The development and evolution of Saccharomyces interspecific hybrids for improved, industry relevant, phenotypes.

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A thesis submitted for the degree of Doctor of Philosophy

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Table of contents

Thesis summary i

Declaration iii

Publications iv

Panel of Supervisors v

Acknowledgements vi

Chapter 1

Review of the literature and summary of research aims

Chapter 2

Newly generated interspecific hybrids introduce flavour and aroma diversity to wines.

Chapter 3

Introducing a new breed of wine yeast: interspecific hybridisation between a commercial *Saccharomyces cerevisiae* wine yeast and *Saccharomyces mikatae*.

Chapter 4

Designing and creating *Saccharomyces* interspecific hybrids for improved, industry relevant, phenotypes.

Chapter 5

A novel approach to isolating improved industrial *Saccharomyces* interspecific wine yeast using chromosomal mutations as a marker for increased fitness.

Chapter 6

Concluding remarks and future perspectives

Thesis Summary

This thesis describes a progression of experimental work from proof of concept (ie can laboratory generated interspecific yeast strains be developed for industrial application) through to realisation of the potential of novel interspecific wine yeast for improved outputs in the winery.

A competitive market requires winemakers to not only be aware of production costs, but also to find market niches by differentiating their wine styles. Developing new yeast with improved fermentation traits and/or potential to produce diverse wine flavours and aromas can provide tools to the winemaker that are readily and easily utilised in the winery without any extra (or costly) processing intervention.

With consumer reluctance to the acceptance of genetically modified organisms, yeast breeding remains an important technique for yeast strain development. Traditionally, yeast breeding programs have centred around mating between different *Saccharomyces cerevisiae* yeast strains. Incorporating a higher level of genomic diversity into a *Saccharomyces cerevisiae* wine yeast by hybridisation with other *Saccharomyces* species has the potential to deliver novel flavour and aroma profiles through the production of a wider range of yeast-derived, flavour-active metabolites.

This research reports on the development of laboratory-generated yeast interspecific hybrids created by natural breeding techniques. Initially, interspecific hybrids between species most closely related to *S. cerevisiae* were assessed. Grape juice fermentation by hybrids from crosses between a commercial *S. cerevisiae* wine yeast and either *Saccharomyces paradoxus* or *Saccharomyces kudriavzevii* showed that the hybrids had robust fermentation properties and produced wines with different concentrations of aromatic products relative to the commercial wine yeast parent.

Progeny from crosses utilising a more divergent species (*Saccharomyces mikatae*) were fermentation competent and could deliver wines with novel flavours and aromas, including flavour compounds more commonly associated with non-*Saccharomyces* species.

Next, a targeted approach to determine whether hybrids could be generated with a predictable phenotype that could address an explicit problematic fermentation trait was used. Elevated volatile acidity levels when producing dessert wines from high-sugar juices pose a challenge to winemakers. Hybrids from a mating with a *S. cerevisiae* wine yeast and *Saccharomyces uvarum* (a species previously reported to produce wines with low concentrations of acetic acid) displayed the desired targeted phenotypes; strong fermentation properties in high-sugar juice and wines with low volatile acidity.

Subsequent experiments indicated that the hybrids were less robust in grape juice than their *S. cerevisiae* wine yeast parent. With this in mind, it was decided to attempt to increase fitness of one *S. cerevisiae* x *S. uvarum* hybrid by an adaptive evolution approach in grape juice. To avoid the problem of selecting end-point collateral mutations that shape phenotypes in addition to that which is targeted, isolates were progressively screened from the evolving population.

An evolved isolate with loss of *S. uvarum* Chromosome 14 (the overriding chromosomal alteration) but no other detectable changes in karyotype demonstrated that loss of *S. uvarum* Chromosome 14 alone conferred increased fitness. Fermentation kinetics showed that the evolved strain had an increased fermentation performance relative to the original hybrid and retained the desirable fermentation trait of the parent: wines with low volatile acidity.

This research establishes that *Saccharomyces* interspecific hybridisation can deliver tools to the winemaking industry in the realm of wine style differentiation through the formation of novel yeast volatile fermentation metabolite profiles, and improved yeast fermentation properties. In addition, adopting an evolutionary approach in a fermentative context can deliver increased fitness to a wine yeast interspecific hybrid.

For a thesis that contains publications.

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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06/07/2018

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DATE

Publications

This thesis is a collection of manuscripts published or submitted for publication in various peer-reviewed journals during candidature. The manuscripts were published in the journals 'Applied Microbiology and Biotechnology' (impact factors of 3.425 in 2011 and 3.376 in 2015), Plos One (impact factor of 3.23 in 2013) and submitted to Frontiers in Microbiology (impact factor of 4.079 in 2018).

The text and figures in the different chapters have different formatting, according to the various journal requirements. A Statement of Authorship, signed by all authors, listing individual contributions to the work is included at the beginning of each chapter.

The thesis is based on the following referred publications:

Chapter 2. Jennifer R. Bellon, Jeffery M. Eglinton, Tracey E. Siebert, Alan P. Pollnitz, Louisa Rose, Miguel de Barros Lopes and Paul J. Chambers. Newly generated interspecific wine yeast hybrids introduce flavour and diversity to wines. Appl. Microbiol Biotechnol. **2011**, 91:603-612. DOI:10.1007/s00253-011-3294-3

Chapter 3. Jennifer R. Bellon, Frank Schmid, Dimitra Capone, Barbara L. Dunn and Paul J. Chambers. Introducing a new breed of wine yeast: Interspecific hybridisation between a commercial *Saccharomyces cerevisiae* wine yeast and *Saccharomyces mikatae*. PLoS ONE **2013**, 8(4): e62053. DOI:10.1371/journal.pone.0062053

Chapter 4. Jennifer R. Bellon, Fei Yang, Martin P. Day, Debra L. Inglis and Paul J. Chambers. Designing and creating *Saccharomyces* interspecific hybrids for improved, industry relevant, phenotypes. Appl. Microbiol Biotechnol. **2015**, 99:8597-8609 DOI:10.1007/s00253-015-6737-4

Chapter 5. Jennifer R. Bellon, Anthony R. Borneman, Christopher M. Ford and Paul J. Chambers. A novel approach to isolating industrial *Saccharomyces* interspecific wine yeasts using chromosomal mutations as a marker for increased fitness. Submitted to Front. Microbiol. March **2018**

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Chapter 1

LITERATURE REVIEW

This literature review covers the literature up to May 2009, which was within the first six-months equivalent of candidature. Relevant literature beyond this date is included in the publications covered in Chapters 2 to 5.

Saccharomyces yeasts and fermentation

The *Saccharomyces* genus is a group of genetically related yeasts that are recognized for their ability to ferment sugars; the Latin term *Saccharomyces* means sugar fungus. The genus was defined by Rees in 1870 but it was earlier, in 1838, that Meyen first proposed the name *Saccharomyces* for bread and beer yeasts (Refer to Rainieri 2003). The species name of the most well-known of the *Saccharomyces* yeasts, *S. cerevisiae*, has its origin in the Gaelic word kerevigia and the old French word cervoise; both ancient terms for beer. An important characteristic of *Saccharomyces* yeasts is their propensity to ferment hexoses to ethanol, even under aerobic conditions, due to a glucose repression circuit which represses aerobic respiration under high levels of glucose; this is known as the Crabtree Effect (Johnston 1999). *Saccharomyces* yeast are able to both produce and consume ethanol, and, because of their tolerance for high ethanol concentrations, they have a competitive advantage in an environment containing high sugar content, such as rotting fruit and industrial fermentations.

Identified by Pasteur as the causative agent for alcoholic fermentation and known to the layperson as Baker's yeast, *Saccharomyces cerevisiae*, is the pillar of alcoholic fermentation industries including winemaking, brewing and spirit production. A degree of specialisation has evolved in different domesticated lineages; for example ale yeasts are not well suited to making wine, and baking yeasts are generally not good at making alcoholic beverages. This is borne out in comparative genomics studies which have shown that there is a clear clustering of sub-groups of *S. cerevisiae* strains, linked to the industry that they serve (Legras et al. 2007). Several hundred different compounds determine the flavour and aroma of wine, and, particularly for inoculated fermentations, it is the action of yeast on grape compounds via metabolism and bio-transformations that is the main contributor in building the complex chemistry of wine (Rapp and Mandery, 1986).

Saccharomyces species

Molecular studies have established seven distinct species within the *Saccharomyces* spp. complex (Barnett 1992; Kurtzman 2003; Wang and Bai 2008) as well as identifying an eighth species, *S. pastorianus*, as a natural hybrid between *S. cerevisiae* and a *S. bayanus*- type yeast (Nilsson-Tillgren et al. 1981; Vaughan-Martini and Martini 1987; Vaughan-Martini 1989; Naumov 1996; Naumov in 2000). All *Saccharomyces* spp. are physiologically similar to *S. cerevisiae*, have a similar karyotpye and are able to mate with each other, however interspecific hybrids are sterile, having less than one percent spore viability (Naumov 1987).

There is considerable genetic variation in the *Saccharomyces* clade. For example, comparative genomics have shown that the sequence divergence between *S. cerevisiae* and its most distant relative, *S. bayanus* is similar to that

between human and mouse; 11% indels (insertions and deletions) and 62% nucleotide identity in aligned positions compared to 12% indels and 66% nucleotide identity in aligned positions respectively (Kellis et al. 2003).

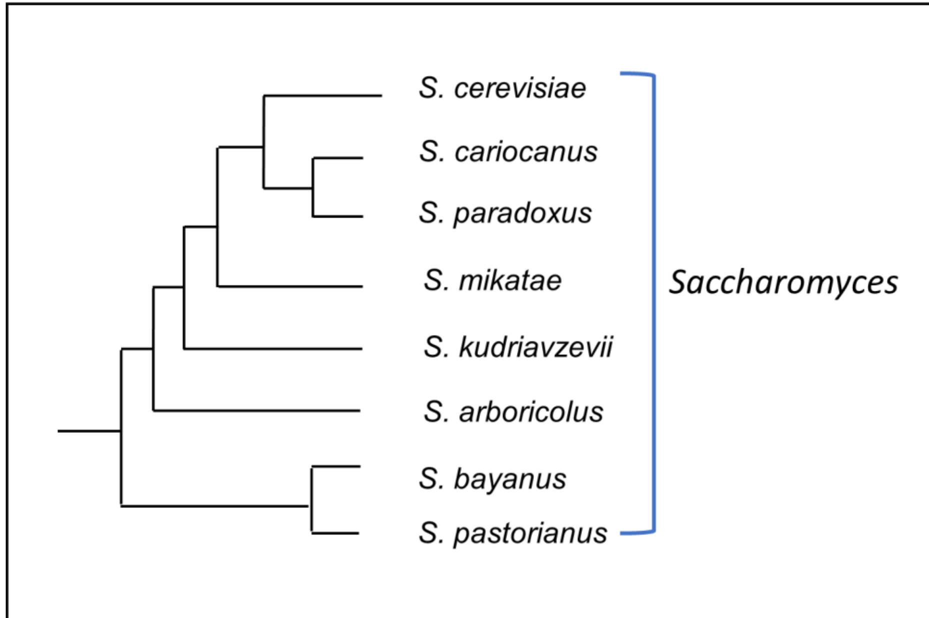

Figure 1. A representation of the *Saccharomyces* Phylogenetic Tree (adapted from the Washington University in St. Louise web page; http://128.252.233.3/genome/yeast/map.html)

Life cycle of the *Saccharomyces* spp.

The life cycle of *Saccharomyces* species includes sexual and asexual stages (Refer to Figure 2). Mitosis, asexual growth, involves replication of nuclear DNA and the packaging of the resultant daughter nucleus and associated organelles into a small bud that forms on the cell wall. Cytokinesis occurs, releasing the new daughter yeast cell.

Haploid yeast cells are of either an ‘a’ or ‘α’ mating type, and can mate with cells of the opposite mating type to form diploids (Herskowitz I 1988). Meiosis, the sexual cycle, requires the expression of both mating type alleles and consequently, only cells with a diploid (or polyploid) complement can undergo meiosis, producing a tetrad of spores enclosed within an envelope, (the ascus) and joined by interspore bridges (Coluccio and Neiman 2004). As diploids are heterozygous for the mating type locus, MAT, meiosis produces two spores of each mating type allele. Self-fertilisation can occur upon germination, with spores tending to mate with another

spore from the same ascus. Alternatively, an unfertilised spore can divide by mitosis and subsequently undergo a mating-type switch. The daughter cell now can mate with a previous mitotically derived cell of the opposite mating type to form a diploid cell homozygous at every allele except for the Mat locus.

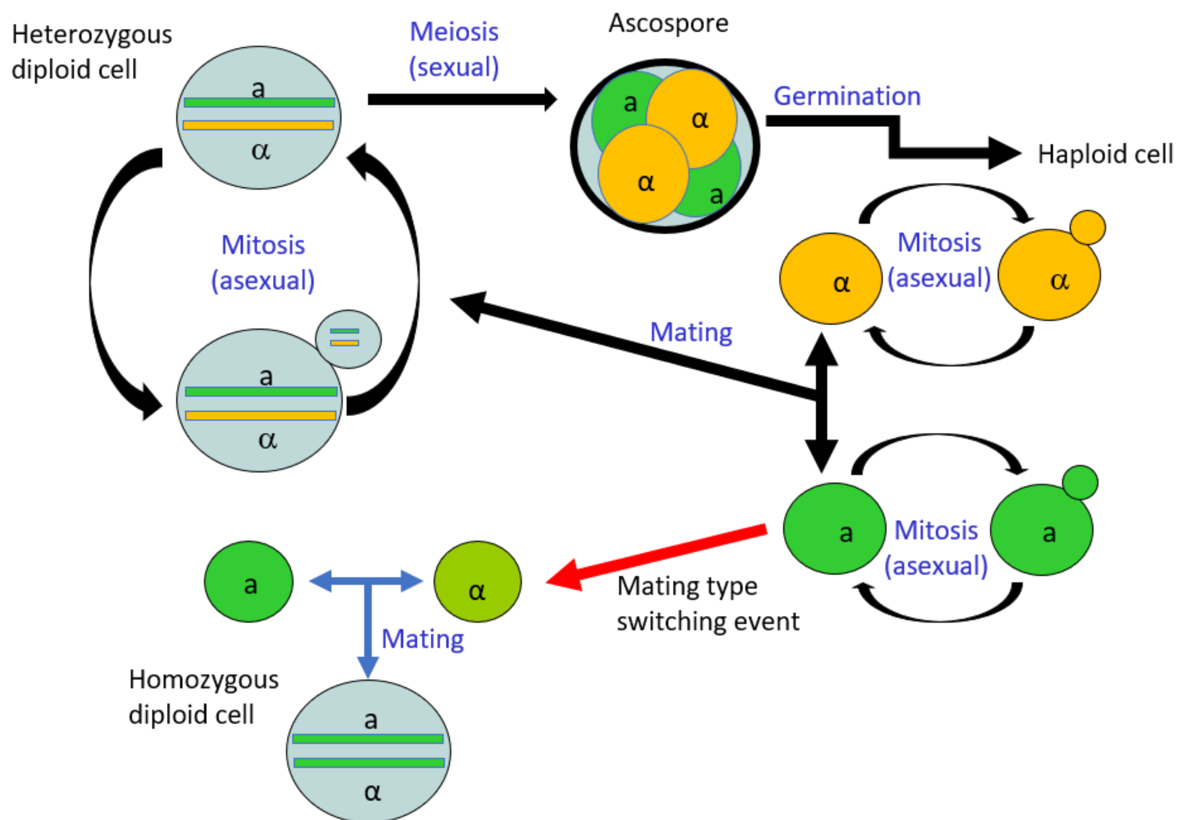

Figure 2. Life cycle of *Saccharomyces* yeast detailing sexual and asexual cycles with mating type switching event

Rare mating

In nature, yeast mating is activated by the presence of pheromones produced by haploid yeast. Rare mating relies upon an infrequent event (1×10^{-6} cells) whereby mating type switching within the diploid genome leads to a cell homozygous at the mating type loci, either a/ a or α/α (Gunge and Nakatomi 1972). These homozygotes are able to enter the mating pathway and can conjugate with a cell of the opposite mating type, resulting in a triploid progeny. When a homozygous diploid cell conjugates with a haploid cell of a different *Saccharomyces* species an interspecific hybrid is formed.

