School of Science
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Diversity of yeast populations naturalised in Margaret River vineyards

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DECLARATION

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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ABSTRACT

The fermentation of grape must by the action of microbial species naturalised within the vineyard and/or winery environment, rather than the use of monoculture inoculation, is practiced within some sections of the wine industry. From a technological perspective this represents a retrograde step, and is driven by actual or perceived consumer demands for culturally and ethically responsible food products, and unique products with optimal and diversified sensory complexity.

The vineyard environment houses a complex microbiota, potentially including naturalised yeast genotypes. The aim of this thesis is to identify the diversity of the naturalised yeast population in Margaret River vineyards, targeting the Saccharomycotina sub-phylum. In addition, it aims to improve existing methods for their identification and characterisation, and discuss the potential oenological implications of these species, and their role in naturalised fermentation.

A diverse microbiota of fungal species capable of fermentation was identified within grape-derived samples sourced from Margaret River vineyards. The potential for pleasant and complex (from a sensory perspective) wines to be produced by these species is highlighted. However, the limits of current knowledge indicate there is still future research to be conducted.

The application of appropriate barcodes for the culture-independent amplification of fungal species capable of fermentation was elucidated, and reinforces the validity of a two-gene approach. In addition, the modification of an appropriate plating regime for the application of culture-dependent analyses can now reduce the risk of cultivation bias in qualitative mucosal colony isolation from grape samples.

The aims of this thesis were addressed in full, and the application of naturalised fermentation utilising diverse yeast populations naturalised in Margaret River vineyards, evaluated.

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CHAPTER ONE: General Background

1.0: The evolution of fermentation and the role of monocultures and naturalised yeast

1.0.1: The evolution of winemaking

Traditional winemaking began when the civilised world exploited the natural process of spontaneous fruit fermentation (Figure 1.0.1). Wine production spread throughout history alongside the formation and dissemination of major civilizations (e.g. the Roman Empire (Pretorius 2000, This et al. 2006)) and religions (e.g. the Catholic Church (This et al. 2006)). It was during the expansion of wine production that humanity began to experiment and optimise winemaking practices, and the beginning of this period is discussed here as early industrialization (16th-19th Century) and late industrialization (20th Century to today).

The early and late industrialization eras accompanied significant scientific breakthroughs and technological advances. The pivotal turning point for the optimisation of wine fermentation occurred with the invention of the microscope, observation of *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (1883) (1680 AD), and the identification of the fermentative activity of microbial populations (1863 AD) (Pretorius 2000). These spurred the domestication of *S. cerevisiae* (Martini 1993), and subsequent cultivation and hybridisation of strains ideal for wine fermentation (Sicard and Legras 2011). Thereby allowing the inoculation of grape must with specific strains in order to guide fermentation (Blanco et al. 2011). Advances in scientific understanding and technological capabilities led to the significant alteration of winemaking practice, and while the use of spontaneous and naturalised wine fermentation remained in some areas, most new-world production became typified by the use of commercial inoculum for fermentation.

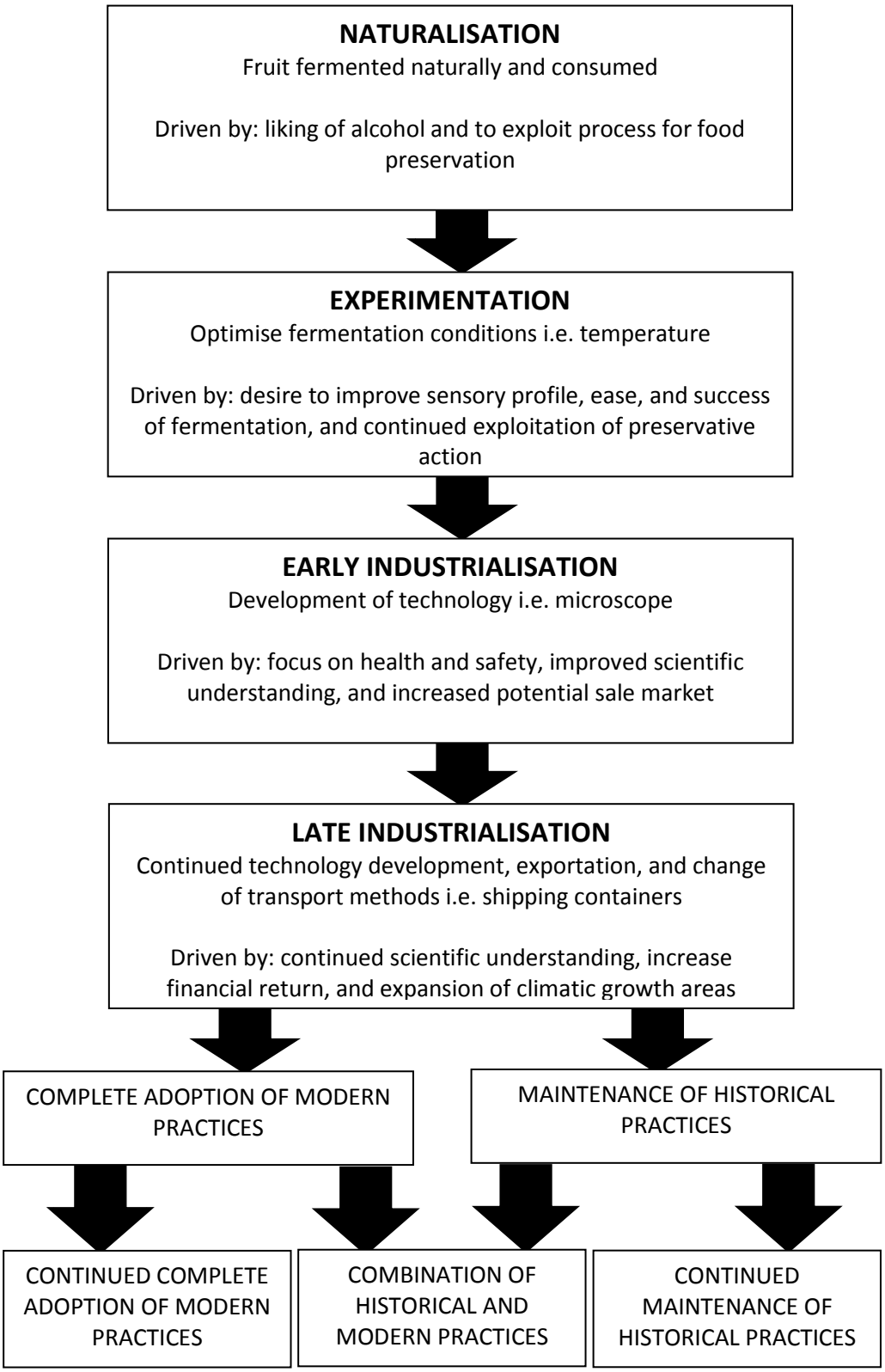

Figure 1.0.1: The progression of changes in wine production practices throughout history

Modern winemaking practices stem from three foundations: 1) complete adoption of modern techniques, 2) continued implementation of traditional techniques, 3) blended application of modern and traditional techniques. The majority of modern wine production is conducted via the application of modern techniques developed through experimentation and optimisation (Blanco et al. 2011) (e.g. the forced-inoculation of grape must with wine specific *S. cerevisiae* strains (Domizio et al. 2007)). Conversely, many sections of the wine industry maintain traditional practices (i.e. spontaneous fermentation of grape must with naturalised yeast populations (Domizio et al. 2007)). In order to capitalise on the demand for authentic and natural food products currently exhibited within the consumer market (Beverland 2005), a number of producers are experimenting with, and adopting aspects of traditional practice, including fermentation with microbes naturalised within the vineyard and/or winery. However, such producers rarely turn away from other modern winemaking protocols and engineering. Indeed there is a strong argument for scientific understanding and experimentation to assist producers in the transition to naturalised fermentation in the context of a winery and, with an appropriate level of control and risk management.

1.0.2: Traditional fermentation approaches

1.0.2.1: Naturalised fermentation consists of a dynamic microbial community

In the absence of a dominating monoculture inoculum fermentation is dependent on naturalised microbial species, and is driven by a complex interplay of multiple yeast species and strains (Jolly et al. 2014). As such, within a naturalised fermentation yeast species derived from the vineyard (predominantly non-*Saccharomyces* (Bokulich et al. 2014, Taylor et al. 2014)) initiate fermentation before combining with yeast derived from the winery (*Saccharomyces* Meyen ex Reess (1870) and non-*Saccharomyces* populations (Ocón et al. 2010, Bokulich et al. 2013)) to complete fermentation (Fleet 2003).

In naturalised ferments non-*Saccharomyces* species actively participate in the first few days of fermentation before succumbing to competitive exclusion factors, such as increased substrate competition with *Saccharomyce*s species (Pretorius 2000, Fleet 2008) and potential ethanol intolerance (levels greater than 5 to 6% (Romano et al. 2003)). After these first few days the population density of the highly-efficient *Saccharomyces* fermenters approaches a level whereby their activity dominates fermentation, and drives it to completion (Domizio et al. 2007).

1.0.2.2: The adoption of mono-cultures

One of the key limitations of naturalised fermentation is the risks associated with the interplay and succession of species (Jolly et al. 2014). If there are undesirable species (spoilage microbes), inadequate genotypic diversity, or low initial cell density, a slow or incomplete fermentation (Bisson and Butzke 2000) takes place, and the excessive production of undesired sensory compounds (Zoecklein et al. 1999, Jackson 2008) may result. This becomes particularly apparent in the absence of *Saccharomyces* species. To prevent these issues many wine producers adopt the practice of inoculating fermentations with prepared mono-cultures of known identity. The result is an improved rate, scale and predictability of fermentation.

During fermentation, the metabolic activity of yeast assist secondary flavour development via the extraction and modification of grape-derived sensory precursors, and the production of over 1000 sensory metabolites (Romano et al. 2003). The addition of a known species at a high cell density (mono-culture) standardises the range of sensory volatiles produced under certain processing conditions (Cavazza et al. 1989, Grando et al. 1993). Thus, the application of mono-cultures increases the level of control the winemaker possesses over fermentation, reducing any potential risks associated with product quality decline or loss.

1.0.3: Drivers for the regression towards traditional fermentation approaches

1.0.3.1: The pursuit of sensory complexity and aromatic pungency

Whilst naturalised fermentation will increase the risk profile, it can also infer favourable attributes, and is known to produce wines of increased complexity and aromatic pungency (Henick-Kling et al. 1998). As naturalised fermentations are typified by a dynamic microbial population, it is the diversity of this population that produces a range of sensory volatiles, particularly amongst the non-*Saccharomyces* species (Jolly et al. 2014). The non-*Saccharomyces* species possess greater bioactivity (Eglinton et al. 2000, Domizio et al. 2007, Jackson 2008) and have been associated with wines of significantly higher levels fruit and floral characters, compared to their inoculated counterparts (Henick-Kling et al. 1998, Egli et al. 1999, Domizio et al. 2007). However, many non-*Saccharomyces* species may also produce excessive acidity during fermentation (Sadoudi et al. 2012, Magyar et al. 2014), and the anonymity of the present naturalised community must be resolved to enable the sensory potential of a fermentation to be estimated. If successful, naturalised fermentation can culminate in a wine with 'multi-dimensional' sensory complexity and/or increased fruit and floral character (Henick-Kling et al. 1998, Medina et al. 2013).

1.0.3.2: The shifting social perceptions and attitudes of consumers

The consumer driven demand for natural and authentic foods is evidenced by the growing popularity of locally produced, minimally processed foods, and food production techniques that are culturally and socially sensitive. (Harper and Makatouni 2002, Lockie et al. 2002, Schneider 2008). Marketing approaches focused on food authenticity are also highly valued by consumers (Beverland 2005). Therefore, naturalised foods are potentially highly marketable as they are locally produced alternatives, which preserve cultural identity by reflecting traditional consumption behaviours.

Greater awareness of cultural and social issues in relation to food production is a driver for the consumer demand for natural and authentic food products (Guy 2002, Lockie et al. 2002, Schneider 2008). In a wine context cultural preservation and the desire for unique products is evidenced within the Champagne industry. A number of French Champagne houses have banded together to champion 'respect for tradition' as the primary driver behind their winemaking practices (Guy 2002). This collective argued the production of sparkling wine via traditional techniques originated in the Champagne region of France (Guy 2002), suggesting a unique product which engages cultural traditions. The increasing popularity of ethically and culturally responsible food choices is evident (Wandel and Bugge 1997, Magnusson et al. 2003, Aertsens et al. 2009), and naturalised fermentation allows the capitalisation on current market trends.

1.0.3.3: Showcasing rare and unique wine products through geographic originality

Each geographic location has a unique resident yeast profile due to the interplay between environmental conditions and time. In addition to variation across the macro-scale (inter or intra-regional variation), the yeast population colonising any point will vary due to ecological niche adaptation (Polsinelli et al. 1996, Ribereau-Gayon et al. 2006). Furthermore, this variation increases with extensive climatic and other environmental differences (i.e. founding microbial population) (Renouf et al. 2005, Nisiotou and Nychas 2007, Li et al. 2010). Therefore, it is reasonable to assume that the population profile of naturalised yeast species existing in any given ecological niche, may produce a wine product that is uniquely reflective of its origin.

In the current area of study (Western Australia) only one yeast species is known to be endemic (Ellis and Pfeiffer 1990). This species is identified as *Cryptococcus neoformans* var. *gattii* Vanbreuseghem & Takashio (1970), and its presence as an air-borne contaminant coincides with the flowering of the most widely distributed eucalyptus trees in Australia, *Eucalyptus camaldulensis* Dehnhardt (Ellis and Pfeiffer 1990). Therefore, we are adopting the assumption that the identified yeast species have been naturalised in Western Australian vineyards.

Microbial ecological niche adaptation is not to be confused with the broader term of 'terroir' currently used within some sections of the wine industry. Some winemakers believe 'terroir' encompasses the interplay of all environmental factors (soil, climate and microbiota) and often less concrete factors (age of a region or its culture), to collectively influence the final wine product and pinpoint it to a geographical location. This thesis refers to the specific scientific determination of microbial ecological niche adaptation, and may result in a locally authentic product as variances in the potential community population of environmental niches enable a unique wine to be produced.

1.0.4: Initial problem statement

The anonymity and complexity of the yeast community exacerbate the risks of naturalised fermentation within the existing environment. A greater understanding of this microbial community can assist in the exploitation of fermentation advantages and improve risk management.

1.1: Yeast ecology and the vineyard environment

1.1.1: Resident versus Transient species

In the viticultural environment yeast species can be classified either as 'resident' or 'transient' (Davenport 1976), and their colonisation is largely the result of competitive exclusion through resistance to environmental stressors. The residential yeast species are those which are consistently persistent over time, within one locale (Davenport 1976). These may be a foundation species initially colonising a particular locale, or an introduced species, which has historically achieved dissemination and persisted within the environmental conditions to consistently re-colonise a locale (Davenport 1976). To illustrate, a study of four geographical regions in South Africa observed a population of 13 different *S. cerevisiae* strains. Of these, 6 strains were noted within the same geographic region in 2 out of 4 years. This strain repetition was either successive, or with a 1 to 2 year gap between appearances (van der Westhuizen et al. 2000), thereby hypothesized to be resident within that region.

Yeast are non-motile, therefore to recolonise a vineyard consistently resident yeasts rely on the ability to enter a viable but non-cultivable state (Fleet 1999). Through the viable but non-cultivable state resident yeasts may minimise metabolism until favourable conditions arise (Fleet 1999) as evidenced in bacterial wine systems (Millet and Lonvaud-Funel 2000), and thereby achieve re-colonisation in new locales (Goddard et al. 2010). Through this mechanism resident yeast species may survive harsh environmental conditions to achieve a continued presence on the grape berry surface at harvest.

Conversely, transient yeast species do not repeatedly persist within the vineyard environment (Davenport 1976). Therefore, their colonisation is dependent on transfer to the vineyard, and maintenance of optimum conditions for population establishment. Wine yeast dissemination can be achieved through the action of insects (Goddard et al. 2010, Stefanini et al. 2012), birds (Francesca et al. 2012), landscape orientation, and human activity. For instance, pathogenic *Botyrosphaeria* Ces & De Not (1863) spores causing fungal grapevine dieback are evidenced to be transmitted via contaminated water run-off (Amponsah et al. 2009), wind-blown rain, and beetle activity (Epstein et al. 2008). If the environmental conditions are conducive this pathogen may invade the vineyard for a single vintage, or persist to become a resident species over time.

1.1.2: The influence of vineyard age on yeast ecology

The earliest record of cultivated grapevines exist from the early Bronze age in 'old world' viticultural regions such as Italy, France and Spain, (Zohary and Spiegal-Roy 1975). Conversely, a large number of the 'new world' viticultural regions such as North America, South Africa and Australia (Banks and Overton 2010), were cultivated with vines comparatively recently, with Australia's first vines being planted with white settlement in 1788 (Oag 2001). The resident and transient yeast species colonising the viticultural environment may vary, due to the planting of grapevines in largely 'new world' regions as introduced species (This et al. 2006), and the reduced timeframe for evolutionary adaptations to occur within these regions. The yeast ecology identified in vineyards worldwide varies (Francesca et al. 2010, Li et al. 2010, Gayevskiy and Goddard 2012, Bokulich et al. 2014), but whether this is primarily due to the age of the vineyard in question, and its classification as a 'new world' or 'old world' regional vineyard, remains to be elucidated.

1.1.3: The influence of microclimates on vineyard yeast ecology

It is well established that many microclimates exist within the vineyard and consequently the rate of berry ripening and yeast accumulation is variable. As such, environmental adaptation throughout vineyard niches and berry ripening is evident. Trought et al. (2011) demonstrated all berry quality indices (i.e. soluble solids, pH, acidity, phenolic and anthocyanin's) varied within a single block (Trought and Bramley 2011). This variation was the smallest at harvest for soluble solids (approximately 3%), then increased slightly for pH and acidity (Trought and Bramley 2011). The qualitative and quantitative profile of a yeast ecological community is therefore closely related to ripening, with the fermentative species experiencing fast accumulation in ripe fruit (Renouf et al. 2005). These factors will impact within block microclimate niche colonisation, with the yeast population of a vineyard block (van der Westhuizen et al. 2000) and single vine (Polsinelli et al. 1996) demonstrated to vary.

The impact of zonal microclimates on yeast communities within a block has not been studied extensively and is not thoroughly understood. Conclusions are thus far based on extrapolations from vineyard zonal management (Trought and Bramley 2011, King et al. 2014), ripening (Renouf et al. 2005) and vineyard population studies (Polsinelli et al. 1996, van der Westhuizen et al. 2000), therefore requiring further investigation. However, the preference of unique yeast species for differing environmental conditions drives ecological niche adaptation and vineyard microbial variation.

1.1.4: The influence of disturbance on vineyard yeast ecology

External disturbances which exert changes on a community of organisms can drastically alter the existing ecology of a microbial community. These disturbances can be natural in origin, e.g. the *Phylloxera* Fonscolombe (1834) insect outbreak in the 1870's which threatened grapevines in Europe (Pretorius 2000); or human in origin, e.g. the large-scale replacement of the *Phylloxera* infected grapevines with disease resistant cultivars (Pretorius 2000) thus providing an ecological 'blank slate'. Microbial ecological disturbance can come in many forms, but its impact on the current and evolving microbiome of the vineyard is considerable.

1.1.4.1: Precipitation and temperature

The highly efficient fermenter, *S. cerevisiae*, is rarely identified within the vineyard (Radler et al. 1990, van der Westhuizen et al. 2000, Sabate et al. 2002, Comitini and Ciani 2005, Renouf et al. 2005, Cafarchia et al. 2006, Ribereau-Gayon et al. 2006, Konig 2009, Francesca et al. 2012, Sun et al. 2014). Vineyard samples that have detected *S. cerevisiae* tend to be collected from locations that exhibited a warm and dry growing season (van der Westhuizen et al. 2000, Schuller D. 2005, Valero et al. 2005, Mercado et al. 2007, Chavan et al. 2009, Francesca et al. 2010). Similarly, the biomass and diversity of non-*Saccharomyces* species is increased in vineyard samples from warm and dry growing seasons (Longo et al. 1991, Yanagida et al. 1992, Rementeria et al. 2003). The cause of this environmental preference may be a consequence of the warm temperature tolerance and preference for high sugar environments of fermentative *Saccharomyces cerevisiae* and non-*Saccharomyces* species (Kurtzman et al. 2011). One conflicting report isolated viticultural *S. cerevisiae* in a warm, but monsoonal summer climate (Sun et al. 2014), thereby exhibiting high rainfall. This variation may be a result of two outside influences; 1) human activity within the vineyard acting as a dissemination vector, or 2) demonstration of a well-established resident species in an ecological niche of the vineyard. It can be deduced warm, dry and sugar dense environments act as an encouraging colonisation factor for fermentative population establishment.

Although a general trend is evident, the preference for warm and dry climates may not be universal for all fermenting species. For instance, the most common yeast species reported within vineyards, *Hanseniapora uvarum* (Niehaus) Shehata, Mrak & Phaff ex M.Th. Smith (1984), does not appear to display any climatic preference (van der Westhuizen et al. 2000, Sun et al. 2014). Additionally, a location and climate specific study across four geographic regions in South Africa found no significant correlation between environmental conditions and berry microbiota (Jolly et al. 2003). It can therefore be deduced that warm climatic conditions correlate with fermentative yeast colonisation. However, this may not be true for all fermenting species located within vineyards.

In addition, current temperature and precipitation studies evaluating vineyard microbial ecology are not without error, with many climatic studies spanning only one or two seasons, lacking a consistent fungicide application regime, and are present in insufficient numbers to draw definitive conclusions. In addition, it is common for an individual species identified from vineyard sampling to be unique to a specific geographical location i.e. *Candida valida* (Leberle) van Uden & H.R. Buckley (1970) has been only isolated in South African vineyards (Jolly et al. 2003) and *Kloeckera lindneri* (Klöcker) Janke (1928) has been only isolated in Spanish vineyards (Radler, Schmitt et al. 1990). Therefore, it is difficult to compare inter-regional studies and any conclusions must be made via the long term monitoring of successive vintage conditions.

1.1.4.2: Fungicide application

There is substantial conjecture around the influence of fungicides on fermentative yeast populations in the vineyard. Two studies have reported a beneficial influence and increase in the diversity and biomass of yeast populations seen in grape samples subjected to conventional fungicide treatments (Čadež et al. 2010), and copper and sulphur organic treatments (Milanovic et al. 2013). In contrast, one study disputed both findings, reporting untreated grape samples house greater yeast biomass and species diversity compared to conventional fungicides and organic treatments (Comitini and Ciani 2008). Similarly, laboratory trials have reported a negative effect of conventional fungicide residues on the bioactivity of several fermenting yeasts (Calhelha et al. 2006), supporting the assertion of a negative effect of conventional fungicide. However, despite this inhibition only one fungicide treatment (Benomyl) delayed fermentation initiation, and the species most resistant to the effect of fungicides were known fermenting species (*S. cerevisiae* and *Zygosaccharomyces rouxii* (Boutroux) Yarrow (von Arx et al. 1977)) (Calhelha et al. 2006). Thus no overall conclusions can be drawn from the current level of information.

The most likely explanation for the conflicting impact of fungicide application within laboratory versus field trials, is the presence of external factors unable to appropriately controlled during field trials. For example, Čadež et al. (2010) suggested yeast losses observed within laboratory trials were not noted in the field due to a resident microbial population re-colonizing the vineyard after fungicide application and demonstrating high persistence within an ecological niche. In addition, field trails are subject to the varying, and largely unpredictable climatic conditions which may adversely influence the recorded results. For example, the results obtained by van der Westhuizen et al. (2000) and Comtini and Ciani (2008) may be a reflection of the heavy rainfall recorded during the study period, and not fungicide application alone. A need for more extensive and long-term investigation is evident.

1.1.4.3: Winery establishment and the dissemination of commercial inoculants

The establishment of a winery in a new location changes the immediate physical environment and is thereby hypothesized to disturb the surrounding microbial community. The increased human activity, modification of the surrounding environment for a new wine yeast habitat, and introduction of new yeast species via cultivation or commercial inoculant use (Clavijo et al. 2011), are just a few factors that are commonly introduced with the establishment of a new winery. In addition, through the action of microbial vectors the new yeast flora developing within a winery may be transported to other locations, such as local vineyards (Goddard et al. 2010, Francesca et al. 2012, Stefanini et al. 2012).

The activity of insects and birds as vectors for yeast transportation has been established (Goddard et al. 2010, Francesca et al. 2012, Stefanini et al. 2012), but the extent of dissemination and survival of commercial inoculants beyond the confines of the winery warrants discussion. Valero et al. (2005) conducted a study of spatial distribution and ascertained that 94% of isolates identified as commercial yeasts were within a radius of 200 m from a winery building. Furthermore, 78% of these were within 10 to 50 m of the winery boundary, and this microbial density was particularly high at water run-off and the grape marc disposal sites (10 to 20 m) (Valero et al. 2005), suggesting commercial inoculants did not travel far beyond the winery. In contrast, two commercial inoculants were identified at 400 to 1000 m from the winery boundary, within the vineyard. However, these genotypes represented commercial inoculants which were originally cultivated from genotypes indigenous to the surrounding region (Valero et al. 2005) therefore, may either be an endemic species, or adapted to the local environment.

Similarly, directly inoculating vines with a thriving *Saccharomyces* culture and measuring the prevalence on the grape berry surface over an extended time period have proved ineffective to establish *Saccharomyces* populations within the vineyard (Comitini and Ciani 2005, Cordero-Bueso et al. 2011). Therefore, the lack of commercial inoculant survival and colonisation outside a boundary of 200 m from the winery (Valero et al. 2005) may be due to a lack of appropriate environmental adaptations for external environmental survival, or evidence of a reduced competitive advantage to displace the local community.

1.2: Discussion of the relevant methodologies for the identification of naturalised yeast

1.2.1: Morphological and physiological identification of environmental fungal communities

Morphological methods are commonly used in combination with physiological methods for the taxonomic classification of microbial organisms. Morphological methods entail the observation of the cell, colony and sporulation characteristics, such as ascospore formation, to determine the taxonomic identity of microorganisms (Iland et al. 2007, Fugelsang and Edwards 2010, Cappucino and Sherman 2011). Conversely, physiological methods include experimentation and the evaluation of cells and colonies within different environmental conditions (Iland et al. 2007, Fugelsang and Edwards 2010, Cappucino and Sherman 2011), such as assimilation of Nitrogen and Carbon (Iland et al. 2007, Fugelsang and Edwards 2010, Cappucino and Sherman 2011). The data accumulated by both techniques can assist in the taxonomic classification of environmental microbial communities, such as those seen on the ripe grape berry.

