reference	ARS Y-1415
214	Nakazawaea holstii (tentative)	Isolated in this Study	-
329	Kazachstania aerobia	Isolated in this Study	-
330	Nakazawaea holstii	Isolated in this Study	-
332	Kazachstania aerobia	Isolated in this Study	-
333	Brettanomyces bruxellensis	Isolated in this Study	-
335	Ogataea polymorpha	Isolated in this Study	-
336	Brettanomyces bruxellensis	Isolated in this Study	-
337	Nakazawaea holstii	Isolated in this Study	-
339	Ogataea polymorpha	Isolated in this Study	-
340	Nakazawaea holstii	Isolated in this Study	-
341	Brettanomyces bruxellensis	Isolated in this Study	-
342	Nakazawaea holstii	Isolated in this Study	-
343	Brettanomyces bruxellensis	Isolated in this Study	-
344	Brettanomyces bruxellensis	Isolated in this Study	-
345	Brettanomyces bruxelensis	Isolated in this Study	-
346	Unconfirmed	Isolated in this Study	-
347	Brettanomyces bruxellensis	Isolated in this Study	-
348	Brettanomyces bruxellensis	Isolated in this Study	-

Y#	Species	Origin	Other Identifiers
349	Brettanomyces bruxellensis	Isolated in this Study	-
350	Brettanomyces bruxellensis	Isolated in this Study	-
351	Brettanomyces bruxellensis	Isolated in this Study	-
352	Brettanomyces bruxellensis	Isolated in this Study	-
353	Brettanomyces bruxellensis	Isolated in this Study	-
355	Nakazawaea holstii	Isolated in this Study	-
356	Nakazawaea holstii	Isolated in this Study	-
357	Lodderomyces elongisporus	Isolated in this Study	-
358	Ogataea polymorpha	Isolated in this Study	-
359	Brettanomyces bruxellensis	Isolated in this Study	-
360	Kazachstania aerobia	Isolated in this Study	-
361	Nakazawaea holstii	Isolated in this Study	-
362	Nakazawaea holstii	Isolated in this Study	-
363	Ogataea parapolymorpha	Isolated in this Study	-
429	Kazachstania aerobia	Isolated in this Study	-
430	Kazachstania aerobia	Isolated in this Study	-
432	Hanseniaspora uvarum	Isolated in this Study	-
433	Kazachstania aerobia	Isolated in this Study	-
435	Kazachstania aerobia	Isolated in this Study	-
446	Nakazawaea ishiwadae	Isolated in this Study	-
449	Kazachstania aerobia	Isolated in this Study	-
450	Uncomfirmed	Isolated in this Study	-
451	Uncomfirmed	Isolated in this Study	-
452	Kazachstania aerobia	Isolated in this Study	-
453	Kazachstania aerobia	Isolated in this Study	-
454	Hanseniaspora valbyensis	Isolated in this Study	-
455	Kazachstania aerobia	Isolated in this Study	-
456	Kazachstania aerobia	Isolated in this Study	-
457	Kazachstania aerobia	Isolated in this Study	-
470	Nakazawaea ishiwadae	Isolated in this Study	-
471	Kazachstania aerobia	Isolated in this Study	-
472	Hanseniaspora uvarum	Isolated in this Study	-
474	Kazachstania aerobia	Isolated in this Study	-
476	Hanseniaspora valbyensis	Isolated in this Study	-
477	Nakazawaea holstii	Isolated in this Study	-
478	Kazachstania aerobia	Isolated in this Study	-
479	Kazachstania aerobia	Isolated in this Study	-

4 POPULATION GENOMICS OF THE WINE SPOILAGE YEAST *BRETTANOMYCES BRUXELLENSIS* ISOLATED FROM NEW ZEALAND WINERIES

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4.1 Introduction

The idea of "terroir" has long existed in winemaking. Terroir is the notion that wines have a flavor profile unique to the region in which the grapes are grown and wine is made. Microbial populations in vineyards and wineries have, over recent years, been shown to differ geographically and represent an important component of terroir (Knight et al., 2015). Nonetheless there are some microbial influences on wine style that are widely considered undesirable, regardless of whether the offending species is part of the natural winemaking microflora. These undesirable traits can overpower any positives traits a wine may have, resulting in consumer rejection and economic loss for the winemaker. An organism that is almost universally considered negative in wine is the budding yeast species *Brettanomyces bruxellensis*. In this context, *Brettanomyces* spoilage remains one of the most important microbiological issues facing winemakers across the globe, leading to significant loss of quality in premium, barrel-aged red wines (Curtin et al., 2015).

Brettanomyces yeasts cause spoilage by converting aroma-less hydroxycinnamic acid precursors, found ubiquitously in grapes and wine, into potent odorant ethylphenols. The aromas imparted by mixtures of the two most important compounds, 4-ethylphenol and 4-ethylguiacol, range from "bandaid" and "medicinal" to "clove" and "barnyard" (Chatonnet et al., 1995). Known as 'Brett' taint, these odors are universally undersirable in wine, and formation of these compounds is directly linked to *B. bruxellensis* growth, which generally occurs during barrel maturation. Strategies to control 'Brett' therefore revolve around good fermentation management, barrel sanitization, pH management, and appropriate use of the preservative sulfur dioxide (SO_2) (Coulter et al., 2004). Despite the existance of control strategies, B. bruxellensis is still a major issue that winemakers face. Although recently

there has been development of alternatives (e.g. fungal chitosan; (P. Taillandier et al., 2015), most winemakers rely heavily upon SO_2.

Variation in sulfite tolerance has been observed amongst *B. bruxellensis* isolates (Conterno et al. 2005), and in Australian (Curtin et al. 2012b) and French (Avramova, Cibrario, et al., 2018; Cibrario et al., 2019) studies, shown to be dependent upon genotype. Comparisons of Australian and French genetic groups seem to indicate that the SO_2 tolerant strains are quite common, and potentially distributed around the world (Avramova, Cibrario, et al., 2018; Curtin et al., 2007). Recent genomic studies of B. bruxellensis in wine and beer suggest that isolates from wine are divergent from isolates of other substrates (Colomer et al., 2020). Unequal distribution of genetic groups within the wine isolates were observed, suggest that some factor, possibly related to SO_2 practices is shaping the distribution of these groups (Cibrario et al., 2019).

The geographic isolation of New Zealand coupled with its relatively short history of human settlement have made it a fruitful location to study population structure and gene flow of *Saccharomyces cerevisiae* (Goddard et al., 2010), and it is one of a handful of locations where one of the progenitors of lager yeast, *S. eubayanus*, has been found (Gayevskiy & Goddard, 2016). On the other hand, to date only a single study from 1974 has reported isolation of *Brettanomyces* from New Zealand (Wright & Parle, 1974). In this study we obtained *B. bruxellensis* isolates from across the majority of New Zealand's winegrowing region and utilized whole-genome sequencing to evaluate population structure. While our results reinforce the apparent global dispersal of *B. bruxellensis* wine strains, sulfite tolerance does not appear to be as strong a driver of population structure in New Zealand as observed elsewhere.

4.2 Materials and Methods

4.2.1 Chemicals and laboratory media

All chemicals were obtained from Sigma Aldrich (location) unless otherwise specified. Wallerstein Laboratory agar (supplier) containing 10 mg/l cycloheximide (WL+C) was used for direct-plating of wine samples. Yeast extract Peptone Dextrose (YPD) agar (amounts of each ingredient) was used for propagation of received cultures, and YPD broth for liquid cultures used in cryopreservation (combined with glycerol to final concentration of 15% w/v) or as starters for physiological screening experiments.

4.2.2 Sampling, isolation and identification of Brettanomyces yeasts.

Anonymized samples coded by region and winery were received from three commercial laboratories in New Zealand (Pacific Rim Oenology Services, location; WineWorks, location; dNature, location) who used direct-plating techniques to presumptively identify *Brettanomyces* in wine samples from across New Zealand. A small number of wines suspected to harbor *Brettanomyces* were received directly from wineries, and filter-plated onto WL+C. Presumptive *Brettanomyces* yeasts from all sources were identified by Internal Transcribed Spacer region Polymerase Chain Reaction Restriction Fragment Length Polymorphism (ITS-PCR-RFLP) (Guillamón et al., 1998), using 2x Econotaq plus green (Lucigen, Middleton, USA) and products run on 2% TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) agarose gels, using 6x Gel-red loading dye (supplier). Agarose gels were visualized under UV light and photographed Biorad Gel Doc XR+ gel imager (Biorad, Hercules, USA) and PCR product size compared with a known control, Y78 (AWRI1499)

Yeast isolates obtained from New Zealand wineries, along with reference isolates representative of known *B. bruxellensis* strain groups, are summarized in Supplementary Table S4.1.