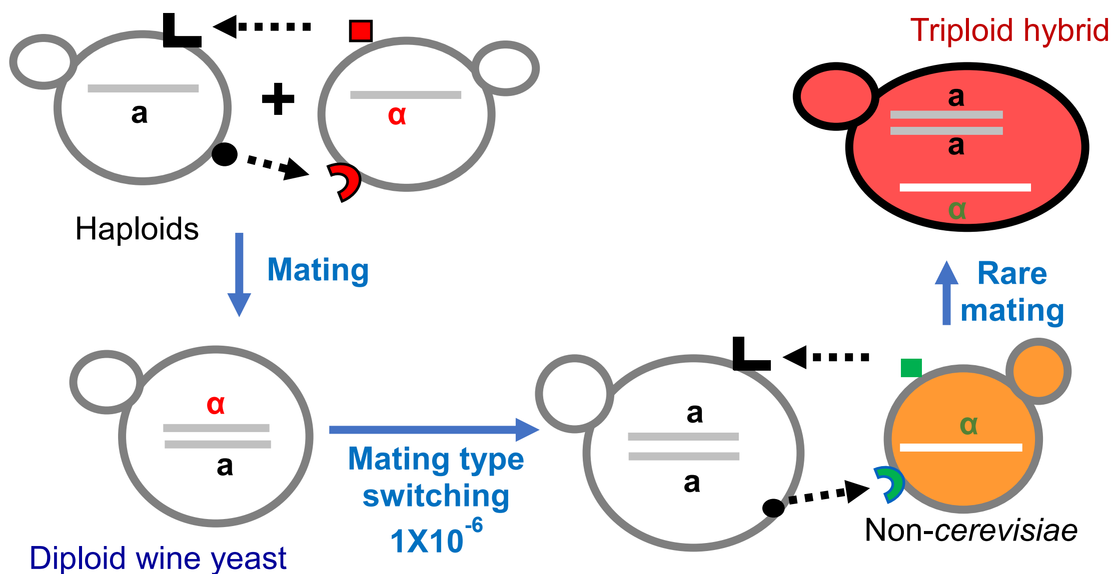

Figure 3. Rare mating in *Saccharomyces* (see accompanying text)

Interspecific hybrids

The "biological species concept" was proposed by Ernst Mayr in his book on evolutionary biology, 'Systematics and the origin of the species', published in 1942 where he defined biological species in terms of interbreeding; "species are groups of interbreeding natural populations that are reproductively isolated from other such groups." Separate, interbreeding populations that produce infertile or sterile offspring are different species under this definition.

Most commonly, interspecific hybrids are formed when two species from within the same genus are crossed and the offspring display traits and characteristics of both parents. In nature, interspecific hybridisation of closely related species is common, and tends to occur in narrow geographic regions called hybrid zones where the ranges of two closely related species meet, and individuals can mate and produce hybrid offspring. Plant, bird, reptile and fish hybrids are commonly found in such regions. One well-known avian hybrid zone is that between the Hooded Crow *Corvus corone cornix* and Carrion Crow *C. c. corone,* which extends throughout much of Europe (Haas and Brodin, 2005).

Humans have utilised animal husbandry for the breeding of interspecific animal hybrids for domestic use. Mules, arising from crosses between horses and donkeys, have played an important role as work animals down throughout history; they have the patience, endurance and surefootedness of a donkey and display the vigour, strength and courage of a horse.

Plant species hybridise more readily than animals, and this has been used to advantage in modern times to generate a plethora of new varieties of grains, fruits and flowering plants with improved seed or fruit size, or with disease resistance traits. For example, the tangelo is a hybrid between a mandarin orange and a grapefruit, while most ancient and modern wheat breeds are hybrids between different species of grasses.

Polyploidism (possessing more than two complete sets of chromosomes) and aneuploidism (an abnormal number of chromosomes caused by missing or extra chromosomes) is also common amongst plants (Blakeslee 1920; Ramsey and Schemske 1998). Polyploidism has played an important role in plant evolution as many ferns and flowering plants, including both wild and cultivated species, have been shown to be polyploids. Polyploids can be autopolyploids (the doubling of a genome of the same origin) or allopolyploids (a combination of two genetically distinct genomes as found in interspecific hybrids).

Hybridisation between *Saccharomyces* spp.

Natural hybrids

There are many examples of natural hybrids between the closely related *Saccharomyce*s clade. The lager brewing yeast, *S. pastorianus* (synonym *S. carlsbergensis),* currently accepted as a member of the *Saccharomyces* clade, is a hybrid between two species of *Saccharomyces*, a *S. cerevisiae* and a *S. bayanus*-like yeast. (Kielland-Brandt et al. 1995; Dunn and Sherbrook 2008).

The wine making yeast, S6U, has been identified as a hybrid between *S. cerevisiae* and *S. bayanus* strains (Masneuf et al. 1998), and a small number of commercial wine yeast and wine yeast isolates have been identified as being hybrids between *S. cerevisiae* and *S. kudriavzevii* (Bradbury et al. 2005; González et al. 2006), while isolates from oak exudate and soil have been identified as hybrids between *S. cerevisiae* and *S. paradoxus* (Liti et al. 2005).

The cider yeast, CID1, and a wine isolate from Switzerland have been identified as natural hybrids between three different *Saccharomyces* yeast species; *S. cerevisiae*, *S. bayanus* and *S. kudriavzevii* all contribute to their hybrid genomes (Groth et al. 1999; Gonzalez et al. 2006).

Laboratory-made hybrids

Since the work of Winge and Lausten (1938), yeast researchers have been able to utilize the mating pathway of *S. cerevisiae* for breeding new strains of brewing yeast with improved characteristics. Such techniques were subsequently used on bread,

wine and sake yeast strains to generate intraspecies hybrids with traits pertinent to these industries (Spencer and Spencer 1983).

The fact that members of the *Saccharomyces* complex share the same mating pathway and utilise very similar molecular processes for mating, has allowed researchers to create interspecific hybrids in the laboratory.

With the exception of *S. pastorianus, Saccharomyces* interspecific hybrids can be produced between all species, (eg. Naumov 1987; Zambonelli et al. 1993; Hunter et al. 1996; Naumov 2000; de Barros Lopes et al. 2002).

The ability of *Saccharomyces* spp. to undergo multiple hybridisation events was confirmed by the successful mating of existing natural interspecific hybrid strains with either a *S. cerevisiae* or *S. paradoxus* strain (de Barros Lopes et al. 2002).

Researchers have even utilized the hostile environment of an invertebrate's digestive tract in order to produce interspecific hybrids between *S. cerevisiae* and *S. uvarum*[1] strains. This unusual procedure was devised in order to verify that animals can promote the formation of new yeast strains. The experiments relied on the digestive tract's ability to degrade the ascus wall of an ascospore thus liberating individual spores. Different species (freshwater worms and drosophila flies) were fed a diet of different sporulated yeasts and their faeces were collected. Yeast propagated from the faeces were analysed to confirm their hybrid nature (Pulvirenti et al. 2002).

The potential for interspecific hybrids to deliver wines with novel flavours and aromas

To date, the suitability of the non-*cerevisiae Saccharomyces* species for winemaking has not been explored fully and few examples of laboratory-made wine yeast interspecific hybrids have been reported.

The usefulness of *S. bayanus* var. uvarum for improvements in winemaking traits has been investigated by researchers as this yeast has been shown to produce wines with higher concentrations of glycerol and phenylethyl alcohol (floral, rose aroma) whilst lowering acetic acid levels relative to several commercial *S. cerevisiae* wine yeast strains studied. On the other hand, *S. bayanus* can produce unacceptably high levels of succinic and malic acid, thus making wines excessively acidic. Interspecific hybrids between *S. cerevisiae* and *S. bayanus* var. *uvarum* have been successful in lowering the volatile acidity of wines whilst producing higher concentrations of the volatile flavour compounds phenylethyl alcohol, isoamyl acetate and phenylethyl acetate (Kishimoto 1994), increasing the glycerol content relative to the *S. cerevisiae*

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[1] *S. uvarum* is a sub group of the *S. bayanus* species and some taxonomists propose that there should be a formal phylogenetic distinction between the two groups)

parent and reducing the production of malic acid relative to the *S. bayanus* parent (Zambonelli et al. 1997; Rainieri et al. 1999).

The capacity for *S. paradoxus* to have a favourable oenological impact was proposed by Majdak et al. 2002 when *S. paradoxus*-made wines were shown to have different chemical and sensory properties relative to *S. cerevisiae*-made wines.

Whilst *S. kudriavzevii* has not been utilised in winemaking to date, putative hybrids between *S. cerevisiae* and *S. kudriavzevii* isolated in Austrian grape-growing regions have been shown to produce wines with higher concentration of fruity esters relative to other *S. cerevisiae* isolates from close locations and may influence wine quality favourably (Lopandic et al. 2007).

Genetic stability and incompatibility in interspecific hybrids

Plant studies have shown that the evolution of polyploid genomes is a dynamic process and that extensive intra- and intergenomic changes occur rapidly; increasing the copy number of a gene provides redundancy and allows for divergence of function, potentially accelerating evolution. However, in many species, gene copy number above the normal diploid complement can give rise problems associated with gene dosage. An example in the human arena is Down syndrome, the gene dosage disease caused by trisomy of Chromosome 21. Two ways for a polyploidy organism to deal with the duplication of genes are to lose or silence extra copies.

Gene loss and the silencing of genes involved in metabolism, disease resistance and cell cycle regulation, as well as rRNA genes, have been identified in wheat allotetraploids (Kashkush et al. 2002). It is thought that these changes not only deal with gene-dosage consequences, but lessen gene redundancy or genome incompatibility. As well as allowing hybrid genome stability, these changes can provide genetic variation (Soltis and Soltis 1999). A study using synthetic *Brassica* polyploids showed that chromosomal rearrangement involving homeologous recombination is a major factor in genome alterations and that the degree and frequency of genomic change in the resultant polyploidy is directly related to the degree of divergence between the two parental species (Song et al. 1995).

Hybrid incompatibility is a term used to describe the phenomenon by which closely related species can mate, but their progeny are inviable or sterile. Hybrid incompatibility has the effect of forming an isolating barrier to reproduction and is thought to be an important vehicle for speciation (Coyne and Orr 1998). Hybrid incompatibility might be due to chromosomal differences between species. A different number of chromosomes, or chromosomal translocation, may mean that homeologous chromosomes cannot pair leading to non-disjunction during meiosis (Otto and Whitton 2000; Pikaard 2001).

In addition to gross chromosomal changes that impact on stability of newly formed hybrid genomes, some genes (termed ‘speciation’ genes) may be incompatible

between the different species, leading to loss of hybrid viability or reproductive isolation. A small number of speciation genes have been identified in *Drosophlila* that lead to male sterility (Ting et al. 1998) and hybrid inviability (Watanabe 1979; Hutter and Ashburner 1987; Presgraves et al. 2003).

In plant breeding, the formation of auto-polyploids is a common technique used to overcome sterility of a hybrid (Chen and Kirkbride, 2000). After polyploidisation, each chromosome will have an identical partner to pair with during meiosis, thus, the hybrid becomes fertile and can be further propagated.

Genetic stability, compatibility and evolution in yeast interspecific hybrids

What determines the viability and stability of the product of an interspecific hybridisation event when two *Saccharomyces* yeast are mated? Studies in plants suggest important factors include:

- Polyploidism
- DNA sequence divergence and chromosomal rearrangements
- Incompatability and speciation genes

Yeast polyploidism

Although plant researchers became aware of the polyploidy nature of plants early in the 1900's, only recently have researchers unveiled a similar evolutionary phase in yeast. Advances in DNA sequencing and comparative genomics led to the proposal that the *Saccharomyces* spp. are paleopolyploids, having a common ancestor that went through a polyploidal phase followed by regression of the genome (Wolfe and Shields, 1997). It is thought the common ancestor had a genome complement of eight chromosomes which underwent a complete duplication event followed by massive gene loss of nearly 90% of the duplicated genes, leaving a remnant genome consisting of sixteen separate chromosomes. Polyploidism relaxes the constraints on DNA sequence conservation and the post-duplication divergence of gene pairs enables neo-functionalisation with the evolution of specialised functions and expression. This is what is thought to have happened in the ancestor of *Saccharomyces* yeasts (Kellis et al. 2004).

DNA sequence divergence and chromosomal rearrangements

The mismatch repair system, responsible for detecting and correcting mismatched DNA base pairs during replication plays a role in meiosis (Selva et al. 1995; Wang et al. 1999) and has been shown to contribute significantly to hybrid spore inviability in *S. cerevisiae* x *S. paradoxus* hybrids (Hunter et al. 1996). Two outcomes are

possible when recombination is initiated between divergent chromosomes: gene conversion occurs when mismatches are repaired, or recombination is aborted (Borts et al. 2000). The degree of sequence divergence and type of recombination (mitotic vs meiotic) influences which outcome occurs (Borts and Haber 1987; Chen and Jinks-Robertson 1999; Harfe and Jinks-Robertson 2000); the more divergent the DNA sequences from parental species the more likely it is to lead to reproductive isolation.