The physiological and morphological methodologies for the taxonomic identification of isolates harbour few advantages and numerous disadvantages. In terms of the advantages, morphological and physiological techniques allow a greater understanding of the microbial organisms and their cellular function (Kurtzman et al. 2011), and these techniques provide valuable data for a preliminary taxonomic diagnosis before targeted genetic sequencing can occur. However, the application of these techniques is time-consuming (Fugelsang and Edwards 2010). In addition, microbial organisms experience rapid evolution under adverse environmental conditions (Nevo 2001), therefore their environmental tolerances (temperature of growth and sulphur dioxide tolerance for wine related species) may continue to evolve (Gasch and Werner-Washburne 2002). As such, the appropriate physiological classifications for these species when originally characterised, may not be the same as the researcher is currently observing, leading to incorrect taxonomic assignment. To illustrate, during microscopic cell evaluation the size, shape and appearance of yeast cellular structure is often altered by the age of the organism and the environmental conditions in which culturing occurs. For example, mature *Klockera* Janke (1928) and *Hanseniaspora* Zikes (1912) yeast cells which have experienced multiple budding appear lemon-shaped. However, young cells yet to experience reproduction appear spherical (Fugelsang and Edwards 2010). Thereby, it can be easy to mischaracterise microbial species based on observational techniques alone.

The study of the microbial ecology of environmental samples traditionally lies in the culturing and cultivation of samples and subsequent genetic assessment of the resulting community. These culturing and cultivation techniques rely on the ability of microorganisms to be viable and grown in the laboratory conditions, separating target isolates from other community members for monoculture study (Iland et al. 2007, Cappucino and Sherman 2011). Culturing and cultivating not only requires an approximate knowledge of the community to be studied so the optimal growth environment can be duplicated (Fleet 1999), quite often difficult to achieve in environmental experimentation in novel locations, but adequate initial cell numbers or cell viability to achieve colonisation (Fleet 1999, Giraffa and Neviani 2001).

It is widely accepted the majority of the environmental microbiome is not cultivable (Fleet 1999, Giraffa and Neviani 2001) with the level of uncultivable populations reported to contain up to 95% of the microbiota (Taylor et al. 2014). Consequently, culture-dependent evaluation portrays a distorted representation of the microbial community present. The uncultivable community exists as species unable to achieve colonisation on any microbiological medium due to damage during cultivation, a lack of essential nutrients, or unknown problems (Millet and Lonvaud-Funel 2000, Giraffa and Neviani 2001). These organisms are then forced into a viable but non-cultivable state (Fleet 1999), a phenomenon evidenced to occur within bacterial wine systems (Millet and Lonvaud-Funel 2000) as a result of abiotic stress factors, predominately nutrient depletion (Fleet 1999, Millet and Lonvaud-Funel 2000). The lack of cultivability of some microbial species significantly hinders the isolation of the complete microbial community. As such, genetic verification is required for accurate taxonomic identification, and to reduce the impact of cultivation bias.

1.2.2: Genetic sequencing and the identification of environmental fungal communities

DNA sequencing entails the identification of all the specific base nucleotides making up a DNA region or genome in the correct order (Alphey 1997). Microbial isolates vary though their genetic make-up, and once the sequence of genetic nucleotides has been established, the scientific community can align this data to draw comparisons between isolates down to individual base pair differences. Thus enabling the tracking of the evolutionary history of isolates, and ancestry-linked taxonomic identification (Alphey 1997).

Next-generation sequencing enables a large volume of sequencing data to be produced daily, at a much lower cost than low throughput methods (Metzker 2010). Older sequencing techniques, such as Sanger sequencing, are too slow and expensive for the sequencing of the long reads and large sample sizes (Kircher and Kelso 2010) required for microbial ecological investigation. Next-generation technologies incur high acquisition, maintenance and running costs (Kircher and Kelso 2010). However, the superior read length generation, increased volume of data, low cost per read (Metzker 2010), low error rate (0.4% reported for the Illumina MiSeq platform (Quail et al. 2012)), and ability to sequence heterogeneous species without culturing (Thomas et al. 2012), have proven revolutionary to the ease of scientific analyses and applicability to vineyard and winery environmental microbial study.

1.2.2.1: The Illumina MiSeq platform

Next-generation sequencing technologies encompass platforms designed to achieve high through-put sequencing, and Illumina exist at the forefront of these technologies, including the bench-top Illumina Miseq system (Quail et al. 2012). The Miseq platform is limited to short fragment size single reads (< 250 bp), yet the commonly employed taxonomic identifiable fungal DNA regions of isolates (barcodes) are commonly much larger (500 to 600 bp), and the appropriate use of these barcodes under debate (Kiss 2012, Schoch et al. 2012).

To circumvent the short read length able to sequenced by the Illumina Miseq platform, paired-end sequencing can be employed, and requires the DNA template to be sequenced from both ends of the DNA strand (Glenn 2011). If 250 bp sequencing is conducted from either end of the DNA strand, the produced sequences can be aligned and stitched together to provide a replicated DNA sequence of long reads (~500 bp). This allows greater taxonomic discretion and full sequencing of the chosen target DNA regions to be conducted to counter the short read length dilemma. The Illumina Miseq system reportedly generates a high volume of data with 1.1% more coverage being able to achieved from paired-end sequencing (Quail et al. 2012), thus outperforming Sanger sequencing 100 to 1000 times in daily throughput (Kircher and Kelso 2010).

1.2.3: Role of culture-dependent techniques in naturalised yeast study

Culture-independent genetic sequencing techniques are unquestionably more scientifically appropriate for the evaluation of microbial ecology; yet culture-dependent techniques are utilised in a wide range of specific applications. Morphological and physiological techniques entail the cultivation of mono-cultures of microorganisms present within our environment (Fugelsang and Edwards 2010). Subsequent testing of these pure isolates allows the assessment of the fermentative potential of various organisms (Medina et al. 2013, Magyar et al. 2014), resistance to fungicides (Čadež et al. 2010, Milanovic et al. 2013) and environmental niche adaptation (Cordero-Bueso et al. 2011). Downstream application of this data enables the researcher to further understand the natural environment and the appropriate human modifications to curb undesired growth or volatile production. In addition, these techniques provide valuable data for a preliminary taxonomic diagnosis before targeted genetic sequencing can occur, addressing the issues of primer bias and specificity. The relevance of culture-dependent techniques is still apparent in the scientific investigation and the appropriate technique refinements are required to improve the ease of microbial analysis.

1.2.4: Addressing the challenges of cultivation

Culturing and cultivation can result in a limited level of understanding of the environmental ecology of organisms isolated via culture-dependent analyses; yet it is possible to modify microbiological mediums to target specific groups of microorganisms. As such, the supplementation of agar with other substrates is a common practice, particularly antibiotics (i.e. chloramphenicol) and filamentous fungi inhibitors (i.e. Biphenyl) (Kurtzman et al. 2011).

Filamentous fungi (moulds) are a particular issue in vineyard studies, being noted on grapes and other ripe, sugary fruit (Tournas and Katsoudas 2005), and within aerobic and humid environments, such as the surface of grape berries within bunches, and incubated agar plates (Fugelsang and Edwards 2010). Filamentous fungi inhibitors proven to be effective include Rose-Bengal and Dichloran (Addis et al. 1998, Viljoen et al. 2004). However, the qualitative isolation of yeast colonies on microbiological media supplemented with Biphenyl is reported to be superior (Viljoen et al. 2004). Although proven to be a highly effective mould inhibitor, little research has been reported on the appropriate level of Biphenyl to be supplemented within microbiological media, and this warrants investigation in order to increase the ease of fermentative yeast cultivation.

1.3: Problem statements

1.3.1: Problem statement one

The lack of data on grape and vineyard microbial ecology combined with the limitations of the previous analytical techniques, limits our knowledge of the diversity and detail of the microbial ecology within Margaret River vineyards in Western Australia.

This thesis aims to:

1. Establish the level of taxonomic resolution achievable using two putative fungal barcodes. Analyses will target naturalised fungi capable of fermentation.
2. Identify the diverse range of fungal organisms present within Margaret River vineyards, targeting those capable of fermentation.

1.3.2: Problem statement two

Although commonly employed in grape and wine microbial analyses, there is no published record of an optimal rate of Biphenyl supplementation required to selectively cultivate a diverse range of mucosal colony forming grape and wine isolates.

This thesis aims to:

1. Determine the appropriate level of Biphenyl to ensure the maximum genetic diversity of mucosal colonies isolated on microbiological media from macerated grape samples.

CHAPTER TWO: The taxonomic discrimination of the D2 domain of the 26S gene and the second region of the Internal Transcriber Space (ITS2) within grape samples

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2.0: Statement of Contribution

I, as first author conducted all laboratory work, data analysis and authored the first and final drafts of the experimental chapter. My co-authors assisted with important laboratory tasks, stages of data analysis and chapter review. The laboratory protocols were conducted in collaboration with the Trace and Environmental DNA Laboratory, with Dr. Nicole White assisting with the sample extraction, qPCR cycling, Illumina Miseq sequencing, and chapter development and review, particularly within materials and methods. Prof. Mark Gibberd is the director of the Centre for Crop and Disease Management and primary supervisor of this thesis, providing valuable feedback during Chapter development and review.

Elizabeth Nugent

2.1: Abstract

The yeasts capable of fermentation occur as naturalised species within the local environment and drive ethanol production during grape must fermentation, whilst playing important roles in the development of sensory character. The ecological characterisation of these species within the vineyard is in its infancy, with high-throughput sequencing and the application of next-generation sequencing technologies at the forefront of genetic identification. High-throughput sequencing requires the application of DNA barcodes for taxonomic assignment, with the most appropriate fungal barcode varying between taxa. This study utilized high-throughput sequencing to evaluate the discriminatory power of two barcodes (the D2 domain of the 26S gene and Internal Transcriber Space 2 (ITS2)), with a primer set targeting fungal organisms of known fermentation activity. The D2 domain demonstrated a greater ability to taxonomically assign sequences to the species level, classifying 28% of the operational taxonomic units (OTUs) compared to 22% for the ITS2 region. This is concordant with other literature of species of fermentative fungi, and likely to be a reflection of reference database coverage. Conversely, the ITS2 region classified more OTUs to the genera level (21% of OTUs versus 13% of OTUs for the D2 domain) and less OTUs to the family and high taxonomic levels, demonstrating an increased ability to classify sequence in the absence of species level assignment. This is hypothesized to be a consequence of the increased number of nucleotide polymorphisms housed within the ITS2, and broader application of this region to general fungal investigation, particularly amongst the filamentous fungi. The increased ability of the D2 domain to taxonomically-classify sequences to the species level and leave a lower number of unclassified sequences, suggests it is more appropriate for species level characterisation when applying a primer set targeting organisms capable of fermentation. However, the high capacity of the ITS2 for inter-species classification is relevant, and with the continued deposition of sequences on public reference databases, this region may outperform the D2 domain in the future.

2.2: Introduction

Wine production is a result of the metabolic activity of fungal organisms capable of fermentation (yeasts) (Ribereau-Gayon et al. 2006). The variation among yeast species and strains in the development of sensory character in wine has been established (Fleet 2003, Romano et al. 2003), with oenologically-relevant fungi found as naturalised within the local environment i.e. vineyard (Pinto et al. 2014, Taylor et al. 2014) and winery (Bokulich et al. 2013). The characterisation of these naturalised yeast species and their importance in oenological processes is in its infancy, with the application of developing technologies requiring continued refinement to address scientific aims.

High-throughput sequencing (HTS) enables a culture-independent and metagenomic approach to identify genotypes within communities (O'Brien et al. 2005), such as those encountered in soil (Schmidt et al. 2013) and vineyards (Taylor et al. 2014). The microbiome within a given environment is challenging to analyse due the difficulty in identifying non-cultivable organisms (Fleet 1999, Epstein 2013). HTS circumvents these challenges by sequencing species directly from the environment (Nevo 2001), and identifying heterogeneous microbial communities via the application of metabarcoding (Murray et al. 2013). HTS data can then be analysed either via taxonomy-dependent means, where reads are assigned to the closest reference in a taxonomically annotated database, such as GenBank (Ribeca and Valiente 2011, Santamaria et al. 2012, Murray et al. 2013); or via taxonomy-independent means, where genetic diversity can be classified via the grouping of similar sequences (Ribeca and Valiente 2011), such as Operational Taxonomic Units (OTUs) (Edgar 2013, Murray et al. 2013). Through the identification of OTUs further detail into ancestral hybridisation and sample diversity, particularly amongst species unable to be taxonomically assigned, is achieved. Thus, these analyses provide an unprecedented depth of environmental characterization.

The current next-generation sequencing technologies which employ HTS are limited to the sequencing of small genetic regions (< 250 base pairs (bp)) of inter-species variability (genetic barcodes) (Quail et al. 2012). The barcode for the characterisation of yeast proposed to be first utilised was the D2 domain of the 26S gene (~250 bp) (Peterson and Kurtzman 1991, Kurtzman 1994). With the advancement of genetic characterisation and the development of low throughput sequencing techniques, this was later expanded to encompass the entire D1/D2 domain (~550 bp) (Kurtzman and Robnett 1998, Kurtzman 2010), with the

identification of longer DNA sequences generating more opportunity for taxonomic differentiation (Bokulich and Mills 2013). However, it has been demonstrated that the D2 domain houses the majority of polymorphisms required for inter-species taxonomic assignment (Peterson and Kurtzman 1991, Hinrikson et al. 2005, Kurtzman 2010, Stockinger et al. 2012), and very little to no additional taxonomic information is found by sequencing the D1 domain (Kurtzman 1994). Therefore, due to the requirement of short barcodes for the application of HTS, the D2 domain possesses the greatest relevance for fungal taxonomic discrimination and is a suitable barcode, but is there a better candidate?

The internal transcriber space (ITS) has recently been proposed as a robust barcode for the identification of fungi (Schoch et al. 2012), and has been widely employed in the identification of filamentous fungi (Ksuaba and Tsuge 1995, Takamatsu et al. 1998, Hinrikson et al. 2005). Similar to the D1/D2 domain, the length of the entire ITS region (ITS1-5.8S gene-ITS2) is too long for the application of many next-generation sequencing technologies (~600 bp (Kurtzman 2010)). As the 5.8S gene is highly conserved and possesses little to no taxonomic information (Kurtzman 2010), the ITS1 or the ITS2 regions are utilised in HTS analyses with the preferred region for species assignment reported as taxa-dependent (Monard et al. 2013).

The ITS1 region of DNA is a rapidly-evolving region, whereas ITS2 is moderately rapid to rapidly evolving (Nilsson et al. 2008). This slight difference in evolutionary changes suggests the ITS1 region possesses increased genetic variation, allowing for greater taxonomic discrimination universally within the fungi (Nilsson et al. 2008, Monard et al. 2013). However, both regions evolve dependently (Nilsson et al. 2008) and the degree of genetic variation between each ITS region is dependent on the taxa under investigation (Monard et al. 2013). Previous research has been unable to classify this variation to a specific phylum (Nilsson et al. 2008). However, recent investigation has noted a greater distinction of the Ascomycota phylum (housing the fermentative isolates) within the ITS2 region compared to the ITS1 (Monard et al. 2013). The ITS2 region has been utilised for wine yeast differentiation (Bokulich and Mills 2013, Ženišová et al. 2014), with 250 bp reads showing an increased rate of fungal species level classification than ITS1 (Bokulich and Mills 2013).

The application of a two-gene approach has been previously validated within environmental ecological research (Scorzetti et al. 2002, Garner et al. 2010, Taverna et al. 2013, Porras-Alfaro et al. 2014); yet due to the taxa-dependent nature of barcodes in relation to taxonomic discrimination (Kurtzman 2010), the level of taxonomic assignment obtained for each barcode has not been elucidated for grape and wine samples. This study aimed to undertake HTS analysis and the application of primers targeting fungal species capable of fermentation, in order to evaluate the level of taxonomic assignment of the D2 and ITS2 barcodes within samples of grape origin.

2.3: Materials and Methods

2.3.1: Sampling procedure

Cabernet Sauvignon grape bunch samples were aseptically collected within 24 hours prior to commercial harvest, from six vineyard sites spread ~60 km (North to South) across the Margaret River region. Five grape bunches were collected from each of the six sites (*n*=30) and all grape bunches were transferred to the laboratory on ice. Each grape bunch was homogenized, and glycerol was added to a final concentration of 18 to 20%, prior to being held at -80 °C until further analyses. Hereafter, homogenized grape bunches are referred to as samples.

2.3.2: DNA extraction and quantification

Samples were thawed at room temperature in a sterile biological cabinet immediately prior to sampling for DNA extraction. DNA was extracted using a Yeast DNA Extraction kit (Thermo Scientific) and modified protocol (overnight digestion). Approximately 3 ml from each sample yielded a pellet (60 to 130 mg) after centrifugation for 5 mins at 5,000 g. The resulting pellets, in addition to two extraction controls, were incubated in a rotating hybridization oven overnight at 56 °C and then followed the Yeast DNA Extraction kit manufacturer's instructions. Genomic DNA (gDNA) and extraction controls (*n*=32) were frozen at -20 °C until further analyses.

The DNA extracts were then quantified via real-time quantitative polymerase chain reaction (qPCR) to assess for quality and quantity of gDNA, in addition to the assessment of PCR inhibition. Each gDNA extract was assessed at three DNA dilutions (undiluted, 1/10, 1/100) using a primer set designed for this study (Q1_F 5' GTTGTTTGGGAATGCAGCTC 3' and QB3_R 5' AGTGCTTTTCATCTTTCCCTCAC 3') that targeted a highly conserved region of the 26S gene between the D1/D2 domains. The qPCR setup for samples and controls were prepared in a physically separate ultra-clean laboratory and were carried out using each primer set in 25 µL reactions containing 1X PCR Gold Buffer, 2.5 mM $MgCl_2$, 0.4 mg/mL BSA, 0.25 mM of each dNTP, 0.4 µM of forward and reverse primer, 0.25 µL AmpliTaq Gold, 0.6 µL SYBR Green and 2 µL of gDNA. The cycling conditions for qPCR included an initial heat denaturation at 95 °C for 5mins, followed by 40 cycles of 95 °C for 30 s; 52 °C for 30 s; 72 °C for 45 s followed by final extension at 72 °C for 10 mins. From the qPCR results an optimal DNA concentration was selected for DNA sequencing which was free of inhibition and yielded DNA of sufficient quality. It has been advocated assessment of gDNA extracts in this way can facilitate reproducible quantitative data (Murray et al. 2011).

2.3.3: High-throughput DNA Sequencing

The D2 domain and ITS2 regions were amplified and sequenced on an Illumina MiSeq system utilizing previously published primers that were modified with a unique 8bp Multiplex Identifier tag (MID-tag) and MiSeq adaptors for paired-end sequencing. For the D2 domain primers U1_F (Putignani, Paglia et al. 2008) and NL4_R (Kurtzman and Robnett 1998) were utilized and for the ITS2 region fITS7_F (Ihrmark et al. 2012) and ITS4_R (White et al. 1990) (*see Appendix SI 1 for primer selection details*).

Independent MID-tagged qPCR setup for samples and controls were prepared in a physically separate ultra-clean laboratory and were carried out using each primer set in 25 µL reactions containing 1X PCR Gold Buffer, 2.5 mM $MgCl_2$, 0.4 mg/mL BSA, 0.25 mM of each dNTP, 0.4 µM of forward and reverse MID-tag primer, 0.25 µL AmpliTaq Gold, 0.6 µL SYBR Green and 2 µL of gDNA. The cycling conditions for qPCR using the U1_F/NL4_R (52 °C annealing) and fITS7_F/ITS4_R (54 °C annealing) primer sets were as follows: initial heat denaturation at 95 °C for 5 mins, followed by 40 cycles of 95 °C for 30 s; 52 °C or 54 °C for 30 s (annealing step); 72 °C for 45 s followed by final extension at 72 °C for 10 mins. Multiplex Identifier-tagged PCR amplicons were generated in duplicate for each sample and pooled together to minimise the effects of PCR stochasticity. The pooled amplicons were purified using Agencourt AMPure XP PCR Purification Kit (Beckman Coulter Genomics) following the manufacture's protocol. Purified amplicons were electrophoresed on 2% agarose gel and pooled in approximately equimolar ratios based on ethidium-stained band intensity to form a MID-tagged DNA sequencing library. For each MID-tagged qPCR assay, extraction and PCR controls were included and if found to contain amplifiable DNA these reactions were incorporated into the pooled MID-tagged DNA sequencing library. Illumina MiSeq sequencing was performed using a MiSeq Reagent Kit v2 (500 cycles) 250 bp paired-end protocol as per manufacture's instruction.

2.3.4: DNA Sequence quality filtering and analyses

Sequences were sorted into sample batches based on MID-tags using Geneious v7.1.7 (Drummond et al. 2011). MID-tags, gene specific primers and sequencing adaptors were trimmed from the sequences allowing for no mismatch in length or base composition. Batched and trimmed sequences were then de-replicated (i.e. clustering sequences of exact identity and length) using USEARCH (Edgar et al. 2011) and then de-replicated sequence files were then searched for chimeras using the *de novo* method and were removed. After the above sequence filtering, de-replicated files that contained four or less sequences were removed from further analysis.

Further analysis of yeast sequences were conducted by determining Operational Taxonomic Units (OTUs) using USEARCH (UPARSE pipeline (Edgar 2013)) due to the volume of DNA sequences generated and time required to manually confirm the level of taxonomic assignment for each sequence. The UPARSE pipeline has demonstrated greater generation of named OTUs compared to the MOTHUR and QIIME methods (Edgar 2013) commonly employed in other publications (Bokulich et al. 2014, Pinto et al. 2014, Taylor et al. 2014). The UPARSE pipeline also proved capable of handling 10,000 to greater than 2,000,000 raw reads for bacterial 16S and fungal ITS data sets (Edgar 2013), thus was utilized in this taxonomic discrimination study. Once complete, each reference OTU sequence was searched using BLASTn (Altschul et al. 1990), against the National Centre for Biotechnology Information (NCBI) GenBank nucleotide database (Benson et al. 2006) to enable the identification of reads utilizing the program YABI (Hunter et al. 2012).

Each taxonomic assignment followed a 97% minimum similarity cut-off (Kurtzman and Robnett 1998, Nilsson et al. 2008) and was further investigated to assess scientific validity and taxonomic matches from unpublished studies or improbable sources were classified as unknown. The UNITE database was used to randomly confirm taxonomic assignment for the OTUs of the ITS2 region. UNITE was not utilised as the primary database for ITS2 taxonomic assignment due to a specificity for ectomycorrhiza fungi and underrepresentation of yeasts in comparison to GenBank, for example two ITS sequences of the genera *Pichia* E.C. Hansen (1904) have been deposited on UNITE as opposed to 3,000 on GenBank.

2.4: Results and Discussion

The total number of Operational Taxonomic Units (OTUs) generated for each gene was 187 for D2 domain and 188 for the ITS2 region. However, two OTUs of the ITS2 region were classified as non-fungi and discarded from further analyses (one plant OTU of the genera *Vitis* L. (1753), commonly known as the grapevine (Jackson 2008), and one algal OTU classified as *Trebouxia* Puymaly (1924), a common tree bark algae (Anderson 2014)). Of the fungal OTUs the overall taxonomic classification of the species of grape origin, utilising primers known to amplify fermentative species (*see Appendix SI 1*) was successful, classifying 122 out of 187 fungal OTUs generated for the D2 domain, and 117 out of 186 fungal OTUs for the ITS2 region.

The number of OTUs able to be assigned to the species level was highest for the D2 domain (Figure 2.4.1). The D2 domain classified 28% of the OTUs to the species level, representing 67% of the total sequences obtained, whereas the ITS2 region classified 22% of the number of OTUs to the species level, representing 51% of the total sequences obtained (Figure 2.4.1). The following result suggests the D2 domain superior to the ITS2 region for identification of fermentative fungal OTUs with the primer set chosen (*see Appendix SI 1*).

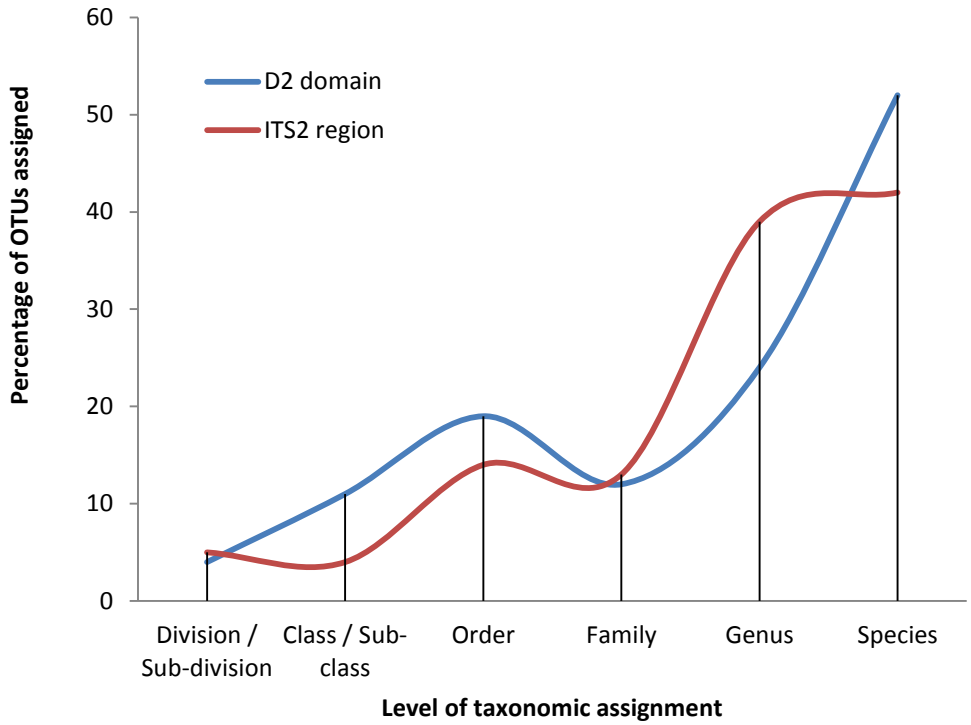

Figure 2.4.1: The number of Operational Taxonomic Units (OTUs) classified to each taxonomic level for the D2 domain and ITS2 region barcodes utilising ripe grape must samples from the Margaret River region after paired end sequencing on an Illumina Miseq platform

The taxonomic assignment of organisms is only as good as the reference database available, and the integrity of the database utilised is reported to have greatest impact on the taxonomic resolution of fungal species (Porras-Alfaro et al. 2014). The NCBI GenBank public database for the comparison of fungal reference sequences must be used with caution, as it has been found to contain sequencing and annotation errors (Nilsson et al. 2006). Additionally, it experiences infrequent updating of existing sequences, and sequences are continually reclassified due to mislabelling (Nilsson et al. 2006, Taverna et al. 2013). However, GenBank houses the largest selection of reference sequences from novel species worldwide (Taverna et al. 2013), as such, is particularly useful for the environmental fungal characterisation of novel samples and locales.