4.2.3 DNA extraction and whole-genome sequencing

Each isolate was retrieved from cryo-storage and grown on solid YPD agar plates, and then in YPD broth liquid culture to obtain sufficient biomass for DNA extraction. DNA was extracted from pelleted cells (1 ml liquid culture) using the Puregene Yeast/Bact kit (Qiagen, details), according to manufacturer's instructions. Purified genomic DNA was quantified using AccuBlue High Sensitivity reagents (Biotium, details) according to manufacturer's instructions on a M2 Spectramax microtiter plate reader (Molecular Devices). Each DNA extract was diluted to 6 ng/µl and re-quantified, then DNA concentration was adjusted if necessary

A modified Illumina Nextera library preparation protocol (Baym et al., 2015) was used to generate sequencing libraries, using Nextera reagents (Illumina, San Diego, USA) unless otherwise specified. Briefly, 25ng of genomic DNA was tagmented in 1/5 size reactions, then purified using AMPure XP beads (Beckman Coulter). Duplicate 9-cycle PCR reactions were performed using the KAPA Library Amplification Kit (Roche, Basel, Switzerland and Illumina Nextera i5 and i7 indexing primers. Resultant PCR products were again purified using AMPure XP beads, quantified (as described above), and pooled at equimolar concentrations. The final pooled library was size-selected by agarose-gel electrophoresis and excision of a product size range of 350-550bp, then purified using MinElute Gel extraction kit (Qiagen, Hilden, Germany) and submitted to the Oregon State

University Centre for Genome Research and Biocomputing for sequencing. Following library quantification by qPCR, whole-genome sequencing was performed in two 2x150bp Hi-Seq 3000 lanes, according to standard Illumina protocols.

4.2.4 Bioinformatics

Raw fastq files were de-multiplexed according to i5/i7 dual-index combinations to obtain data for each individual yeast isolate, and trimmed using Trimmomatic v0.36 (Bolger et al., 2014) (parameters LEADING:3, TRAILING: 20, SLIDINGWINDOW:4:15, MINLEN:50). Reads were mapped using bwa-mem (BWA v0.7.15) (Li 2013) against three different genome references. First, the data was mapped onto a composite of genome sequences for all available *Brettanomyces* species (Supplementary Table S4.2), and the percentage of each covered by at least 10 reads used to determine presence of non-*bruxellensis* isolates in the dataset and/or presence of interspecies hybrids. This screening approach enabled us to exclude one *Brettanomyces anomalus* isolate from subsequent analysis. Reads were then mapped against *B. bruxellensis* assemblies for strains UCD2041 (Roach & Borneman, 2019) and CBS11270 (Tiukova et al. 2019), and BAM files from forward- and reverse-reads merged using samtools v1.7 (Li et al., 2009). Non-primary alignments and non-properly paired reads were filtered out and duplicate reads were marked using Picard Tools (Wysoker et al. 2013). Before variant calling, reads were locally realigned in order to eliminate false positives due to misalignment of reads. Variants were called for all BAM files simultaneously, using Freebayes v1.1 (Garrison & Marth, 2012), and saved in vcf format (Danecek et al. 2011). The combined vcf file was used for base quality score recalibration, using the Broad Institute Genome Analysis Toolkit (GATK)

(McKenna et al., 2010). The re-calibrated BAM files were used for variant prediction using Freebayes, generating final vcf files for further analyses.

Read-depth across aligned regions of the *B. bruxellensis* reference genome was estimated from each BAM file using genomecov (Bedtools) (Quinlan, 2014), and multi-intersect-bed (Bedtools) was used to identify common regions across all isolates that were represented by at least 10 sequencing reads. FastaAlternateReferenceMaker (GATK) was then used to generate an isolate-specific fasta sequence file incorporating variants from each vcf file, and these fasta-files were used to construct a neighbour-joining tree in Seaview (Gouy et al. 2009).

Additional analyses were performed on a merged vcf file of all variants. First, the merged vcf file was filtered using VCFtools v0.1 (Danecek et al., 2011) with following parameters: mac 3, maf 0.05, minQ 30, max-missing, 0.95, minDP 3, min-meanDP 20) Filtering reduced the number of variants from 913,329 to 20,860. This high-quality SNP dataset was analyzed in R 3.4.1 (R-core team, 2013) using the package snpRelate (Zheng et al., 2012). Identity-by-state analysis on 17,941 biallelic sites was used to generate a dissimilarity matrix (snpgdsHCluster) and determine the number of statistically meaningful groups of isolates (snpgdsCutTree: z-threshold 15, outlier threshold 5, 5000 permutations). Eigen-analysis (snpgdsEIGMIX) was performed and the eigenvectors used to evaluate admixture (snpgdsAdmixProp) between genetic groups.

VariantsToTable (GATK) and custom scripts were used to calculate the Variant Allele Fraction (VAF) at each polymorphic site for each sequenced strain, in order to estimate ploidy. A distribution ratio centred around 0.5 was classified as diploid (2n) while a

bimodal distribution with centres at 0.33 and 0.66 was classified as triploid (3n) (see Supplementary Figure S4.1 for examples).

4.2.5 Screening of isolates for SO_2 tolerance

All isolates were grown in YPD and transferred into two 96-deepwell plates along with reference strains, and cryo-preserved in 15% glycerol as described above. A pintool was used to transfer each isolate from cryo-stock into YPD in a standard 96-well plate, which was sealed with a BreatheEasy membrane (Diversified Biotech) and incubated at 30ºC until all isolates reached stationary phase. These wells were used to inoculate a 96-deepwell plate containing 600µl Yeast Nitrogen Base (YNB) supplemented with 20g/l glucose (pH 3.5) per well, which was sealed with a BreatheEasy membrane and incubated at 30ºC for 4 days. A sub-sample of each well was diluted in peptone water (0.1%) and absorbance (600nm) measured in a M2 multiwell spectrophotometer. A second 96-deepwell plate was prepared by transferring and diluting the contents of the original plate with sterile water (pH3.5) to a final density of 10^7 cells/ml, based upon absorbance readings and a calibration curve.

Quadruplicate standard 96-well plates containing 180µl of 1.1x YNB (pH3.5, 20g/l glucose) and different concentrations of SO_2, were inoculated by transferring 20µl of each starter culture. Each plate was sealed using a BreatheEasy membrane and incubated at 30ºC for 7 days, and absorbance (600nm) read every 24 hours. SO_2 tolerance was defined as the maximum concentration that allowed growth of the isolate to exceed a blank-subtracted value of 0.1 OD_{600} units by day 7.

4.2.6 Screening of isolates for ethanol tolerance

Deepwell cryocultures were transferred to fresh YPD for a subset of 55 isolates, as described above, and grown to stationary phase. A pintool was then used to transfer each culture in quadruplicate to solid YPD agar that was supplemented with different concentrations of ethanol just prior to the agar being poured. Plates were dried for 30minutes in a laminar flow and used immediately.

Images of plates were taken every 24 hours using the spImager (S&P Robotics inc.) and colony size calculated using gitter (Wagih & Parts, 2014) as implemented in the R statistical programming language (R-core team, 2013). Ethanol tolerance was evaluated as the colony size after 7 days growth at 11% ethanol, relative to colony size after the same time on control plates, where a value of 1 means the isolate grew as well in the presence of ethanol as it did without.

4.3 Results and discussion

4.3.1 Isolation and identification of *Brettanomyces* yeasts from New Zealand wine samples

Of the 256 samples received for this study from commercial service laboratories, or directly from wineries, 170 were confirmed as *B. bruxellensis*. The majority of these were obtained from the North Island wine producing regions (Tables 4.1 & 4.2), whereas the bulk of wine produced in New Zealand originates from the Marlborough wine region of the South Island. This result is, however, representative, as it reflects the North Island's focus on wine made from red grape varietals, that are more at risk of 'Brett' spoilage. Interestingly, a single *B. anomalus* isolate was obtained from the year 1 sampling. *B. anomalus* is much more commonly associated with spoilage in soft drinks and beer than in wine (Cocolin et

al., 2004). Because the focus of this study was population genomics of *B. bruxellensis*, no further work was performed on the *B. anomalus* isolate.

Table 4.1: Summary of all samples received, and the number that yielded *B. bruxellensis*.