DNA sequences that are highly conserved within the *Saccharomyces* are more likely to undergo gene conversion. rDNA sequences have been identified as undergoing ‘concerted evolution’ in *S. cerevisiae* x *S. kudriavzevii* hybrids where ITS sequences were found to be derived solely from the *S. kudriavzevii* species (Liti et al. 2005). Concerted evolution events, where each gene locus in a gene family comes from the same variant, have also been reported in rDNA sequences of plants (Koch et al. 2003) and fungi (James et al. 2001; Teyessier et al. 2003). Gene conversion reduces the DNA sequence divergence in an interspecific hybrid genome and is one way that a genome may be stabilised.

Chromosomal translocations are implicated as a mechanism for species barriers in plants (Lai et al. 2005), and restoring genome co-linearity between *S. cerevisiae* and *S. mikatae* chromosomes increased the ability of hybrids between these two species to produce viable spores (Delneri et al. 2003).

Incompatibility and speciation genes

To date, the search for incompatibility genes in *Saccharomyces* has focused on substitution lines, replacing a single *S. cerevisiae* chromosome in a haploid cell with the homeologous chromosome of another *Saccharomyces* species. Two studies have been reported; the first reported no incompatibility between *S. cerevisiae* and *S. paradoxus* (Greig 2007), but this was an incomplete study with only nine of the sixteen *Saccharomyces* chromosomes investigated. A second study using the same approach screened *S. cerevisiae* with *S. bayanus*. Here, a *S. bayanus* gene was found to be incompatible with the *S. cerevisiae* mitochondrial genome. The strain generated had dysfunctional mitochondria, leading to reproductive isolation in a homozygous diploid because functional mitochondria are necessary for the cell to undergo meiosis (Lee et al. 2008).

Transcriptional differences between genomes of different *Drosophila* species have also been shown to have an impact on hybrid viability (Barbash et al. 2003). Similarly, differences in transcriptional regulation have been identified across the *Saccharomyces* spp. with *S. cerevisiae*, *S. mikatae* and *S. bayanus* showing extensive differences in binding sites of a selected group of transcription factors (Borneman et al. 2007). For example, the target genes of transcription factor Ste12 are activated under mating conditions in *S. cerevisiae*, but are activated under pseudohyphal-inducing conditions (low nitrogen) in both *S. mikatae* and *S. bayanus*.

If genes are activated in response to diverse pathways, this may lead to completely new physiological traits, or have a deleterious effect on hybrid viability.

Genomes of natural interspecific yeast hybrids

Natural interspecific hybrids are a resource that can be utilised to inform end-point genomic changes that have led to stable hybrid strains. A study investigating the genomes of a large group of lager yeast (*S. pastorianus*) strains (Dunn and Sherlock 2008) showed that *S. bayanus* was the major contributor to the relic genome with only partial *S. cerevisiae* genomes retained. Also, the strains investigated formed two distinct groups with the authors concluding that two separate hybridistation events had participated in the generation of *S. pastorianus*.

Another study examined the genomes of a small number of natural *S. cerevisiae* x *S. kudriavzevii* hybrids (Belloch et al. 2009). The researchers believe that these hybrids were very closely related, probably arising from a single hybridisation event. These hybrid strains retained the *S. cerevisiae* genome but lost varying portions of the *S. kudriavzevii* genome.

Whilst determining the genomic complement of natural hybrids gives an indication of the plasticity of interspecific genomes, there is no way of determining the order of changes, whether the changes were stabilising or de-stabilising, what ploidy the parental strains were, or the parentage of inherited traits.

Summary of Research Aims

The aim of the research submitted for this thesis was to develop a range of wine yeast interspecific hybrids with divergent genomes by hybridising a robust *S. cerevisiae* wine yeast with other members of the *Saccharomyces* genus. Incorporating a higher level of genomic diversity into a *S. cerevisiae* wine yeast has the potential to deliver novel wine flavour and aroma profiles through the production of a wider range of yeast-derived, flavour-active metabolites. Wine yeast intended for commercial purposes must be robust, providing reliable and consistent fermentations, and be genetically stable. Thus, the genomic stability of newly-formed interspecific hybrids arising from this work was established to ensure that the strains were suitable for industrial application.

Proof of concept: can *Saccharomyces* interspecific hybrids with improved wine-relevant traits be generated?

In order to test whether improved wine yeast strains can be developed from interspecific *Saccharomyces* hybrids a robust commercial *S. cerevisiae* wine yeast strain will be mated with other members of the *Saccharomyces* clade. However, as sporulation of the wine yeast parent to produce haploid spores might lead to the loss of important wine fermentation traits, rare mating will be used to generate triploid interspecific hybrids with a genome complement of diploid *S. cerevisiae* and haploid non-*S. cerevisiae*.

Investigations will centre on assessing the stability of interspecific hybrids made by crossing closely-related versus more distantly-related species. Mitotic segregation of hybrid genomes will be followed and individual clones (colonies formed from a single cell) will have their genomic content analysed to determine parental chromosomal composition.

To determine if interspecific *Saccharomyces* spp hybrids deliver novel flavour and aroma wine profiles

Progeny from interspecific hybridisations between a robust *S. cerevisiae* wine yeast and different members of the *Saccharomyces* species will be assessed for fermentation proficiency in grape juice and the resultant wines will be analysed for yeast-derived flavour-active volatile fermentation compounds.

To utilise interspecific hybridisation as a strategy to introduce targeted, wine-related phenotypes to improve a commercial *S. cerevisiae* wine yeast.

Whilst phenotypic studies of non-*cerevisiae Saccharomyces* species have shown reasonable sugar tolerance, many have poor ethanol tolerance when compared to *S. cerevisiae* thus limiting their usefulness in commercial winemaking. However, members of the non-*cerevisiae* species may have particular traits that can enhance wine fermentation performance to meet a special challenge when mated to a *S. cerevisiae* wine yeast.

To determine whether selective pressure can drive hybrid evolution towards new, positive wine traits and/or improve fitness

A study into the evolution of yeast interspecific genomes by following chromosomal changes from the initial fusion of genomes to a stable hybrid endpoint will give insights into the sequence of events that led to the remnant genomes of natural *Saccharomyces* interspecific hybrids. Hybrid progeny will undergo multiple, successive fermentations and individual cells sampled from each fermentation stage will be analysed for genomic content. Chosen evolved hybrid strains will be assessed for fitness improvements in a fermentation context.

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Contribution to the Paper	Generated and confirmed interspecific hybrid yeast strains, performed fermentation and chemical profiling analyses in synthetic medium, interpreted data, wrote manuscript and acted as corresponding author. The generation of interspecific hybrid yeast strains reported in this manuscript and some of the data shown in Figures 1, 2 & 3 were from experimental work performed prior to candidature for this thesis. They are therefore not submitted for thesis examination. All other data in this manuscript was from experiments performed following candidature.
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Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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By signing the Statement of Authorship, each author certifies that:

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Chapter 2

Newly generated interspecific wine yeast hybrids introduce flavour and diversity to wines.

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This manuscript addresses the generation of interspecific hybrids between *S. cerevisiae* and closely related *Saccharomyces* and their ability to produce wines with novel flavour and aroma profiles.

Interspecific hybrids were generated between a *S. cerevisiae* wine yeast and two other *Saccharomyces* species that are closely-related to *S. cerevisiae* (*S. paradoxus* and *S. kudriavzevii*). It was reasoned that closely related species are more likely than distant relatives to produce hybrids that are stable.

This work generated hybrid progeny that displayed desirable winemaking traits (growth in high sugar medium and tolerance to high ethanol concentrations) and produced wines with concentrations of aromatic fermentation compounds that were different to that found in wines made by using the commercial wine yeast parent. The results demonstrated that the introduction of genetic material from a non-*S. cerevisiae* parent into a wine yeast background can impact significantly and potentially desirably on wine-relevant traits of a commercial *S. cerevisiae* wine yeast.

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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Newly generated interspecific wine yeast hybrids introduce flavour and aroma diversity to wines

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Abstract Increasingly, winemakers are looking for ways to introduce aroma and flavour diversity to their wines as a means of improving style and increasing product differentiation. While currently available commercial yeast strains produce consistently sound fermentations, there are indications that sensory complexity and improved palate structure are obtained when other species of yeast are active during fermentation. In this study, we explore a strategy to increase the impact of non-*Saccharomyces cerevisiae* inputs without the risks associated with spontaneous fermentations, through generating interspecific hybrids between a *S. cerevisiae* wine strain and a second species. For our experiments, we used rare mating to produce hybrids between *S. cerevisiae* and other closely related yeast of the *Saccharomyces* sensu stricto complex. These hybrid yeast strains display desirable properties of both parents and produce wines with concentrations of aromatic fermentation products that are different to what is found in wine made using the commercial wine yeast parent. Our results demonstrate, for the first time, that the introduction of genetic material from a non-*S. cerevisiae* parent into a wine yeast background can impact favourably on the wine flavour and aroma profile of a commercial *S. cerevisiae* wine yeast.

Keywords *Saccharomyces* sensu stricto · Interspecific hybrids · Metabolites · Fermentation products · Wine yeast

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Introduction

The *Saccharomyces* sensu stricto complex consists of a number of closely related species (Naumov 1987; Vaughan-Martini and Martini 1987; Naumov et al. 2010). Of these, *Saccharomyces cerevisiae* has been utilised by humans down through the ages, culminating in recent decades in a large number of industrial *S. cerevisiae* wine yeast strains being available to commercial winemaking. These strains show robust growth characteristics in grape juice, tolerating both the initial high sugar concentration at the onset of fermentation and high ethanol concentrations towards the end. In contrast, other *Saccharomyces* species generally ferment more slowly than *S. cerevisiae* and are often unable to tolerate the high alcohol concentrations encountered. However, there are indications that sensory complexity and more rounded palate structure is obtained when other species of yeast are active during fermentation, as in the case of traditional, spontaneous fermentations. Spontaneous fermentations allow the many different species of indigenous microorganisms that populate the vineyard, grape-picking equipment and winery to contribute to vinification. Studies on spontaneous ferments have identified a number

of non-*Saccharomyces* species present at the early stages of fermentation (Fleet and Heard 1993), and products of the metabolism of these species are thought to contribute to more complex aroma and flavour profiles in the wine. Nonetheless, because of their unpredictable nature, the desirability of spontaneous fermentations is a source of debate, many winemakers preferring to inoculate with a proven *S. cerevisiae* industrial strain.

Experiments using inoculations of mixtures of *S. cerevisiae* strains in a grape juice show dynamic population fluctuations between strains (Howell et al. 2004; King et al. 2008), with unpredictable fermentation outcomes. The situation is even worse when less robust non-*S. cerevisiae* strains are used. Fermentations using co-inocula of *S. cerevisiae* and non-*Saccharomyces* strains typically have limited success, with the non-*Saccharomyces* strain having only a minor impact on wine aroma and composition (Soden et al. 2000). The dominance of *S. cerevisiae* over other species in spontaneous fermentations is due mainly to their tolerance of high sugar and high ethanol concentrations (Pretorius 2000) and perhaps for some *S. cerevisiae* strains the capacity to produce 'killer' compounds that trigger cellular death of non-*Saccharomyces* strains (Heard and Fleet 1987; Perez-Navzdo et al. 2006).

An alternative to co-ferment that avoids growth competition between species is to use an interspecific hybrid strain, where the genomes of different species are contained within the one cell. Species of the *Saccharomyces* sensu stricto clade are able to mate with each other to form interspecific hybrids, but the hybrids formed are sterile, having non-viable ascospores (Naumov et al. 2000). This occurs in nature and, in fact, one member of the *Saccharomyces* sensu stricto complex, *Saccharomyces pastorianus*, the lager making yeast, has been identified as a stable, natural hybrid from an evolutionary timeframe (Groth et al. 1999) resulting from a cross between *S. cerevisiae* and a *Saccharomyces bayanus*-type yeast (Masneuf et al. 1998; Marinomi et al. 1999; Dunn and Sherlock 2008).

We have used a rare-mating strategy (Spencer and Spencer 1996) to generate interspecific hybrids between a robust diploid *S. cerevisiae* commercial wine strain, AWRI 838, and strains of either *Saccharomyces paradoxus* or *Saccharomyces kudriavzevii*. AWRI 838 is an isolate of EC 1118 and genomic sequencing has revealed that it is a diploid (Novo et al. 2009).

In nature, yeast mating is activated by the presence of pheromones produced by haploid yeast, but sporulation of the wine yeast parent to generate haploid spores might lead to the loss of important wine fermentation traits. Rare mating relies upon an infrequent event (1×10^{-6} cells) whereby mating type switching within the diploid genome leads to a cell homozygous at the mating type loci, either **a**/**a** or α/α (Gunge and Nakatomi 1972). These homozygotes are able to enter the mating pathway and can conjugate with a cell of the opposite mating type, leading to an interspecific hybrid.

In order to establish an experimental precedent and for ease of selection, the diploid wine yeast strain was first mated with a haploid, auxotrophic *S. paradoxus* strain. Metabolite analysis was performed on the resultant interspecific hybrid to confirm that the addition of the *S. paradoxus* genome had an impact on the parental wine yeast metabolome. Additional interspecific hybrids were then generated using random spores of wild-type strains of either *S. paradoxus* or *S. kudriavzevii*. Hybrids resulting from each of the wild-type crosses were chosen for grape juice fermentation and the wine analysed for important wine fermentation compounds.

Materials and methods

Yeast strains and media

Parental strains are *S. cerevisiae* AWRI 838 (an isolate of the commercial wine yeast strain EC 1118), *S. paradoxus* strains N17–78–*Mat**a** ho ura3 lys2 met13* provided by Rhona Borts (Hunter et al. 1996) and 52–153 (Herman J. Phaff Yeast Culture Collection, University of California Davis) and *S. kudriavzevii* type strain NCYC 2889. Strains generated from this study are listed in Table 1. All yeasts were grown in YEPD medium (1% (*w*/*v*) yeast extract, 2% (*w*/*v*) peptone, 2% (*w*/*v*) glucose) with shaking (100 rpm) at 25°C. Mitochondrial mutants of AWRI 838 were isolated by treating cells for 8 h in synthetic complete medium containing 10 μg mL^{-1} ethidium bromide. Cells were then diluted in water, and due to their inability to utilise glycerol as a carbohydrate source, the mitochondrial mutants were revealed by their petite colony growth on YPDG (1% (*w*/*v*) yeast extract, 2% (*w*/*v*) peptone, 3% (*w*/*v*) glycerol and 0.1% (*w*/*v*) glucose; Sherman et al. 1986).