It is reported the level of taxonomic discrimination of the D2 and ITS2 region are taxa-dependent (Kurtzman 2010), as such previous authors whom highlighted the ITS2 region as possessing superior taxonomic differentiation to the species level (Hinrikson et al. 2005, Taverna et al. 2013) and of unknown sequences (Scorzetti et al. 2002), did so utilising samples of the Basidiomycota phylum (Scorzetti et al. 2002), or within a single genera (Hinrikson et al. 2005, Taverna et al. 2013). Conversely, a study by Garner et al. (2010) classified 87% of isolates, including known non-*Saccharomyces* weak fermenters, to the species level with the D1/D2 domain, compared to 79% with the ITS2 region. Thus, demonstrating superior species level taxonomic resolution amongst the relevant taxa. In addition, Garner et al. (2010) further identified the reduced characterisation of sequences of the ITS2 region as attributed to *"low sequence identity to the sequences present in the NCBI database"*, whereas any uncharacterised D2 species were attributed to *"a lack of adequate separation between the two most similar species"* (Garner et al. 2010). As this study targeted diverse fungal organisms capable of fermentation within the Ascomycota phylum, it can be hypothesized the integrity of the NCBI GenBank reference database for the characterisation of these species utilising D2 is better equipped than the ITS2 region. This hypothesis is further supported by the determination of all known D1/D2 sequences of *Saccharomyces* and non-*Saccharomyces* species (Kurtzman and Robnett 1998, Kurtzman et al. 2011) and their sequence deposition on public databases, thereby providing a vast pool of targeted reference sequences, a project currently unmatched for the ITS region. Therefore, whilst the high evolutionary rate (Ihrmark et al. 2012) and increased ability to differentiate between species indicate ITS region is the ideal barcode for fungal species characterisation (Schoch et al. 2012), the proposed reduced integrity of the targeted ITS sequences on public reference databases (Nilsson et al. 2008) compared to the D2 domain (Kurtzman and Robnett 1998, Kurtzman et al. 2011), highlight D2 as the preferred region for species level characterisation amongst fungal organisms capable of fermentation.

Table 2.4.1: Overview of the OTU taxonomic assignment for both the D2 domain and the ITS2 region with the associated number of DNA sequences classified to each taxonomic level using the NCBI public reference database obtained from ripe Cabernet Sauvignon grape must extracts in the Margaret River region

	D2 domain		ITS2 region	
	Number of OTUs	Number of sequences	Number of OTUs	Number of sequences
Division	2	943	3	2479
Sub-division	2	34328	2	559
Class	10	183225	3	128
Sub-class	1	5	1	5
Order	19	195219	14	5912
Family	12	350059	13	336078
Genus	24	63582	39	2006271
Species	52	2998145	42	2593074
Unclassified	65	655045	69	107741

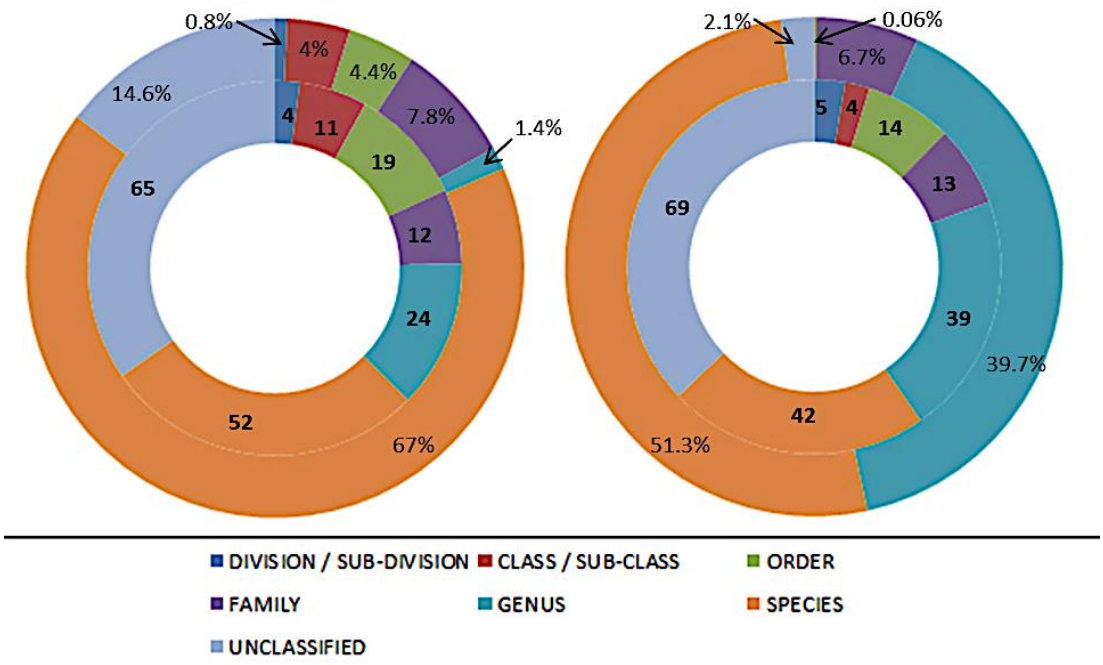

Figure 2.4.2: Taxonomic discretion of the D2 domain (left) and ITS2 region (right); number of OTUs (inner circle) versus percentage of sequences obtained (outer circle) for each taxonomic level for paired end sequences sourced from ripe Cabernet Sauvignon grape must samples in the Margaret River region

The number of OTUs able to be taxonomically assigned at the genera level was greater for the ITS2 region than the D2 domain (21% and 13%, respectively, encompassing 39.7% of the total number of sequences for the ITS2 region, and 1.4% of total of sequences for the D2 domain (Table 2.41 and Figure 2.4.2)). These results suggest the ITS2 region may provide a greater level of higher taxonomic description in the absence of species identification, and is supported by the previous illustration of the ITS region as able to classify 2-3% more fungal genera than the D2 domain (Porras-Alfaro et al. 2014), reduced high-level taxonomic assignment observed within the ITS2 region, compared to the D2 domain in this study (19% of the OTUs and 7% of the total sequences for the ITS2 region classified between family to division levels, versus 25% of the OTUs and 17% of the total sequences for the D2 domain (Table 2.41 and Figure 2.4.2)). For example, within this study a lack of taxonomic assignment existed for the second most abundant OTU within the D2 domain data (*see Appendix SI 6, Table S4*). This OTU was taxonomically suggested to be of the Erysiphaceae family, and represented 14.5% of total sequences (99.1% of the unclassified sequences). However, the sequence similarity (94.0%) was below the required 97.0% for a definitive match (Nilsson et al. 2008), and was therefore unable to be taxonomically assigned to the species level. However, a similar highly abundant OTU within the ITS2 data taxonomically classified as the genera *Erysiphe* R. Hedw. ex DC. (1805) (33.2% of total sequences), and exists within the Erysiphaceae family. Therefore, for this genera, the ITS2 region was superior for taxonomic assignment, and may be a reflection of the broader application of the ITS region in relation to environmental fungal ecology (O'Brien et al. 2005, Taylor et al. 2008, Buée et al. 2009, Schoch et al. 2012), and therefore characterization and deposition of reference sequences to databases, particularly the filamentous and pathogenic species (Glass and Donaldson 1995, Ksuaba and Tsuge 1995, Takamatsu et al. 1998, Smith et al. 2007). Conversely, an increased sequence polymorphisms may be present (Ksuaba and Tsuge 1995, Hinrikson et al. 2005, Garner et al. 2010) and allowing greater taxonomic discretion in the absence of species classification. However, it should be recognised the ITS2 region is multi-copy (West et al. 2014), and has a higher incidence of nucleotide polymorphisms within the same genome than the D2 domain (protein coding in nature) (Solieri et al. 2007). Therefore, it is possible a number of the ITS sequences grouped as individual species or clades of species may be a result of intra-genomic heterogeneity, not species variance, as discussed in detail in Chapter 3.

The number of unclassified OTUs represented approximately 35% of the total number of OTUs obtained for the D2 domain, and 44% for the ITS2 region, accounting for 14.6% and 2.1% of the number of total sequences respectively (Table 2.41 and Figure 2.4.2). These results indicate the undiscernible fungal clusters predominately consisted of low abundance OTUs (except for one aforementioned OTU within the D2 data, in its absence the unclassified D2 OTUs represented 0.13% of the total number of DNA sequences). The persistence of low abundance unclassified OTUs may be a consequence of rarity in nature or laboratory cultivation difficulty, leading to a lack of characterisation and subsequent deposition of reference sequences on public databases, such as GenBank.

The application of high throughput sequencing for the analysis of grape and wine samples is in its infancy, with only one previous study focusing exclusively on the grape fungal biome (Taylor et al. 2014). As a consequence this study is the first application of this technique within Australian vineyards, and it is likely that fungi which have rarely, or not previously, been isolated from nature may be present, and as such are uncharacterised on public databases. In addition, these OTUs may be unclassified due to laboratory-related cultivation difficulty (many unknown OTU clusters identified as 'uncultured fungal clone' on public database comparison), and thereby a lack of scientific knowledge of existence and subsequent deposition of species on reference databases may exist.

2.5: Conclusions and future directions

With the application of a primer set targeting fungal species capable of fermentation, the discretion of the ITS2 region was superior to the D2 domain in relation to genera classification; yet the D2 domain proved superior for species classification, and left fewer unclassified OTUs.

As many of the unclassified OTUs were of low abundance and potentially a result of intra-genomic heterogeneity within the ITS2 data, these OTUs were best characterized by the D2 domain. Whilst the ITS2 region is reported to house greater sequence polymorphisms for taxonomic identification, the reduced rate of species level characterisation is likely to be a reflection a lack of characterisation of relevant ITS sequences deposited on the NCBI public reference database (including multiple copies from a single genome).

The ease of conducting high throughput genetic sequencing and the continued characterization of the complete ITS region will continue to improve taxonomic resolution; subsequently the level of taxonomic assignment within the ITS2 region may exceed the D2 domain in the future. The current investigation suggests a two gene approach as the superior methodology to maximize the level of taxonomic resolution of fermentative fungi from environmental samples of grape and wine origin, and recommends continued investigation into phylum specific taxonomic resolution amongst the ITS2 region and D2 domain.

2.6: Acknowledgements

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CHAPTER THREE: Culture-independent genotyping of yeast populations in Margaret River vineyards

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3.0: Statement of Contribution

I, as first author conducted all laboratory work, data analysis and authored the first and final drafts of the experimental chapter, as prepared as an independent manuscript for submission to a publication. My co-authors assisted with important laboratory tasks, stages of data analysis and chapter review. The laboratory protocols were conducted in collaboration with the Trace and Environmental DNA Laboratory with Dr. Nicole White assisting with primer selection strategies and all laboratory tasks, including sample extraction, qPCR cycling and the Illumina Miseq sequencing, data analysis and chapter drafting. Prof. Mark Gibberd is the director of the Centre for Crop and Disease Management and primary supervisor of this dissertation, providing valuable feedback at the project development and manuscript stages.

Elizabeth Nugent

3.1: Abstract

Fermentation with naturalised yeast is known to impart beneficial sensory attributes to wine, and aligns with the societal attitude shift towards authentic food products. However, due to the poorly defined and variable microbial populations found on grapes significant risks occur within naturalised fermentation, such as unpredictable rates of substrate conversion and unsatisfactory aroma production. This study used culture-independent high-throughput DNA sequencing targeting the D2 domain of the 26S ribosomal DNA (rDNA) gene and the Internal Transcribed Spacer 2 (ITS2) region, to classify the structure and diversity of naturalised fermentative fungal communities within an iconic wine region, in order to elucidate fungal species of oenological importance. Over 14.5 million DNA sequences were obtained for investigation. Taxonomy-dependent analyses identified Ascomycota and Basidomycota from 10 classes, 14 orders, 15 families, 19 genera, and 4 species, and our taxonomy-independent analyses, Operational Taxonomic Units (OTUs), classified 183 and 187 OTUs for ITS2 and D2, respectively. This study was able to classify six Saccharomycotina yeasts (*Saccharomyces cerevisiae, Starmerella bacillarus, Candida parapsilosis*, *Candida tropicalis, Pichia mexicana* and *Zygoascus*) not previously reported in Australian vineyards, and confirmed a presence of the previously detected *Hanseniaspora* genera as classified to the species level (*H. uvarum* and *H. vineae*). The most abundant OTUs belonged to Pezizomycotina (filamentous fungi), whereas Saccharomycotina (fermentative yeast) represented six OTUs for the D2 domain and five OTUs for the ITS2 regions, encompassing 0.44% and 0.01% of the total number of DNA sequences, respectively. Filamentous fungi clearly dominated our results of the grape berry surface and only with the unprecedented depth of sequencing obtained were the small number of fermentative species identified. Studies such as this are providing exceptional exploration of regional fermentative fungal characterization, thus providing clarity for the feasible application of naturalised wine fermentation based upon within-vineyard sampling.

3.2: Introduction

The food and wine production industries have experienced rapid evolution of technology and process throughout recent history. However, these processes are now de-evolving in some instances as the popularity of authentic food products increases. The fermentation of food products (including wine) is utilised for its impact on sensory quality, alcohol production, and for preservation (Pretorius 2000, Bokulich and Mills 2012). The early and late industrialisation of civilisation led to the manipulation and optimization of fermentation, largely enabled by scientific advancement (Pretorius 2000), with a key enabling step for the wine industry being the commercial production of active-dry yeast monocultures for wine production. However, some segments of the food and wine industries are currently experiencing a retro gradation of manufacturing processes, representing a larger societal attitude shift towards authentic food products, as evidenced by the increased popularity of organic foods (Jolly et al. 1989, Huang 1996), and the advancing 'slow food' movement, aimed to preserve cultural food processes, like naturalised fermentation (Schneider 2008). The permanency of authentic food demands is yet to be determined. However, demand appears to be driven by the continued development of the consumer social conscience (Harper and Makatouni 2002, Lockie et al. 2002) and desire for rare or unique products. This is particularly relevant within the wine industry as naturally fermented wines are coveted for their sensory complexity (Henick-Kling et al. 1998), adherence to the consumer 'natural' food and wine ideology, and unique geographic origins.

The evolution of the wine fermentation technology and processes and, in particular, the implementation of inoculation with mono-culture dry-active yeast, are approaches adopted to counter the potential disadvantages related to naturalised fermentations (Henick-Kling et al. 1998). This practice overcomes the risk of natural fermentation incompletion and unpredictability by introducing a large population of known, efficiently-fermenting species of high biomass, to dominate fermentation and drive specific outcomes (Cordero-Bueso et al. 2011). This approach improves fermentation management whilst reducing the probability of undesirable sensory character production (Zoecklein et al. 1999, Jackson 2008). However, the popularity of naturally fermented wines is increasing in spite of these risks, and producers are seeking to exploit naturalised fermentative species within wine regions, often accompanied by claims of regional-specific wine character.

The vineyard is a typically harsh environment for fermentative yeast colonisation and the fungal biome collectively influenced by; climate and geographical location (Longo et al. 1991, van der Westhuizen et al. 2000, Jolly et al. 2003), the fungicide regime implemented (Milanovic et al. 2013), grape variety (Cordero-Bueso et al. 2011), grape berry ripening stage (Renouf et al. 2005), bird and insect activity (Francesca et al. 2012, Stefanini et al. 2012), and vineyard management strategies (Cordero-Bueso et al. 2011, Pancher et al. 2012). In contrast, the winery is an environment supporting more favourable conditions for a dynamic community of fermentative yeast from many origins and subject to; (1) periodic influxes of substrate in the form of grape must, variable ethanol and sulphur dioxide concentration and duration of exposure (Ribereau-Gayon et al. 2006); (2) commercial inoculant presence (Blanco et al. 2011, Clavijo et al. 2011), and thus increased opportunity for inter-species hybridization; (3) imported and humid barrels (Goddard et al. 2010); (4) exposure to cleaning agents (Ocón et al. 2010); (5) increased human and potentially insect activity, and (6) a contaminated microbial community if fruit is processed from neighbouring regions. Therefore, vast differences exist between the vineyard and winery microbial community structures (Pretorius et al. 1999, Sabate et al. 2002). Consequently, in order to target analyses towards naturalised yeast species of the Margaret River wine region in Western Australia, this study focused on grape berry samples obtained from the vineyard immediately prior to harvest.

Previous literature has evaluated the microbial community of the vineyard and winery environment; yet significant gaps in understanding remain. Community identity studies largely fail to elucidate the complete fungal community, utilizing culture-dependent techniques, which disregard ~95% of the fungal community (Taylor et al. 2014). Few studies have utilized culture-independent techniques to study viticulture and oenology-related microbial communities (Bokulich et al. 2013, Bokulich et al. 2014, Pinto et al. 2014, Taylor et al. 2014), and even fewer still focusing on regional characterization of wine grapes sourced from the vineyard (Taylor et al. 2014).

High-throughput DNA sequencing (HTS) is widely utilized in culture-independent techniques and requires DNA metabarcoding to genetically characterise complex fungal communities. HTS has enabled the genetic characterization of complex microbial communities of environmental origin, such as soil (Schmidt et al. 2013) and grape must (Bokulich et al. 2014) through the rapid and low cost generation of a large volume of heterogeneous sequencing data (Metzker 2010). During the exploration of HTS data organisms can be identified in a taxonomy-dependent manner, where reads are assigned to the closest reference in a taxonomically annotated database, such as GenBank (Ribeca and Valiente 2011, Santamaria et al. 2012, Murray et al. 2013). In addition, data can be further analysed in a taxonomy-independent manner, where genetic diversity can be classified via the grouping of similar sequences (Ribeca and Valiente 2011), such as Operational Taxonomic Units (OTUs) (Edgar 2013, Murray et al. 2013), thus providing an unprecedented depth of potential environmental characterization.

The next-generation technologies utilizing HTS approaches include the Illumina Miseq platform and 454-Pyrosequencing, and are revolutionizing the environmental characterization of metagenomics samples. HTS technologies incur high acquisition, maintenance and running costs (Kircher and Kelso 2010). However the ability to utilize DNA metabarcoding to sequence uncultivable species of mixed samples, increased volume of data, low cost per read (Metzker 2010), and low error rate (0.4% reported for the Illumina MiSeq platform (Quail et al. 2012)) have proven revolutionary to the ease of scientific analyses, and their applicability to vineyard and winery environmental microbial studies.

The Illumina Miseq platform requires the sequencing of short genetic regions of high taxonomic resolution (< 250 bp) for the identification of fungal isolates, otherwise known as genetic barcodes (Quail et al. 2012). The appropriate barcode for the identification of fungi is reported to be taxa-dependant (Monard et al. 2013). As a consequence, the previous chapter evaluated the taxonomic resolution of two common barcodes (D2 and ITS2) for the identification of fungal diversity from grape–derived samples (Chapter 2). A two-gene approach (i.e. sequencing of the D2 and ITS2 barcodes) was determined to be most conservative when applying a primer set which targets the amplification of fermentative species (Chapter 2).

The aims of this study were to use culture-independent and HTS techniques to identify the fermentative fungal community and characterize their community structure on Cabernet Sauvignon grapes, obtained from six Margaret River vineyard sites, in order to reduce community anonymity and to provide a first step towards elucidation of potential oenological outcomes during naturalised fermentation. To conduct this a HTS method was employed using two primer set targeting the D2 domain of the 26S ribosomal DNA (rDNA) gene and the Internal Transcribed Spacer 2 (ITS2) region between the 5.8S and 26S rDNA genes, which are commonly used for fungal species identification and references databases have been established for comparative purposes.

3.3: Materials and Methods

3.3.1: Sampling procedure

Samples were collected from six site locations spread ~60 km (North to South) across the Margaret River Wine region according to the same methodology as Chapter 2.

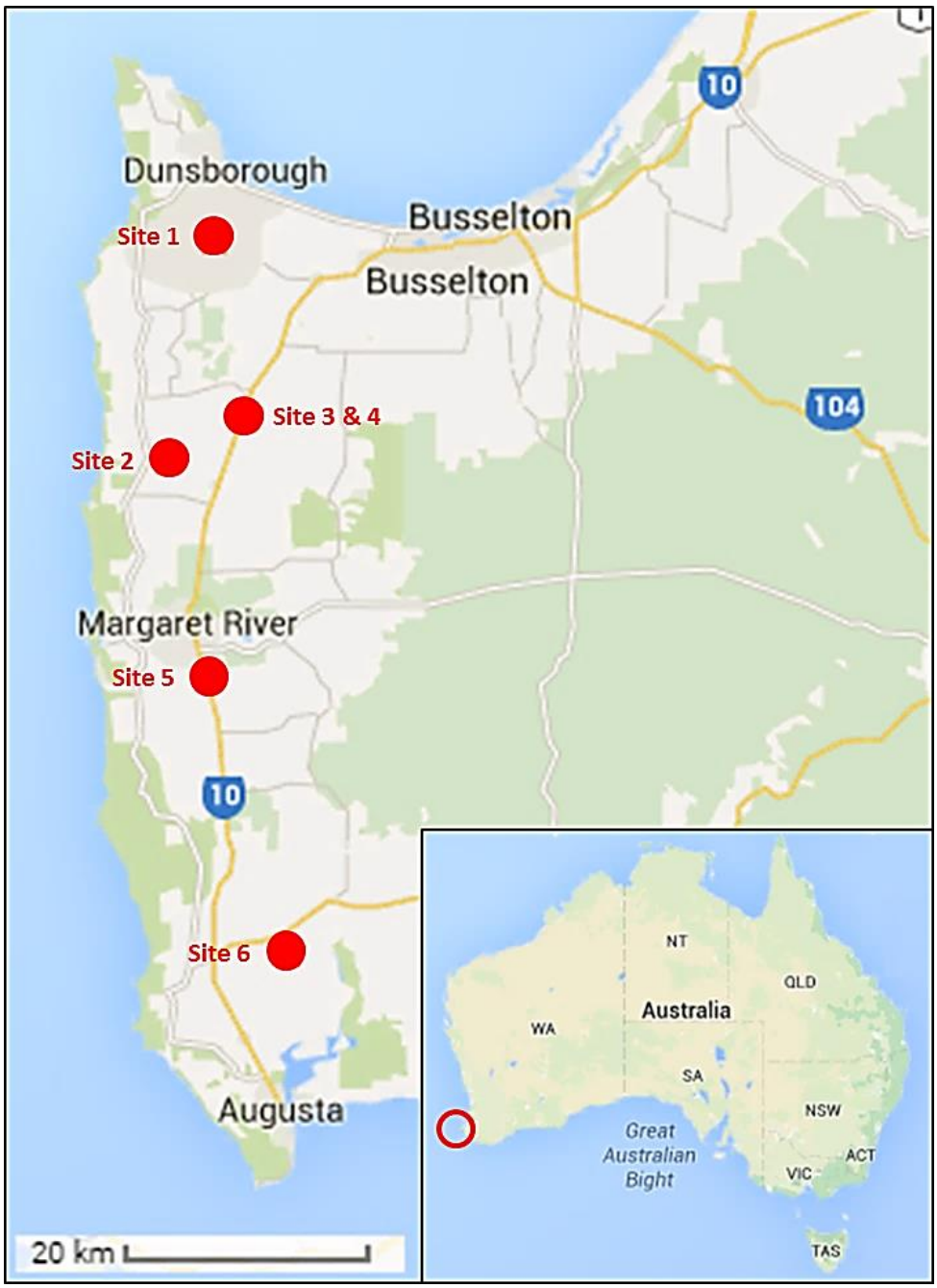

Figure 3.3.1: Map of vineyard sampling sites within the Margaret River wine region for the collection of Cabernet Sauvignon grape samples a maximum of 24 hours before commercial harvest

3.3.2: DNA extraction and quantification

Samples (*n*=30) were digested and DNA extracted utilizing the same Yeast DNA Extraction kit (Thermo Scientific) and modified protocol (overnight digestion) out lined in Chapter 2. Similarly, the DNA extracts (*n*=32) were then diluted and quantified via real-time quantitative polymerase chain reaction (qPCR) to assess for quality and quantity of gDNA using the previously designed and tested primer set (Q1_F 5' GTTGTTTGGGAATGCAGCTC 3' and QB3_R 5' AGTGCTTTTCATCTTTCCCTCAC 3') (*see Appendix SI 1 for further information*).

3.3.3: High-throughput DNA Sequencing

The D2 domain and ITS2 regions were amplified and sequenced in a paired-end configuration on an Illumina MiSeq platform utilizing previously published primers as outlined in Chapter two. (U1_F (Putignani et al. 2008) and NL4_R (Kurtzman and Robnett 1998) for the D2 domain; and fITS7_F (Ihrmark et al. 2012) and ITS4_R (White et al. 1990) for the ITS2 region. Similarly, independent MID-tagged qPCR setup for samples and controls were prepared and amplified as previously described (Chapter 2) with purified amplicons then electrophoresed on 2% agarose gel and pooled in approximately equimolar ratios based on ethidium-stained band intensity to form a MID-tagged DNA sequencing library (*n*=1). Illumina MiSeq sequencing was performed using a MiSeq Reagent Kit v2 (500 cycles) 250 bp paired-end protocol as per manufacture's instruction.

3.3.4: DNA Sequence quality filtering and analyses

Sequences were sorted into sample batches and analysed as previously described (Chapter 2). The BLAST results obtained were imported into MEtaGenome Analyzer v5.2.3 (MEGAN (Huson et al. 2007)), where they were taxonomically assigned using the LCA-assignment algorithm (min. bit score = 65.0, top percentage = 10%, min. support = 1). Further analysis of yeast sequences were conducted by determining Operational Taxonomic Units (OTUs) using USEARCH (UPARSE pipeline (Edgar 2013)) (for further information refer to Chapter 2). The representative sequence of each OTU was taxonomically classified using the NCBI GenBank database as previously described (Chapter 2), with the UNITE database used to randomly confirm taxonomic assignment for the OTUs of the ITS2 region.