Source	**Received**	**Identified as *B. bruxellensis***
North Island	157	117
South Island	99	53
Total	256	170

Table 4.2: Regional breakdown of *B. bruxellensis* isolates

Region	**Number of *B. bruxellensis* isolates obtained**		
	Year 1	**Year 2**	**Total**
North Island			
Gisborne	-	-	-
Hawkes Bay	59	-	59
Kumeu	-	-	-
Matakana	5	-	5
Northland	1	-	1
Waiheke	12	-	12
Waikato	-	-	-
Wairarapa	34	19	53
Total	***111***	***19***	***130***
South Island			
Canterbury/Waipara	4	3	7
Central Otago	1	9	10
Marlborough	22	23	45
Nelson	16	-	16
Waitaki	-	-	-
Total	***43***	***35***	***78***
Combined Total	***154***	***54***	***208***

4.3.2 Population structure of New Zealand *B. bruxellensis* isolates

Reference-mapped whole-genome sequencing data used to generate a phylogenetic tree of *B. bruxellensis* isolates from New Zealand alongside reference isolates from other origins (Figure 4.1). Five distinct clades were observed, and all NZ isolates could be classified within these clades with the exception of Y0224.

The first three clades (A,B,C) correspond to previously sequenced strains (Curtin et al. 2012a, Borneman et al. 2014) from Australia (AWRI1499, AWRI1608 and AWRI1613, respectively), and made up 65% of sequenced NZ isolates. Consistent with published data, all NZ isolates in clades A & B exhibited triploid genomes, while all clade-C isolates were classified as diploid. These three clades were well supported across the different phylogenetic trees (Supplementary Figures S4.2, S4.3, S4.4). While clades D1 and D2 were distinguished by IBS-clustering, on the neighbor-joining tree they clustered extremely closely together (Figure 4.1), and the maximum likelihood tree did not support separation between the two clades. *B. bruxellensis* Y224 was the only New Zealand isolate to be classified by IBS-clustering as an outlier, positioned equidistant to clades B, C and D1/D1 on the neighbor-joining tree. Interestingly, based upon analyses of variant allele fraction across the genome (Supplementary Figure S4.1) the Y224 genome displays regions of aneuploidy. To investigate the nature of this isolate further, we used IBS-derived ancestry proportions (Figure 4.2). The IBS tree suggests that Y224 clusters by itself between clades B, C and D2. The IBS-ancestry analysis shows that Y224 shares common SNPs with mostly clades C and D2, suggesting that it could be a degenerative hybrid of parents from these clades. The possible hybrid origins of triploid strains AWRI 1499 and 1608 has been previously hypothesized (Borneman et al., 2014). It is interesting to note that isolates from the triploid clades A and B appear to have different ancestry with regard to their diploid

genome donors. Isolates from Clade A share a significant proportion common SNP's with clade D1. Most isolates from clade B share SNPs with clade D2, though some share a larger proportion with D1. This extends previous thoughts that the haploid genome donor of 1499 and 1608 were different by showing that the diploid donor was also likely different (Borneman et al., 2014).

Based upon previous genetic characterization using amplified fragment length polymorphism (AFLP), clades A, B & C represented ~98% of isolates found in Australian wineries (Curtin et al., 2007). Similarly, a microsatellite PCR-based population genetics study of *B. bruxellensis* (Avramova, Cibrario, et al., 2018) found similar main groups, with 89% of isolates belonging to equivalent of clades A, B & C. While not directly relatable due to a lack of overlapping reference strains, recent whole genome sequencing of wine *B. bruxellensis* isolates from multiple countries revealed 5 clusters (Gounot et al. 2019). The authors' linked three of these (G3N2, G3N1, and G2N3) to reference strains belonging to our clades A, B & C, respectively, and were able to link their final two clusters (G2N1 and G2N2) to additional diploid groupings identified by Avramova et al. (2018).

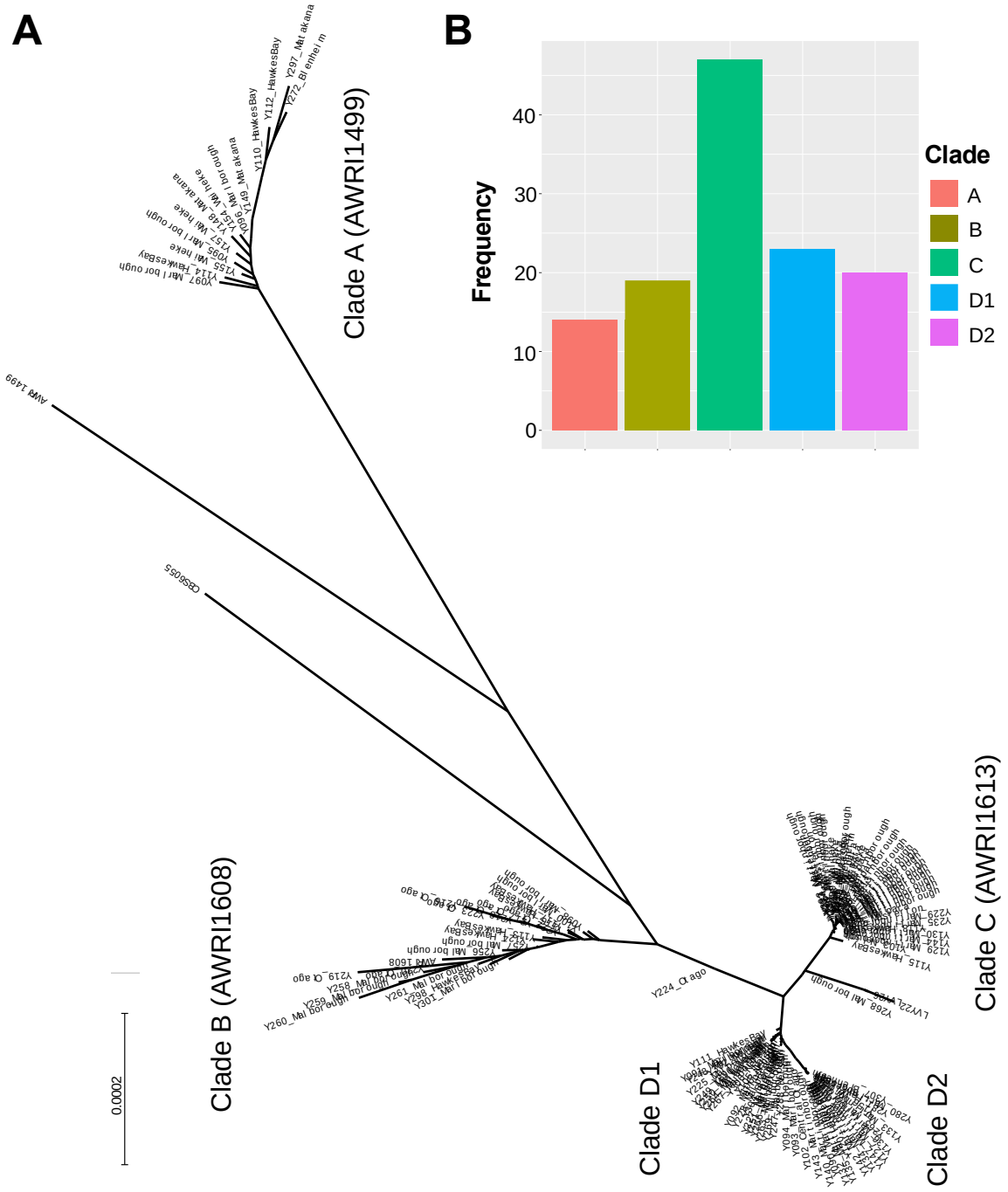


Figure 4.1. Population structure of New Zealand wine isolates of *B. bruxellensis*. Unrooted neighbour-joining tree (A) based upon ~400kb of aligned whole-genome sequencing data mapped to the *B. bruxellensis* UCD2041 genome. Clades assigned according to significance determined through IBS-analysis and labelled with previously sequenced reference strains that fall within each clade. Unshaded isolates were outliers in this analysis. Frequency of assignment for New Zealand *B. bruxellensis* isolates (B) to clades.