Generation of interspecific hybrid yeast

Rare-mating, essentially as described by Spencer and Spencer (1996), was used throughout. Strains were grown to stationary

Table 1 Hybrid strains generated in this study

Cross	Hybrids	AWRI number
AWRI 838 × N17–78	A1–A5	A2 = AWRI 1519
AWRI 838 × 52–153	B1–B5	B2 = AWRI 1501
AWRI 838 × NCYC 2889	C1–C7	C1 = AWRI 1503

phase in YEPD at 27°C. Spores of strains 52–153 and NCYC 2889 were generated by inoculating the equivalent of 2 mL of a washed YEPD culture into 5 mL of sporulation medium (1%, *w/v* potassium acetate). After sporulation, cells were washed and re-suspended in sterile water. In a 250-mL conical flask, 1 mL of each parent strain was added to 20 mL of fresh YEPD and incubated for 7 days at 27°C. Appropriate numbers of cells were washed in sterile water and plated onto selective plates. Wild-type strains were assayed under several phenotypic conditions to determine selection criteria for hybridisation. Selection in mating experiments was performed on YNB–glycerol–ethanol plates (0.67% (*w/v*) yeast nitrogen base without amino acids, 3% (*w/v*) glycerol, 3% (*v/v*) ethanol, 2% (*w/v*) agar) for the auxotrophic strain cross and YEP–glycerol–ethanol plates (1% (*w/v*) yeast extract, 2% (*w/v*) peptone, 3% (*w/v*) glycerol, 14% (*v/v*) ethanol, 2% (*w/v*) agar) for wild-type strain crosses.

PCR confirmation of hybrids

For all strains, DNA was purified using mechanical breakage with glass beads (Ausubel et al. 1994). Yeast cells were disrupted using a Mini-Beadbeater® (BioSpec) for 3 min with glass beads. Genomic DNA was used as template for PCR analyses, with amplification using the δ transposon primer set MLD1 5′-CAAAATTCACCTAAA/TTCTCA-3′ and MLD2 5′-GTGGATTTTTATTCCAACA-3′ (Ness et al. 1993) and the intron primer set EI1 5′-CTGGCTTGGTGTATGT-3′ and LA2 5′-CGTGCAGGTGTTAGTA-3′ (de Barros Lopes et al. 1996). PCR fragments were resolved on a 1.5% (*w/v*) agarose gel. rDNA PCR-RFLP was conducted using the rDNA Internal Transcribed Spacer unit primer pair ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ and the Restriction Enzyme *Hae*III and fragments were resolved on a 2% (*w/v*) agarose gel (Esteve-Zarzosa et al. 1999).

Fermentation stress assay plates

Assay plates containing 14% (*w/v*) ethanol were produced by addition of a requisite volume of 99% (*w/v*) ethanol to cooled YEPD. The plates were wrapped in parafilm during storage and after plating were incubated at 22°C. Glucose assay plates of YEP plus 25% (*w/v*) glucose were also incubated after plating at 22°C. Strains were grown to stationary in liquid YPD (2 days), and 5 µL of 10-fold serial dilutions was spotted to plates.

Chemical profiling of volatile metabolite products

Hybrid strains generated from the *S. cerevisiae* × *S. paradoxus* N17–78 cross were screened for robust growth in YEPD, and a single strain, AWRI 1519, was selected for further study. Parent and hybrid strains were inoculated in triplicate at 1×10^6 cells from a pre-culture (2 days growth in YEPD) into 50 mL of Synthetic Complete medium with 4× amino acid mix (Sambrook and Russel 2001) and 8% (*w/v*) glucose. On completion of fermentation (<0.25% residual sugar as determined with Clinitest® tablets, Bayer, Switzerland), duplicate samples were analysed for volatile metabolites using gas chromatography–mass spectroscopy (GC-MS; Eglinton et al. 2002).

Fermentation product analysis of hybrid-generated wines compared to their commercial yeast parent

Small-scale industrial ferments were carried out at a commercial winery in 240 L barrels. Chardonnay grapes were machine harvested with no sulphur dioxide added. Fruit was tank-pressed, homogenised and transferred to barrels. Triplicate fermentations were conducted at 11–17° C using either the commercial wine yeast parent AWRI 838, the *S. cerevisiae* × *S. paradoxus* hybrid strain, AWRI 1501 or the *S. cerevisiae* × *S. kudriavzevii* hybrid strain, AWRI 1503. Wines were not produced by the non-*S. cerevisiae* parents, as neither was able to grow in the Chardonnay grape juice. At completion of fermentation (determined with Clinitest® tablets), wines were settled with sulphur dioxide and ascorbic acid added and treated with 250 µg/L copper. Triplicate wines were then pooled, filtered and bottled. Chemical analysis of target compounds, previously identified as important for wine flavour and aroma, was undertaken from duplicate samples of the resultant wines using GC-MS preceded by a headspace solid-phase micro-extraction (HS-SPME), with polydeuterated internal standards for stable isotope dilution analysis (Siebert et al. 2005).

Statistical analysis

A one-way analysis of variance and Student's *t* test (<0.05) were used to determine significant differences of compound concentrations between media and wines fermented by each yeast strain.

Results

Rare matings

Colonies formed on selection plates following interspecific matings were scored (Table 2) and subsequently picked onto new selection plates. Mating efficiency in the AWRI 838 × N17–78 cross was 30-fold greater than the AWRI 838×52–153 cross, while the less closely related *S.*

Table 2 Frequency of interspecific hybridisation

Strain cross	Frequency of hybridisation
AWRI 838 × N17–78	2×10^{-6}
AWRI 838 × 52–153	6×10^{-8}
AWRI 838 × NCYC 2889	2×10^{-8}

kudriavzevii strain had the lowest mating frequency of the crosses at 100-fold less than the *S. cerevisiae* × *S. paradoxus* haploid cross. The differences in mating efficiency between the crosses could be due to a number of factors; for example, *S. paradoxus* 52–153 spores may have mated with each other reducing the pool of spores available to mate with *S. cerevisiae* whereas N17–78 is a stable haploid; there may be inherent differences in sporulation efficiency between the two non-*S. cerevisiae* species; and the greater evolutionary distance between *S. cerevisiae* and *S. kudriavzevii*.

Confirmation of hybrid status of mating products

The hybrid nature of colonies from the *S. cerevisiae* × *S. paradoxus* crosses was confirmed by PCR analysis of genomic DNA utilising amplification with δ transposon primers and intron primers (Figs. 1a, b and 2a, b). A control PCR using DNA from both parents revealed that the transposon primers showed a bias towards *S. cerevisiae* targets, while the intron primers showed a bias towards *S. paradoxus* targets.

The AWRI 838 × N17–78 hybrids showed a transposon PCR pattern with specific bands from both parents, however, not all of the parent-specific bands were observed in all hybrids. For instance, hybrid strains A1, A2 A3 and A4 contain all five major bands amplified from the *S. cerevisiae* parent, while hybrid strain A5 is missing the lowest *S. cerevisiae* specific band (Fig. 1a). The intron PCR pattern for this cross showed a bias towards the *S. paradoxus* genome, with all hybrids having the complete set of *S. paradoxus* bands but only faint *S. cerevisiae* specific bands (Fig. 1b).

The AWRI 838×52–153 hybrids showed a transposon PCR pattern with mainly *S. cerevisiae* specific bands, but, again, not all bands were amplified in each hybrid (Fig. 2a), as hybrid strains B1 and B3 are missing both of the two lowest *S. cerevisiae* specific bands whereas B5 is missing only the lowest band. Three *S. paradoxus* specific bands were amplified strongly in the intron PCR (Fig. 2b), with all five hybrid strains amplifying the middle band, but not the top band. Hybrid B1 alone amplified the lowest *S. paradoxus* specific band. Collectively, PCR analyses confirmed the hybrid nature of the putative hybrid strains.

The hybrid nature of products from the *S. cerevisiae* × *S. kudriavzevii* cross was unable to be confirmed by transposon or intron PCR, as both analyses showed a fragment pattern attributed to the AWRI 838 parent only (Fig. 3a, b). ITS PCR-RFLP targeting the rDNA tandem repeat loci, however, revealed the existence of rDNA from both species within these hybrid strains (Fig. 3c).

Interspecific hybrids inherited wine-relevant traits from the wine yeast parent

Two confirmed interspecific hybrids were chosen for grape juice fermentation studies: AWRI 1501 from the *S. cerevisiae* × *S. paradoxus* (wild-type) cross and AWRI 1503 from the *S. cerevisiae* × *S. kudriavzevii* cross. Assay plates were designed to test the tolerance of hybrids to two major stresses encountered during fermentations: high sugar and high ethanol concentrations. Medium incorporat-

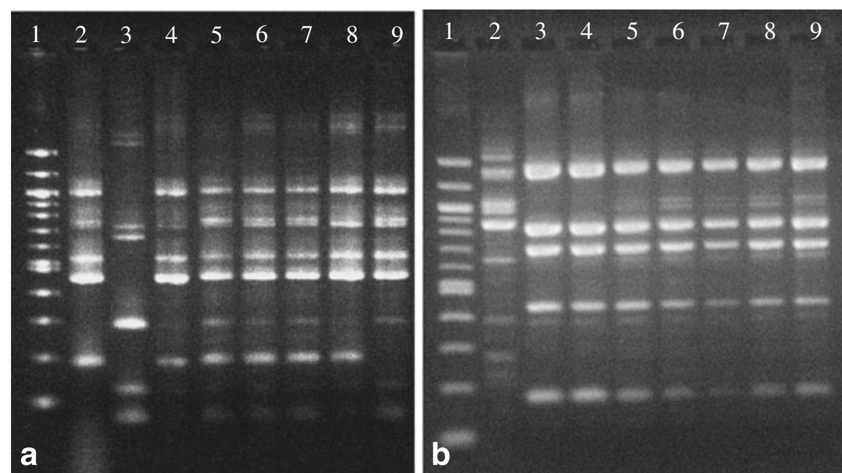

Fig. 1 Transposon PCR (**a**) and intron PCR (**b**) of AWRI 838 × N17–78 interspecific hybrid strains. *Lanes*: *1* 100 bp ladder, *2* AWRI 838 (*S. cerevisiae* parent), *3* N17–78 (*S. paradoxus* parent), *4* DNA from both AWRI 838 and N17–78, *5* to *9* hybrids A1 to A5

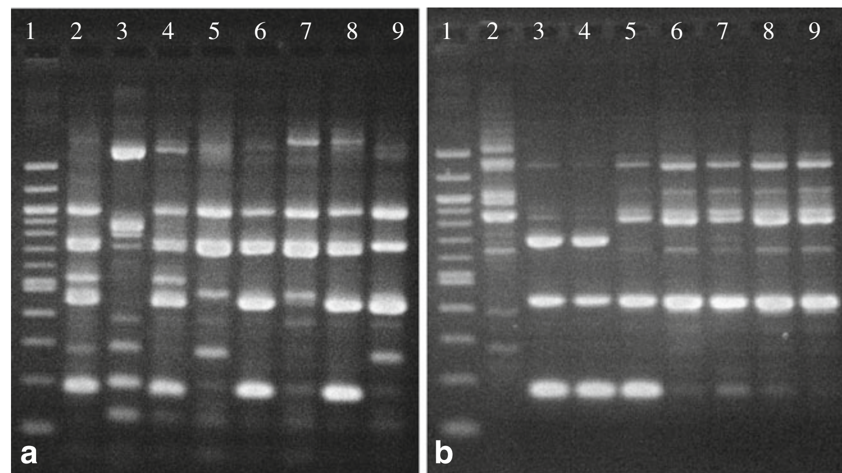

Fig. 2 Transposon PCR (**a**) and intron PCR (**b**) of AWRI 838× 52–153 interspecific hybrid strains. *Lanes*: *1* 100 bp ladder, *2* AWRI 838 (*S. cerevisiae* parent), *3* 52–153 (*S. paradoxus* parent), *4* DNA from both AWRI 838 and 52–153, *5* to *9* hybrids B1 to B5

ing a high concentration of glucose (25%, *w*/*v*) allowed robust growth of both hybrids and the *S. cerevisiae* parent, while the *S. kudriavzevii* parent showed less robust growth and the *S. paradoxus* parent no growth at all (Fig. 4). Neither the *S. paradoxus* nor the *S. kudriavzevii* parent was able to grow on high ethanol (14%, *v*/*v*) plates, but both hybrid strains grew well, although AWRI 1503 showed slightly weaker growth than the *S. cerevisiae* parent.

Chemical analysis of volatile metabolites from hybrid AWRI 1519 and parent strains in defined medium

After the completion of fermentation (<0.25% residual sugar), GC-MS analysis of defined medium fermented by AWRI 1519 and its parent strains identified 32 compounds (Table 3), 13 of which showed a significant changed concentration for the hybrid relative to the *S. cerevisiae* wine yeast parent. The chemical concentration profile of the hybrid volatile metabolites followed the gamut of all possible outcomes. In some cases, the hybrid strain produced a compound at the higher-producing parent level, but on other occasions produced a compound at the lower-producing parent level. For example, in the case of benzaldehyde, the hybrid generated 13.0 μg/L, an amount equivalent to 85% of the *S. paradoxus* parent (14.9 μg/L), while the *S. cerevisiae* parent generated considerably less (2.44 μg/L). Conversely, for dodecalactone, the hybrid generated 3.51 μg/L, a level similar to the *S. cerevisiae* parent (6.5 μg/L), whereas the *S. paradoxus* parent generated a far greater amount (30.9 μg/L). Some compounds were produced by the hybrid at an intermediate level between the two parental levels (e.g. 2-phenylethyl acetate), while two compounds were produced by the hybrid at remarkably lower levels than for either parent (*cis*-4-hydroxymethyl-2-methyl-1,3-dioxolane and *cis*-5-hydroxy-2-methyl-1,3-dioxane). A third compound, ethyl hexanoate, was produced by the hybrid at a concentration much higher than the cumulative total of the parents.