3.4: Results and Discussion

3.4.1: Fungal Taxonomic Identity and Diversity

A total of 14,908,537 raw DNA sequences were obtained and after stringent post-filtering for each gene, the amplicon length for the D2 domain was 214 base pairs (bp) and the number of DNA sequences per sample ranged from 29,479 to 244,382. For ITS2, the amplicon length varied, ranging from 234 to 336 bp and the number of sequences per sample ranged from 64,226 to 284,985. Excluding the controls, the de-replicated DNA sequence files (i.e. a minimum of five or more identical sequences were required to remain as a unique sequence; hereafter referred to as unique) showed a higher number for ITS2, ranging from 536 to 1,608 unique sequences per sample, compared with 142 to 1,231 unique sequences per sample for D2 domain. This increase in the number of unique sequence isolated for the ITS2 region may be a consequence of greater intra-genomic variation in comparison to the D2 domain. For instance, the large ribosomal sub unit (LSU) housing the D2 domain and ITS2 sequences is illustrated to be paralogus across fungal taxa (Alvarez and Wendel 2003, Nilsson et al. 2008, West et al. 2014), with *S. cerevisiae* reported to house 50 to 354 copies within a single genome (West et al. 2014). The homogeneity of sequences of this multi-copy LSU is higher in encoding regions, such as the 26S gene housing the D2 domain, than the transcriber spaces (Solieri et al. 2007), therefore an increase in unique sequences is seen within the ITS2 region. This is supported by previous authors determining ITS intra-genomic fungal variability (Aanen et al. 2001, Okabe et al. 2001, Wang and Yao 2005, Fell et al. 2007, Solieri et al. 2013), specifically three taxonomically divergent sequences with polymorphisms spread across the ITS1 and ITS2 regions have been isolated within single strains of *Z. rouxii*. However, no heterogeneity existed within the D2 domain of the same strain (Solieri et al. 2007). This intra-genomic heterogeneity is particularly evident in yeasts and species of fermenting origin, due to frequent hybridization (West et al. 2014) and rapid evolutionary rates.

In line with the aims of this study, the genetic diversity among the fungal community identified on Margaret River Cabernet Sauvignon wine grapes showed an increased diversity amongst the Phylum Ascomycota, comprising 6 Classes, 12 Orders, 13 Families, 15 genera, and 3 species. The Phylum Basidiomycota was less diverse and encompassed a smaller proportion of genetic variation in the fungal grape biome (4 Classes, 3 orders, 2 families, 4 genera and 1 species (*see Appendix SI 2 for phylogenetic trees*)). The Ascomycota are known for housing the filamentous, pathogenic and fermentative fungi (Taylor et al. 1999) and Basidiomycota the non-fermentative species; hereafter our results and discussion will focus on the Ascomycota phylum.

The taxonomic identification of vineyard fungal species, not to mention the intra-species diversity, can be a complicated process given the vast diversity of species that exist in the physical environment (estimated at 3.5 to 5.1 million species within global soil communities (O'Brien et al. 2005)). Through ecological research it is now becoming apparent a large number of species obtained from environmental samples (particularly uncultivable organisms) are insufficiently characterized (Nilsson et al. 2006), or absent from public reference databases. Thereby leading a researcher to taxonomically assign an uncultivable organism to either; a higher taxonomic level (i.e. Family or Order) based on the similarity of a match sourced from a reference database, or classify the species as unknown. In such circumstances the application of Operational Taxonomic Unit (OTU) analyses allows for the classification of fungal community structure and diversity in a taxonomy-independent manner through grouping of DNA sequences that represent a species or clade (Edgar 2013). This approach is considerably more robust and in many instances still allows for taxonomic analysis of known species.

3.4.2: Fungal Community Structure as Operational Taxonomic Units

An equivalent number of OTUs were identified for each gene region amplified (187 for D2 and 188 for ITS2), although on subtraction of three ITS2 OTUs classified from the experimental controls, one OTU classifying as the grapevine, and one OTU classifying as algae, subsequently leaving 183 ITS2 OTUs for further investigation.

Within the Ascomycota OTUs were dominant and an extraordinary diversity was classified, comprising 84.5% and 86.9% of the total number of DNA sequences within D2 and ITS2, respectively (Table 3.4.1). Our ability to generate diverse OTUs and classify Ascomycota was higher than what has been previously reported, regardless if they were culture-independent or culture-dependent studies (Renouf et al. 2005, Raspor et al. 2006, Barata et al. 2008, Gayevskiy and Goddard 2012, Setati et al. 2012, Taylor et al. 2014). This is likely attributable to the *in silico* analyses undertaken during the experimental design and sequencing depth achieved, resulting in ~60-fold increase of Ascomycota DNA sequences compared to the previous authors.

Table 3.4.1: Operational Taxonomic Units classified with the number of DNA sequences assigned to sub-phylum for the D2 domain and ITS2 region obtained from next-generation sequencing of ripe Cabernet Sauvignon grape must extracts sampled within the Margaret River region.
Data in brackets represent the proportional distribution of sequences among the Phylum/sub-Phylum categories

	D2 domain		ITS2 region	
Phylum and Sub-Phylum	Number of OTUs	Number of sequences	Number of OTUs	Number of sequences
Ascomycota: Saccharomycotina	6	19582 (0.44%)	5	92 (0.01%)
Ascomycota: Pezizomycotina	74	3761941 (84.0%)	65	1154060 (86.5%)
Ascomycota: Other sub-phyla	2	3940 (0.09%)	6	4199 (0.3%)
Basidomycota: All sub-phyla	40	40043 (0.9%)	26	68643 (5.1%)
Unclassified OTUs	65	655045 (14.6%)	81	107723 (8.1%)

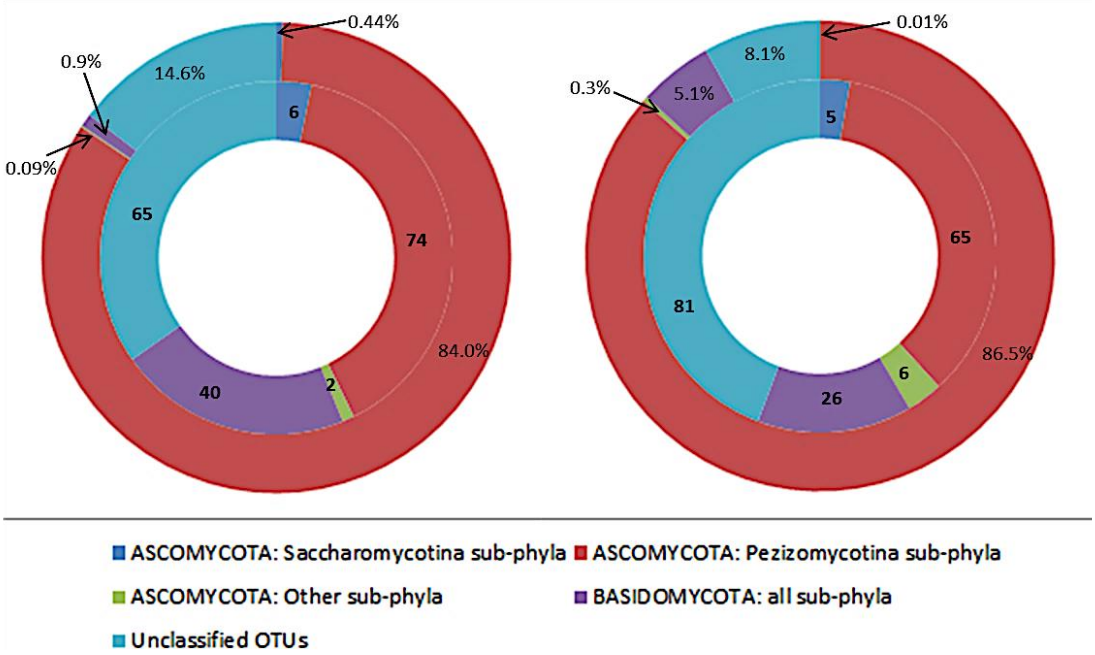

Figure 3.4.1: Distribution of number of OTUs classified (inner circle) versus percentage of total number of DNA sequences (outer circle) generated by the D2 domain (left) ITS2 region (right) for each sub-phylum for sequences generated from ripe Cabernet Sauvignon grape must samples of the Margaret River wine region

Despite the application of primers targeting fermentative fungi the OTUs classifying to the Pezizomycotina sub-phylum (filamentous fungi) represented the predominant proportion of OTUs generated (Table 3.4.1 and Figure 3.4.1), and encompassed the OTUs of highest abundance for both genes. Conversely, the Saccharomycotina sub-phylum, housing the fermentative species, represented only six OTUs for the D2 domain and five for the ITS2 region, and their relative proportion to other OTUs classified was 0.44% and 0.01% of the total DNA sequences, respectively. This predominant classification of the Pezizomycotina sub-phyla compared to Saccharomycotina is concordant with other viticulture and oenology studies utilizing culture-independent techniques (Bokulich et al. 2014, Pinto et al. 2014, Taylor et al. 2014).

The number of OTUs unable to be taxonomically classified was high at 65 for D2 and 69 for ITS2; yet these were generally low abundance OTUs, representing 14.6% and 8.1% of the total DNA sequences, respectively. These OTUs may be unclassified due to the recent implementation of next-generation sequencing in the evaluation of fungal environments (Bokulich and Mills 2013) and thus limited characterization and deposition of new sequences on reference databases (less than 1% of 1.5million extant ITS reference sequences present (Nilsson et al. 2006)), particularly uncultivable species. Conversely, this may be a reflection of database integrity hindering taxonomic assignment as it is reported that greater than 10% of ITS fungal taxonomic annotations are compromised, and many sequences lack publication and specific identifying information (i.e. specimen country of origin) within GenBank (Nilsson et al. 2006). However, the generation of OTUs illustrates the extensive diversity of fungal microbiota obtained from Margaret River grapes regardless of taxonomic classification, and as the diversity and integrity of reference databases increases the opportunity for taxonomic assignment similarly increases.

3.4.3: The predominant classified OTUs – the Pezizomycotina sub-phyla

The Pezizomycotina sub-phylum houses the filamentous fungi and those of pathogenic and non-pathogenic activity, and has previously been demonstrated to be dominant on the grape berry surface (Pancher et al. 2012, Bokulich et al. 2014, Taylor et al. 2014). Within D2 the filamentous fungi *Aureobasidium pullulans* (De Bary) G. Arnaud ex Cif., Ribaldi & Corte (1957) classified as the largest OTU (65.5% of the total DNA sequences), followed by an unclassified OTU suspected to be the Erysiphaceae family (14.5% of the total DNA sequences) (*see Appendix SI 6, Table S4*). Similarly, the ITS2 amplification highlighted *Aureobasidium pullulans* as the most abundant OTU, followed by filamentous *Cladosporium* Link (1816); yet both of these OTUs were discarded due to control contamination and discounted from further analyses (*see Appendix SI 3*). Subsequently, the most abundant ITS2 OTU classified as the genera *Erysiphe* of the Erysiphaceae family (33.2% of total DNA sequences), followed by an OTU classifying within the filamentous Sclerotinaceae family (24.6% of total DNA sequences).

The most prevalent OTU of the D2 data, *A. pullulans*, is a yeast-like black mould and has been identified on grape berry surfaces previously (Sabate et al. 2002, Raspor et al. 2006, Gayevskiy and Goddard 2012, Setati et al. 2012, Bourret et al. 2013, Milanovic et al. 2013, Pinto et al. 2014, Sun et al. 2014, Ženišová et al. 2014), including one Australian study (Prakitchaiwattana et al. 2004) and one culture-independent study sampling vineyard leaves (Pinto et al. 2014). *Aureobasidium pullulans* does not possess any fermentative or directly oenological-relevant behaviour. However, is known to exhibit epiphyte and endophyte antimicrobial activity, effectively deterring rot in ripened fruit to the same extent as chemical preparations (Dimakopoulou et al. 2008). Therefore, this organism may play a keystone role in viticultural community dynamics and subsequent grape berry and wine quality.

The genera *Erysiphe* was the most abundant OTU for the filtered ITS2 data and houses a large portion of the plant pathogenic and disease-causing fungi, specifically those responsible for powdery-mildew, such as *Erysiphe necator* Schwein. (1832) in grapevines (Jones et al. 2014). Powdery-mildew is a common grapevine disease flourishing in maritime climates (Huang et al. 2000), such as exhibited within the Margaret River vineyard region of Western Australia, therefore the detection of *Erysiphe* is not unexpected in this study. It is responsible for a significant alteration in the total soluble solids of grape berries, hindering sugar accumulation and lowering bunch weight (Stummer et al. 2003, Stummer et al. 2005). Wines made from 1 to 20% bunch infected Cabernet Sauvignon fruit reportedly display a decrease in flavour intensity, herbaceous and vegetative sensory characteristics (Stummer et al. 2003), thus demonstrating a reduction in fruit ripeness and wine quality of powdery mildew infected fruit.

The high incidence of pathogenic and disease causing Pezizomycotina fungal species is in contrast with the observations at sampling for this study – all fruit appeared to be healthy and intact. This could possibly be explained as DNA presence does not necessarily mean the species is biologically active to the point where it was visually apparent or influenced grape berry quality. It may be more suitable to interpret the findings of this study as 'potential disease load' as described by Taylor et al. (2014). Similarly, abductive reasoning dictates the identification of yeast capable of conducting fermentation does not guarantee they are active participants in the process, but provides an indication of 'potential fermentation outcomes'.

3.4.4: The fermentative grape berry ecology – the Saccharomycotina sub-phyla

The fermentative fungal grape berry ecology of the Margaret River wine region has not been previously investigated, with Prakitchaiwattana et al. (2004) being the only published study from Australian sourced grape extracts. Therefore, this investigation was able to identify five yeast species (*Candida tropicalis* (Castellani) Berkhout (1923), *Candida parapsilopsis* (Ashford) Langeron & Talice (1932), *Starmerella bacillaris* Kroemer & Krumbholz (2012), *Saccharomyces cerevisiae*, and *Pichia Mexicana* Miranda, Holzschu, Phaff & Starmer (1982)) and one yeast genera (*Zygoascus* M.Th. Smith (1986)) of the Saccharomycotina sub-phyla as a novel classification within Australian vineyards. In addition, *Hanseniaspora* although previously noted within an Australian vineyard, could be further taxonomically classified to two species (*H. uvarum* and *H. vineae* van der Walt & Tscheuschner (1957)). Therefore, this study is a significant step forward to elucidate the fungal anonymity of the Margaret River vine region, which has been operating for less than 50 years (ABS 2003).

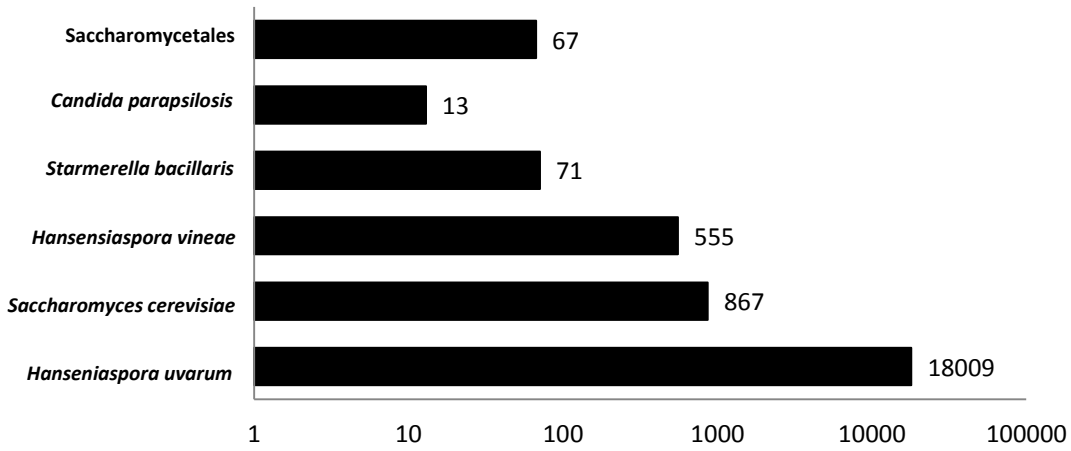

Figure 3.4.2: Each classified Saccharomycotina clade of the D2 domain and subsequent number of DNA sequences on a logarithmic scale obtained from the representatives sequences of OTUs generated from ripe Cabernet Sauvignon grape must samples within the Margaret River wine region.

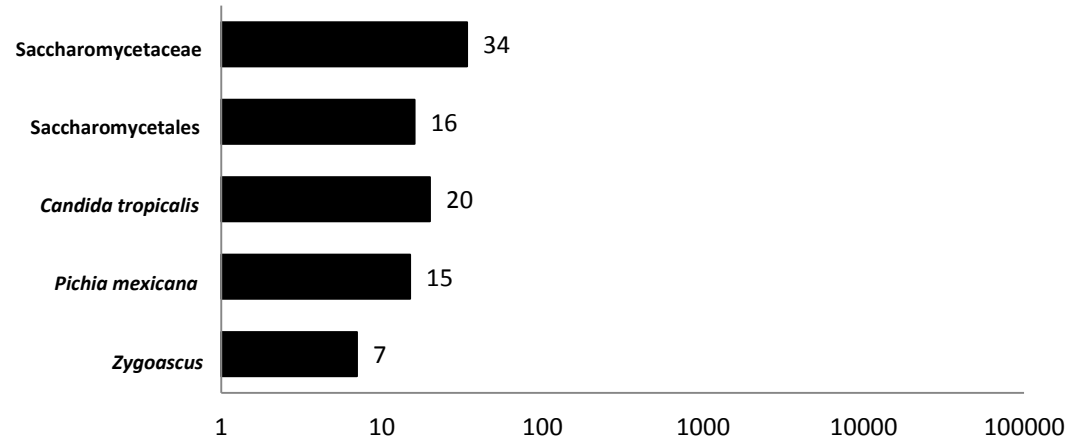

Figure 3.4.3: Each classified Saccharomycotina clade of the ITS2 region and subsequent number of DNA sequences on a logarithmic scale obtained from the representatives sequences of OTUs generated from ripe Cabernet Sauvignon grape must sampled within the Margaret River wine region.

The D2 domain taxonomically classified 2.5-fold more species of the Saccharomycotina sub-phylum over the ITS2 region (Figure 3.4.2 and 3.4.3). In addition, the D2 domain only classified one OTU to a higher taxonomic level (order), whereas the ITS2 region classified three OTUs at the order, family and genera level. Thus, the taxonomic discrimination of known fermentative yeast appeared to be greater within the D2 domain, and this may be representative of the integrity of the GenBank 26S reference sequences, with Kurtzman et al. (1998) sequencing the 26S gene of all known non-*Saccharomyces* species. No such yeast targeted endeavour has been conducted within the ITS2 region, highlighting the validity of a two-gene approach used for this study (Chapter 2).

Saccharomyces cerevisiae (Figure 3.4.2) and the detection of this species within the Margaret River vineyards increase the likelihood of a complete naturalised fermentation. Successful naturalised fermentation is dependent upon the interplay and succession of a diverse range of yeast species (Jolly et al. 2014), as such inadequate diversity or initial cell density, particularly of the highly fermentative *Saccharomyces* species, may result in incomplete fermentation (Jackson 2008). *Saccharomyces cerevisiae* is the most important of the oenological-relevant yeast species, being largely ethanol tolerant and a highly efficient sugar consumer, thus acting to drive fermentation to completion (Goddard 2008).

Saccharomyces cerevisiae is reported to be rarely identified within the vineyard environment via culture-dependent techniques (Tofalo et al. 2014). Similarly, a culture-independent analysis focusing on the grape berry determined that *S. cerevisiae* comprised only ~1:20,000 (0.00005%) of the total population (Taylor et al. 2014), much lower than the 0.02% of total DNA sequences for the D2 obtained in this study. This may be a reflection of the sampling time-point at which Taylor et al. (2014) collected their grape bunch samples.

The vast accumulation of fermentative species on the grape berry surface is noted to occur over the same period as berry sugar accumulation (Renouf et al. 2005). Fermentative species are particularly pronounced if the skin integrity is compromised, allowing the increased release of grape sugars and a fermentative environment favouring *Saccharomyces* colonization (Mortimer and Polsinelli 1999, Barata et al. 2008). Similarly, overripe fruit is conducive to *Saccharomyces* colonisation due to these physiological berry factors and preferable ecological niche conditions (Barata et al. 2012). The increased sugar concentration and berry vulnerability also encourage bird and insect activity, which subsequently act as vectors for yeast transfer (Francesca et al. 2012, Stefanini et al. 2012) potentially from the *Saccharomyces* rich winery, to the vineyard. Thereby birds and insect activity potentially contribute to increasing *Saccharomyces* abundance during ripening. Taylor et al. (2014) harvested grape samples 1 week before harvest, much earlier than the maximum of 24 hours before harvest experienced in our sample collection. The harvest sampling point in this investigation allowed for greater ripening and associated activity, leading to an ecological niche more favourable for *Saccharomyces* colonization, and therefore may explain some of the increased *S. cerevisiae* comparatively obtained.

Conversely, the proportion of Saccharomycotina sub-phyla, including *S. cerevisiae* is notably lower in our study, than those obtained by Bokulich et al. (2014) (< 10% of the total number of reads). This may be explained by hypothesized regional variations such as climate, geographic location and farming practices; but more importantly sampling methodology, as sampling was conducted by these authors at the winery press. The winery environment, including the press, harbours a conglomerate of fermentation inducing parameters, such as increased sugar availability. These parameters increase the potential number of fermentative species obtained, particularly *S. cerevisiae* (Bokulich et al. 2013). Therefore, it is expected an investigation sampling directly from the vineyard would incur a lower proportion of the Saccharomycotina sub-phyla, including *S. cerevisiae*, compared to a winery-derived study.

The most abundant of all Saccharomycotina OTUs were classified as *Hanseniaspora uvarum* (Figure 3.4.2) and may play an important role in the sensory character of naturalised fermentation. *Hanseniaspora* is a weakly fermentative organism, and its use as a starter culture has been recently presented with positive organoleptic potential and thereby fermentative importance (Hong and Park 2013). *H. uvarum* was originally thought to only exhibit spoilage characteristics in the form of ethyl acetate. It is now determined ethyl acetate is deleterious to wine aroma in concentrations 150 to 200 mg/L, and can in fact add organoleptic complexity at lower levels (Zoecklein et al. 1999). Therefore, it is hypothesized *H. uvarum* may positively contribute to the sensory perception of wine in controlled conditions. Significant strain variation in relation to this organoleptic volatile production exists (Hong and Park 2013). However, the use of *H. uvarum* as a starter culture has recently been determined to positively contribute to the sensory character of wine (Hong and Park 2013).

Hanseniapora uvarum is commonly identified in quantities greater than 10%, and is the most widely detected organism in viticultural environments, being previously noted in Japan (Yanagida et al. 1992), Spain (Sabate et al. 2002), Slovenia (Raspor et al. 2006), Greece (Nisiotou and Nychas 2007), Italy (Francesca et al. 2010), India (Chavan et al. 2009), China (Sun et al. 2014), New Zealand (Gayevskiy and Goddard 2012), and Slovakia (Ženišová et al. 2014).

The identification of *H. vineae* (Figure 3.4.2) in the Margaret River vineyard region of Australia may help to convey positive sensory characters to locally produced wine, despite the small proportion (0.00014% of the total DNA sequences (Figure 3.4.2)). There is currently only one other report of *H. vineae* within the viticultural environment (Chavan et al. 2009), therefore this species may either not be widely distributed throughout the viticultural environment, or unable to be identified utilising previous techniques.

It has been evidenced co-fermentations with *H. vineae* and *S. cerevisiae* exhibit significantly greater banana, pear and apple, and lower humid earth, sensory characteristics than *S. cerevisiae* monocultures (Medina et al. 2013). In addition, the co-fermentation exhibited increased cell lysis, contributing to body and texture, whilst demonstrating increased flavour complexity and fruit intensity (Medina et al. 2013).

The *Pichia* genera are frequently identified within viticultural and oenological environments (Sabate et al. 2002, Prakitchaiwattana et al. 2004, Raspor et al. 2006, Gonzalez et al. 2007, Chavan et al. 2009, Gomes et al. 2009, Francesca et al. 2010, Gayevskiy and Goddard 2012, Bourret et al. 2013, Sun et al. 2014, Ženišová et al. 2014), with *P. mexicana* CBS5815 isolated from grape must in Italy (CBS 2015). However, identification of this species in the vineyard is rare, and this instance is second in recent published literature (Bourret et al. 2013). The isolation of *P. mexicana* (Figure 3.4.3) may be a reflection of its' rare occurrence in nature, or the sensitivity of the sequencing technology employed (15 out of 1,334,717 DNA sequences (Figure 3.4.3)), as no other culture-independent viticulture study has recorded the same depth of sequencing, with the closest relatives achieving 95,104 (Taylor et al. 2014) and 79,398 DNA sequence reads (Pinto et al. 2014). Therefore an increased potential to identify minor colonizing communities exists within the utilized culture-independent approach.

The *Pichia* genera demonstrate a wide range of oenological impacts with species noted to; produce killer toxins capable of inhibiting the growth of vulnerable spoilage yeasts (Comitini et al. 2004), increase β-glucosidase activity to enhance the fruity and floral aroma of wine (Swangkeaw et al. 2011), and have been identified as prominent wine spoilage yeasts (Loureiro and Malfeito-Ferreira 2003, Saez et al. 2010) and film-formers (Rankine 1966, Zoecklein et al. 1999). The rare reported isolation of *P. mexicana* has resulted in a lack of scientific study. However, the CBS5815 strain isolated in Italy has demonstrated 29 to 36% killer toxins susceptibility (Yap et al. 2000) making it moderately susceptible to killer toxin presence. Thereby unlikely to contribute to fermentation if the relevant yeast species, such as the two *Pichia anomala* strains tested by these authors (Yap et al. 2000), were present and producing relevant killer toxins within a naturalised fermentation.

The potential sensory character induced by the fermentative activity of *Starmerella bacillaris (Syn. Candida zemplinina)* is variable (Figure 3.4.2), and sensory volatile production should be assessed on a strain by strain basis. The taxonomic classification of *S. bacillaris* has a long and complicated history, being previously thought to be synonymous with *Candida stellata* (Kroemer & Krumbholz) S.A. Meyer & Yarrow (Yarrow & Meyer 1978). However, recent examination of the *Starmerella* Rosa & Lachance (1998) clade determined that the two were closely related, but *S. bacillaris* is reported to be synonymous with *Candida zemplinina* Sipiczki (2003), and some oenological strains have since been reclassified (Csoma and Sipiczki 2008, Duarte, Pimentel et al. 2012). *Candida zemplinina* is widely studied and characterized throughout geographical regions, such as Greece, China, New Zealand, Slovakia and

Italy (Nisiotou and Nychas 2007, Zhang et al. 2010, Gayevskiy and Goddard 2012, Milanovic et al. 2013, Sun et al. 2014, Ženišová et al. 2014), unlike *S. bacillaris*, therefore it will be focus of this discussion. *Candida zemplinina* has been demonstrated to be fructophillic in nature and exhibit variable sensory character production in monoculture and co-fermentation with *S. cerevisiae* (Sadoudi et al. 2012, Magyar et al. 2014). Similar to *H. uvarum*, extensive strain variation exists in relation to *C. zemplinina* glycerol, acetic acid and sensory thiol production (Zott et al. 2011, Sadoudi et al. 2012, Magyar et al. 2014), and the capacity of the relevant strain should be examined to confirm a positive sensory influence.