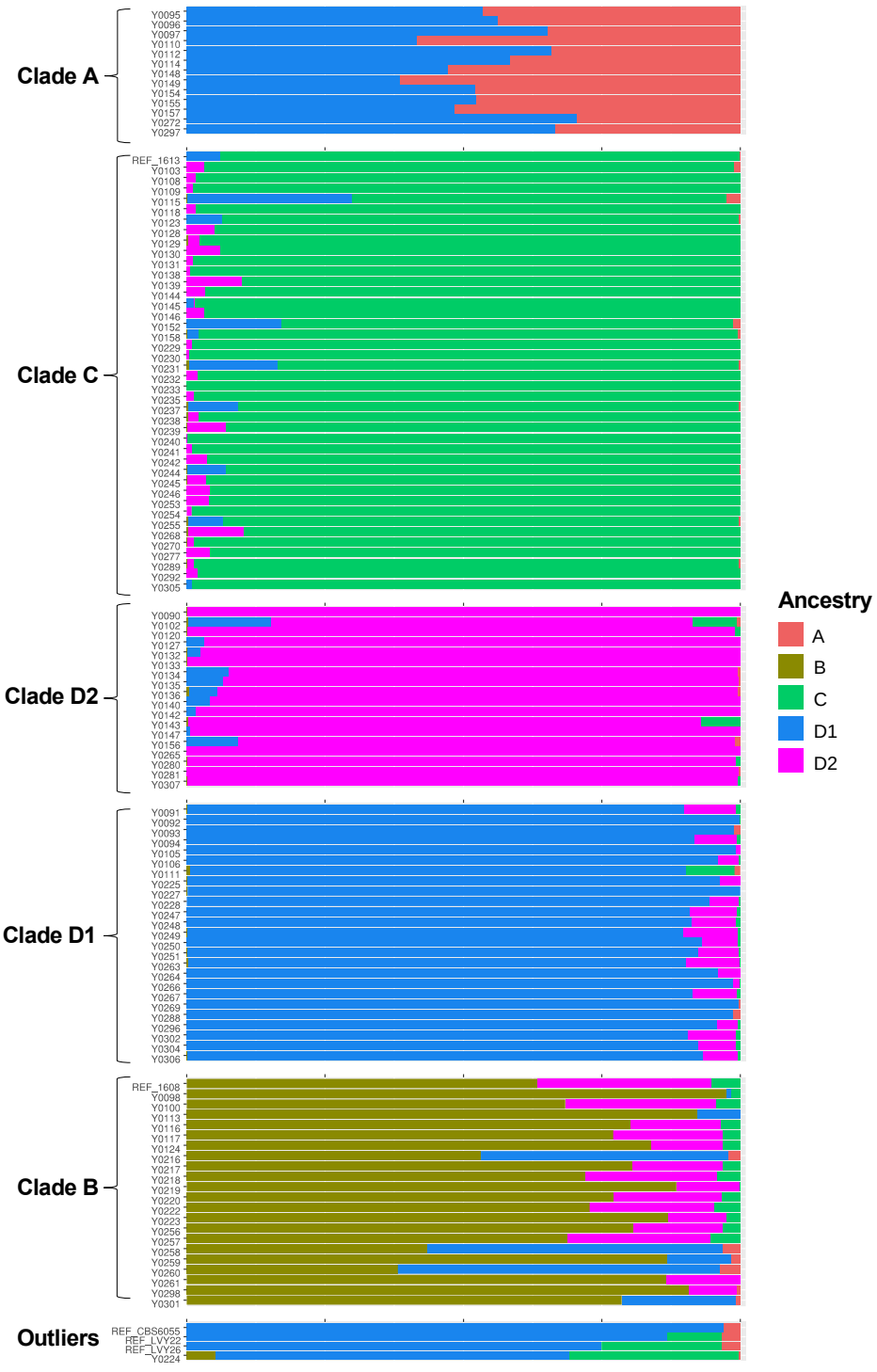


Figure 4.2. Admixture amongst *B. bruxellensis* populations. Relative proportion of SNPs predominantly associated with clades indicated by shading within barplot, as determined by IBS-analysis of 17,491 biallelic SNPS using R package snpRelate.

Based upon comparison of tree topology our clades D1 and D2 likely correspond to these clusters, though without overlapping reference isolates we cannot unambiguously make this determination. Each of the NZ *B. bruxellensis* isolates from D1 and D2 were assigned diploid status, consistent with this inference. Interestingly, compared to data for strain distributions from Australia (Curtin et al. 2007) and around the world (mostly France) (Avramova et al. 2018), New Zealand winery B. bruxellensis populations comprise a relatively low frequency of clade-A isolates (Table 4.3). Furthermore, in both the Australian and French datasets (Cibrario et al., 2019; Curtin et al., 2007), clade-A was observed to have increased in relative abundance over the past 20-30 years.

Table 4.3. Comparison of clade-A relative abundance in NZ wine regions relative to other datasets

Source	Number of isolates genotyped	Clade-A (AWRI1499)	References
Australia	244	87%	Curtin et al., 2007
Global (mostly France)	1488	37%	Avramova, Cibrario, et al., 2018
New Zealand	123	11%	This Study

4.3.3 Evaluating spoilage-related traits of NZ *Brettanomyces* isolates

In light of previous results highlighting variation in SO_2 tolerance of *B. bruxellensis* strains (Curtin et al. 2012b, Avramova et al. 2019), 142 New Zealand isolates were subjected to high-throughput screening in laboratory media to evaluate this wine-spoilage phenotype. In addition, we evaluated ethanol tolerance, to explore to what extent strain-level variation exists for this phenotype.

Median SO_2 tolerances of New Zealand isolates from clades A, B and C (Figure 4.3A) were consistent with previous observations (Curtin et al., 2012) and positive controls, though there was a wide distribution of individual isolate tolerances for clade-C in particular. Nevertheless, clade-A exhibited significantly greater tolerance to SO_2 than all other clades. Clade-D2 was significantly more tolerant than clades B and C, but was not distinct from clade-D1. Ethanol tolerance was evaluated on a subset of isolates (n=55), representative of all five clades (Figure 4.3B). The only significant difference observed was between clades A and C. Variation between isolates for ethanol tolerance has been noted previously in the literature, but not robustly linked to genetic groupings. That said, slower growth in wine medium (that contains 10% ethanol) was observed for AWRI1499 relative to AWRI1613 (Curtin et al., 2013), consistent with our results showing that clade-A isolates did not grow as well on solid media at 11% ethanol.