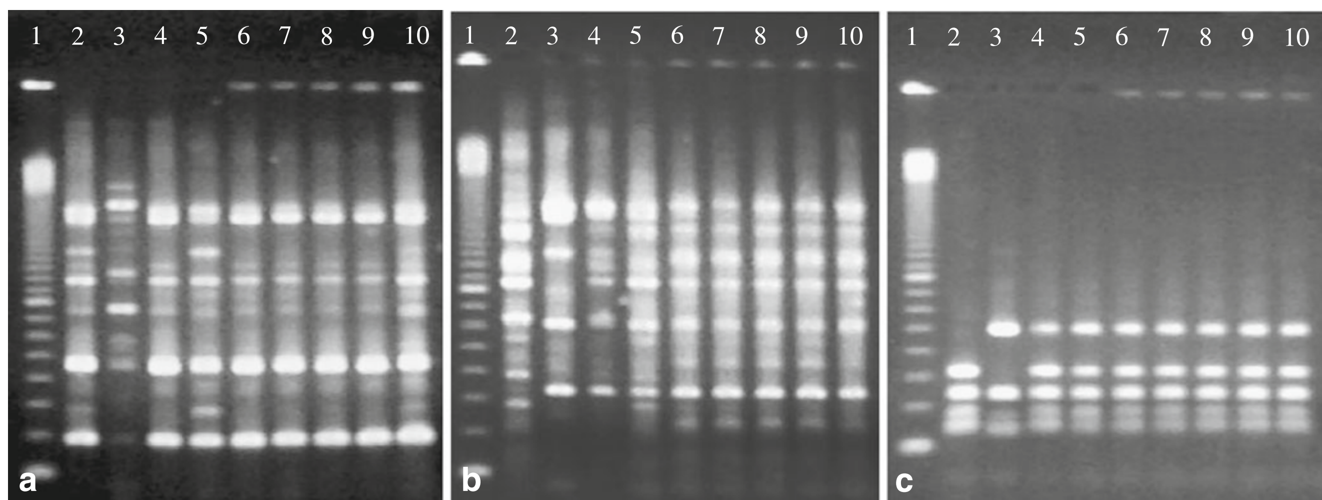

Fig. 3 Transposon PCR (**a**), intron PCR (**b**) and ITS PCR-RFLP (**c**) of AWRI 838 × NCYC 2889 interspecific hybrid strains. *Lanes*: *1* 100 bp ladder, *2* AWRI 838 (*S. cerevisiae* parent), *3* NCYC 2889 (*S. kudriavzevii* parent), *4* to *10* hybrids C1 to C7

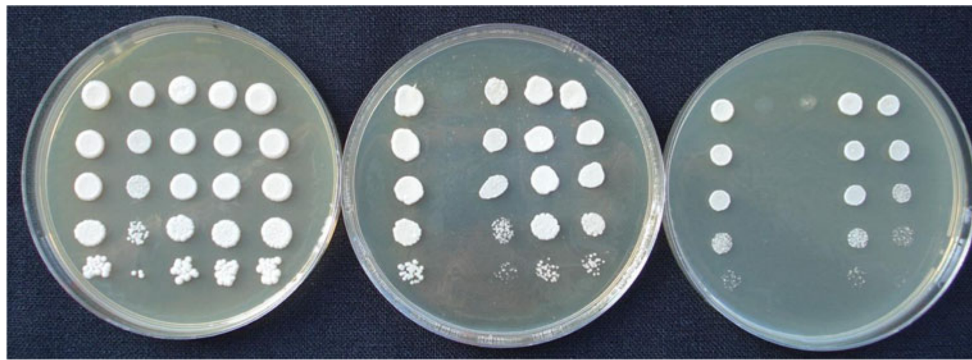

Fig. 4 Fermentation stress assay plates. Assay plates *left* to *right*: YEPD control, YEP-25% glucose, YEPD-14% ethanol. Strains, *left* to *right*, are AWRI 838 (*S. cerevisiae* parent), 52–153 (*S. paradoxus* parent), NCYC 2889 (*S. kudriavzevii* parent), AWRI 1501 (*S. cerevisiae* × *S. paradoxus* interspecific hybrid) and AWRI 1503 (*S. cerevisiae* × *S. kudriavzevii* interspecific hybrid). Spotted cultures are in 10-fold serial dilutions from *top* to *bottom*

Chemical analysis of fermentation products of hybrids AWRI 1501 and AWRI 1503 from small-scale grape juice industrial ferments

Small-scale (240 L) industrial ferments of Chardonnay juice were carried out using interspecific hybrid strains AWRI 1501 and AWRI 1503 and the wine yeast parent AWRI 838 alone, as the non-*S. cerevisiae* parents were unable to grow in the juice. The resultant wines (all having fermented to completion with <0.25% residual sugar) were analysed for volatile compounds using HS-SPME-GC-MS targeting 31 compounds (Table 4) previously established as important flavour and aroma compounds in wine (Siebert et al. 2005).

Relative to the *S. cerevisiae* parent strain, AWRI 1501 showed noteworthy differences in the concentration levels of 17 of the compounds analysed, with six compounds increasing and 11 compounds decreasing. Similarly, AWRI 1503 produced considerable differences in 20 compounds relative to the *S. cerevisiae* parent; seven showed an increase and 13 showed a decrease in concentration. A number of compounds that can have a negative effect on wine aroma and flavour were produced at much lower concentrations by the hybrid yeasts: acetic acid (vinegar), 3-methylbutanoic acid (blue cheese) and ethyl acetate (nail polish) decreased to 35%, 50% and 60%, respectively, of the concentrations produced by the *S. cerevisiae* parent. On the other hand, a number of compounds that contribute to fruity aromas had increased levels in the hybrid yeast wines. Ethyl hexanoate (green apple) levels increased to 120% for both hybrid yeast wines while the fruity aroma compounds, ethyl butanoate and ethyl propanoate, also showed increases, 117% and 160% (AWRI 1501) and 123% and 124% (AWRI 1503), respectively. 2-Methylpropyl acetate (banana) was produced in higher amounts by hybrid strain AWRI 1503. Compounds associated with more complex characters were also produced at increased levels to the parent wine yeast: Hexanoic acid (sweaty) levels were 137% (AWRI 1501) and 135% (AWRI 1503) and butanol (fusel) were 122% and 110%, respectively.

Discussion

Rapid and consistent fermentations are essential in large-scale, commercial wine production, and the majority of wineries worldwide rely upon inoculating their ferments with active dried yeast products from a yeast-manufacturing company. These ADY products are commonly strains of *S. cerevisiae*, although a small number have been identified as natural hybrids between members of the *Saccharomyces* sensu stricto species (Masneuf et al. 1998; Groth et al. 1999; Gonzalez et al. 2006; Bradbury et al. 2006). Different wine yeasts vary in their efficiency and reliability when fermenting grape juice (Pretorius 2000) and can impart different sensory properties to wine (King et al. 2008). This variation in yeast strain performance and delivery of product quality gives winemakers options when attempting to tailor their products to the preferences of different market segments. Development of new yeast strains with improved and/or desirable novel flavours is of growing importance for winemakers needing to produce wines that are differentiated from others in a competitive, over-crowded market.

Traditional breeding techniques are commonly used for yeast strain improvement (Winge and Lausten 1938; Pretorius 2000), and typically, these strain development programmes involve hybridising yeast of the same *Saccharomyces* species (i.e. *S. cerevisiae*), to produce intraspecific hybrids. This manuscript describes, for the first time, laboratory-based interspecific matings between a *S. cerevisiae* wine yeast strain and strains from two other *Saccha-*

Table 3 Chemical analysis of volatile metabolites from hybrid AWRI 1519 and parent strains in defined medium

Compound	RT (min)	AWRI 838 (μg/L)	N17–78 (μg/L)	AWRI 1519 (μg/L)
cis-4-Hydroxymethyl-2-methyl-1,3-dioxolane	6.06	397 (±51) b	1,620 (±593) a	37.8 (±27.0) c
Ethyl hexanoate	6.20	7.6 (±3.4) b	1.78 (±0.8) c	25.4 (±3.4) a
trans-4-Hydroxymethyl-2-methyl-1,3-dioxolane	6.47	39.0 (±15) b	1,421 (±376) a	58.0 (±10) b
Benzaldehyde	6.57	2.44 (±0.2) b	14.9 (±5.4) a	13.0 (±0.2) a
Dihydro-2-methyl-thiophenone	6.95	3.56 (±1.5) b	62.4 (±34.9) a	6.4 (±1.9) b
3-Methylthiopropanol	7.28	981 (±137) a	134 (±67) b	1,121 (±686) a
Hexanoic acid	7.39	47.1 (±21) a	44.5 (±36) a	50.5 (±23) a
Ethyl heptanoate	7.47	31.8 (±8.1) a	39.4 (±8.7) a	29.8 (±8.3) a
cis-5-Hydroxy-2-methyl-1,3-dioxane	7.55	95.0 (±13) b	604 (±247) a	5.3 (±1.3) c
Phenylacetaldehyde	7.81	30.7 (±5.9) b	44.4 (±4.2) a	47.5 (±4.3) a
Ethyl octanoate	8.69	37.1 (±3.1) b	31.6 (±3.5) b	45.6 (±3.8) a
2-Phenylethanol	8.95	49,487 (±6,254) a	13,842 (±2,971) b	46,883 (±28,682) a
2-Hydroxy-3,3-dimethyl γ butyrolactone	9.10	45.9 (±4.9) a	64.6 (±24.0) a	22.0 (±7.9) b
Succinic anhydride	9.72	1.74 (±0.4) b	110 (±83) a	41.0 (±37.4) a
4-Ethyl benzaldehyde	9.91	60.8 (±8.0) a	58.4 (±23) a	69.2 (±13.2) a
Benzothiazole	9.96	78.0 (±33.0) a	89.3 (±10.2) a	49.7 (±27.3) a
2-Phenylethyl acetate	10.08	60.3 (±6.0) a	7.8 (±5.3) c	30.3 (±4.4) b
4-Hydroxy-5-oxohexanoic acid lactone	10.43	14.5 (±3.0) b	1,183 (±247) a	7.1 (±7.0) b
Ethyl decanoate	10.92	18.6 (±2.0) ab	23.9 (±6.6) a	12.2 (±7.5) b
5-Hydroxymethyl furfural	11.64	56.2 (±9.0) a	36.9 (±20.0) a	48.5 (±28.8) a
4-(1-Hydroxyethyl) γ butanolactone	11.85	178 (±23) b	709 (±306) a	484 (±283) ab
3-Hydroxy-4-phenyl-2-butanone	12.06	1,145 (±200) a	254 (±88) b	294 (±108) b
Nerolidol	12.94	17.7 (±4.9) a	24.7 (±7.7) a	25.5 (±12.7) a
Di-*tert*-butylphenol	13.14	28.2 (±5.2) b	40.0 (±12.7) b	86.1 (±15.2) a
2-Propylphenol	13.48	28.6 (±4.2) a	10.7 (±4.1) b	43.6 (±9.7) a
4-Hydroxyphenyl ethanol	13.94	15,823 (±1,997) a	5,483 (±1,304) b	13,629 (±3,696) a
Tyrosol acetate	14.85	16.0 (±4.2) a	2.45 (±1.8) b	3.49 (±1.6) b
Dodecalactone	15.58	6.5 (±1.4) b	30.9 (±7.3) a	3.51 (±0.6) b
2-Hydroxybenzothiazole	16.50	64.4 (±20.9) b	298 (±63) a	23.9 (±7.4) b
Tryptophanol	16.95	2,073 (±279) a	615 (±306) b	984 (±178) b
1-Acetyl-β-carboline	17.98	32.2 (±4.5) a	28.9 (± 0.9) a	27.6 (±5.1) a
3-Formyl indole	18.41	33.6 (±8.5) b	60.0 (±21) a	20.3 (±9.1) b

Levels not connected by same letter are significantly different

romyces sensu stricto species—*S. paradoxus* and *S. kudriavzevii*—in order to generate wine yeast that produce novel wine and flavour aroma profiles.

Initially, for ease of selection, to optimise mating conditions, and for proof of concept experiments, *S. cerevisiae* wine yeast hybrids were generated using a genetically modified (GM) laboratory *S. paradoxus* haploid strain carrying auxotrophic markers. However, as only non-GM yeast are used by the Australian wine industry, interspecific hybrids were subsequently generated using non-genetically modified, natural isolates of *S. paradoxus* and *S. kudriavzevii*. Yeast mating is activated by the presence of pheromones normally produced by haploid yeast, and so the parent *S. paradoxus* and *S. kudriavzevii* strains were sporulated to generate haploid spores. To minimise the risk of potential loss of important wine yeast fermentation properties, the wine yeast parent was not sporulated; rare matings (Spencer and Spencer 1996) were used to form presumptive triploid interspecific hybrids.

Strain-specific and species-specific banding patterns generated using primers that target δ-transposon regions, introns and rDNA regions were used as markers to confirm the presence of each parental input in the resultant hybrid strains. Although a degree of preferential amplification of the *S. cerevisiae* parent genome was observed with the transposon primers, the intron primers showed a preference for *S. paradoxus* genomic sequences in the *S. cerevisiae* × *S. paradoxus* hybrids. However, both δ-transposon and intron primer sets showed a preference for *S. cerevisiae* DNA sequences in the *S. cerevisiae* × *S. kudriavzevii*

Table 4 Fermentation products in wines made using hybrids (AWRI 1501 and AWRI 1503) or parent (AWRI 838) yeast strains

Compound	Descriptor	AWRI 838	AWRI 1501	AWRI 1503
Acetic acid, mg/L	Vinegar	386 (±7) a	141 (±4) b	128 (±4) b
Ethyl acetate, mg/L	Nail polish	73.4 (±0.1) a	41.2 (±0.3) c	45.4 (±0.3) b
Ethyl butanoate, μg/L	Acid fruit	504 (±3) c	592 (±8) b	624 (±6) a
Ethyl-2-methylbutanoate, μg/L	Sweet fruit	10.4 (±0.1) a	8.3 (±0.3) b	6.7 (±0.1) c
Ethyl-3-methylbutanoate, μg/L	Berry	9.2 (±0.1) a	9.2 (±2.0) a	7.9 (±0.5) c
Ethyl-2-methylpropanoate, μg/L	Fruity	71.9 (±1) a	49.0 (±0.5) b	47.2 (±0.1) b
Ethyl propanoate, μg/L	Fruity	190 (±1) c	307 (±6) a	237 (±5) b
2-Methylbutyl acetate, μg/L	Banana, fruity	97.0 (±3) a	52.8 (±4.9) c	72.5 (±5.8) b
2-Methylpropyl acetate, μg/L	Banana, fruity	56.9 (±0.5) b	55.4 (±1.0) b	66.0 (±0.1) a
2-Methylbutanol, mg/L	Nail polish	26.5 (±1) a	30.0 (±0.9) a	25.1 (±2.8) a
Propanoic acid, μg/L	Vinegar	1,368 (±44) a	965 (±300) b	373 (±195) c
2-Methylpropanoic acid, μg/L	Cheese, rancid	493 (±31) a	583 (±11) a	523 (±108) a
2-Methylbutanoic acid, μg/L	Cheese, sweaty	236 (±80) a	239 (±14) a	189 (±12) a
2-Phenylethanol, mg/L	Roses	45.5[a]	48.0[a]	45.5[a]
2-Phenylethyl acetate, μg/L	Flowery	404 (±1) a	239 (±10) c	290 (±1) b
Hexanoic acid, mg/L	Cheese, sweaty	5.1 (±0.1) b	7.0 (±0.3) a	6.9 (±0.2) a
Decanoic acid, mg/L	Fatty	3.46 (±0.3) a	3.21 (±0.4) a	2.77 (±0.3) a
Octanoic acid, mg/L	Rancid, harsh	9.3 (±0.4) a	5.2 (±0.4) b	8.9 (±0.6) a
Hexyl acetate, μg/L	Sweet, perfume	142 (±1) a	41.6 (±0.2) c	60.5 (±0.2) b
Ethyl lactate, mg/L	Strawberry	34.7 (±3.5) a	27.7 (±2.3) a	16.5 (±0.8) b
3-Methylbutanoic acid, μg/L	Blue cheese	492 (±18) a	266 (±26) b	235 (±15) b
2-Methylpropanol, mg/L	Fusel, spirituous	28.0 (±0.3) a	28.0 (±0.2) a	24.8 (±0.1) b
3-Methylbutyl acetate, mg/L	Banana	2.14 (±0.01) a	2.02 (±0.01) a	2.06 (±0.04) a
Butanol, μg/L	Fusel, spirituous	810 (±10) c	990 (±30) a	890 (±5) b
Hexanol, mg/L	Green, grass	2.43 (±0.01) a	2.31 (±0.01) b	2.19 (±0.03) c
Ethyl octanoate, mg/L	Sweet, soap	1.52 (±0.01) a	1.55 (±0.08) a	1.39 (±0.03) a
Ethyl decanoate, μg/L	Pleasant, soap	677[a]	600[a]	551[a]
Ethyl dodecanoate, μg/L	Soapy, estery	150[a]	256[a]	287[a]
3-Methylbutanol, mg/L	Harsh, nail polish	140[a]	165[a]	171[a]
Ethyl hexanoate, mg/L	Green apple	1.00 (±0.01) b	1.24 (±0.02) a	1.24 (0.03) a
Butanoic acid, mg/L	Cheese, rancid	2.24 (±0.04) b	3.73 (±0.10) a	3.37 (±0.14) a

Levels not connected by same letter are significantly different

[a] Data based on a single determination

hybrid. Nonetheless, the hybrid status of all progeny used in fermentative work was confirmed using the above primer sets, or by targeting the rDNA ITS region that, upon restriction digestion, generated species-specific banding patterns.