Candida parapsilosis and *Candida tropicalis* (Figure 3.4.2 and 3.4.3) are infrequently identified in nature, as such their oenological importance in fermentation has not been fully characterised. *Candida parapsilosis* prevalence exists in palm wine (Stringini et al. 2009) and rice wine (Jeyaram et al. 2008), but has only been detected as a low abundant species in two wine growing regions, Spain (Gonzalez et al. 2007) and South Africa (Setati et al. 2012). In addition, this species did not survive to beyond the middle of the fermentation (Gonzalez et al. 2007), therefore may actively contribute to aromatic sensory complexity in the beginning of naturalised fermentation. *Candida parapsilosis* has been classified as a spoilage yeast (Loureiro and Malfeito-Ferreira 2003). However, volatile characterization during grape fermentation has not been conducted, and classifications standards are continually changing, such as evidenced by *H. uvarum*.

Candida tropicalis is difficult to cultivate within the laboratory, commonly detected only via culture-independent techniques (Xie et al. 2007) or only on specialized media (Hierro et al. 2006), thus the rare isolation of *C. tropicalis* in this and previous studies may be a reflection of difficulty cultivating the isolate in a laboratory setting combined with a rare occurrence in nature. *Candida tropicalis* has been identified in two oenological related studies, at a prevalence of 0.6% of all experimental fermentations including under-ripe, ripe and overripe fruit in Italy (Hierro et al. 2006), and only present on sour rot infected grapes in a single vintage of Portugal (Barata et al. 2008). Coincidentally, the fermentative importance of *C. tropicalis* has not been elucidated, but its presence highlights the generic and species diversity present within the Margaret River grape samples.

Zygoascus remains an ill-studied organism and to our knowledge has no oenological importance (Figure 3.4.3). Although *Z. hellenicus* M.Th. Smith (1986) is prevalent on sour rotten fruit, bioactivity of this genera as spoilage yeast has not been ascertained (Barata et al. 2008). This lack of scientific study may again arise from cultivation difficulty as Ocon et al. (2010) had isolated *Z. hellenicus* on general media from the first 48 hours of spontaneous wine fermentations in Spain. However, other authors only isolated *Z. hellenicus* on sour rot fruit and with the application of specialized cultivation media (Guerzoni and Marchetti 1987, Barata et al. 2008). The rare isolation of the genus *Zygoascus* in previous culture-dependent methods (Barata et al. 2008, Romancino et al. 2008, Chavan et al. 2009), low proportion in this study (7 out of 1,334,717 DNA sequences (Figure 3.4.3)), and our inability to taxonomically identity to the species level, supports the hypothesis of reduced cultivability of this genera, and our detection may be due to the depth of sequencing and the approach employed.

3.5: Conclusions and future directions

The depth and quality of analysis able to be achieved by our two-gene investigation has not been realised hitherto by any other reported investigation, and provided a broad characterization of the fungal community present at wine grape harvest. The taxonomic assignment of yeast species to a reference sequence is dependent on adequate genetic variation with the targeted region to enable accurate taxonomic assignment. The D2 domain increases the opportunity for yeast taxonomic discrimination by reducing intra-genomic heterogeneity and providing a vast reference database for characterization. However, the ITS2 region houses a high evolutionary rate allowing for maximal inter-species discrimination. Therefore, the application of both genes significantly increased the diversity of DNA sequences generated and opportunity for interrogation.

Our findings indicate after the targeted analysis of fermenting organisms, the fungal grape biome within the Margaret River wine region consisted of a predominant proportion of filamentous fungi of the Pezizomycotina sub-phylum, such as and yeast-like *A. pullulans* and pathogenic *Erysiphe*. The number of naturalised fermentative fungal species classified and their OTU abundances from ripe Cabernet Sauvignon berries were minor, but consisted of diverse species of both non-*Saccharomyces* and *Saccharomyces* origins. These species may have important implications within naturalised fermentation. However, a significant lack of scientific study of the oenological implications of these organisms is apparent, largely due to difficulty cultivating some organisms in the laboratory.

This preliminary understanding of the fungal community structure, including fermentative organisms and their role in oenological processes, assists in the evaluation of the potential of naturalised yeast species from Australian vineyards. These results have assisted in reducing the anonymity of the regional fungal community present within Margaret River fermentations. Future investigations can assist in the development of strategies to exploit the advantages of naturalised fermentation whilst managing potential risks.

3.6: Acknowledgements

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CHAPTER FOUR: The development of an appropriate microbiological media for the qualitative isolation of yeast in the vineyard fungal microbiome

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4.0: Statement of Contribution

I, as first author conducted all laboratory work, data analysis and authored the first and final drafts of the experimental chapter, as prepared as a manuscript for submission to a publication. My co-authors assisted with protocol design and chapter review. Dr. Ayalsew Zerihun assisted in the design of sampling and microbiological plating methodology, statistical data analysis, and provided comments on numerous drafts of the chapter. Prof. Mark Gibberd assisted in the drafting of the research question and provided valuable feedback at the manuscript stage.

Elizabeth Nugent

4.1: Abstract

In order to evaluate the organoleptic potential and fermentative efficiency of vineyard-resident yeast genotypes, a need to selectively culture viable cells directly from the physical environment is apparent, and as such the microbiological media must contain inhibitors to deter the growth of competitive microbiota. The application of Biphenyl as an inhibitor of filamentous fungi is widely applied within grape and wine studies. However, the appropriate rate of application for maximal yeast diversity is not established. Therefore, this study aimed to determine the appropriate concentration of Biphenyl necessary to culture diverse mucosal (yeast) colonies directly from the vineyard environment. To achieve this, half bunches of Chardonnay fruit were crushed and samples of must plated on Wallerstein's laboratory agar, supplemented with Chloramphenicol and differing concentrations of Biphenyl solubilised in alcohol. At low Biphenyl concentrations in media (0 to 0.005%), the filamentous fungal colonies dominated microbiological media, and although effectively deterred with increasing Biphenyl treatment, the qualitative isolation of mucosal isolates was also inhibited at high concentrations (0.020 to 0.025%). Considering all samples from different vineyards it is concluded that the range of 0.010 to 0.015% Biphenyl concentrations in media was most effective for the qualitative isolation of mucosal colonies, suspected to be of yeast origin, from grape-derived samples. This plating technique can be applied to the environmental cultivation of diverse yeast isolates for grape- and wine-related studies.

4.2: Introduction

The vineyard fungal microbiome comprises a variety of mucosal and filamentous genotypes. Of these the mucosal colony formers, and specifically yeast, are the most important micro-organisms in relation to fermentative potential and the sensory character of wines (Ribereau-Gayon et al. 2006, Jackson 2008, Carrascosa et al. 2011). This is because yeast metabolism is responsible for wine fermentation and influences the sensory character by assisting in the extraction of compounds from solids present in grape must, modifying grape-derived compounds to more flavoursome forms (Lambrechts and Pretorius 2000) and producing over 1000 other organoleptically relevant metabolites (Romano et al. 2003).

Both the vineyard and winery environment are known to harbour naturalised yeast species, including the efficiently fermenting *Saccharomyces* genus, as determined in Chapter 3 and previous literature (van der Westhuizen et al. 2000, Mercado et al. 2007, Goddard 2008). However, many non-*Saccharomyces* species reside within vineyards (Pinto et al. 2014, Taylor et al. 2014) and these can also be responsible for influencing volatile production (Zott et al. 2011, Jolly et al. 2014). However, the vineyard environment is complex and the vineyard fungal microbiome is also home to other fungal species, such as the filamentous fungi (moulds) (Bokulich et al. 2014, Taylor et al. 2014).

The filamentous fungi (Zoecklein et al. 1999) consist of species of pathogenic and non-pathogenic activity. The pathogenic organisms are largely attributed to plant and vineyard diseases, such as powdery mildew, and culminate in a depreciation of fruit hygiene and subsequent wine quality (Stummer et al. 2003, Stummer et al. 2005). The non-pathogenic filamentous fungi are important to vineyard production as they may produce toxins known to be deleterious to human health, such as Ochratoxin A (Serra et al. 2006), play keystone roles in fungal community dynamics deterring the growth of pathogenic filamentous fungi (Dimakopoulou et al. 2008), or exhibit no functional effect on fruit and wine quality. Whilst the importance of the filamentous fungi in terms of grape and vine health is established, to evaluate the fermentative potential of vineyard- or grape-borne organisms, there is a need to inhibit filamentous fungal growth in order to selectively culture multiple mucosal yeast genotypes.

The high concentration of filamentous isolates in the vineyard and their environmental niche tolerances, enable them to dominate the microbial ecology of vineyard and grape must derived samples (Bokulich et al. 2014, Pinto et al. 2014, Taylor et al. 2014). In addition, filamentous fungi are prevalent as airborne

contaminants (Garijo et al. 2008), and due to their tolerance to aerobic conditions and a broad humidity range (Fugelsang and Edwards 2010) often outcompete yeast on the surface of incubated agar plates during cultivation (Addis et al. 1998). Similarly, the phylogenetic lineage of yeasts and filamentous fungi indicate that both clades are commonly susceptible to the same fungicidal chemicals (Addis et al. 1998). Therefore, an appropriate plating technique for the isolation of yeast colonies to achieve maximal diversity must include effective selection against filamentous fungi.

In the elucidation of cultivable yeast colonies, previous authors have utilized Rose-bengal, Dichloran and Biphenyl as filamentous fungal (mould) inhibitors (Mislevic et al. 1992, Pitt et al. 1992, Beuchat 1993), and in grape and wine studies (Combina et al. 2005, Mercado et al. 2007). However, more recent publications evaluating the efficiency of both inhibitors, determined Biphenyl to be superior for qualitative yeast isolation (Addis et al. 1998, Viljoen et al. 2004).

Biphenyl ($C_{12}H_{10}$) is known to inhibit mould growth whilst preserving yeast colony diversity at low concentrations (Addis et al. 1998, Viljoen et al. 2004). Viljoen et al. (2004) conducted an inter-laboratory evaluation to determine the optimal type of mould inhibitor to isolate yeast colonies from blue cheese samples. Viljoen's study is particularly relevant as the primary mould they investigated the filamentous fungi *Penicllium* Link (1809), a known grape must contaminant (Battilani and Pietri 2002, Serra, Mendonca et al. 2006). They found that although yeast enumeration was significantly less on malt extract agar supplemented with Biphenyl (MEB) than 10 other treatments, three out of the five laboratories listed MEB to produce superior qualitative results, due to the substantial inhibition of mould growth. Other inhibitors which were effective included Rose-bengal, Dichloran and Sodium chloride; yet their qualitative isolation of distinctive yeast colonies and mould inhibiting action were vastly inferior to MEB (Viljoen et al. 2004). This is further supported by the finding of malt extract agar supplemented with Biphenyl preserved all yeast species diversity, such as *Saccharomyces cerevisiae* and *Candida* spp. Berkhout (1923), in various cheese samples (Addis et al. 1998). Whilst we can expect the yeast populations within cheese to differ from grape and wine samples, the preservation of *S. cerevisiae* and species of the *Candida* genera, both commonly present within Australian vineyards (see Chapter 3), are promising. The superiority of Biphenyl for relevant filamentous fungal reduction and maximal diversity in yeast isolation is highly appropriate for samples of grape and wine origin.

Biphenyl has previously been used in grape and wine related experimentation (Renouf et al. 2005, Nisiotou and Nychas 2007, Zott et al. 2010, Setati et al. 2012). However, these studies are limited by their use of a single concentration of Biphenyl. The findings are further confounded by variation in application varies among the studies (0.15 mg/L (Guezenec, Aguera et al. 2008) to 500 mg/L (Barata et al. 2008)) and this 3333-fold concentration range is too wide to be of practical use to future researchers. Therefore, there is a need to determine the appropriate level of Biphenyl to ensure the maximum amount of genetic mucosal yeast diversity via limiting filamentous fungal growth on microbiological media from grape must samples. The study aims to determine the optimal rate of Biphenyl supplementation for the isolation of maximal diversity of grape-related mucosal (yeast) colonies on microbiological media.

4.3: Materials and Methods

4.3.1: Sampling procedure

Half bunches of mature Chardonnay grape berries were taken from three vineyards spread across the Margaret River region (Western Australia) at the 2012 harvest. All samples were taken within three days of commercial harvest and picked between 7 and 10 am. The half bunch samples were aseptically snipped from their respective bunches and transferred on ice to the laboratory in sterile plastic containers.

4.3.2: Laboratory procedure

4.3.2.1: Sample processing

The samples were processed in an aseptic environment to reduce outside contamination. Eight to ten berries were randomly selected from each half-bunch and homogenised in 10 ml eppendorf tubes before addition to a final glycerol concentration of 18 to 20% (Sigma-Aldrich, Sydney, Australia). The sample homogenate was stored at -80°C for future analysis.

4.3.2.2: Media preparation

Microbiological plates for the trial were prepared by supplementing Wallenstein's laboratory (WL) agar (with 100 mg/l chloramphenicol and varying additions of Biphenyl solution (the chloramphenicol and Biphenyl crystals (Thermo Fischer Scientific, Scoresby, Victoria, Australia) were solubilized in analytical reagent grade ethanol (Sigma-Aldrich, Sydney, New South Wales, Australia)) (Table 3). Separate tests were conducted to evaluate the influence of 3% ethanol solutions on qualitative mucosal colony formation and no impact was noted (*see Appendix SI 3*).

Table 4.3.1: Composition of the agar-based media for the evaluation of the influence of Biphenyl treatments on the cultivation of fungal isolates derived from Chardonnay grape extracts sampled in Margaret River vineyards

Biphenyl concentration (w/v)	Chloramphenicol concentration (mg/L)	Ethanol approximate concentration (w/v)
0% (control)	100	0.5%
0.005%	100	1.0%
0.010%	100	1.5%
0.015%	100	2.0%
0.020%	100	2.5%
0.025%	100	3.0%

4.3.2.3: Microbiological plating

The homogenised grape samples were de-frosted and vortexed. A 100 µl sample of the homogenate was spread onto prepared media in triplicate. The samples were then incubated at 25°C for 4 days.

4.3.2.4: Colony counting

Post–incubation, the distinct colonies were classified via accepted colony descriptors (Fugelsang and Edwards 2010, Cappucino and Sherman 2011), and counted down the centre of a single agar plate on contact with a background reference grid (*see Appendix SI 5, Table S6 and S7*). It was common for the initial growth of mucosal or filamentous forming colonies to be extremely pronounced, preventing distinct colonies formation. As such, it was not possible to accurately classify these isolates and hence these were not counted.

4.3.3: Data analysis

The colony count data were examined by a two-way analysis of variance. Prior to analyses, the data were checked for meeting the assumptions of parametric analyses (variance homogeneity between samples and Biphenyl supplementation rates; and for normal distribution of residuals). All declared treatment effects are significant at $p < 0.05$ or lower. Optimum Biphenyl supplementation rates were determined from the first derivative of second degree polynomial functions relating colony counts to substrate concentrations. The statistical analyses were carried out using IBM SPSS Statistics for Windows, v22.0 (IBM Corp, Armonk, NY, USA)

4.4: Results and Discussion

4.4.1: Effect of Biphenyl concentration on the selective growth of mucosal colonies

Biphenyl concentration of agar-based media had a significant effect ($p < 0.001$) on the number of distinct total and mucousal colony types isolated (Table 4.4.1 and 4.4.2). Overall, the optimal Biphenyl concentration in media for the elucidation of the maximum diversity of distinct total and mucosal colonies was 0.010 to 0.015% (Figure 4.4.1).

Table 4.4.1: Analysis of variance of effects of sample-origin and the concentration of Biphenyl supplementation to agar media on the total number of distinct colonies (i.e. total diversity) isolated from plated samples of grape must extract obtained from the Margaret River region

Source of variation	df	Sum of Squares	Mean Square	F	Sig.
Rep stratum	2	5.44	2.72	3.87	
Biphenyl_conc	5	27.72	5.54	7.89	<.001
Sample	2	27.11	13.56	19.29	<.001
Biphenyl_conc x sample	10	31.33	3.13	4.46	<.001
Residual	34	23.89	0.70		
Total	53	115.50			

Table 4.4.1: Analysis of variance of effects of sample-origin and the concentration of Biphenyl supplementation to agar media on the number of distinct mucosal colonies (i.e. mucosal diversity) isolated from plated samples of grape must extract obtaiend from the Margaret River region

Source of variation	df	Sum of Squares	Mean Square	F	Sig.
Rep	2	10.04	5.02	6.57	
Sample	2	45.48	22.74	29.78	<.001
Biphenyl_conc	5	34.59	6.92	9.06	<.001
Biphenyl_conc x sample	10	35.63	3.56	4.67	<.001
Residual	34	25.96	0.76		
Total	53	151.70			

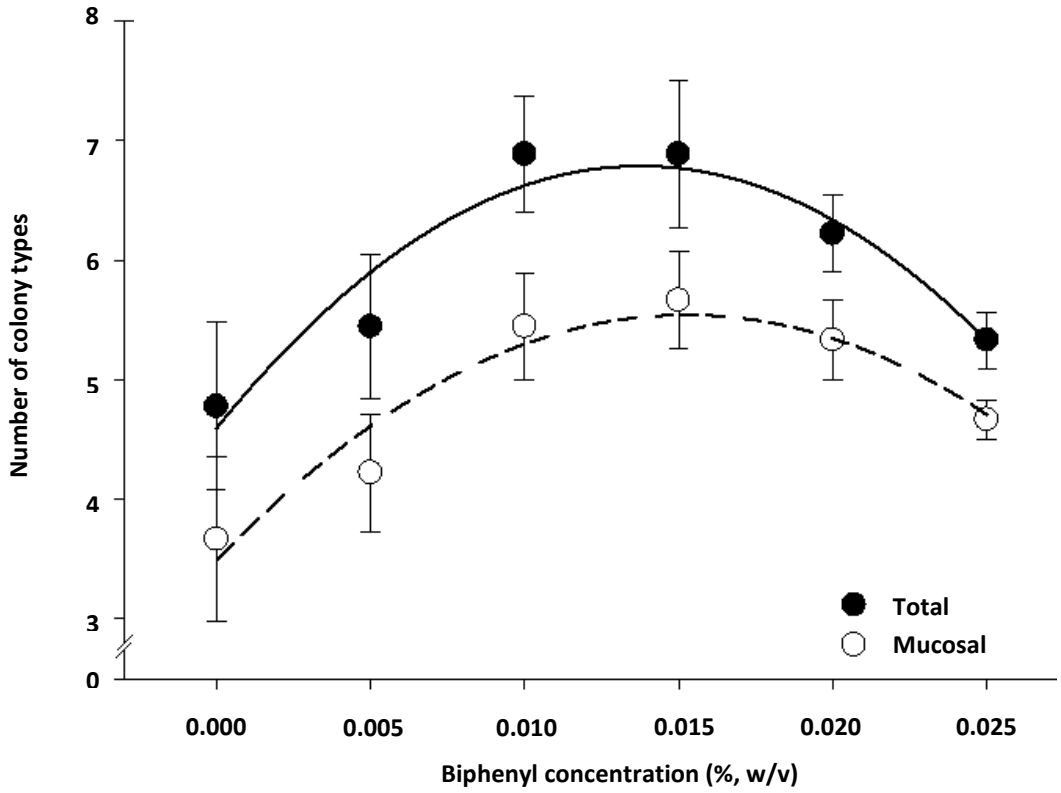

Figure 4.4.1: Number of diverse colony types for must extracts from mature Chardonnay grapevine bunches sampled in the Margaret River region and plated across a range of Biphenyl concentrations in media. Data are means (n=3) with standard errors. Number of colony types were assessed following 4 days of incubation at 25 ^{o}C. Lines are of best fit for the number of total (solid line) and mucosal (broken line) colonies isolated on the media.

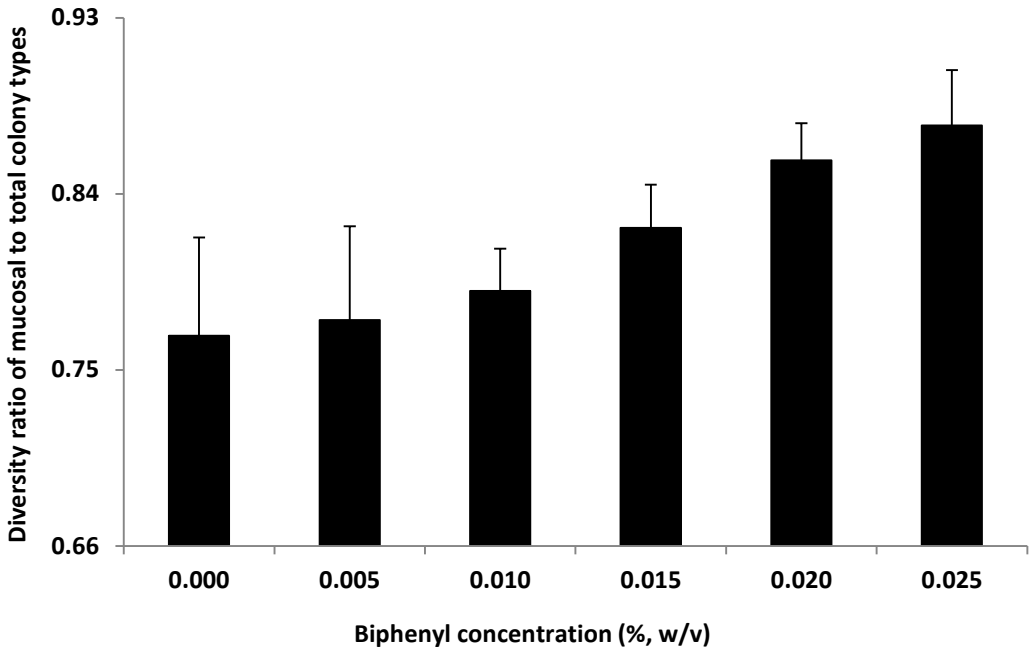

Figure 4.4.2: The ratio of the number of mucosal to total colony types isolated from Chardonnay grape must sampled in the Margaret River region and spread plated on microbiological media which was treated with a range of Biphenyl concentrations. Data are means (n=3) with standard errors.

At low or zero Biphenyl concentrations (0% (control) to 0.005%), a low rate of mucosal diversity was observed (Figure 4.4.1). The low rate of mucosal diversity is likely a result of a high population density of filamentous fungi within the grape must extract before culturing, as indicated by findings in Chapter 3. In addition, the filamentous species experience increased growth in the aerobic and humid conditions experienced on agar plates (Addis et al. 1998), thereby contributing to a dominating presence on the media, and inhibiting the growth and diversity of mucosal isolates (Figure 4.4.2).

At the highest Biphenyl concentration (0.025%) the filamentous fungal diversity was reduced by 40% (Figure 4.4.2). However, the apparent diversity of mucosal colonies established was also inhibited (Figure 4.4.1). The reduction in filamentous colony diversity with Biphenyl supplementation is consistent with the inhibitory action of Biphenyl, and is expected to be concentration dependent. However, this concordant loss in mucosal diversity at high concentrations of Biphenyl (0.020 to 0.025%) suggests the mode of action of Biphenyl is not specific to filamentous species and some loss of diversity amongst susceptible mucosal species also occurs. These results are is in contrast to one study observing no inhibitory action of Biphenyl on qualitative yeast isolation (Addis et al. 1998). However, the previous investigation was limited as it did not analyse multiple rates of Biphenyl supplementation (Addis et al. 1998), therefore the qualitative inhibition of yeast may have occurred if the rate of supplementation was analysed incrementally.

The best selectivity and qualitative isolation of mucosal yeast fungal isolates is highest at 0.010 to 0.015% Biphenyl supplementation (Figure 4.4.1). 0.010 to 0.015% biphenyl supplementation allowed for diverse filamentous inhibition whilst preserving the metabolic activity, isolating the highest diversty of mucosal isolates.

4.4.2: Variation of total and mucosal colony types between samples

The variation among samples suggests that the initial diversity of each population (Figure 4.4.3) is specific to a sample location. Whilst the level of microbial variation within the sampled blocks, and wider wine region remains unknown, the hypothesis of location specific fungal diversity is supported by the variability of communities obtained in previous vineyard studies of differing sampling locales (Polsinelli et al. 1996, Jolly et al. 2003, Gayevskiy and Goddard 2012, Bokulich et al. 2014). The variance between the sampling locations and mechanism of ecological niche adaptation for mucosal fungi has been previously well characterised and may be attributed to differences in climate (Longo et al. 1991, van der Westhuizen et al. 2000, Jolly et al. 2003), geography (Gayevskiy and Goddard 2012, Bokulich et al. 2014), vineyard management (Cordero-Bueso et al. 2011, Pancher et al. 2012), vector exposure (Francesca et al. 2012, Stefanini et al. 2012), or fungicide application (Milanovic et al. 2013). In addition, berry chemistry factors such as, pH or titratable acidity may contribute to the variation among samples (Renouf et al. 2005). However, it should be noted sample ripeness measured as total soluble solids (TSS), showed no relationship (21.9 °Brix for vineyard 1, 24.0 °Brix for vineyard 2, and 21.8°Brix for vineyard 3) in this study.