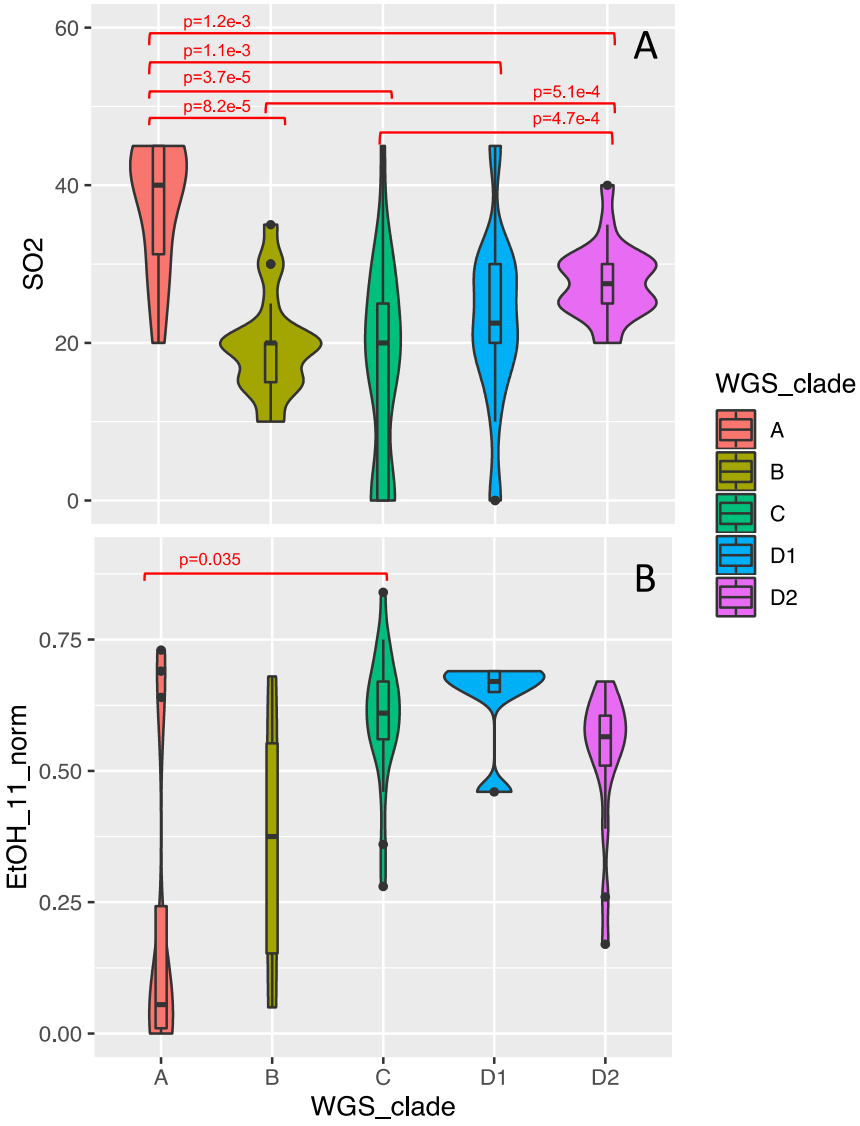
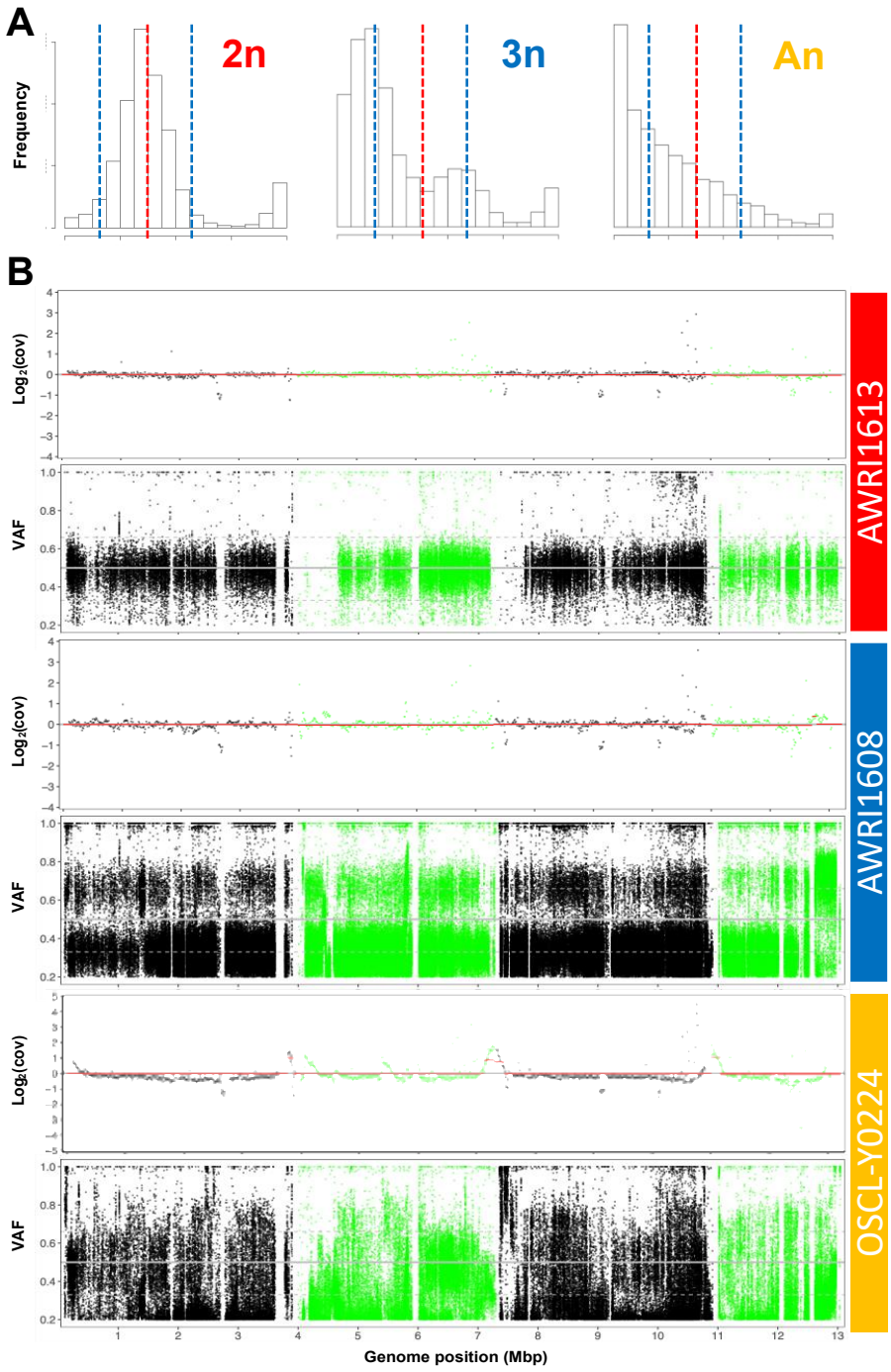


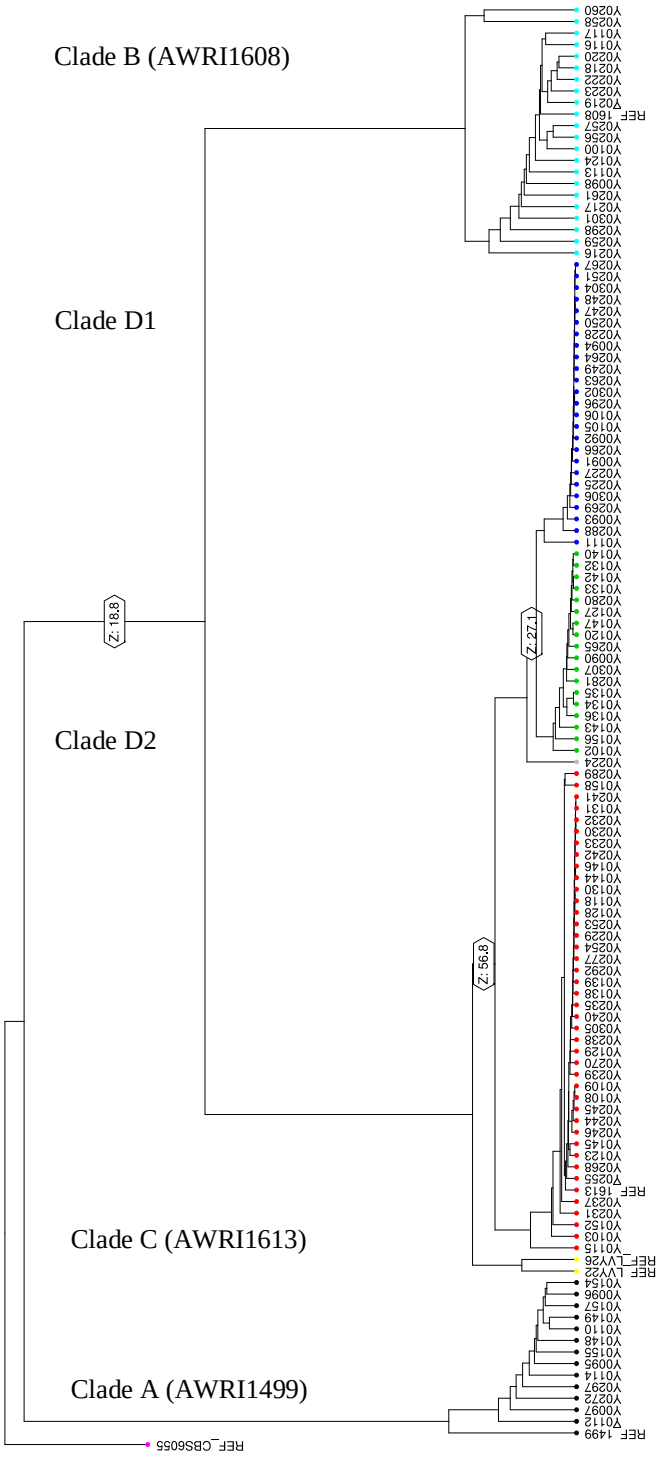
Figure 4.3. Violin plots summarizing high-throughput screening of NZ *B. bruxellensis* isolates for SO_2 tolerance (A) and ethanol tolerance (B). SO_2 tolerance for all isolates was the maximum concentration of free SO_2 in laboratory media at pH 3.5 that allowed growth within 7-days of incubation, evaluated in quadruplicate. Ethanol tolerance (ETOH_11_norm) was assessed for 55 isolates, and is defined as the ratio of colony size of quadruplicate colonies grown on solid agar containing 11% ethanol, relative to colonies grown on agar without ethanol. Because of differences in sample numbers and uneven variance the non-parametric Kruskal-Wallis test was performed to evaluate significance, followed with post-hoc Wilcoxon-rank testing. Significant differences are denoted by braces.