Interestingly, different hybrids generated from the same cross did not give identical banding patterns. This may be due to genome loss or rearrangement during the incipient stages of interspecific hybrid evolution. In previous work, genomic analyses of natural interspecific yeast hybrids have identified loss of varying portions of parental genomes (Dunn and Sherlock 2008; Belloch et al. 2009), and plant studies have shown that changes within newly formed interspecific hybrid genomes occur rapidly leading to extensive inter- and intra-genome rearrangements and gene loss (Song et al. 1995; Kashkush et al. 2002).

Genetic stability analysis (using the same PCR approach as for confirmation of hybridisation) was carried out on each hybrid strain generated in this study. Twenty individual isolates from each hybrid strain were assessed after 50 generations in YEPD and at the end of model medium and grape juice fermentations. No further change in fingerprint profile was identified (results not shown).

Genome loss and rearrangement in newly formed wine yeast hybrids might lead to loss of industrially important traits, such as stress tolerance. Two stresses common to grape juice fermentations are high sugar concentration experienced at the beginning of fermentation and high ethanol concentration that builds towards the end of fermentation. Hence, assay plates designed to select for tolerances to these stresses were used to confirm that

hybrids chosen for further investigation at least retained these traits.

All hybrids generated for this work clearly produce different volatile fermentation product profiles to the wine strain parent. Chemical analysis of volatile metabolites in spent minimal medium for AWRI 1519 showed this yeast to be very different to its parental strains. In some cases, levels of metabolites for the hybrid followed closely that of the 'highest-producing' parent, but, on other occasions, a compound was produced at the level of the 'lower-producing' parent. Moderating effects (where hybrid levels are midway between parents) were also noted. Intriguingly, a small number of compounds were produced by the hybrid in a considerably reduced concentration relative to either parent, or at a level much higher than a cumulative amount. It is possible that flavour-active metabolites of interspecific hybrids, at concentrations not predicted by their parental metabolite profiles, could lead the generation of new yeast strains capable of creating unique wine styles from conventional grape varieties.

Chardonnay wines produced by hybrids AWRI 1501 and AWRI 1503 again showed compound concentrations that were greater or less than produced by the wine yeast parent. Interestingly, the magnitude of differences varied between the two interspecific hybrids, highlighting the potential for different hybrid strains to tailor wines towards different consumer groups (Lattey et al. 2007). The specific contribution of the non-*S. cerevisiae* parents was not assessed, as neither was able to grow in the Chardonnay grape juice. The compounds that were present at altered levels in the hybrid-made wines contribute flavours such as fruits (banana, strawberry and green apple), perfumes and flowers, and compounds with the more pungent attributes of blue cheese, rancid cheese and fusel. High concentrations of flavour or aroma compounds in wine result in a greater sensory impact but may also lead to the masking of less obvious flavours and aromas. Conversely, lowering the level of a particular compound may result in the unmasking of other flavours and aromas within the wine (Saison et al. 2009).

It is important to note that the number of differences in fermentation products between hybrid-made wine and *S. cerevisiae*-made wine will be a conservative estimate, as the fermentation product analysis targeted only compounds that have previously been identified as important contributors to flavour and aroma in wines, wines typically produced by a single industrial *S. cerevisiae* strain (Siebert et al. 2005). Thus, there may be other important flavour and aroma compounds produced by the input of the non-*S. cerevisiae* genome component of the hybrid strains that were not considered in this study.

Metabolite differences between hybrid and parental strain(s) were identified in both model medium and grape juice fermentations. These differences in metabolite levels may be the direct result of polyploidy (Hull-Sanders et al. 2009); the additive effect of an extra genome; synergistic genetic interactions (Mani et al. 2008); heterosis, whereby the hybrid displays superior growth and yield over both parents (Lippman and Zamir 2006); or differences in gene expression. Differences in gene expression could be explained by the observations that divergence of transcription factor binding sites across the *Saccharomyces* species far exceeds the interspecies variation in orthologous genes (Borneman et al. 2007); alterations in transcription factor binding within the hybrid genome could lead to differences in gene regulation effecting metabolite production. All, or any, of the above genomic effects would potentially contribute to the novel wine flavour and aroma profiles produced by interspecific wine yeast hybrids.

Performance of interspecific wine yeast hybrids in an industrial setting

Informal blind tastings on wines made using interspecific wine yeast hybrids described in this manuscript concluded that the hybrid yeast wines were more complex, with a wider range of flavour and aroma attributes (results not shown). The above hybrids have since been used to produce award winning wines and are now available commercially having been adopted by winemakers internationally.

In conclusion, this manuscript describes a new strategy for developing wines with greater complexity. By combining the genomes of a commercial *S. cerevisiae* wine yeast strain and other *Saccharomyces* sensu stricto yeast, we have successfully bred new commercial wine yeast strains capable of producing novel wine aroma and flavour profiles. These new hybrid yeasts can assist winemakers in their search for tools that introduce flavour and aroma diversity to their wines.

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Contribution to the Paper	Generated and confirmed interspecific yeast hybrid strains, performed Array-comparative hybridisation analysis, fluorescence flow cytometry analysis, phenotypic assessment and laboratory grape juice fermentations, interpreted data, wrote manuscript and acted as corresponding author.
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 3

Introducing a new breed of wine yeast: Interspecific hybridisation between a commercial *Saccharomyces cerevisiae* wine yeast and *Saccharomyces mikatae*.

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This manuscript describes the generation of a new breed of wine yeast by interspecific hybridisation between a commercial *S. cerevisiae* wine yeast strain and a distantly-related member of the *Saccharomyces* clade hitherto not associated with industrial fermentation environs, *S. mikatae*. Although mating between spores of *S. cerevisiae* and *S. mikatae* had previously been performed to determine species boundaries (Naumov et al. (2000) *Int. J Syst. Evol. Microbiol.* 50:1931-1942), no natural interspecific hybrids between these species, and no hybridisations events of diploid *S. cerevisiae* cells with *S. mikatae* spores had been reported.

Hybrid progeny were identified with robust fermentation properties and winemaking potential. Chemical analyses showed that, relative to the *S. cerevisiae* parent, hybrids produced wines with different concentrations of volatile metabolites that are known to contribute to wine flavour and aroma. The impact of the introduction of a more divergent genome on yeast flavour-active metabolites was revealed with increased concentrations of some flavour compounds more commonly associated with non-*Saccharomyces* species. Genetic stability analysis of 300 end-of-fermentation hybrid isolates revealed only minor chromosomal alterations in a small number of isolates (approximately 6%) and no loss of overall ploidy.

Introducing a New Breed of Wine Yeast: Interspecific Hybridisation between a Commercial *Saccharomyces cerevisiae* Wine Yeast and *Saccharomyces mikatae*

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Abstract

Interspecific hybrids are commonplace in agriculture and horticulture; bread wheat and grapefruit are but two examples. The benefits derived from interspecific hybridisation include the potential of generating advantageous transgressive phenotypes. This paper describes the generation of a new breed of wine yeast by interspecific hybridisation between a commercial *Saccharomyces cerevisiae* wine yeast strain and *Saccharomyces mikatae*, a species hitherto not associated with industrial fermentation environs. While commercially available wine yeast strains provide consistent and reliable fermentations, wines produced using single inocula are thought to lack the sensory complexity and rounded palate structure obtained from spontaneous fermentations. In contrast, interspecific yeast hybrids have the potential to deliver increased complexity to wine sensory properties and alternative wine styles through the formation of novel, and wider ranging, yeast volatile fermentation metabolite profiles, whilst maintaining the robustness of the wine yeast parent. Screening of newly generated hybrids from a cross between a *S. cerevisiae* wine yeast and *S. mikatae* (closely-related but ecologically distant members of the *Saccharomyces* sensu stricto clade), has identified progeny with robust fermentation properties and winemaking potential. Chemical analysis showed that, relative to the *S. cerevisiae* wine yeast parent, hybrids produced wines with different concentrations of volatile metabolites that are known to contribute to wine flavour and aroma, including flavour compounds associated with non-*Saccharomyces* species. The new *S. cerevisiae* x *S. mikatae* hybrids have the potential to produce complex wines akin to products of spontaneous fermentation while giving winemakers the safeguard of an inoculated ferment.

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Introduction

The *Saccharomyces* sensu stricto complex is a group of closely related yeast species that can mate to form interspecific hybrids. Natural *Saccharomyces* interspecific hybrids have been isolated from various fermentation environs. The lager yeast *Saccharomyces pastorianus*, (syn *Saccharomyces calsbergensis*), first described in 1883 by Emil Christian Hansen, is a stable, natural hybrid between *S. cerevisiae* and *Saccharomyces eubayanus* [1,2,3]. A small number of wine yeast and cider yeast strains have also been identified as natural interspecific hybrids between the *Saccharomyces* species, *S. cerevisiae*, *S. bayanus* and *S. kudriavzevii* [1,4,5,6]. Although no natural hybrids between *S. cerevisiae* and *S. mikatae* have been reported to-date, two ale strains have been shown to contain a small (4.5 kb) *S. mikatae* introgressed non-coding region corresponding to the right end of chromosome VI [7].

Here we describe for the first time, the generation of an interspecific hybrid between a commercial *S. cerevisiae* wine yeast strain and *S. mikatae*, a species not previously associated with alcoholic fermentation and isolated only from soil and decaying leaf litter [8,9]. Although members of the *Saccharomyces* sensu stricto group are considered to be closely related yeast, DNA sequence variation between the most distantly related species within this group corresponds roughly to that between man and mouse [10].

The driver for this work comes from a desire in the wine industry to develop novel yeast strains that bring greater complexity to wine than strains currently available to the industry. Winemakers grapple with many issues when deciding their winemaking practices including consistency in wine style and quality across vintages, and dealing with the risk of spoilage by indigenous microorganisms. With these concerns in mind, the process of inoculating grape must with a single, proven commercial strain, (typically *S. cerevisiae*), has become the backbone of modern winemaking. Commercial yeast strains have robust growth properties in demanding conditions (low pH, osmotic stress due to the initial high sugar concentration of grape must and accumulation of alcohol in the later stage of fermentation), and out-compete indigenous microorganisms to carry out fermentation in a timely manner while producing reliable, quality wines.

Whilst there are indications that contributions from the many different indigenous microorganisms in uninoculated spontaneous fermentations build a more complex palate structure and greater

diversity of flavour profiles [11], the unpredictable nature of spontaneous fermentations leads many winemakers to prefer an inoculation regime where the microorganism population is controlled. One approach to reaping the benefits of spontaneous fermentations while minimising risk of spoilage is to use inoculations with multiple *S. cerevisiae* wine yeast strains or *S. cerevisiae* and non-*Saccharomyces* strains. However, studies show that growth variability can occur between strains with unpredictable results [12,13,14], presumably due to the differential fitness of strains in highly variable grape juice compositions. A strategy that avoids the problem of competition between strains is to hybridise the genomes of two different species, generating an interspecific hybrid yeast strain capable of producing a wide range of flavour-active metabolites.

Mating in *Saccharomyces* spp. is typically between haploid cells of the opposite mating type (**a** and **α**). For the purpose of generating novel interspecific wine yeast however, it was decided to retain the full complement of the wine yeast parent diploid genome in the new hybrids; diploid wine yeast were therefore mated with haploid *S. mikatae*. This can be achieved because diploid *S. cerevisiae* cells can undergo a low frequency (1×10^{-6}) mating type switch that results in a diploid cell homozygous at the mating type locus, **a/a** or **α/α** [15]. These homozygotes can enter the mating pathway and conjugate with a cell of the opposite mating type, leading to the generation of polyploid interspecific hybrids.

Hybrid progeny from rare matings between *S. cerevisiae* and *S. mikatae* were screened for fermentation traits and their wines analysed for basic fermentation chemistry. Subsequently, two hybrid strains were selected for further study and the wines produced by these hybrids and the parent wine yeast were analysed for volatile and solvent extractable fermentation products as well as phenolic content. The genetic stability of these two hybrid strains was also assessed.

Materials and Methods

Yeast strains and media

Parental strains: *S. cerevisiae* AWRI838 (an isolate of the commercial wine yeast strain EC1118), *S. mikatae* type strain NCYC2888 (designated AWRI1529); a diploid, prototrophic, heterozygous and homothallic wild yeast strain [16]; and hybrid strains generated from this study, CxM1 – CxM5 (CxM1 designated AWRI2526), were grown in YEPD medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) with shaking (100 rpm) at 25°C for one day. Mitochondrial mutants of AWRI838 were generated by ethidium bromide mutagenesis [17]. Ploidy control strains for fluorescence flow cytometry analysis were: BY4742 mat alpha, haploid and BY4743, diploid, (Euoroscarf®) and 53–7 tetraploid [18].