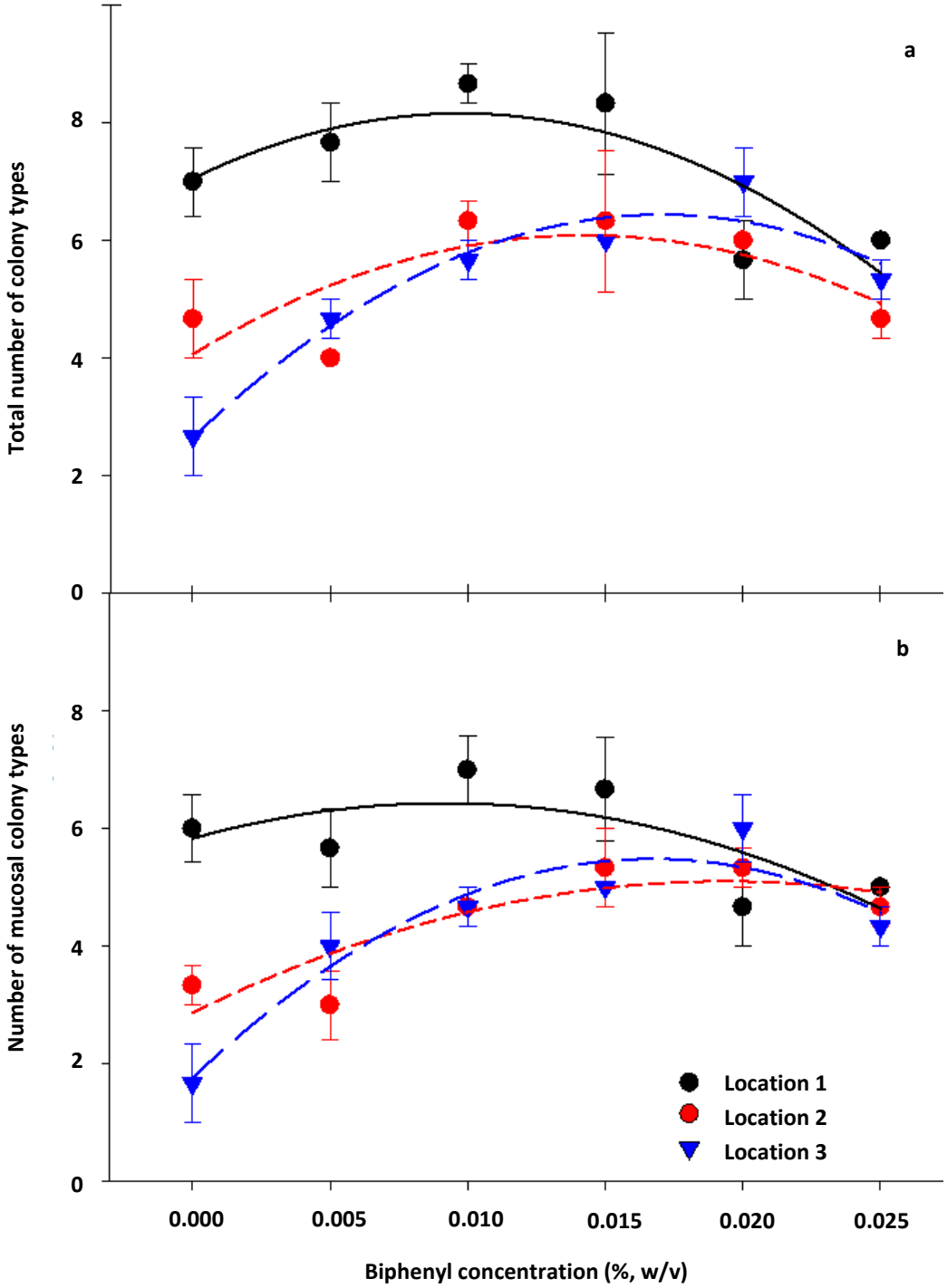

Figure 4.4.3: Average number of total and mucosal colony types isolated from grape must samples from mature Chardonnay grapevine bunches sampled in the Margaret River region and plated across a range of Biphenyl concentrations in media. Data are means (n=3) with standard errors. Number of colony types were assessed following 4 days of incubation at 25 ^{o}C. Lines are of best fit for a) total number of colony types, and b) number of mucosal colony types, per vineyard.

The optimum level of Biphenyl required to elucidate the maximum number of distinct colonies was dependent on the diversity of the population of the sample in question, i.e. the optimum Biphenyl media concentration varied between samples (Figure 4.4.3). However, similar to the overall fungal observation, all samples demonstrated a central peak in qualitative colony isolation (Figure 4.4.3), where competitive growth and filamentous fungi inhibition was optimised. The shifting of the optimal Biphenyl concentration obeserved between samples may be a reflection of the taxonomic identities of the sample population, with eight diverse yeasts of the fermentative Saccharomycotina sub-phylum previously isolated within Margaret River vineyards (Chapter 3).

Although no prior investigations have assessed genetic diversity among Saccharomycotina yeast species in tolerance to Biphenyl exposure, the peak in mucosal diversity at supplementation rates of 0.010 to 0.015% Biphenyl, suggest the metabolic tolerances and characteristics of the mucosal colony-forming species play a large role in determining qualitative colony counts. This is further evidenced as the initial sample colony types established showed no discernible correlation with Biphenyl optimum preferences (i.e. sample location 1 possessed the largest diversity in mucosal colony types, but demonstrated the lowest mucosal tolerance to Biphenyl (0.008%), whereas sample 3 possessed the lowest number of mucosal colony types but demonstrated the mid-range qualitative mucosal tolerance to Biphenyl (0.0157%) (Figure 4.4.3). Therefore, the initial diversity of the sample population may be more important than the effect of Biphenyl concentration on mucosal colony formation, and Biphenyl treatment will optimise conditions for selective growth of mucosal species.

4.5: Conclusions and future directions

Biphenyl is a suitable supplement for the selective culture of grape-derived biological samples. Biphenyl effectively inhibits growth of filamentous fungi and allows for selective the isolation of yeast from vineyard- and/or grape-derived samples. While the greatest level of colony diversity was dependent on the microbiological composition of each sample, overall Biphenyl supplementation rates at 0.010 to 0.015% achieved the best qualitative results. The over or under-supplementation of microbiological media can pose detrimental effects on the quantitative and qualitative composition of filamentous and mucosal colony counts. The above range was successfully shown to be suitable for ecological qualification of samples of grape origin; its suitability for samples of other origins (e.g., grains) would need to be evaluated.

In future investigations it would be worthwhile to taxonomically identify all mucosal isolates and analyse their tolerance to Biphenyl on a case-by-case basis. However, the predictable inhibition of Biphenyl on filamentous rather than mucosal colonies highlights its applicability.

This study has demonstrated the role of Biphenyl supplementation to optimise the selective cultivation of mucosal fungal isolates, including those of yeast origin. This technique can be applied to culture diverse mucosal isolates of grape-origin for downstream processing, such as monoculture fermentative performance evaluation.

4.6: Acknowledgements

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CHAPTER FIVE: General Discussion

5.0: The application of high throughput sequencing to environmental DNA

The environment encompasses a wide range of microbial species (Roesch et al. 2007), many have yet to be fully characterised (Taylor et al. 2014). However, the recent advances in metagenomics allow the acquisition and analyses of DNA directly from the physical environment (eDNA), we are beginning to get a greater understanding of (1) the complexity and diversity of the environmental biome, and (2) the impacts of natural factors and human activity on it.

A breakthrough in the characterisation of eDNA involved the application of high throughput sequencing (HTS) via next-generation technologies (O'Brien et al. 2005), with which the DNA profile of heterogeneous communities could be amplified (Riesenfeld et al. 2004, Tringe and Rubin 2005). This breakthrough enables the characterisation of a broad and unprecedented range of microorganism. As a result, we are beginning to understand that the cultivable component of the microbiome typically consists of less than 5% of the environmental microbial community (Epstein 2013, Taylor et al. 2014). Importantly, HTS is increasing our ability to characterise these difficult to cultivate organisms (Riesenfeld et al. 2004, Tringe and Rubin 2005), such as *C. tropicalis*, *C. parapsilosis* and *Zygoascus* identified in Chapter 3, and enables an estimation of diversity of eDNA to be established in the absence of taxonomic classification (Ribeca and Valiente 2011, Edgar 2013). This was clearly demonstrated in Chapter 2: while a broad range of eDNA was found, only 56 to 65% of sequences grouped as OTUs, could be taxonomically assigned.

The increased depth and integrity of data obtained from eDNA has been evidenced (Hill et al. 2002, O'Brien et al. 2005, Buée et al. 2009). The results from Chapter 2 and 3 of this thesis demonstrate that the same holds true for the microbiome of the viticultural environment. In summary, eDNA is enabling the scientific community to extract a high level of information from the environmental microbiome (O'Brien et al. 2005), and with continued technological and bioinformatic advances the value of this data will increase.

5.1: The application of barcodes for the characterisation of vineyard yeast

The application of HTS revolutionised the characterisation of eDNA, including vineyard fungi; yet there are still limits to current technologies, such as sequence length, technology acquisition and running costs, and total laboratory hours required (Metzker 2010, Quail et al. 2012). As such, it remains impractical to sequence whole genomes for the taxonomic resolution of complex heterogeneous communities. As a result, only short DNA segments of high taxonomic resolution (barcodes) (Stoeckle and Hebert 2008) are sequenced for the large-scale characterisation of eDNA (O'Brien et al. 2005).

The selection of barcodes for the characterisation of diverse fungi sourced from eDNA is under much discussion (Kiss 2012, Schoch et al. 2012) with the D2 domain of the 26S gene (Peterson and Kurtzman 1991) and the ITS2 region (Schoch et al. 2012) widely utilised. The determination of the appropriate candidate is reported to be taxa-dependent (Kurtzman 2010, Monard, Gantner et al. 2013). This is demonstrated in two ways in this thesis for the DNA sequences amplified by a primer set targeting environmental species capable of fermentation. Firstly, by the identification of different species, or clades of species, with each barcode (Chapter 3), and secondly, by the variable level of taxonomic resolution obtained with each barcode (Chapter 2).

The identification of different species or clades of species of each barcode reflects the specificity and amplification integrity of each primer set. Each primer set was hypothesized to bind to Saccharomycotina species, based on reference sequence alignment during the primer selection process (*see Appendix SI 1*); yet in practice, two different data sets were obtained (Chapter 3). This highlights the specificity and preferential amplification of each primer set towards particular species (Chapter 2). Conversely, the variable level of taxonomic resolution obtained by each primer set is hypothesized to be a partial reflection of reference database scope and integrity (Chapter 2), as shown in previous studies (Garner et al. 2010, Porras-Alfaro et al. 2014).

The increased depth of data and verification of results obtained by comparing both barcode data from this work validates and recommends the application of a two gene approach for large-scale, heterogeneous eDNA characterisation.

5.2: The naturalised fermentative species within Margaret River vineyards and their proposed influence on naturalised fermentation

5.2.1: Sensory character and fermentation vigour

The naturalised non-*Saccharomyces* and *Saccharomyces* species and strains contribute to the sensory character of a naturalised fermentation by producing a wide range of sensory compounds, converting existing grape substrates to more flavoursome forms, and extracting flavour compounds from grape must (Fleet 2003, Romano et al. 2003). The identity and subsequent potential sensory compound production is integral to ensuring a successful end product, as each species and strain is known to contribute characteristic sensory profiles during fermentation (Jolly et al. 2014). Many of the species and genera identified within Margaret River vineyards (Chapter 3) have previously been shown to differ in their capacity for the production of fruit intensity (Medina et al. 2013), aromatic esters (Hong and Park 2013), volatile acids (Magyar et al. 2014), sensory thiols (Zott et al. 2011), and fermentation vigour (Hong and Park 2013). The combination of this information allows the winemaker to make a more informed decision about the nature of the ferment, the vigour with which it may progress, and the potential sensory advantages and risks.

It can be hypothesized that a naturalised fermentation from the Margaret River region may produce a pleasant sensory character from the bioactivity of *H. uvarum* (Hong and Park 2013) and *S. bacillaris* based on the reported synonym with *C. zemplinina* (Duarte et al. 2012, Magyar et al. 2014). However, if these organisms become stressed or an adverse strain is present, a high production of volatile acidity may result (Sadoudi et al. 2012), spoiling the wine product.

In addition, whilst *H. uvarum* consisted of the largest number of DNA sequences OTUs of the fermentative organisms identified in Chapter 3, it was present in only 5 out of 30 samples spread across three sites. Similarly, *S. bacillaris* was present in 3 out of 30 samples spread across two sites (*see Appendix SI 5*). Therefore, whilst a potential positive sensory impact is apparent (Zott et al. 2011, Sadoudi et al. 2012, Hong and Park 2013), an initial population was not present within all regional samples.

A number of the species identified in samples from Margaret River vineyards have not been thoroughly investigated. This may be due to the difficulty cultivating some organisms as previously demonstrated (*C. tropicalis* (Hierro et al. 2006, Xie et al. 2007) and *Zygoascus* (Guerzoni and Marchetti 1987, Barata et al. 2008). Similarly, the rarity of these species in nature (*P. mexicana* and *H. vineae*) may reduce scientific interest in characterisation due to a lack of perceived importance, or focus on other research areas, i.e. *S. cerevisiae* characterisation. Additionally, two species or clades of species for the ITS2 barcode and one for the D2 barcode were only able to be classified at taxonomic family or order level (Chapter 3). As such, the contributions to fermentative vigour and sensory character within wine have not been elucidated for some naturalised species, and the influence of all these organisms within naturalised fermentation is unknown, and requires further scientific investigation.

5.2.2: The importance of fermentative species derived from the naturalised vineyard population for winemaking

This investigation targeted naturalised vineyard species to identify geographical specific residents, not winery-microbiota which may consist of multiple forcefully introduced species (Zhang et al. 2010, Clavijo et al. 2011), and hybrids of naturalised and introduced organisms (Blanco et al. 2011). In addition, the non-*Saccharomyces* species were targeted due to their reported increased fruity, floral and complexity contributions (Ciani and Comtini 2011, Jolly et al. 2014), which are presumed benefits of naturalised fermentations.

Chapter 3 identified predominately non-*Saccharomyces* species in Margaret River vineyards. Although non-Saccharomyces species contribute to wine sensory character (Jolly et al. 2014), their rate of substrate utilisation (Ciani and Picciotti 1995) and survival in high ethanol environments is lower than *Saccharomyces* species (Soden et al. 2000), thus it is *Saccharomyces* species which complete fermentation (Heard and Fleet 1985). Therefore, whilst there may be the accumulation of desirable sensory compounds in the beginning of fermentation, without a persistent *Saccharomyces* population the fermentation is unlikely to reach completion (Ciani and Picciotti 1995, Ciani and Ferraro 1996).

The level of *Saccharomyces* isolated within the vineyard was high compared to previous vineyard-origin studies (Taylor et al. 2014), but low compared to winery-origin studies (Bokulich et al. 2014). This finding emphasizes the importance of the winery-derived microbiota for the provision of *Saccharomyces* species to naturalised fermentations. However, whilst a vineyard source of *Saccharomyces cerevisiae* was confirmed within Margaret River vineyards (Chapter 3), this was not true for all samples (4 out of 30 samples across three sites (*see Appendix SI 5*)), and subsequently, these fermentations are dependent on the winery-sourced microbiota for completion.

Successive interaction and maintenance of fermentation diversity may be responsible for the most coveted sensory characteristic of naturally fermented wine, complexity. Successful naturalised fermentation is dependent on the successive dominance of naturalised species, with the ability of organisms to out-compete those which become stressed, inactive or in danger of producing excessive undesirable sensory volatiles (Jolly et al. 2014). Through the tailoring of undesired organism growth and the combination of a range of sensory volatiles produced from multiple species and genera, pleasant sensory wine complexity can be evolved (Soden et al. 2000, Medina et al. 2013). The current investigation identified up to 4 vineyard species of fermentative capability per sample although most had none (*see Appendix SI 5*). This suggests the vineyard microbiota may contribute to the sensory character of wine. However, to increase diversity and the likelihood of achieving sensory complexity, the winery microbiota play an important role, with winery-derived naturalised fermentations reported to house between five and seven species (Bezerra-Bussoli, Baffi et al. 2013).

A number of the yeast species capable of fermentation, particularly with the ITS2 data set, were present in very low numbers of DNA sequences. For example, *P. mexicana* contributed to 0.001% of the total DNA sequences obtained (Chapter 3). Whilst we attributed the detection of these isolates to the superiority and sensitivity of the next-generation sequencing technique (Chapter 3), and specificity of the primer set employed (*see Appendix SI 5*), the likelihood of impact on fermentation of these low abundant OTUs is worth discussion.

The environmental tolerances and preferences of fungi capable of fermentation directly impact their biomass and bioactivity. This is evident in the accumulation of species capable of fermentation identified on damaged fruit, as the release of sugar induces an environment more favourable for fermentation (Renouf et al. 2005, Barata et al. 2008). This is also true for the fermentation of wine, for instance during the cold maceration of fruit, an increased biomass and bioactivity of cold tolerant non-*Saccharomyce*s species is evidenced, whilst upon warming, these species are quickly outcompeted and *S. cerevisiae* establish dominance and fermentation control (Hierro et al. 2006). Therefore, it can be extrapolated that as the fruit harvested from Margaret River vineyards is transported to the winery and crushed, a more favourable environment for the continued establishment of these populations is probable (Jackson 2008). However, if a winery-resident species is better established and more capable in the existing environmental conditions, the growth and bioactivity of vineyard microbiota during fermentation, particularly of low abundant species, may be outcompeted.

5.3: The application of culture-dependent and culture-independent techniques

5.3.1: The appropriate application of culture-dependent and independent techniques

Culture-dependent and -independent techniques are both utilised within microbial characterisation in different scientific applications. Culture-dependent techniques require the cultivation of microbiota on or within microbiological media before further analysis can be undertaken. These applications are required for the study of pure microbiological cultures and the characterisation of acidity and sensory compound production (Sadoudi et al. 2012, Magyar et al. 2014), evaluation of environmental tolerances and fermentation kinetics (Ciani and Ferraro 1996, Hierro et al. 2006), cloning of microorganisms genes and investigation of expression genes (Torsvik and Øvreås 2002), and determination of the role of species in community dynamics i.e. killer toxin expression (Comitini et al. 2004). These applications allow researchers to identify the morphological and physiological characteristics of individual microbial species. However, such techniques can be time-consuming, and incur cultivation bias due to difficulty of cultivating unknown or uncultivable organisms (Epstein 2013).

Conversely, culture-independent techniques enable the study of isolates directly from their environment, circumventing the errors of cultivation bias and time-consumption. The application of these techniques are best suited for untargeted ecological characterisation of the community at present (Hill et al. 2002), enabling the large-scale determination of microbial environments. In addition, these techniques can be useful in the study of unknown or uncultivable organisms (O'Brien et al. 2005), heterogeneous microbial communities (Riesenfeld et al. 2004), and monitoring community adaptations within the external environment (Bokulich et al. 2014). For instance, the application of highly sensitive culture-independent techniques as illustrated in Chapter 3, allows for in-depth screening of microbial populations to isolate species of targeted oenological purposes, e.g. release of specific flavour precursors, textural contributions, fermentation efficacy, or level of ethanol production. These applications highlight the scope of research able to be achieved utilising culture-independent techniques, and the potential influence of this research on future winemaking practices. However, there is still a prominent role for the application of

culture-dependent techniques in scientific study, and it is the role of current research to minimise cultivation challenges, such as the culturing of undesired organisms.

The application of culture-dependent techniques requires an approximate understanding of the targeted community, in this investigation wine fungi capable of fermentation. To increase the likelihood of culturing the coveted community the microbiological media must resemble the existing or coveted environmental conditions, and tailor unwanted growth of competitive microorganisms (Fleet 1999). In this manner, the likelihood of cultivating targeted organisms increases and the time required to conduct culture-dependent techniques can be minimised.

5.3.2: The application of Biphenyl in the cultivation of fermentation fungi

The application of Biphenyl assists in the cultivation of some targeted organisms within microbiological media, minimising the risk of the unsuccessful or undesired cultivation of organisms (Addis et al. 1998, Viljoen et al. 2004). As illustrated in Chapter 4, Biphenyl is effective at deterring the growth of filamentous fungi, thereby reducing spatial and nutrient competition to encourage the colony establishment and continued growth of targeted species.

In addition, the determination of an appropriate level of Biphenyl in order to elucidate the maximum diversity of targeted organisms as possible reduced the likelihood of unsuccessful cultivation of the targeted population (Chapter 4). The method developed in Chapter 4 enhances our current capability, and reduces the time required to apply culture-dependent techniques. It also, reduces the risk of cultivation error, by providing an environment conducive to isolation of a diverse range of targeted fermentative fungi from grape samples.

CHAPTER SIX: Concluding Remarks and Future Directions

6.0: Concluding remarks for naturalised yeast research

The popularity of naturally fermented food and beverages is increasing, with the demand for wines produced by naturalised fermentation being a case in point. The successful application of these fermentations is dependent on the fungal community present, particularly the naturalised yeast organisms capable of fermentation.

The evaluation of the fungal biome of Cabernet Sauvignon from the Margaret River Wine Region, demonstrates that there is potential for the production of naturalised fermentations with pleasant oenological characteristics. However, the extensive sensory compound variation previously noted between strains of the identified species, and reduced diversity compared to winery-derived fermentations, highlight areas for future study and the important role of the winery microbiota.

The determination of the fungal community present within Margaret River vineyards has enabled the successful evaluation of the oenological potential of these organisms, and likely outcomes of naturalised fermentation. In addition, the evaluation and modification of existing culture-dependent and independent techniques, has enabled the targeted application of methodologies in order to elucidate data of specific relevance for the naturalised fungi organisms capable of fermentation in a timely-manner.

6.1: Future directions for naturalised yeast research and the application of naturalised fermentation

The evaluation of the naturalised fungal populations within vineyards is in its' infancy, as such this thesis focused on the identification of vineyard species in one region within a single vintage. The characterisation of vineyard species as either 'resident' or 'transient' via the undertaking of study from successive vintages would allow transferable information between harvest years, and provide insight to the influence of vintage conditions, i.e. climate and fungicide regime, on the naturalised community elucidated. In addition, the investigation of a larger number of samples within harvest sites would provide valuable data regarding the distribution of naturalised populations throughout the vineyard, enabling the refinement of current representative sampling techniques, and evaluation of the impact of microclimate variation on yeast diversity.

In relation to the successful application of naturalised fermentations, the knowledge of the identities determined within Margaret River, their approximate fermentation contributions, and an appropriate method for their successful cultivation, enable future studies to further evaluate strain variability, including sensory compound production. In this way high throughput sequencing and metagenomic analyses can provide valuable information regarding the community composition at each stage within a naturalised ferment, and culture–dependant techniques can assist in the determination of cell viability, and likelihood of significant fermentation contributions of individual species. As such, future analyses can now be targeted to specific species in order to reduce the duration of study, and chase the most significant results for naturalised species, i.e. evaluation of succession dynamics, fermentation efficacy, sensory output, and environmental niche preferences.

The synthesis of data obtained enables the winemaker to make evidenced-based decisions regarding the probable outcomes of naturalised fermentation, and develop strategies to further manage fermentation risk. In addition, it enables the continued development and optimisation of non-conventional monoculture preparations, and the alteration of winemaking practices to target specific fermentation outcomes.

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APPENDIX

SI 1: Quantitative primer design and primer selection for each barcode

SI 1.0: Selection of high-throughput sequencing primers for a fungal community

The application of high-throughput sequencing to the identification of fungal isolates is in its infancy, arguably due to an extended fungal amplicon length required for reliable taxonomic assignment (Bokulich and Mills 2013). High-throughput sequencing primers are required to accurately amplify short amplicons which contain adequate coverage of the selected gene and taxonomic resolution (Bokulich and Mills 2012, Bokulich and Mills 2013). Two genetic regions believed to possess adequate fungal taxonomic differentiation are the D1D2 domain for yeasts (Kurtzman and Robnett 1998) and the ITS region for all fungi (Schoch et al. 2012).

The amplification of community fungal samples is complicated by the anonymity and diversity of the ecological genotypes, leading to primer and sequencing bias. ITS primers have been tested for their accuracy of quantitative fungal community isolation (Ihrmark et al. 2012, Bokulich and Mills 2013), establishing adequate semi-quantitative community profiling, not perfect profiling (Ihrmark et al. 2012, Bokulich and Mills 2013). This is expected due to primer bias from competition between primers and diverse sequence templates (Ihrmark et al. 2012), and minor mismatches in primer sequences potentially disfavouring the amplification of some taxa (Bellemain, Carlsen et al. 2010, Ihrmark et al. 2012). Sequencing bias may also be a factor due to amplification length bias in uni-directional studies (Bokulich and Mills 2013), and computational bias (Bokulich and Mills 2013). This study attempted to minimise all bias by conducting paired-end sequencing to ensure the entire amplicon length was sequenced, and targeting oenologically relevant yeast sequences utilising a multiple species alignment.

The successful application of semi-quantitative community amplification relies upon the utilisation of primers targeted towards a specific goal, such as fermentative yeast amplification. To reduce any potential primer bias a multiple alignment was done on known yeast species of oenological relevance within the Saccharomycotina sub-phylum (Table S1). The alignment of the Genbank sequences acquired from the NCBI database of the D1D2 domain was straightforward, due to high complementarity between species sequence length, and the highly conserved nature of the middle section and two regions flanking the D1D2 domain. However, despite the similar conserved nature of the 5.8S gene between the ITS1 and ITS2, and outside regions flanking the ITS, the extensive size variation amongst deposited sequences of the ITS region lead to the separation of sequences and sub-manual alignment within Geneious v7.1.7 (Table 1). In addition, the diversity of targeted species sequences deposited on Genbank was lower than the D1D2 domain. This is evidenced by the reduction in total sequences aligned, and a reflection of the large undertaking by Kurtman et al. (2011) to sequence the D1D2 domain of all known Ascomycetous yeasts (Kurtzman, Fell et al. 2011). Upon completion of both alignments a computational landing of primers was conducted, to ensure amplification of the targeted sequences would occur.

Table S1: Summary of the sequence alignment conducted for both target regions

D1D2		ITS	
Alignment sub-group	**Number of sequences**	**Alignment sub-group**	**Number of sequences**
Hanseniaspora, Kloeckera & Saccharomyces	49	Hanseniaspora	9
Metschnikowia	12	Saccharomyces	11
Candida	27	Metschnikowia	3
Pichia & Issatchenkia	28	Pichia	13
		Torulaspora & Issatchenkia	7
		Candida (1)	7
		Candida (2)	6
Total	**116**	**Total**	**56**

SI 1.1: Selection of primers for the D1D2 domain

It was established that closely related yeasts could be taxonomically separated via sequencing of the D2 domain, and this later expanded to the entire D1D2 domain. However, due to the amplicon length restrictions of next-generation sequencing technologies, the sequencing of the 600 to 800 base pair (bp) domain (Kurtzman, Fell et al. 2011) is unrealistic and a single domain is once again targeted. The D1 domain houses lower genetic variation leaving more taxonomically unresolved species than the D2 domain (Kurtzman, Fell et al. 2011, Stockinger, Kruger et al. 2012), therefore this D2 domain was the focus of the investigation.

Two D2 forward primers were investigated, each binding within the highly conserved region at the middle of the D1D2 domain (NL-3A (Kurtzman and Robnett 1998) and U1 (Putignani et al. 2008)). Both forward primers bound to all aligned genetic sequences, yet the NL-3A primer bound at ~50 bp before the U1 primer, to produce a longer amplicon. In order to ensure the 250 bp paired-end sequencing technique was successful the amplicon length needed to contain the primer sequence, sample index barcode and adequate sequence overlap for the accurate stitching of paired reads post sequencing. The U1 primer was therefore selected due to amplicon length suitability and target population specificity.