4.4 Conclusions

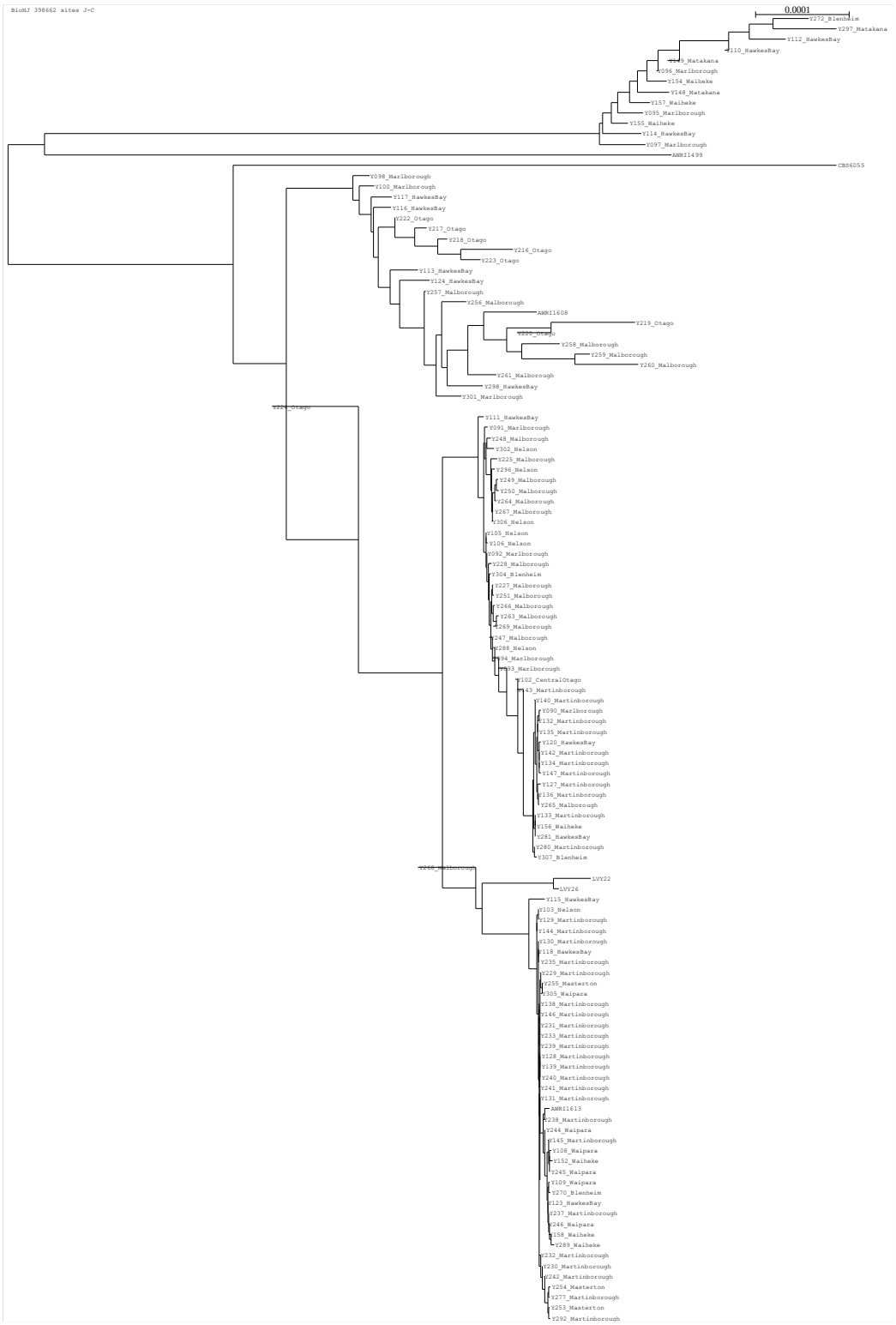
Using Whole Genome Sequencing and assaying for ethanol and sulphite tolerance, we investigation the phenotype and genotype of the New Zealand *B. bruxellensis* population. This has revealed the same genetic groups seen elsewhere in the world are present in New Zealand. However, the proportion of isolates in each group was different in New Zealand to what has been observed in other geographic locations. Phenotype assays are consistent with what has previously been reported for each group. Analysis of phylogeny reveals one isolate, Y224, that appears to be a degenerative hybrid two previously observed populations. Furthermore, Identity by State analysis of suggests that from two of the triploid strains suggests that they have originated from different diploid genome donors.



Supplementary Figure S4.1. Prediction of isolate ploidy based upon variant allele fraction (VAF) ratios. Histograms of VAF (A), with vertical lines at ratio of 0.5 (red) and 0.33/0.66 (blue), indicative of diploid (2n) or triploid (3n) genomes. Examples provided exhibit histograms consistent with 2n, 3n and aneuploid (An) genomes. Coverage and VAF by genome position (B), for isolates predicted to have 2n (red)), 3n (blue) and An (orange) genomes. In VAF plots, solid grey horizontal line indicates VAF ratio of 0.5, while dotted horizontal lines indicate VAF ratios of 0.33/0.66.



Supplementary Figure S4.2. Population structure of New Zealand wine isolates of *B. bruxellensis*. Unrooted IBS dendrogram generated using R-package snpRelate, based upon 17,491 polymorphic sites mapped to the *B. bruxellensis* UCD2041 genome. Clusters defined by minimum Z-score of 15, indicated by alternating shades, and outliers (clusters with n<5) from this analysis shaded red. Reference isolates denoted as REF.



Supplementary Figure S4.3. Population structure of New Zealand wine isolates of *B. bruxellensis*. Unrooted neighbour-joining tree based upon ~400kb of aligned whole-genome sequencing data mapped to the *B. bruxellensis* UCD2041 genome.

Supplementary Figure S4.4. Population structure of New Zealand wine isolates of *B. bruxellensis*. Maximum-likelihood tree (PhyML) based upon ~400kb of aligned whole-genome sequencing data mapped to the *B. bruxellensis* UCD2041 genome. Node support based upon 100 bootstrapped trees.

Supplementary Table S4.1. Isolates obtained from New Zealand.

Y#	Region	Median coverage	WGS clade	Ploidy	SO2_max	EtOH_11_norm
90	Marlborough	37	D2	2	25	0.67
91	Marlborough	79	D1	2	30	0.65
92	Marlborough	82	D1	2	25	0.69
93	Marlborough	101	D1	2	25	0.46
94	Marlborough	61	D1	2	30	0.69
95	Marlborough	58	A	3	40	0.01
96	Marlborough	77	A	3	45	0.11
97	Marlborough	43	A	3	45	0.69
98	Marlborough	49	B	3	15	0.10
100	Marlborough	80	B	3	15	0.05
102	Central Otago	30	D2	2	25	0.59
103	Nelson	70	C	2	25	0.36
105	Nelson	70	B	2	10	0.51
106	Nelson	64	D1	2	25	0.67
108	Waipara	73	C	2	25	0.54
109	Waipara	65	C	2	15	0.67
110	Hawke's Bay	115	A	3	30	0.00
111	Hawke's Bay	35	C	2	20	0.64
112	Hawke's Bay	39	A	3	35	0.01
113	Hawke's Bay	56	B	3	35	0.59
114	Hawke's Bay	58	A	3	45	0.73
115	Hawke's Bay	60	C	2	30	0.46
116	Hawke's Bay	70	B	3	20	0.44
117	Hawke's Bay	61	B	3	25	0.31
118	Hawke's Bay	80	C	2	20	0.57
120	Hawke's Bay	110	D2	2	30	0.56
123	Hawke's Bay	73	C	2	15	0.61
124	Hawke's Bay	64	B	3	15	0.68
127	Martinborough	52	D2	2	25	0.64
128	Martinborough	90	C	2	30	0.28
129	Martinborough	54	C	2	30	0.59
130	Martinborough	84	C	2	20	0.68
131	Martinborough	224	C	2	20	0.64
132	Martinborough	113	D2	2	30	0.57
133	Martinborough	62	D2	2	20	0.48