Generation of interspecific hybrid yeast

Rare-mating was used for interspecific hybridisations as described previously by [17]. Cells from the cross were washed in sterile water and plated onto YEP-glycerol-ethanol selection medium (1% w/v yeast extract, 2% w/v peptone, 3% w/v glycerol, 14% v/v ethanol, 2% w/v agar) and incubated at 22°C.

PCR confirmation of hybrids

DNA was isolated from yeast using mechanical breakage with glass beads [19]. Genomic DNA was used as template for PCR analysis, with amplification using the rDNA Internal Transcribed Spacer primer pair ITS1/ ITS4 (**Table 1**) followed by digestion with Restriction Enzyme *Hae*III ; fragments were resolved on a 3% w/v agarose gel [20].

Table 1. Primer sets and restriction endonucleases used to generate species-specific chromosomal markers.

Primer	R/E	Sequence
ITS1	*Hae*III	TCCGTAGGTGAACCTGCGG
ITS4		TCCTCCGCTTATTGATATGC
ScSm IL	*Alu*I	ATTTCTGAATCGTACTGTGCG
		ACCTCGATGACATTGTCGGAT
ScSm IIR	*Taq*I	CGCATTGGGAAGAATTAGTGG
		TCGTCAACCTGTAAGGAATCG
ScSm IIIR	*Taq*I	TGGCTTTGGAACCTATTGATT
		ATGAAGATTCCGTCATGGAGG
ScSm IVR	*Mse*I	TTTTTGTTCCTGCAGATTTTG
		ACCTGGTAGGGCCCATGAT
ScSm VL	*Taq*I	TTTCAAGTCACTGACGTGGCA
		CATCTGCGATTTCTTGGCAA
ScSm VR	*Taq*I	TTCCGCACTATTATCGCAGA
		TTTGTGCAATAGTGGGTGAGG
ScSm VIL	*Hae*III	GGTGCTGCATTCTGGGAAA
		GGCATCAAACATTTGCTGTG
ScSm VIIL	*Taq*I	TCCATTGGGTTTCACCTTTTC
		AGCAGCAATACCACAAACGGA
ScSm VIIIR	*Taq*I	TCGTTTTGGACACAGGAAAG
		GGAAACCTTTTCGTAGCGTGA
ScSm IXL	*Rsa*I	AACAAGGGGAACAGTCTGTCA
		AGAACACAGCAATGTTCCCA
ScSm XL	*Hae*III	CACTCCAATCAACGCTGAAAA
		TAAATGACCTGGGACATCCA
ScSm XR	*Taq*I	CGTTTATTGTGCCGAGCTTA
		TTGGATATGTCAAAGCCAGG
ScSm XIL	*Taq*I	AAATGCAGTGAACGATCCACG
		AGATGATGGCCAGTATGCAA
ScSm XIIL	*Hae*III	CGGTGAAGGTGCCAAATAC
		AGCAGCATGAATACCCCAGTT
ScSm XIIR	*Mse*I	ATTGGCTCGGTACCCCTTT
		TGCCCACATCTGAGACAAAA
ScSm XIIIR	*Hae*III	TGGACTCCAATGTATTGGACG
		ATGTGGAAATCTTGGCCCTT
ScSm XIVL	*Hae*III	TTTAGCGTGGACGATGATCC
		CCCAATTGTAGAATTGCTGC
ScSm XIVR	*Hae*III	AATGGATTTACGCGGCAATAG
		GGCAGTTTGATTTCTAGCGGT
ScSm XVR	*Taq*I	CAAGGCCAAGATGATGAAGA
		TTCTTTCCCACGTTTGGAAG
ScSm XVIL	*Hae*III	TTCTCCAATCATTGCCACCT
		TTGGCGTTGAAAGATCTCCA
ScSm XVIR	*Hae*III	AAATTCTGGTAATCCATGGGA
		TTCAACCATCTCCTTGGTGTG

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Genomic stability of hybrid isolates

To verify that hybrid strains retained the genomes of both parents following grape juice fermentation, end-of –fermentation

isolates (50 colonies) from each of the triplicate hybrid ferments were analysed with the ITS1/4 primer set and *Hae*III Restriction Enzyme. Subsequently, 50 isolates of each hybrid from one of the triplicate end-of–ferment samples were investigated for genome stability using PCR/RFLP targeting at least one arm of each chromosome; 21 primer sets in total (**Table 1**). Primers were designed with homology to both the sequenced *S. cerevisiae* laboratory strain S288c and *S. mikatae* strain IFO 1815 using the primer design tool accessed from the *Saccharomyces* Genome Database website (http://www.yeastgenome.org/cgi-bin/seqTools). The above ITS PCR program was used except for the annealing temperature that was lowered to 50°C to accommodate a maximum of one missmatched base to either species' DNA sequence in the mid region of a primer. Amplified fragments were then digested with restriction endonucleases to generate species-specific banding patterns. Resultant fragments were resolved on a 3% w/v agarose gel.

Array-Comparative Genome Hybridisation (a-CGH) of *S. cerevisiae* x *S. mikatae* hybrid AWRI 2526 and parent strains

A-CGH hybridization and data analysis was performed as described in [7] using custom microarrays manufactured by Agilent Technologies containing 60-mer oligonucleotides designed to the *S. cerevisiae* S288c and *S. mikatae* IFO 1815 genomes. After quality filtering, data representing 24,000 probes evenly spaced across the *S. cerevisiae* genome and 1,600 probes evenly spaced across the *S. mikatae* genome were used for further examination and analysis.

Fluorescence flow cytometry analysis to determine ploidy of putative interspecific hybrids

Strains were grown in YEPD for five days to late stationary phase and fixed in 70% ethanol. A sample of 1×10^{6} cells was processed by washing with sodium citrate (50 mM), RNA was removed with RNAse A and the sample was stained with propidium iodide (2 µg/ml). Cells were analysed using a FACSCalibur (Becton Dickinson, Australia) instrument equipped with a 15 milliwatt air-cooled argon-ion laser emitting at 488 nm. Cells were detected at 585/42 nm (FL2) using BD FACSFlow™ sheath fluid and fluorescence plotted to a linear scale.

Phenotypic assessment of interspecific hybrids

Ethanol and glucose tolerances were determined as described by [17]. To determine sensitivity to different growth temperatures, standard YEPD plates were incubated at 37°C (high temperature stress), 4°C (low temperature stress) or 22°C (non-stress control). Strains were grown to stationary phase in liquid YEPD (2 days) and 5 µl of 10 fold serial dilutions were spotted to plates.

Grape juice fermentation

Hybrid strains were screened for robust fermentation properties in filter sterilised Chardonnay juice (total sugar, (glucose and fructose) 250 g/L, yeast assimilable nitrogen 227 mg/L, pH 3.01) sourced from The Yalumba Wine Company (South Australia). All strains were initially grown in YEPD for 2 days and then acclimatised by 2 days growth in ½ X Chardonnay grape juice medium (diluted with sterile water), shaking, for 2 days. Triplicate 100 ml fermentations were carried out in Chardonnay juice at 22°C. Juice was inoculated at 2×10^{-6} cells per ml and fermentations carried out in conical flasks fitted with water traps, shaken at 150 rpm. Cell growth was measured using Optical Density (absorption at 600 nm) while utilisation of sugar was measured by Refractive Index using an Atago® Palette Digital Refractometer. Triplicate fermentations were sampled in duplicate for chemical analyses.

Wine chemical analysis

Concentrations of residual sugars (glucose and fructose), ethanol, glycerol, and acetic, succinic, malic, lactic, citric and tartaric acids, were determined by HPLC using a Bio-Rad HPX-87 column [21].

Targeted volatile fermentation products analysis

Samples were analysed using stable isotope dilution combined with gas chromatography/mass spectroscopy (GC/MS) [22]. Wine samples were prepared in 2 dilutions, 1/20 and 3/10, with Model Wine (11% ethanol, 10% potassium hydrogen tartrate, pH adjusted with tartaric acid to 3.1). Analysis was performed on an Agilent 7890A gas chromatograph equipped with Gerstel MPS2 multi-purpose sampler and coupled to an Agilent 5975C VL mass selective detector. Instrument control and data analysis were performed with Agilent ChemStation software.

Solvent-extractable volatile chemical analysis

A 10 mL wine sample was extracted with 3 mL of Pentane:ethyl acetate (2:1) and the organic layer was then transferred to a (2 mL) vial for GC/MS analysis. Samples were analyzed with an Agilent 6890A gas chromatograph fitted with a Gerstel MPS2 auto-sampler and coupled to an Agilent 5973N mass spectrometer. The gas chromatograph was fitted with a 60 m J & W DB-Wax fused silica capillary column (0.25 mm i.d., 0.25 µm film thickness). The auto sampler was fitted to a liquid injector operated in fast liquid injection mode with a 10 µL syringe fitted. The carrier gas was helium and the flow rate was 1.7 ml/min. The oven temperature started at 50°C, was held at this temperature for 1 min., then increased to 240°C at 4°C/min. and held at this temperature for 10 min. The injector was held at 200°C and the transfer line at 240°C. The sample volume injected was 2 µL and the splitter, at 33:1, was opened after 36 sec. Fast injection was performed in pulse splitless mode with an inlet pressure of 45.0 psi maintained until splitting. The liner was borosilicate glass with a plug of resilanised glass wool (2–4 mm) at the tapered end to the column. Positive ion electron impact spectra at 70 eV were recorded in the range m/z 35–350 for scan runs. The identification of compounds was performed by comparison of their retention time and of mass spectra with that of the mass spectral data stored in database libraries; Australian Wine Research Institute, Wiley 275 and NB 275K.

Analyses of wine polyphenolics

Wine samples were scanned in the range 600 nm to 240 nm using a Varian CARY 300 UV-Visible Spectrophotomer. Total Phenolics and Total Hydroxycinnamic Acids were determined spectrophotometrically using the absorbance at 280 nm and 320 nm respectively (10 mm pathlength). Total hydroxycinnamates were quantified as 'caffeic acid equivalents', CAE (mg/L) from the spectral reading at 320 nm:

$$CAE = (E_{320} - 1.4)/0.9 \times 10$$

Total Flavonoid Extracts were determined spectrophotometrically as absorption units (a.u.) at 280 nm (10 mm path), taking into account the contribution of non-phenolics and total hydroxycinnamates by use of the formula:

$$Flavonoid\ Extract = (E_{320} - 4) - \tfrac{2}{3}(E_{320} - 1.4)\ [23]$$

The values 4 and 1.4 are statistically based correction factors for non-phenolics at 280 and 320 nm respectively; and the fraction 2/3 refers to the ratio of hydroxycinnamate absorbance at 280 to that at 320 nm.

Total flavonoids were quantified as 'catechin equivalents', CE (mg/L), from Flavonoid Extract (FE) a.u.:

$$CE = FE \times 70\ [24]$$

Statistical Analyses

A one-way analysis of variance (ANOVA) and Student's t-test ($p<0.05$), were used to determine significant differences between wines.

Results

Generation and phenotypic characterisation of novel *S. cerevisiae* x *S. mikatae* hybrids

Rare mating of the diploid *S. cerevisiae* wine yeast strain, AWRI838, with spores of *S. mikatae* strain NCYC2888, produced five interspecific hybrid colonies (CxM1 - CxM5). Species specific PCR–RFLP of target rDNA confirmed that both parental genomes were present in these hybrids (**Figure 1**). Array-Comparative Genome Hybridisation (a-CGH) was performed on one hybrid strain (CxM1 designated AWRI2526) and the two parental strains. The microarray generated from 1600 *S. mikatae* specific probes and 24,000 *S. cerevisiae* specific probes further confirmed that this hybrid strain's genome contained an entire chromosome set from each parent, and appeared to confirm the expected 2:1 *S. cerevisiae*:*S. mikatae* ploidy ratio (**Figure 2**). (Average *S. mikatae* probe intensity was 2.244 for *S. mikatae* parent NCYC2888 and 0.935 for hybrid strain CxM1, indicating a reduction of *S. mikatae* genome in the hybrid strain from diploid to haploid.)

To determine ploidy levels of hybrids, relative genomic DNA content was assessed by fluorescence flow cytometry analysis using linear plots of cell fluorescence. All cultures generated dual peaks of fluorescence, with the second peak attributed to cells undergoing DNA synthesis. Diploid and tetraploid control strains were easily distinguishable with non-dividing cells giving peaks respectively of approximately double and quadruple fluorescent levels of the haploid strain. Parental yeast strains, AWRI838 and NCYC2888, were confirmed as diploids while all hybrid strains gave fluorescent peaks equivalent to a triploid genome complement (**Figure 3**).

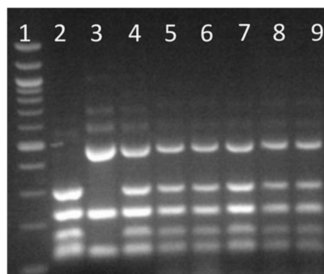

Figure 1. Genetic confirmation of cell hybridization by rDNA ITS PCR-RFLP. Lane 1 100 bp ladder, lane 2 AWRI838, lane 3 NCYC2888, lane 4 DNA from both parents, lanes 5–9 Hybrids CxM1-5.
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Both parental and all hybrid strains were able to grow well on YEPD plates at the non-selective temperature of 22°C. The *S. cerevisiae* parent strain showed strong growth in all conditions except low temperature (4°C). On the other hand, the *S. mikatae* parent grew well at 4°C, poorly on high glucose and was non-viable at both 37°C and high ethanol (14%) concentration. All five hybrid strains were able to grow well in all conditions; high and low temperatures, high glucose and high ethanol concentrations. In fact, a small amount of hybrid vigour is evident at high ethanol concentrations, with three of the hybrid strains showing greater ethanol-tolerance than their *S. cerevisiae* parent (**Figure 4**).

Grape juice fermentation and basic chemical analyses of wines

All five hybrid strains completed fermentation in reasonable time. However, several of them found this medium challenging, with no discernible increase in cell number until the third (CxM2 and CxM3) or fourth (CxM5) day, whereas the wine yeast parent strain and two hybrid strains (CxM1 and CxM4) showed strong growth after the first day following inoculation (**Figure 5a**). No fermentation profile is shown for the *S. mikatae* parent strain as it was unable to grow in Chardonnay juice. Refractive index measurements (an indication of sugar utilisation) showed that the wine yeast parent and the faster-growing of the hybrid strains (CxM1 and CxM4) consumed sugars at a higher rate than other hybrids, and with a shorter growth lag-time (**Figure 5b**). Although final R.I. measurements were similar for all ferments, wines produced by hybrid strains CxM2 and CxM3 had detectable residual fructose (**Table 2**). Wine produced by hybrid strain CxM2 contained 4.5 g/L of fructose, a level considered by winemakers to be too high for the wine to be classed as 'Dry', the maximum for this is less than of 4.0 g/L residual sugar (European Union Commission Regulation EC 753/2002). CxM3 produced wines with the lowest concentration of ethanol (15.8%) while this hybrid strain was also one of the higher glycerol producers, 12.1 g/L compared to the wine yeast parent (16.3% ethanol and 9.6 g/L glycerol). Four of the five hybrids produced wines with no detectable acetic acid; CxM4 produced 0.22 g/L acetic acid, approximately 50% of the parent level (0.41 g/L). In general, the hybrid strains produced wines with equivalent, or slightly higher, levels of citric, malic and succinic acid (97–120%), much higher levels of lactic acid (125–185%) and lower levels of tartaric acid (85–95%).