The two reverse primers (NL-4 (Kurtzman and Robnett 1998) and U2 (Putignani et al. 2008)) were both considered, and NL-4 deemed appropriate. Similarly to the forward, both primers bound effectively to all targeted sequences. However, the U1 primer bound part-way through the NL-4 and as such, the full sequence to check complete binding, were commonly available on Genbank. The amplicon length obtained by the U1 and U2 pair ranged from 120 to 290 bp amongst clinically important yeast isolates, primarily of the *Candida* genera (Putignani et al. 2008). In order to preserve minimal read lengths and avoid primer binding ambiguity U2 was rejected as a reverse primer. The repeated reliability of the NL-4 reverse primer and its wide application as a reverse primer in the sequencing of all known Ascomycetous yeast (Kurtzman, Fell et al. 2011) led to its selection as the appropriate reverse primer.

SI 1.2: Selection of primers for the ITS region

The ITS region possesses a high evolution rate and two highly conserved sections either side of the region optimal for primer binding (Begerow et al. 2010), and has been identified as the universal barcode for all fungi (Schoch et al. 2012). The ITS2 was selected for examination in preference to the ITS1 region due to; similar, if not superior (90 to 95%) accuracy of species level resolution than the ITS1 for reads 250 bp long (Bokulich and Mills 2013), and reduced sequence length variability than the ITS1 (Bokulich and Mills 2013), increasing the ease of alignment.

Five potential forward primers were investigated upon the genetic alignment composed in Geneious (fITS7, fITS9, gITS7 (Ihrmark et al. 2012), 58A2 (Martin and Rygiewicz 2005) and ITS3 (White et al. 1990)). Forward primers ITS3, fITS9 and 58A2 were discarded as candidates, due to a lack of binding to all targeted species on the alignment. The small genetic region with the 5.8S gene all three of these primers bound to was partially or completely absent in 62.5% of the aligned sequences, particularly amongst the *Hanseniaspora* species (0%). Of the remaining forward primer candidates gITS7 demonstrates increased amplification of plant sequences and overrepresentation of Saccharomycotina order, Saccharomycetales, within OTUs, and mismatches upon comparison to the NCBI database in 30% of Saccharomycetale sequences (Ihrmark et al. 2012). Therefore, although the overall mismatch rate was higher in fITS7 (7.9%) compared to the gITS7 (4.5%), the identification of mismatches was largely contained to the Pezizomycotina sub-phylum, primarily *Penicillium* spp. (Ihrmark et al. 2012), and this was considered of lesser importance than Saccharomycetale mismatching. fITS7also displayed superior fungal specificity based on an alignment of 140,000 fungal species (Ihrmark et al. 2012), increased Saccharomycetale taxonomic reliability (Ihrmark et al. 2012) and the ability to land on all targeted and aligned sequences. Therefore fITS7 was identified as the optimal forward primer candidate for diverse Saccharomycotina sequence isolation.

ITS4 is a reverse primer candidate which had been established in 1990 (White, Burns et al. 1990) and widely used within fungal characterisation. A recent publication aimed to improve upon the efficiency of the classic ITS primers, designing ITS4_KY01 and ITS4_KY02 in the place of ITS4 (Toju et al. 2012). The authors stated *"ITS4 was as broad as any other primer, including our newly designed primer*s" and that *"this classic primer is appropriate for fungal DNA barcoding".* Within this publication ITS4 matched with 96.0 to 99.2% of sequences investigated (Toju et al. 2012) and has been used in successful partnership with fITS7 previously (Ihrmark et al. 2012). As such, the

forward primer fITS7 and reverse primer ITS4 were selected for the amplification of fungal species of the Saccharomycotina sub phylum.

SI 1.3: Design of quantification primers

The development of quantification primers was based on the D1D2 oenologically relevant yeast alignment, due to the similarity in deposited sequence length and high complementarity between selected regions. The quantification primers targeted the middle of the D1D2 domain and were designed within Geneious v7.1.7 and expected to yield an approximately 150 bp amplicon.

SI 1.4: Primer annealing temperature optimisation

The annealing temperature for all primer pairs was optimised via qPCR. Each primer set was optimised with gDNA extract of pure fungal DNA (*Botyritis cinerea*) assessed at three DNA dilutions (undiluted, 1/10, 1/100). The qPCR setup for samples and controls were prepared in a physically separate ultra-clean laboratory and were carried out using each primer set in 25 µL reactions containing 1X PCR Gold Buffer, 2.5mM $MgCl_2$, 0.4 mg/mL BSA, 0.25 mM of each dNTP, 0.4 µM of forward and reverse primer, 0.25 µL AmpliTaq Gold, 0.6 µL SYBR Green and 2 µL of gDNA. The cycling conditions for qPCR using the annealing temperature optimisation of each primer set were as follows: initial heat denaturation at 95 °C for 5 mins, followed by 40 cycles of 95 °C for 30 s; 50 °C or 60 °C at 2 °C intervals for 30 s (annealing step); 72 °C for 45 s followed by final extension at 72 °C for 10 mins. The CT values of the PCR amplicons were generated assessed and appropriate annealing temperature determined at 52 °C for the quantification and D2 barcode primer set, and 54 °C for the ITS2 barcode primer set. Additionally, all primers were tested for hair pining and self-dimer formation within Geneious v7.1.7

SI 1.5: References

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SI 2: The phylogenetic trees of sequences obtained from grape berries in a taxonomy- dependent manner

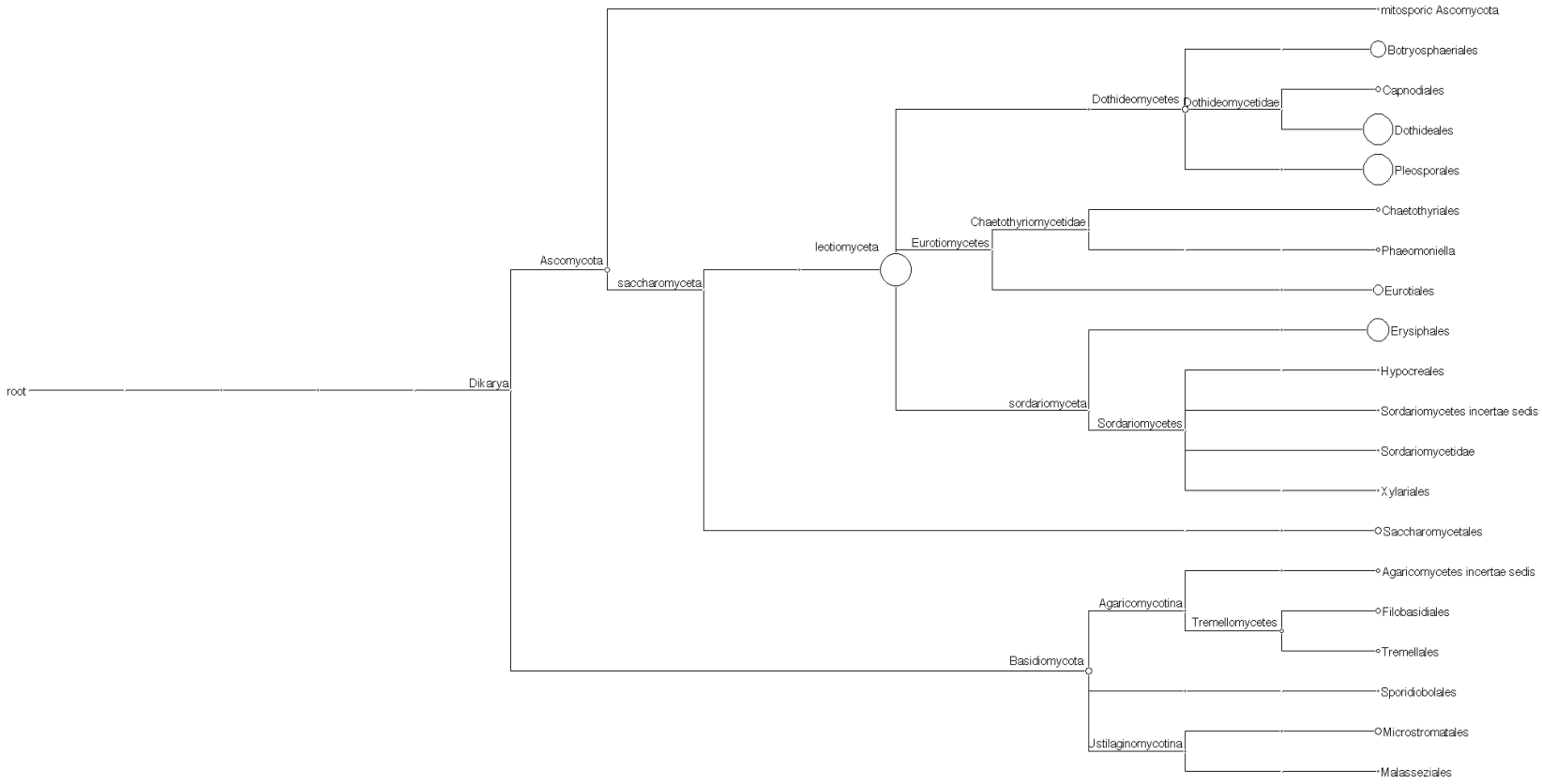

Figure S2.1: The sequences obtained by the amplification of D2 primers from grape must extract sampled within the Margaret River region

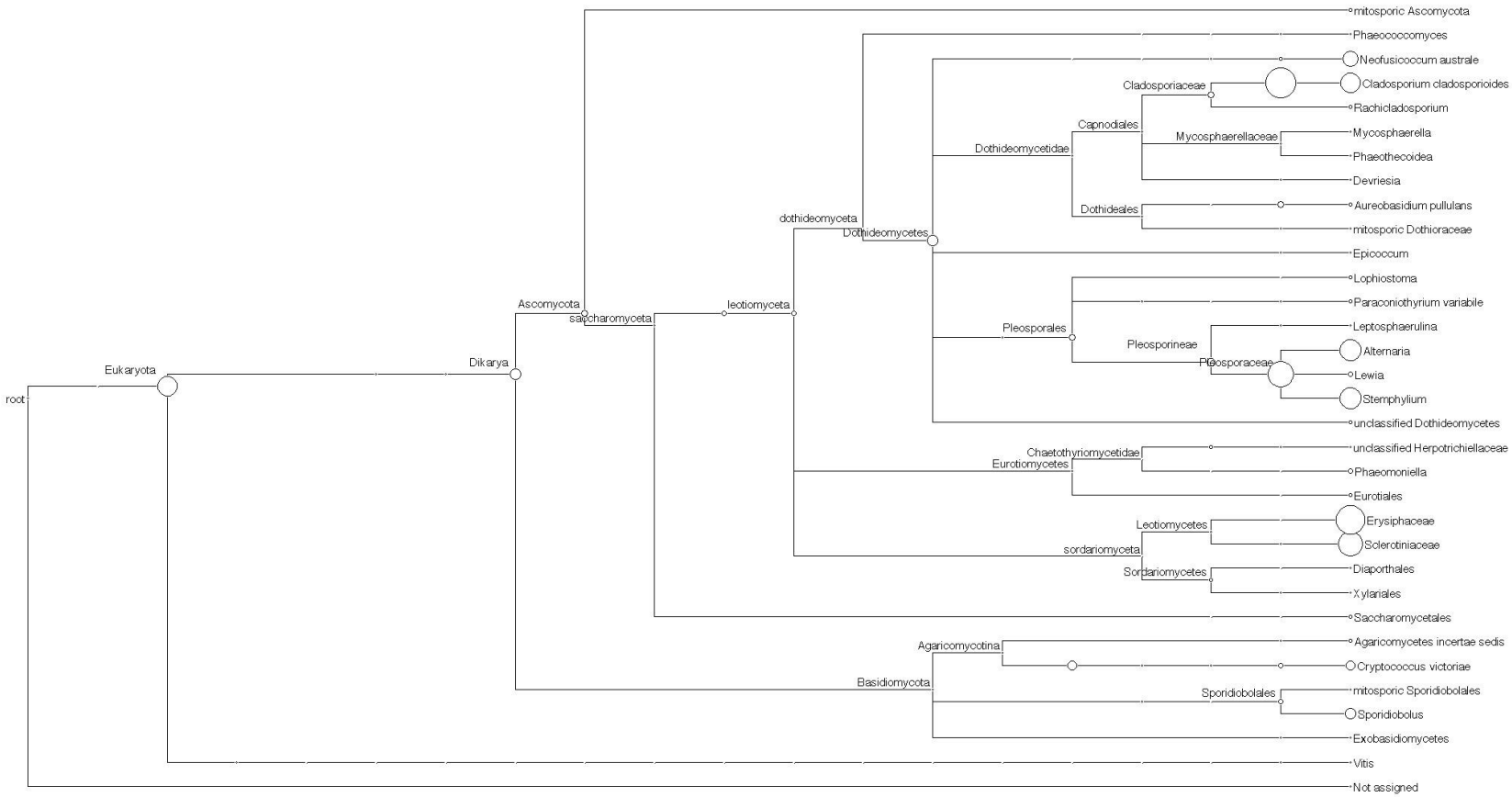

Figure S2.2: The sequences obtained by the amplification of ITS2 primers from grape must extract sampled within the Margaret River region

SI 3: Outline of high throughput sequencing control contamination

The total sequences obtained for the D2 domain and ITS2 region post sequencing error and chimera filtering were 4,596,625 and 5,228,654 respectively (Table S2). After additional filtering and sequences OTU generation the number of sequences obtained per gene changed to 4,480,551 for the D2 domain and 5,052,247 for the ITS2 region, as singletons and any cluster with less than 5 sequences were discarded. However, after investigation of the control samples for each gene, the ITS2 region control sample tested positive for clusters of *A. pullulans, Cladosporium* spp., and *S. cerevisiae*, accounting for approximately 75% of the sequences present within the ITS2 data (Table S2). This control contamination may be a reflection human error, the abundance of these isolates within nature, or the selective sensitivity of the ITS2 primer set and sequencing method as more relative reads per sample were amplified than the D2 domain (Chapter 3). The likely contamination explanation lies behind a combination of these hypotheses.

Table S2: OTUs disregarded from the ITS2 sequencing data of ripe Cabernet Sauvignon grape must extracts sampled within the Margaret River region due to control contamination

OTU taxonomic identity	Number of reads	Number of unique reads	Percentage of total reads
Aureobasidium pullulans	2489961	2675	49.3%
Cladosporium spp.	1227534	2355	24.3%
Saccharomyces cerevisiae	35	2	6.9×10^{4}%

Due to control contamination these three OTUs were discarded from the analysis of the fungal isolates identified within Margaret River vineyards. However, in relation to a discussion of the taxonomic discretion of the two genes and primer sets (Chapter 2), this data is included as its elimination incorrectly alters the balance of taxonomic assignment of the total sequences obtained.

SI 4: Evaluation of the influence of ethanol on mucosal fungi isolation

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SI 4.0: Statement of Contribution

I, as first author conducted all laboratory work, data analysis and authored the first and final drafts of the experimental Chapter, as prepared as a manuscript for submission to a publication. My co-authors assisted with publication drafting, providing comments on drafts of this section.

Elizabeth Nugent

SI 4.1: Abstract

The objective of the primary experiment is to determine the appropriate level of Biphenyl for the qualitative isolation of mucosal colonies. Although previous literature does not indicate an issue with 3% ethanol supplementation and mucosal colony formation, this was independently tested. The supplementation of media with 3% ethanol led to a reduction in the number of colonies formed yet did not alter the qualitative isolation of colonies (mucosal and filamentous). It is concluded that for qualitative isolation, as in objective of experiments described in Chapter 4, supplementation of media with ethanol up to 3% does not alter results and is deemed acceptable.

SI 4.2: Introduction

Biphenyl, a filamentous fungi growth inhibitor (Addis et al. 1998), and chloramphenicol, a bacterial growth inhibitor (Wisseman et al. 1954), require solubilisation in alcohol before they can be incorporated into microbiological media. In this study ethanol was utilised and the resultant concentration monitored for adverse effects. Ethanol concentrations of 4 to 12% have been observed to deter selective filamentous fungal growth, and concentrations of 70% are commonly used to sanitise wet and dry surfaces (Dao and Dantigny 2011). However non-*Saccharomyces* yeast species potentially prevalent in the vineyard, are also inhibited by ethanol concentrations exceeding 5 to 6%, which has been well characterised in text books and publications (Romano et al. 2003, Ribereau-Gayon et al. 2006, Jackson 2008). No recorded evidence states that the qualitative isolation of yeast is inhibited by ethanol concentrations up to 3%. However, due to the anonymity of the flora in our samples, preliminary trials were carried out to establish ethanol concentrations of up to 3% do not impact on qualitative mucosal isolation.

SI 4.3: Experimental Procedures

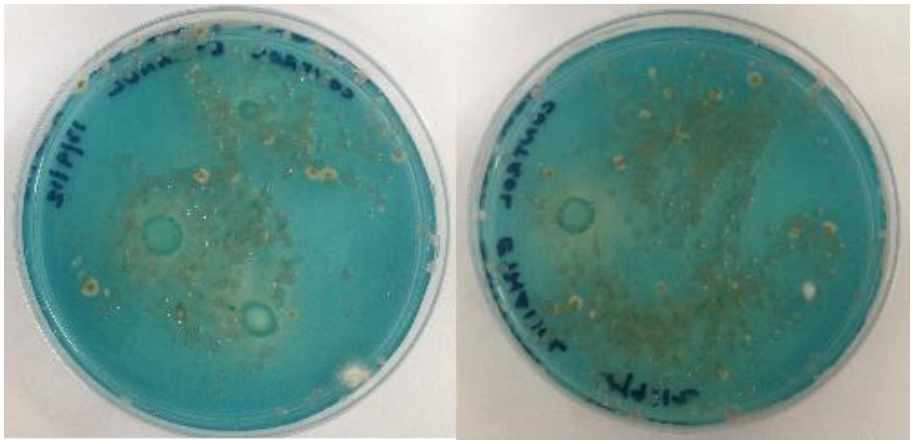
A 100 µl of macerated grape must sample described in Chapter 4 was spread on a Wallerstein's laboratory (WL) control plate, and a WL plate supplemented with 3% ethanol, in duplicate. The plates were then incubated at 25 °C for 4 days, photographed, and the qualitative colonies isolated evaluated.

SI 4.4: Results and Discussion

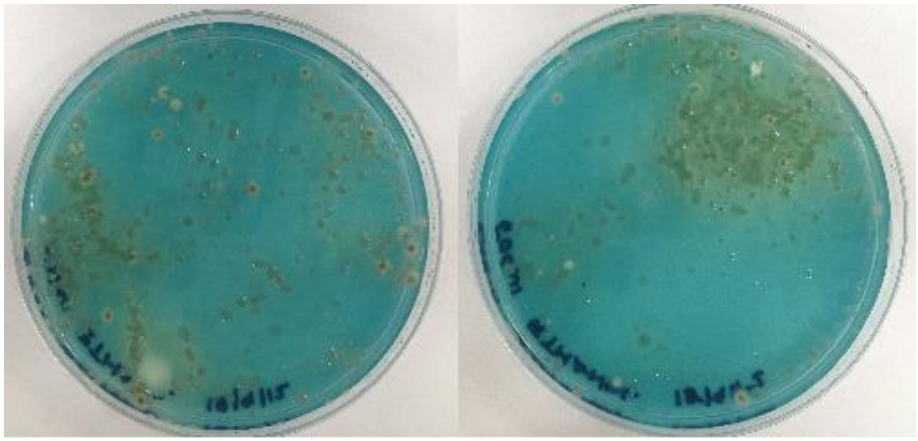
Upon comparison of the control and 3% ethanol supplemented microbiological plates no qualitative reduction (diversity) in mucosal fungal isolates was observed, yet there was a reduction in the total number of mucosal colonies (Figure S4.1). Therefore, in accordance with commonly described in textbooks (Zoecklein et al. 1999, Ribereau-Gayon et al. 2006, Jackson 2008) concentrations of 3% ethanol did not affect qualitative mucosal colony isolation.

One mucosal colony worth mentioning was absent on one plate at the 3% ethanol supplementation compared to the corresponding control. However, as this colony was successfully cultured in the first replicate at the control and 3% ethanol supplementation rate, this absence was attributed to a lack of sample homogeneity and not an inability to be cultured in the presence of 3% ethanol. The future representative sampling techniques were modified to ensure this error was not repeated in the primary experiment.

Wallertstein's Laboratory (WL) agar without ethanol (control)



a b



c d

Figure S4.1: Effect of ethanol concentration on colony diversity and number from Chardonnay grape must samples sourced from the Margaret River region. a and b, control medium; c and d, Wallerstein's Laboratory (WL) agar supplemented with 3% ethanol.

It is of interest to note Biphenyl has a molar solubility of 0.4245 (Khossravi and Connors 1993) , and based on this figure, 100ml of absolute methanol is required for 6.5g of Biphenyl to achieve solubilisation. This high rate is illustrated in a study investigating the solubility of Biphenyl from contaminated citrus fruit samples, requiring 100 ml of a 95% ethanol solution to solubilize 5.1 g of Biphenyl (Davis and Monroe 1979). As such, the concentration utilised, and chemical identity of alcohol (i.e. ethanol or methanol) to solubilise Biphenyl are not mentioned in publications of both yeast and mould enumeration experimental design (Addis et al. 1998, Viljoen et al. 2004) and those of grape and wine ecology (Renouf et al. 2005, Nisiotou and Nychas 2007, Zott et al. 2010, Setati et al. 2012). This is surprising considering the moderately low alcohol tolerance of non-*Saccharomyces* yeast isolates (Romano et al. 2003) and high rate of alcohol required for Biphenyl solubilisation (Davis and Monroe 1979).

Similar to our findings with ethanol exposure, Viljoen et al. (2004) noted a quantitative reduction, but qualitative preservation of yeast isolates obtained on microbiological media supplemented with 0.05% Biphenyl. Due to an absence of scientific clarification, it can be hypothesized this reduction in quantitative yeast previously observed (Viljoen et al. 2004) may have been a combined consequence of the solubilisation agent (i.e. alcohol) and Biphenyl supplementation. This potential synergistic activity warrants further investigation.

SI 4.5: Conclusion

The supplementation of media with 3% ethanol did not impact the qualitative isolation of mucosal fungi, thus considered acceptable for the qualitative isolation of mucosal colonies and independent evaluation of Biphenyl in this context.

SI 4.6: References

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SI 5: Intra-regional diversity and the Saccharomycotina sub-division

SI 5.0: The relevance of intra-regional variation for this dissertation

The aim of this dissertation is to investigate the identities of the naturalised fungal organisms capable of fermentation residing within the Margaret River region, to identify potential oenological outcomes of local naturalised fermentation. To address this aim, grape bunch samples spread across the Margaret River region were harvested in order to capture the maximal amount of oenological species diversity. As such, it was decided a fewer number of samples would be harvested from a broader region to capture site variances, such as climate and conventional farming strategies. The data generated successfully addressed the thesis aim. However, in relation to a discussion of intra-regional site variance the sparse distribution of the Saccharomycotina classified OTUs (generally low abundant), combined with the extensive variance between grape bunch samples and sampling sites of these OTUs makes the additional investigation of intra-regional variance challenging.

SI 5.1: Outline of intra-regional variance for the Margaret River region

The diversity of the Saccharomycotina OTUs identified within the Margaret River wine region varied considerably between the samples within vineyards and the sampling site (Table S3). It is evidenced only 13 out of 30 grape bunches sampled contained Saccharomycotina OTUs able to be taxonomically classified by either the D2 domain or ITS2 region barcodes (Table S3). In addition, one site out of six identified no Saccharomycotina classified OTUs, and in site number 5 only one Saccharomycotina OTU was classified as *Starmerella bacillarius*, from one sample (Table S3).

Table S3: Intra-regional diversity of Saccharomycotina OTUs

	OTU BLASTn assignmnent	Site Origin of Sample	Sample Number
		3	113
		4	118; 119; 120
	Hanseniaspora uvarum	6	131
		1	103; 105
		3	114
	Saccharomyces cerevisiae	4	122
		4	119
	Hanseniaspora vineae	6	132
		3	114
		4	122
	Starmerella bacillaris	5	123
	Candida parapsilosis	3	113
D2	Saccharomycetales	1	105
	Candida tropicalis	1	105
	Pichia mexicana	1	105
	Zygoascus	4	119
	Saccharomycetaceae	6	128
ITS2	Saccharomycetales	1	104

This sparse distribution of Saccharomycotina amongst samples of the same site may be a reflection of variances within microclimates (King et al. 2014), as it is evidenced that even the fungal content within a single vine can be variable (Polsinelli et al. 1996). Further investigations of microclimate impact on the fermentative community within a vineyard require a greater understanding of the naturalised microorganisms present, their role in the community hierarchy, and their distribution throughout a single site.

SI 5.2: Challenges of intra-regional variance microbial evaluation

Two previous studies have utilised culture-independent and high throughput sequencing technologies to address intra-regional diversity (Bokulich et al. 2014, Taylor et al. 2014). Taylor et al. (2014) conducted intra-regional analyses of the fungal

communities present within homogenised grape must, sourced directly from the vineyard utilising three bunches per site (two less than our five). These authors, justified intra-regional variance through dissimilarity distance matrices within their statistical analysis in order to remove random variance. Whilst the study reported successful results concordant with the hypothesis of intra-regional microbial variance, the extensive variation between samples in one site evident within this investigation and supported by others (Polsinelli et al. 1996, van der Westhuizen et al. 2000, Cordero-Bueso et al. 2011, King et al. 2014) brings into question the validity of such a small sampling regime for site characterisation and comparison.

Conversely, Bokulich et al. (2014) conducted sampling at the winery press in order to evaluate intra-regional diversity. This approach would successfully characterise the total microbiota of one site as we know it, providing an appropriate representative sample was obtained. However, sampling at the winery press would include winery-resident microbiota, perhaps unique to that particular winery due to the high rate of hybridisation with commercial inoculants if utilised (Blanco et al. 2011, Clavijo et al. 2011), and the favourable environment for yeast colonisation (Ocón et al. 2010), not sub-regional location. Thus once again challenging the evaluation of intra-regional microbial variation as it currently stands.

In summary, an appropriate sampling regime and methodology would need to either; (1) follow extensive site variation study of the vineyard microbiome to ensure complete representative sampling occurs, or (2) be collected from a sterile press not located within an existing winery, and utilising sterile tools and harvest bins for sampling and transportation. Both of these requirements extend logistical and time-consuming hurdles, such as resource and data availability, providing areas for future investigation.