Y#	Region	Median coverage	WGS clade	Ploidy	SO2_max	EtOH_11_norm
134	Martinborough	86	D2	2	25	0.26
135	Martinborough	78	D2	2	25	0.17
136	Martinborough	48	D2	2	30	0.39
138	Martinborough	40	C	2	0	0.58
139	Martinborough	93	C	2	15	0.56
140	Martinborough	74	D2	2	25	0.62
142	Martinborough	113	D2	2	25	0.54
143	Martinborough	37	D2	2	25	0.63
144	Martinborough	94	C	2	15	0.53
145	Martinborough	48	C	2	15	0.64
146	Martinborough	85	C	2	20	0.66
147	Martinborough	77	D2	2	30	0.58
148	Matakana	67	A	3	25	0.11
149	Matakana	94	A	3	40	0.00
152	Waiheke	38	C	2	25	0.84
154	Waiheke	74	A	3	45	0.64
155	Waiheke	66	A	3	45	0.07
156	Waiheke	35	D2	2	40	0.60
157	Waiheke	71	A	3	40	0.01
158	Waiheke	33	C	2	30	0.74
216	Otago	68	B	3	20	NA
217	Otago	44	B	3	20	NA
218	Otago	107	B	3	20	NA
219	Otago	91	B	3	15	NA
220	Otago	104	B	3	15	NA
222	Otago	114	B	3	20	NA
223	Otago	64	B	3	20	NA
224	Otago	121	Outlier	A	20	NA
225	Malborough	36	D1	2	20	NA
227	Malborough	76	D1	2	20	NA
228	Malborough	74	D1	2	NA	NA
229	Martinborough	80	C	2	15	NA
230	Martinborough	132	C	2	0	NA
231	Martinborough	28	C	2	0	NA
232	Martinborough	373	C	2	0	NA
233	Martinborough	80	C	2	20	NA

Y#	Region	Median coverage	WGS clade	Ploidy	SO2_max	EtOH_11_norm
235	Martinborough	42	C	2	20	NA
237	Martinborough	30	C	2	0	NA
238	Martinborough	39	C	2	0	NA
239	Martinborough	36	C	2	20	NA
240	Martinborough	31	C	2	45	NA
241	Martinborough	104	C	2	45	NA
242	Martinborough	65	C	2	0	NA
244	Waipara	78	C	2	0	NA
245	Waipara	82	C	2	0	NA
246	Waipara	107	C	2	20	NA
247	Malborough	188	D1	2	20	NA
248	Malborough	65	D1	2	15	NA
249	Malborough	44	D1	2	0	NA
250	Malborough	89	D1	2	15	NA
251	Malborough	75	D1	2	20	NA
253	Masterton	104	C	2	0	NA
254	Masterton	49	C	2	0	NA
255	Masterton	38	C	2	20	NA
256	Malborough	83	B	3	20	NA
257	Malborough	124	B	3	20	NA
258	Malborough	57	B	3	20	NA
259	Malborough	43	B	3	30	NA
260	Malborough	37	B	3	30	NA
261	Malborough	64	B	3	10	NA
263	Malborough	68	D1	2	30	NA
264	Malborough	47	D1	2	45	NA
265	Malborough	65	D2	2	35	NA
266	Malborough	56	D1	2	10	NA
267	Malborough	127	D1	2	0	NA
268	Malborough	187	C	2	20	NA
269	Malborough	40	D1	2	30	NA
270	Blenheim	41	C	2	30	NA
272	Blenheim	49	A	3	20	NA
277	Martinborough	180	C	2	0	NA
280	Martinborough	53	D2	2	30	NA
281	Hawke's Bay	43	D2	2	30	NA

Y#	Region	Median coverage	WGS clade	Ploidy	SO2_max	EtOH_11_norm
288	Nelson	139	D1	2	30	NA
289	Waiheke	41	C	2	NA	NA
292	Martinborough	58	C	2	30	NA
296	Nelson	63	D1	2	35	NA
297	Matakana	41	A	3	30	NA
298	Hawke's Bay	39	B	3	10	NA
301	Marlborough	41	B	3	20	NA
302	Nelson	54	D1	2	30	NA
304	Blenheim	74	D1	2	20	NA
305	Waipara	34	C	2	20	NA
306	Nelson	32	D1	2	20	NA
307	Blenheim	38	D2	2	20	NA

Supplementary Table S4.2. Reference genomes used for mapping assemblies.

Species	Strain name	Assembly accession number
B. anomalus	CBS7654	GCA_001754015.1
B. bruxellensis	UMY321	GCA_902155815.1
B. bruxellensis	UCD2041	GCA_011074885.1
B. bruxellensis	CBS11270	GCA_900496985.1
B. custersianus	CBS4805	GCA_011074765.1
B. naardenensis	CBS6042	GCA_011074775.1

5 GENERAL CONCLUSIONS

Brettanomyces yeasts are routinely found associated with wine made across the globe. Is this because they are naturally present "in the wild", or have they been dispersed by humans wherever grapevines have been planted and wineries established? At the outset of this study there was limited evidence that *B. bruxellensis* was a member of the vineyard microbiota, with only two studies twelve-years apart describing enrichment and isolation/detection. Results reported in chapter 3, therefore, represent only the third study to demonstrate that *B. bruxellensis* can be found in the vineyard, and the only one outside of Europe. Very few studies have attempted *B. bruxellensis* enrichment, and even fewer have succeeded. This may be partly explained by interference from other yeast genera found in the vineyard. Future work can draw upon this study to refine enrichment approaches to better exclude these yeasts, and/or make them a target – some of the yeast species isolated have in fact been shown to have enological relevance.

Are *B. bruxellensis* populations the same around the world? There is some evidence for global dispersion, but those studies focused predominantly on French and Australian isolates. New Zealand represents a relatively isolated location that has only recently seen human inhabitants. Whole-genome sequencing and phenotypic data summarized in Chapter 4 shows that strains observed in previous studies in different geographic locations were also found in New Zealand, suggesting a global dispersion of *B. bruxellensis* wine strains. New Zealand Isolates did exhibit differing relative distributions of strains compared to previous studies. This difference could potentially be caused by differing winemaking practices such as SO_2.

The next stage of this work will be to determine if the *Brettanomyces* from the vineyard is the same as in the winery. To do this, whole genome sequencing will be carried out on the Oregon vineyard isolates as well as isolates from the accompanying winery (see Appendix 2). These sequences could then be compared to each other and to the New Zealand isolates that were sequenced. If the vineyard isolates fall into the same clades as the winery isolates, it suggests that there may be movement of *B. bruxellensis* between the vineyard and winery. This would mean that winemakers may have to be aware of grapes as a source of *B. bruxellensis* contamination in the winery. However, if the vineyard isolates do not fall into a previously observed clade, then it would suggest that the vineyard isolates are unique strains that are not the source of *B. bruxellensis* infection wine.

The evolutionary history of *B. bruxellensis* is an interesting parallel to *S. cerevisiae* and is likely a case of convergent evolution. With *S. cerevisiae*, there are clearly wild and domesticated strains. In large scale studies of *S. cerevisiae*, it has been shown that vineyard and wine populations are not distinct and are both in fact part of the same domesticated lineage. We speculate that the same relationship will be true for populations of *B. bruxellensis*. To progress our understanding of this relationship, we would need to identify a truly wild isolate of *B. bruxellensis* as a point of comparison. To do this one would have to look outside of the vineyard to a non-fermentation related niche. It is known that *S. cerevisiae* associates with oak trees and oak galls. No such association has been discovered for *B. bruxellensis* but given its apparent adaptation to coexist with *S. cerevisiae* and ability to metabolize cellobiose, it seems like a reasonable place to start.

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Appendix 1. Competitive spot-plating assays

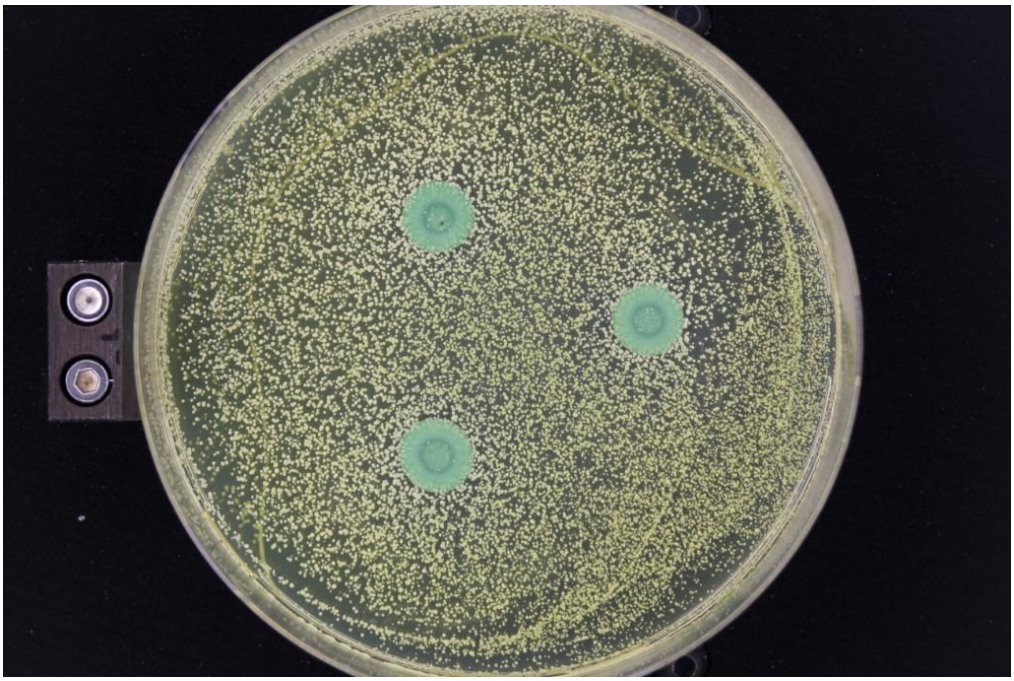

Figure A1: *K. aerobia* spotted onto *B. bruxellensis*. *K. aerobia* culture was spotted onto spread plate *of B. bruxellensis*. Plating shows no evidence of inhibition of *B. bruxellensis* by *K aerobia*.

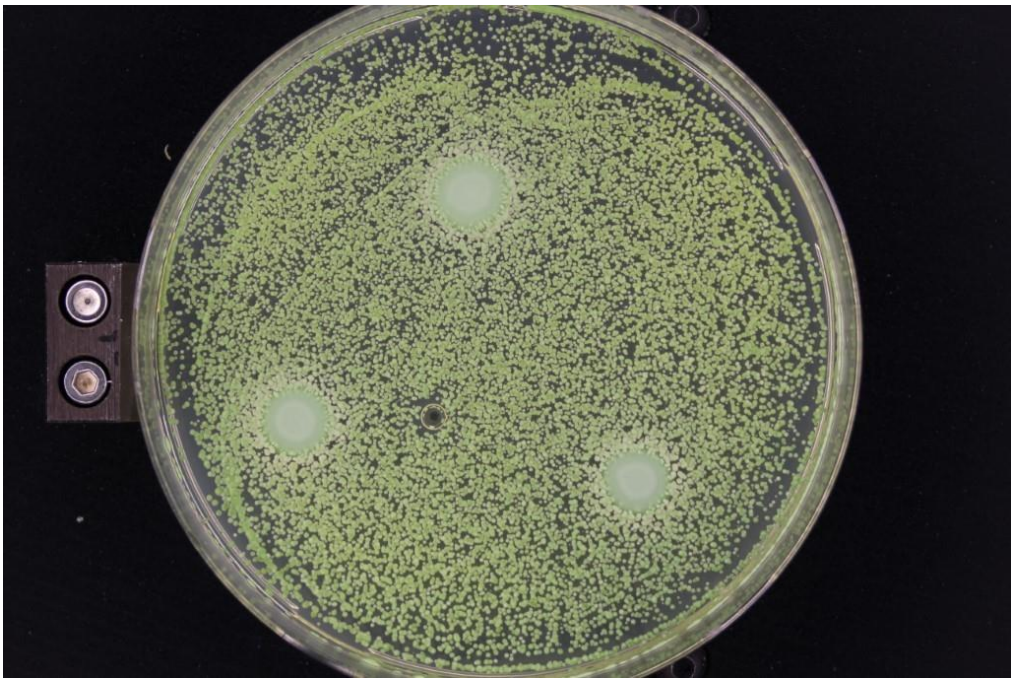

Figure A2: *N. ishiwadae* spotted onto *B. bruxellensis*. *N. ishiwadae* culture was spotted onto spread plate of *B. bruxellensis*. Plating shows no evidence of inhibition of *B. bruxellensis* by *N. ishiwadae*.

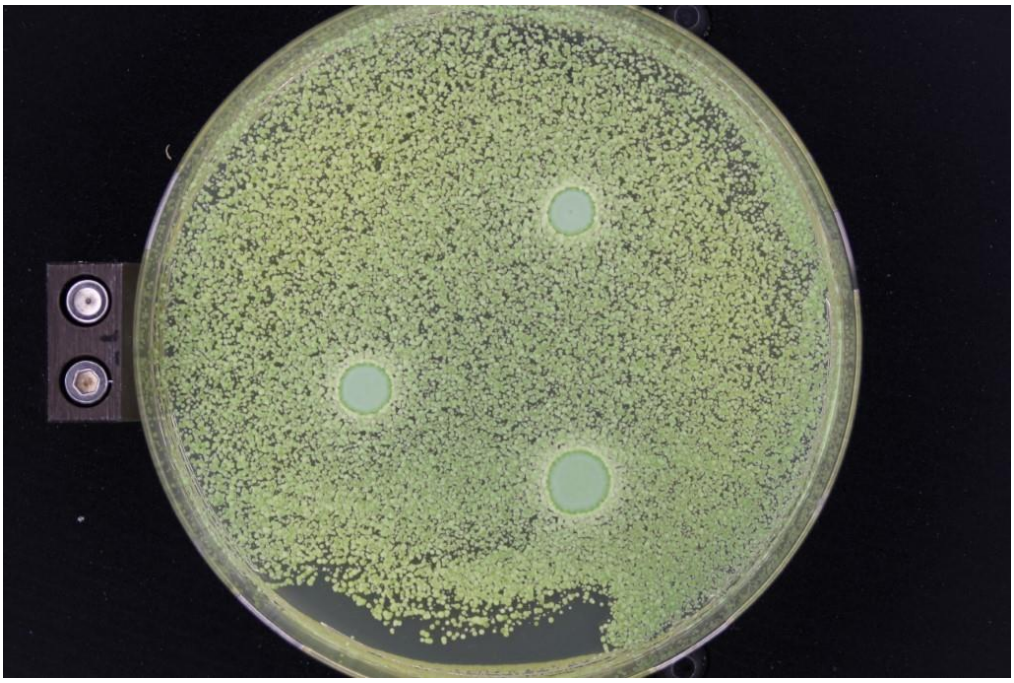

Figure A3: *N. holstii* spotted onto *B. bruxellensis*. *N. holstii* culture was spotted onto spread plate of *B. bruxellensis.* Plating shows no evidence of inhibition of *B. bruxellensis* by *N. Holstii.*

Appendix 2. Isolation of *Brettanomyces* yeast from an Oregon winery

Swabs (3M Quickswab) were taken aseptically from various locations within the vineyard and transported to laboratory for processing. Fluid from swab kit was spread plated on to YPD plates containing cycloheximide and chloramphenicol. Plates were then incubated at 30C for seven days and checked daily for growth. Representatives of each colony and cell morphology were streaked for isolation on YPD and incubated again at 30°C until isolated colonies were visible. Isolates were then prepared for cryogenic storage by preparing a starter culture as previously described, and then adding 1ml of starter culture to 1ml of sterile 30% v/v glycerol solution in water in a cryogenic storage tube. Cultures were then stored at -80C for later use.

Winery sampling resulted in isolation of *B. bruxellensis* from two pinot noir wines in barrel via membrane filter plating (Table 3). Swabs taken of various locations within the winery did not result in isolation of *Brettanomyces*. However, several other yeast species were recovered. These included three isolates of *N. ishiwadae* recovered from bungs of pinot noir barrels, and a single *N. ishiwadae* isolate recovered from the outside surface of a steel wine tank for a total of four isolates, the most of a single yeast species. The other yeast to be isolated more than once was *Meyerozyma guillermondii*, recovered from the top surface of a work table located in the cellar as well as a trench drain on the production floor. Notably, N. ishiwadae and B. bruxellensis were isolated from

both the vineyard and winery, while M. guillermondii was not isolated from the vineyard.

Winery Isolates

Table A1. Recovery of yeast from winery isolation.

Yeast	**Location(s) Recovered From[a]**	**Frequency (% of all samples)[b]**
Brettanomyces Bruxellensis	Barrel (wine)	6.0%
Candida boidinii	Barrel	3.0%
Meyerozyma guillermondii	Table, Drain	6.0%
Nakazawaea ishiwadae	Barrel, Equipment	12.1%
Rhodotorula mucilaginosa	Grape waste container	3.0%
Saccharomyces cerevisiae	Barrel (wine)	9.1%
Yamadazyma sp.	Barrel	3.0%
Unconfirmed Non-*Saccharomyces*	Floor	3.0%

[a] All samples were taken by swabs, with the exception of wine, which was filter plated.

[b] No yeast was recovered from 18/33 (55%) of samples.

Surprisingly, *S. cerevisiae* isolates were recovered from some samples despite the inclusion of cycloheximide in the solid media. In each case, the membrane filter when placed on the agar was not evenly applied, resulting in edges of the membranes being raised slightly from the surface of the agar. Colonies identified as *S. cerevisiae* growth were recovered from these areas, suggesting insufficient contact with the solid media.