SI 5.3: Concluding remarks

Intra-regional variance requires extensive study in order to fully understand all of the influencing factors, and sampling regime and methodology are vital to thoroughly address relevant hypotheses. This dissertation successfully addressed the thesis aims to investigate and evaluate the potential naturalised species, residing within a region never before analysed, in order to elucidate the potential outcomes of the adoption of naturalised wine fermentation.

SI 5.4: References

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SI 6: Tables of supplementary information

List of independent supplementary Tables:

Table S4: Complete list of Operational Taxonomic Units (OTUs) generated for the D2 domain

Table S5: Complete list of Operational Taxonomic Units (OTUs) generated for the ITS2 region

Table S6: Isolated colony descriptors on microbiological media

Table S7: Isolated colony counts for each colony type mapped to sample origin

Table S4: Complete list of Operational Taxonomic Units (OTUs) generated for the D2 domain

OTU No.	No. of DNA sequences	No. of unique sequences	BLASTn assignment	Similarity (%)
1	2936251	1694	Aureobasidium pullulans	100
2	649327	821	Erysiphaceae	94*
3	347861	745	Pleosporaceae	100
4	186363	471	Helotiales	100
5	172244	706	Dothideomycetes	100
6	50947	278	Neofusicoccum	100
7	34268	249	Pezizomycotina	100
8	18009	118	Hanseniaspora uvarum	100
9	11469	65	Penicillium glabrum	100
10	10697	71	Quambalaria cyanescens	100
11	7504	50	Tremellomycetes	100
12	5589	30	Rhodotorula	100
13	5373	34	Cryptococcus victoriae	100
14	5239	28	Phaeosphaeria	98
15	4524	133	Pleosporales	99
16	4421	27	Cryptococcus oeirensis	100
17	3583	33	Cryptococcus carnescens	100
18	3005	17	Phaeomoniella niveniae	100
19	2551	13	Dothideomycetes	100
20	1709	10	Pleosporales	100
21	1141	4	Unknown fungal cluster 1	85*
22	935	9	Ascomycota	100
23	923	5	Penicillium brevicompactum	100
24	867	4	Saccharomyces cerevisiae	100
25	822	7	Sporidiobolaes	100
26	820	9	Dothideales	100
27	582	22	Dothideomycetes	100
28	555	3	Hanseniaspora vineae	100
29	500	11	Penicillium	100
30	485	9	Dothioraceae	99
31	458	1	Amphisphaeriaceae	100
32	422	7	Phoma huancayensis	99
33	393	5	Cladosporiaceae	100
34	360	5	Unknown fungal cluster 2	98
35	353	5	Cryptococcus heveanensis	99
36	347	5	Unknown fungal cluster 8	100
37	340	7	Erysiphe	92*
38	297	3	Cryptobasidiaceae	99
39	296	34	Pleosporaceae	99

OTU No.	No. of DNA sequences	No. of unique sequences	BLASTn assignment	Similarity (%)
40	251	2	Myrothecium gramineum	97
41	245	1	Unknown fungal cluster 3	99
42	231	3	Unknwon fungal cluster 6	87*
43	229	1	Claviceps purpurea	100
44	213	3	Unknown fungal cluster 4	96*
45	199	6	Alternaria	100
46	190	2	Pleosporales	99
47	180	1	Unknown fungal cluster 5	88*
48	179	1	Unknown fungal cluster 7	100
49	177	1	Cytidia salicina	95*
50	175	1	Lophiostoma	100
51	171	1	Aspergillus	100
52	170	3	Unknown fungal cluster 10	99
53	165	2	Cytospora eucalypticola	100
54	155	1	Unknown fungal cluster 9	89*
55	154	1	Bensingtonia	97
56	136	1	Helotiales	97
57	133	2	Malassezia globosa	100
58	133	3	Unknown fungal cluster 11	99
59	129	6	Pleosporaceae	100
60	120	3	Phaeomoniella	89*
61	119	1	Bullera unica	100
62	111	1	Cryptococcus	100
63	111	3	Pleosporales	97
64	105	1	Exobasidium	96*
65	103	4	Aureobasidium pullulans	95*
66	102	2	Hypocreales	99
67	102	3	Dothideomycetes	100
68	99	1	Cryptococcus adeliensis	100
69	97	3	Rhodotorula bacarum	100
70	95	5	Unknown fungal cluster 18	96*
71	93	2	Dioszegia hungarica	100
72	91	1	Sporobolomyces ruberrimus	100
73	88	1	Cryptococcus	100
74	87	2	Penicillium	100
75	86	1	Phaeomoniella	95*
76	84	1	Dothideomycetes	99
77	83	13	Peyronellaea subglomerata	98
78	82	1	Unknown fungal cluster 12	96*
79	81	1	Unknown fungal cluster 13	91*
80	80	8	Dothideomycetes	98

OTU No.	No. of DNA sequences	No. of unique sequences	BLASTn assignment	Similarity (%)
81	79	1	Cryptococcus	100
82	79	1	Xyriales	100
83	79	1	Sporobolomyces	95*
84	79	1	Penicillium cecidicola	97
85	74	2	Unknown fungal isolate 16	97
86	71	1	Starmerella bacillaris	100
87	71	1	Unknown fungal cluster 14	91*
88	71	4	Capnodiales	100
89	70	2	Gloniopsis praelonga	99
90	69	1	Unknown fungal isolate 15	100
91	69	2	Botryosphaeriales	100
92	67	1	Saccharomycetales	100
93	66	1	Rhodotorula	93*
94	65	1	Unknown fungal cluster 17	92*
95	65	4	Cladosporiaceae	98
96	64	4	Chaetosphaeronema hispidulum	100
97	63	10	Dothideomycetes	98
98	60	1	Pezizomycotina	98
99	58	4	Aureobasidium pullulans	96*
100	56	2	Lophiotrema nucula	96*
101	55	2	Cytospora diatrypelloidea	100
102	55	2	Unknown fungal cluster 21	94*
103	54	2	Unknown fungal cluster 19	98
104	50	1	Setophoma sacchari	98
105	49	1	Fusarium	100
106	49	5	Alternaria	99
107	48	3	Teratosphaeria capensis	100
108	45	1	Cryptodiscus pini	98
109	43	1	Pleosporales	100
110	41	1	Dioszegia rishiriensis	97
111	41	1	Unknown fungal cluster 20	94*
112	40	1	Sporidiobolus salmonicolor	100
113	40	2	Unknown fungal cluster 22	93*
114	38	1	Chaetothyriales	99
115	38	1	Pleosporales	100
116	32	1	Unknown fungal cluster 23	92*
117	31	1	Ophiostoma	100
118	31	2	Mycosphaerellaceae	100
119	31	2	Erysiphe	89*
120	29	1	Malassezia furfur	100
121	29	2	mitosporic Capnodiaceae	99

OTU No.	No. of DNA sequences	No. of unique sequences	BLASTn assignment	Similarity (%)
122	27	1	Cystofilobasidium infirmominiatum	100
123	26	1	Resinicium meridionale	98
124	25	1	Unknown fungal cluster 24	100
125	24	2	Tilletiopsis pallescens	99
126	22	1	Chaetothyriales	100
127	22	1	Unknown fungal cluster 25	87*
128	21	1	Unknown fungal cluster 26	92*
129	21	2	Neofusicoccum	96*
130	20	1	Cladosporiaceae	98
131	19	1	Unknown fungal cluster 27	100
132	19	1	Holtermanniella festucosa	100
133	18	1	Unknown fungal cluster 28	86*
134	18	1	Unknown fungal cluster 29	88*
135	17	1	Selenophoma linicola	96*
136	17	1	Unknown fungal cluster 30	88*
137	17	1	Tilletiopsis washingtonensis	100
138	17	1	Exophiala eucalyptorum	100
139	16	1	Penicillium	100
140	16	1	Curreya grandicipis	100
141	16	1	Cryptovalsa ampelina	100
142	15	1	Unknown fungal cluster 31	97
143	15	1	Unknown fungal cluster 32	94*
144	14	1	Unknown fungal cluster 33	98
145	14	1	Unknown fungal cluster 34	100
146	14	1	Coprinellus	100
147	14	1	Cryptovalsa rabenhorstii	99
148	14	2	Unknown fungal cluster 41	92*
149	13	1	Unknown fungal cluster 35	90*
150	13	1	Candida parapsilosis	100
151	13	1	Dothideoraceae	99
152	13	1	Aspergillaceae	100
153	13	1	Bimuria novae-zelandiae	100
154	12	1	Aspergillus	100
155	12	1	Dioszegia	100
156	12	1	Hyphoderma puberum	100
157	12	1	Unknown fungal cluster 36	92*
158	12	1	Cryptococcus	97
159	11	1	Unknown fungal cluster 37	90*
160	10	1	Unknown fungal cluster 38	94*
161	10	1	Sidera vulgaris	100

OTU No.	No. of DNA sequences	No. of unique sequences	BLASTn assignment	Similarity (%)
162	10	1	Laxitextum bicolor	99
163	10	1	Phacidiella eucalypti	100
164	10	1	Arthrinium phragmites	99
165	10	2	Unknown fungal cluster 45	90*
166	9	1	Plectosphaera eucalypti	93*
167	9	1	Plectosphaera eucalypti	95*
168	9	9	Unknown fungal cluster 39	95*
169	9	1	Tremellales	100
170	8	1	Tremellomycetes	100
171	8	8	Unknown fungal cluster 40	96*
172	8	1	Basidomycotina	100
173	8	1	Staninwardia suttonii	95*
174	7	1	Unknown fungal cluster 42	96*
175	7	1	Dothideomycetes	100
176	6	1	Unknown fungal cluster 43	97
177	6	1	Fusarium	100
178	6	1	Hormonema	100
179	6	1	Polyporales	98
180	6	1	Unknown fungal cluster 44	93*
181	6	1	Cryptococcus	96*
182	6	1	Phanerochaete crassa	100
183	5	1	Cryptococcus	100
184	5	1	Hypocreomycetidae	100
185	5	1	Elmerina caryae	100
186	5	1	Unknwon fungal cluster 46	96*
187	5	1	Unknown fungal cluster 47	95*

Legend: less than 97.0% BLASTn similarity*

Table S5: Complete list of Operational Taxonomic Units (OTUs) generated for the ITS2 region

OTU No.	No. of DNA sequences	No. of unique sequences	BLASTn assignment	Similarity (%)
1	2489961	2673	Aureobasidium Pullulans	100
2	1227534	2355	Cladosporium	100
3	443459	1290	Erysiphe	100
4	328821	655	Sclerotinaceae	100
5	189618	642	Stemphyllium	99
6	89959	628	Alternaria	100
7	60535	792	Unknown fungal cluster 1	100
8	49766	472	Cryptococcus victoriae	100
9	31157	283	Epicoccum	100
10	29107	274	Neofusicoccum australe	100
11	26609	320	Unknown fungal cluster 2	100
12	17067	172	Lewia	100
13	9247	101	Sporidiobolus salmonicolor	100
14	6580	97	Dothioraceae	100
15	5344	33	Paraconiothyrium variabile	100
16	4894	37	Pleosporales	100
17	4709	46	Unknown fungal cluster 3	99
18	4473	30	Unknown fungal cluster 5	98
19	3920	41	Cryptococcus stepposus	100
20	3898	30	Phaeomoniella	100
21	2643	14	Unknown fungal cluster 4	99
22	2441	21	Basidomycota	100
23	1674	12	Rachicladosporium cboliae	98
24	1022	10	Unknown fungal cluster 6	91*
25	958	8	Unknown fungal cluster 9	100
26	846	7	Phaeococcomyces aff. Nigricans	99
27	813	10	Leptosphaerulina	100
28	764	6	Unknown fungal cluster 7	90*
29	693	6	Unknown fungal cluster 8	98
30	679	10	Vitis vinifera	97
31	678	4	Chaetothyriales	93*
32	644	6	Cryptococcus oeirensis	100
33	615	4	Sporidiobolales	100
34	601	8	Eurotiales	96*
35	573	7	Herpotrichiellaceae	95*
36	507	5	Phaeothecoidea	99
37	492	4	Pyrenophora leucospermi	100
38	470	2	Pezizomycotina	100
39	458	4	Cryptococcus dimennae	99

OTU No.	No. of DNA sequences	No. of unique sequences	BLASTn assignment	Similarity (%)
40	422	3	Lophiostoma	97
41	289	5	Unknown fungal cluster 15	100
42	280	5	Devriesia fraseriae	100
43	265	1	Cryptococcus	100
44	265	10	Unknown fungal cluster 10	94*
45	217	4	Unknown fungal cluster 11	99
46	216	3	Pleosporales	100
47	213	4	Cladosporium	98
48	207	3	Tremellaceae	99
49	182	3	Teratosphaeria capensis	100
50	170	4	Pleosporaceae	100
51	169	3	Unknown fungal cluster 14	87*
52	166	4	Cryptococcus	100
53	164	3	Unknown fungal cluster 20	94*
54	161	2	Unknown fungal cluster 13	97
55	156	1	Unknown fungal cluster 12	100
56	156	4	Unknown fungal cluster 16	99
57	155	2	Phaeomoniella	99
58	147	4	Cryptococcus	99
59	142	3	Unknown fungal cluster 17	90*
60	129	2	Exophiala	100
61	129	3	Tremellales	98
62	123	1	Unknown fungal cluster 18	96*
63	122	4	Toxicocladosporium leucadendri	99
64	115	5	Cytospora diatrypelloidea	100
65	114	1	Pycnoporus coccineus	100
66	109	1	Unknown fungal cluster 19	100
67	107	2	Lanzia allantospora	100
68	98	1	Bullera unica	100
69	97	1	Pseudoseptoria	98
70	89	2	Agaricomycotina	98
71	88	2	Pseudotaeniolina globosa	100
72	86	2	Unknown fungal cluster 21	91*
73	85	1	Curvibasidium	100
74	77	1	Cryptococcus laurentii	100
75	76	1	Teratosphaeria capensis	93*
76	74	2	Mycosphaerellaceae	100
77	72	1	Phaeospharaceae	100
78	68	1	Unknown fungal cluster 22	86*
79	67	1	Dothideomycetes	100
80	65	2	Saccharata	96*

OTU No.	No. of DNA sequences	No. of unique sequences	BLASTn assignment	Similarity (%)
81	64	1	Diatrypella	100
82	63	4	Unknown fungal cluster 25	97
83	63	2	Unknown fungal cluster 26	92*
84	63	3	Lophiostoma corticola	99
85	62	1	Unknown fungal cluster 23	99
86	61	1	Devriesia	100
87	60	1	Sclerostagonospora	100
88	56	1	Phaeospharaceae	100
89	55	1	Paraphaeosphaeria	100
90	54	4	Thyridaria macrostomoides	99
91	53	1	Unknown fungal cluster 24	100
92	53	2	Dissoconium	99
93	50	1	Sporobolomyces	100
94	49	2	Pleosporales	95
95	47	2	Chaetothyriales	94*
96	44	1	Tremellomycetes	100
97	40	1	Curreya grandicipis	100
98	40	2	Unknown fungal cluster 30	100
99	39	1	Unknown fungal cluster 27	91*
100	38	2	Unknown fungal cluster 31	88*
101	37	1	Diaporthe australafricana	100
102	36	1	Unknown fungal cluster 28	86*
103	36	1	Unknown fungal cluster 29	96*
104	36	3	Claviceps purpurea	100
105	35	1	Mycosphaerella	100
106	35	2	Teratosphaeria keanei	95*
107	35	2	Saccharomyces cerevisiae	100
108	34	2	Unknown fungal cluster 35	91*
109	34	2	Saccharomycetaceae	100
110	31	1	Unknown fungal cluster 32	98
111	31	2	Unknown fungal cluster 37	96*
112	31	2	Hormonena	98
113	31	4	Unknwon fungal cluster 57	100
114	30	1	Ascomycota	97
115	30	4	Alternaria	98
116	29	1	Unknown fungal cluster 33	98
117	28	1	Lophodermium actinothyrium	99
118	27	1	Unknown fungal cluster 34	98
119	27	1	Unknown fungal cluster 36	97
120	26	1	Hormonema	99
121	26	1	Unknown fungal cluster 38	90*

OTU No.	No. of DNA sequences	No. of unique sequences	BLASTn assignment	Similarity (%)
122	25	1	Unknown fungal cluster 39	98
123	25	1	Unknown fungal cluster 40	99
124	25	1	Dioszegia	100
125	25	1	Penicillium	100
126	23	1	Unknown fungal cluster 41	90*
127	23	1	Penicillium	100
128	23	2	Pleosporaceae	99
129	22	1	Cryptococcus paraflavus	99
130	22	2	Unknown fungal cluster 46	91*
131	21	1	Cryptovalsa ampelina	100
132	21	1	Unknown fungal cluster 42	91*
133	20	1	Unknown fungal cluster 43	97
134	20	2	Candida tropicalis	100
135	19	1	Pleosporales	98
136	19	1	Exobasidium	93*
137	18	1	Teratosphaeriaceae	99
138	18	1	Phaeomoniella prunicola	96*
139	17	1	Unknown fungal cluster 44	89*
140	17	1	Dothideomycetes	100
141	17	1	Unknown fungal cluster 45	97
142	16	1	Saccharomycetales	100
143	16	1	Polyporales	94*
144	16	1	Mycosphaerellaceae	100
145	16	1	Unknown fungal cluster 47	100
146	15	1	Pichia mexicana	100
147	14	1	Unknown fungal cluster 48	88*
148	14	1	Unknown fungal cluster 49	98
149	13	1	Unknown fungal cluster 50	100
150	13	1	Pleosporales	100
151	12	1	Unknown fungal cluster 51	99
152	12	1	Unknown fungal cluster 52	84*
153	12	1	Unknown fungal cluster 53	98
154	12	1	Catenulostroma	99
155	11	1	Unknown fungal cluster 54	100
156	10	1	Chaetothyriales	99
157	10	1	Unknown fungal cluster 55	95
158	10	1	Bensingtonia	94*
159	10	1	Unknown fungal cluster 56	90*
160	10	1	Lalaria inositophila	100
161	10	1	Lophiostoma chamaecyparidis	97
162	9	1	Exophiala eucalyptorum	99

OTU No.	No. of DNA sequences	No. of unique sequences	BLASTn assignment	Similarity (%)
163	9	1	Pleosporales	96*
164	9	1	Exophiala	97
165	8	1	Pseudozyma fusiformata	100
166	8	1	Pseudozyma	100
167	8	1	Fusarium tricinctum	100
168	8	1	Unknown fungal cluster 58	100
169	8	1	Basidomycota	98
170	8	1	Unknown fungal cluster 59	97
171	7	1	Zygoascus	100
172	7	1	Pleosporaceae	100
173	7	1	Unknown fungal cluster 60	86*
174	6	1	Unknown fungal cluster 61	87*
175	6	1	Unknown fungal cluster 62	99
176	6	1	Unknown fungal cluser 63	93*
177	6	1	Unknown fungal cluster 64	89*
178	6	1	Fusarium	100
179	6	1	Unknown fungal cluster 65	98
180	6	1	Unknown fungal cluster 66	93*
181	6	1	Pyrenophora semeniperda	98
182	5	1	Unknown fungal cluster 67	99
183	5	1	Unknown fungal cluster 68	97
184	5	1	Limonomyces roseipellis	98
185	5	1	Unknown fungal cluster 69	97
186	5	1	Tremellomycetidae	100
187	5	1	Cryptovalsa rabenhorstii	100
188	5	1	Trebouxia	95*

Legend: less than 97.0% BLAStn similarity*

Table S6: Description of colonies isolated on microbiological media

Sample Number	Colony number	Mucosal or Filamentous	Colony colour	Edge colour	Edge	Shape	Surface	Lustre	Topography
1	1	Filamentous	green/yellow	white	rhizoid	circular	textured	none	rhizoid
	2	Mucosal	blue	white	dentate	circular	smooth	glossy	raised
	3	Mucosal	clear	clear	undulate	irregular	smooth	glossy	convex
	4	Mucosal	blue	blue	entire	irregular	textured	glossy	convex
	5	Filamentous	brown	brown	rhizoid	rhizoid	textured	none	rhizoid
	6	Mucosal	pink	pink	undulate	irregular	smooth	glossy	flat
	7	Mucosal	white	white	entire	circular	smooth	glossy	convex
	8	Mucosal	green/yellow	white	unclear	irregular	textured	matte	flat
	9	Mucosal	cream	white	dentate	irregular	textured	glossy	raised
	10	Mucosal	green/yellow	green/yellow	undulate	circular	textured	glossy	umbonate
	11	Mucosal	blue/green	white	entire	circular	textured	glossy	umbonate
	12	Mucosal	clear	clear	dentate	circular	smooth	glossy	umbonate
	13	Mucosal	green/yellow	yellow/white	entire	circular	smooth	glossy	raised
	14	Mucosal	dark blue	blue	dentate	circular	textured	glossy	umbonate

Sample Number	Colony number	Mucosal or Filamentous	Colony colour	Margin colour	Edge	Shape	Surface	Lustre	Topography
2	1	Mucosal	white	white	dentate	circular	smooth	glossy	raised
	2	Filamentous	white	white	dentate	rhizoid	textured	none	rhizoid
	3	Mucosal	white	white	dentate	irregular	smooth	glossy	raised
	4	Mucosal	clear	clear	entire	circular	smooth	glossy	convex
	5	Mucosal	pink	pink	entire	circular	smooth	glossy	convex
	6	Filamentous	white	white	dentate	rhizoid	textured	none	rhizoid
	7	Mucosal	blue	blue	entire	irregular	smooth	glossy	convex
	8	Mucosal	white	white	dentate	irregular	smooth	glossy	raised
	9	Filamentous	white	white	dentate	rhizoid	textured	none	rhizoid
	10	Mucosal	beige	beige	entire	irregular	smooth	matte	raised
	11	Mucosal	white	white	entire	circular	smooth	glossy	convex
	12	Mucosal	white	white	entire	irregular	smooth	glossy	umbonate
	13	Mucosal	white	white	entire	irregular	smooth	glossy	umbonate
	14	Mucosal	blue	blue	dentate	circular	smooth	matte	convex
	15	Mucosal	white	white	entire	circular	smooth	matte	convex
	16	Mucosal	blue	blue	dentate	irregular	smooth	glossy	umbonate

Sample Number	Colony number	Mucosal or Filamentous	Colony colour	Margin colour	Edge	Shape	Surface	Lustre	Topography
3	1	Filamentous	green/brown	green/brown	rhizoid	rhizoid	textured	none	rhizoid
	2	Mucosal	blue/green	white	dentate	circular	smooth	glossy	convex
	3	Mucosal	clear	clear	entire	circular	smooth	glossy	convex
	4	Mucosal	blue	blue	entire	circular	textured	glossy	convex
	5	Mucosal	pink	pink	entire	circular	smooth	glossy	raised
	6	Mucosal	white	white	dentate	circular	textured	glossy	raised
	7	Mucosal	green	green	entire	circular	smooth	glossy	raised
	8	Mucosal	yellow	cream	undulate	circular	smooth	glossy	raised
	9	Mucosal	cream	cream	dentate	circular	smooth	glossy	convex
	10	Mucosal	blue/green	white	entire	circular	smooth	glossy	umbonate
	11	Mucosal	white	white	entire	circular	smooth	glossy	umbonate
	12	Mucosal	dark blue	dark blue	undulate	irregular	smooth	glossy	flat

Table S7: Colony counts for each colony types isolated on microbiological media

Sample No.	Col. No.	Control (0%)			0.005%			0.010%			0.015%			0.020%			0.025%		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	1	56	32	0	137	177	201	168	239	159	145	190	136	195	229	146	144	169	145
	2	32	13	6	56	18	72	26	80	31	38	52	40	89	91	91	105	96	101
	3	121	118	75	19	80	5	8	11	2	7	5	0	0	0	0	0	0	0
	4	31	9	0	6	20	9	9	22	4	8	6	15	0	1	0	1	1	1
	5	2	0	0	17	7	28	1	16	0	2	3	0	0	0	0	0	0	0
	6	11	14	10	0	18	0	4	16	1	0	0	0	0	0	0	0	0	0
	7	10	25	26	2	8	0	1	1	8	1	17	14	8	7	9	12	11	7
	8	1	3	3	0	2	2	5	2	1	2	0	0	0	0	0	0	0	0
	9	0	8	3	0	5	0	2	0	5	0	2	0	0	0	0	0	0	0
	10	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	11	0	0	0	5	1	3	0	1	3	4	17	7	1	5	6	2	3	1
	12	0	0	0	0	0	3	0	6	1	0	0	0	0	1	0	0	0	0
	13	0	0	0	0	0	0	0	0	0	3	2	0	0	0	0	0	0	0
	14	0	0	0	0	0	0	0	0	0	0	1	2	14	7	8	6	9	27

Sample No.	Col. No.	Control (0%)			0.005%			0.010%			0.015%			0.020%			0.025%		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
2	1	8	6	10	8	3	13	13	5	3	1	1	0	0	0	2	0	1	0
	2	4	1	1	1	0	1	0	1	1	1	1	3	1	2	2	0	0	0
	3	1	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0
	4	15	1	4	5	23	2	3	13	9	35	107	179	393	467	359	403	324	345
	5	1	1	2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	2	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	0	1	0	0	0	0	1	5	0	0	2	1	0	2	1	1	2	0
	8	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	9	0	0	0	0	0	0	1	2	1	0	1	0	0	0	0	1	0	0
	10	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
	11	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
	12	0	0	0	0	0	1	0	4	0	0	6	4	0	1	0	0	0	0
	13	0	0	0	0	0	0	2	2	4	1	3	4	2	2	0	3	7	4
	14	0	0	0	0	0	0	1	0	5	5	1	0	7	3	1	0	0	0
	15	0	0	0	0	0	0	0	10	0	4	2	0	1	0	0	0	0	1
	16	0	0	0	0	0	0	0	0	0	0	0	0	3	6	5	7	4	5

Sample No.	Col. No.	Control (0%)			0.005%			0.010%			0.015%			0.020%			0.025%		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
3	1	244	101	42	59	60	158	93	141	150	69	119	83	105	39	78	70	105	77
	2	0	0	0	15	21	0	91	51	128	77	98	183	148	139	109	232	150	133
	3	34	0	0	4	0	23	0	0	0	0	0	0	0	0	0	0	0	0
	4	2	0	0	0	0	4	3	2	0	0	0	0	1	2	0	0	0	5
	5	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	9	5	0	8	4	34	26	2	1	5	5	7	1	0	1
	7	0	39	15	6	4	1	6	0	0	0	0	0	0	2	2	0	0	0
	8	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	34	37	46	82	132	96	70	25	88	76	97	64
	11	0	0	0	0	0	0	0	2	8	32	1	1	3	1	0	0	1	0
	12	0	0	0	0	0	0	0	0	0	10	8	12	11	12	4	12	21	17