Genetic stability of novel *S. cerevisiae* x *S. mikatae* hybrids

The genetic stability of two hybrid strains considered to have the best fermentation capability, (CxM1 and CxM4), was tested using the same rDNA PCR-RFLP approach as for the confirmation of hybridisation. Fifty end-of-fermentation isolates from each triplicate Chardonnay wine (150 isolates in total for each of the two hybrid yeast strains) were analysed to confirm the retention of rDNA from each species within the hybrid genome. There was no loss of either parental rDNA in isolates of hybrid CxM1 while only one of the 150 CxM4 isolates showed a loss of parental rDNA, with the species specific band of *S. mikatae* missing from the PCR/RFLP pattern (**Figure 6**). Isolates from one of the replicate fermentations of each hybrid strain were further analysed using 21 PCR primer sets targeting at least one arm of all 16 chromosomes.

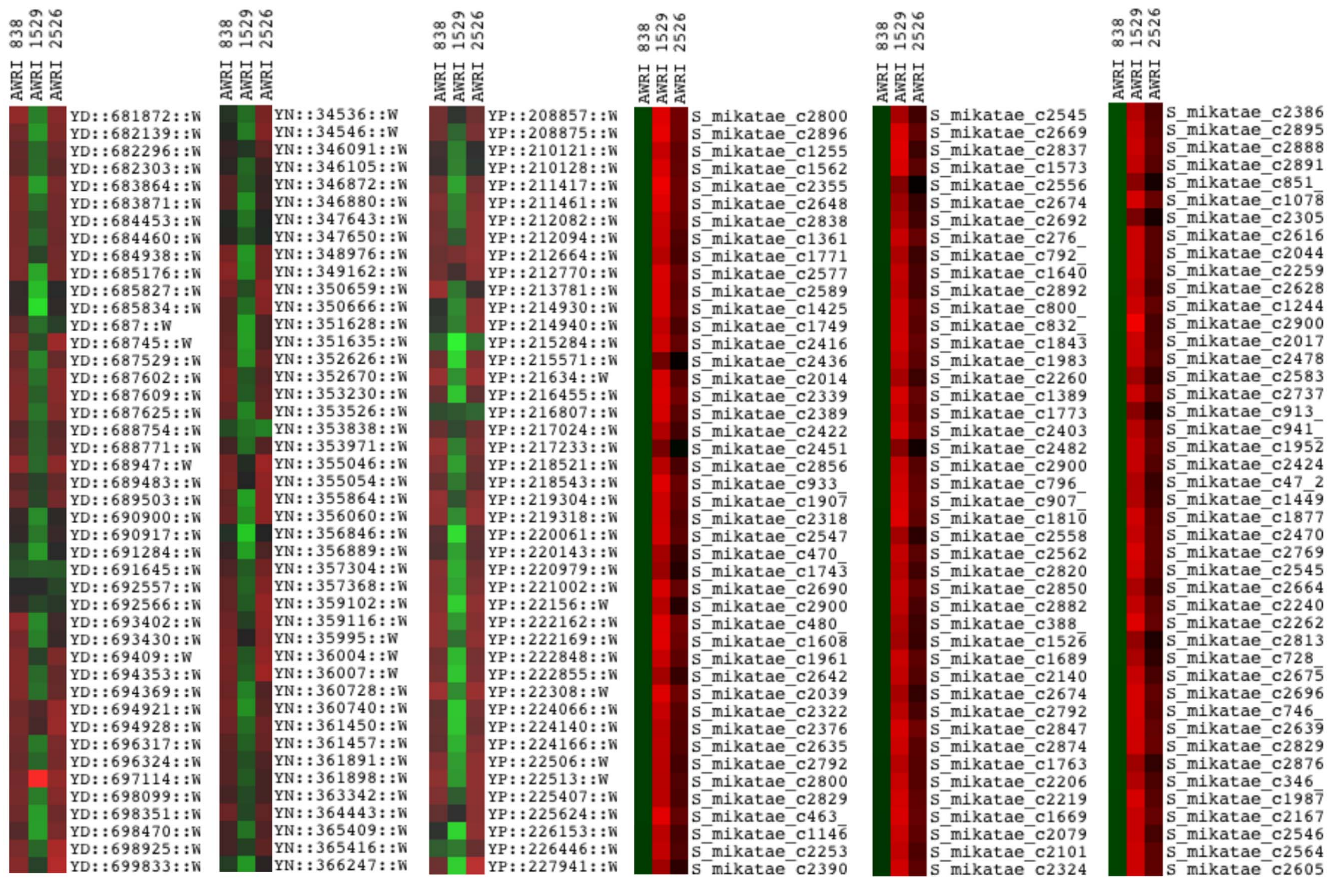


Figure 2. Sample sets of array-CGH data for parents and hybrid strain CxM1. Within each panel of microarray data, each column contains the a-CGH data for a given strain while each row corresponds to a probe for a chromosomal location. The leftmost three panels show the data for probes to the S. cerevisiae genome, located on chromosome V (''YD' followed by chromosome coordinate), XIV ('YN''), and XVI (''YP''); the rightmost three panels show data for probes to various regions (contig ''c'' followed by contig number) of the *S. mikatae* genome. 838 is the *S. cerevisiae* parent strain, AWRI 1529 is the *S. mikatae* parent strain NCYC2888, and AWRI2526 is the hybrid strain CxM1. Red hybridisation intensities for a probe indicate the presence of that species' genome region, while green hybridisation intensities indicate the absence of that species' genome region. The reduced intensity of *S. mikatae* probes in the hybrid dataset indicates a reduced *S. mikatae* ploidy level relative to *S. cerevisiae*, within the hybrid genome.
doi:10.1371/journal.pone.0062053.g002

For hybrid strain CxM1, one isolate, (#41), had lost both left and right arms of *S. mikatae* Chromosome XIV and another isolate, (#10) lost only the right arm of *S. mikatae* chromosome XVI (**Figure 7**). There was no sign of loss in the other isolates of this cross. For strain CxM4, four of the 50 isolates showed chromosomal evolution: one isolate (#4) lost of both arms of *S. mikatae* Chromosome V, another isolate (#6) lost only the left arm of *S. mikatae* Chromosome X. A third isolate (#40) lost the right arm of *S. mikatae* Chromosome XII, (which corresponds to this isolate's loss of rDNA on Chromosome XII observed in the ITS PCR/RFLP), while the fourth isolate (#12) showed a polymorphism at the left arm Chromosome XIV target site (**Figure 8**). No isolate showed loss of DNA on more than one chromosome (**Figure S1**).

Fluorescence flow cytometry analysis on the 50 CxM1 end-of–fermentation isolates showed no discernible loss of ploidy (**Figure S2**).

CxM1 hybrid isolates from the single replicate end-of–fermentation genomic analyses were also screened for the two important fermentation traits of high sugar and high ethanol tolerance. All isolates were able to grow well, however a small reduction of robustness was observed in two of the 50 isolates (**Figure S3**).

Chemical analysis of fermentation products

Additional triplicate laboratory scale fermentations were carried out in Chardonnay juice using the wine yeast parent, AWRI838, and the two hybrid strains that utilised all sugars during the preliminary fermentation trial, CxM1 and CxM4, without the inclusion of the *S. mikatae* parent strain due to its inability to grow in Chardonnay juice. The resultant wines (all having completed fermentation with $<$ 0.25% residual sugar) were analysed using GC/MS for seventeen volatile fermentation-derived compounds previously determined to be important contributors to the aroma and flavour profile of wines [22]. Additional flavour and aroma compounds were identified by GC/MS scan runs and comparing their mass spectra to libraries of known flavour and aroma compounds.

Targeted volatile fermentation products analysis

Both hybrid strains showed differences in the concentration of a number of the compounds analysed relative to the wine yeast parent (**Table 3**). Hybrid strain CxM4 displayed the most differences, producing lower concentrations for 13 compounds and a higher concentration for two compounds; 2-phenylethyl acetate and butanol. Three compounds with the undesirable aroma of 'nail polish', (ethyl acetate, 2-methylbutanol and

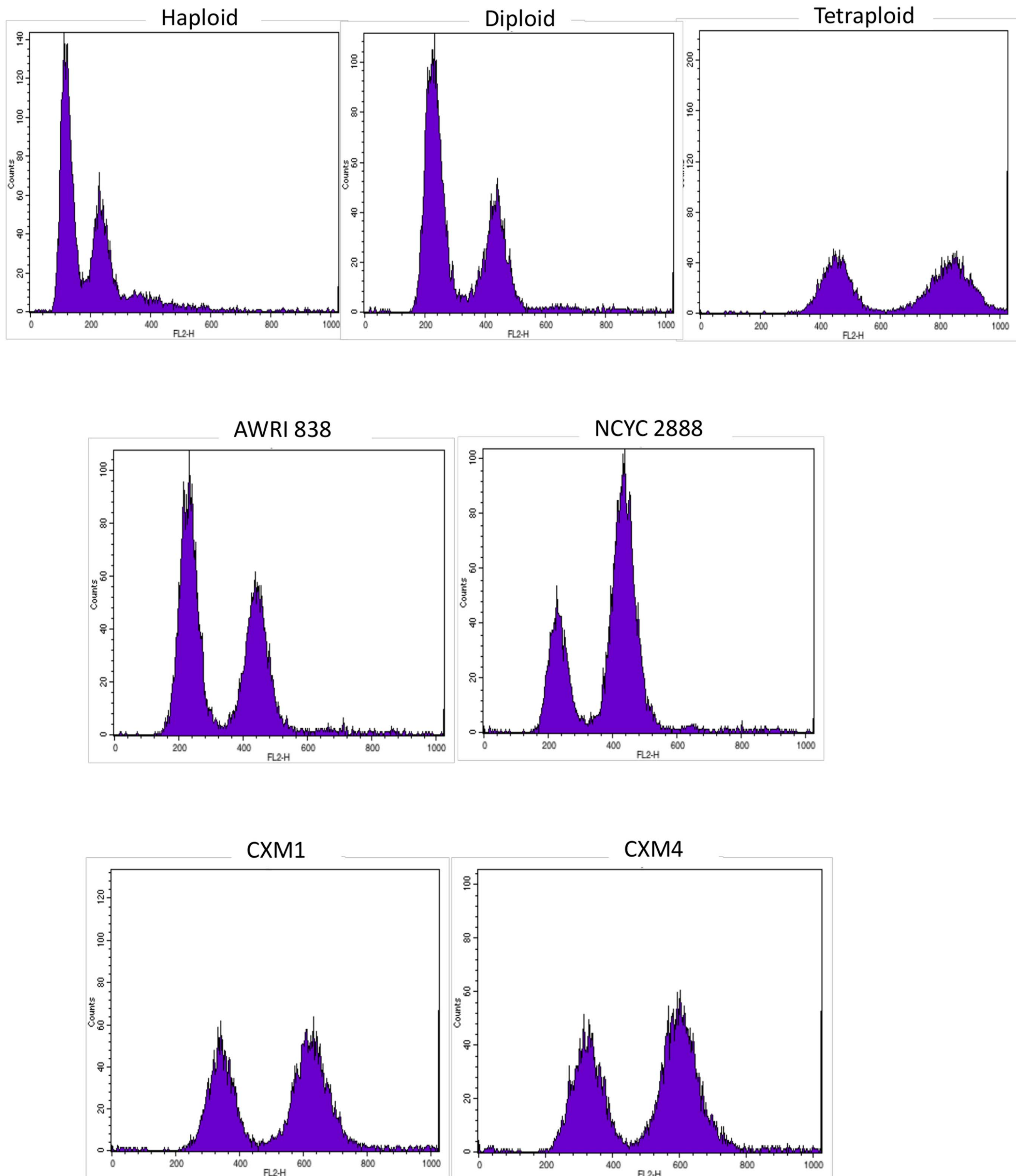


Figure 3. Fluorescence flow cytometry analysis. Top row left to right; Control ploidy strains BY4742 (haploid), BY4743 (diploid) and 53–7 (tetraploid). Middle row left to right; Parent strains AWRI838 and NCYC2888. Bottom row left to right; Hybrid strains CxM1 and CxM4.
doi:10.1371/journal.pone.0062053.g003

3-methylbutanol), were produced at much lower concentrations by this hybrid, ranging from 40% to 65% relative to the wine yeast parent. However, hybrid CxM4 also produced lower concentrations of nine compounds analysed that comprise 'fruity' flavours. In contrast, 2-phenylethyl acetate which elicits a floral aroma, was present at double the concentration compared to the parent. Hybrid CxM1, on the other hand, produced wines with higher concentrations in six compounds, four of which contribute to

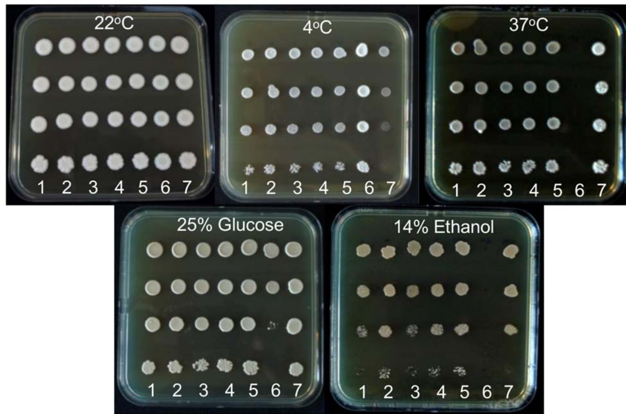


Figure 4. Phenotypic assessment assay plates. Top row plates left to right; YEPD at temperatures 22°C, 4°C and 37°C. Bottom row plates left to right; YEP 25% glucose, YEPD 14% ethanol. Strains are plated in columns at 10 fold serial dilutions from top to bottom; columns 1–5 CxM5-CxM1 in descending order, column 6 NCYC2888, column 7 AWRI838.
doi:10.1371/journal.pone.0062053.g004

'fruit', 'floral' or 'perfume' flavours, while the others contribute 'fusel' and 'nail polish' aromas. Conversely, another compound having a 'nail polish' aroma, (ethyl acetate), was produced at a much reduced level compared to the wine yeast parent (53%).

Solvent-extractable volatile chemical analysis

A total of 27 compounds were identified in the solvent-extractable portion of the Chardonnay wines; compounds such as ethyl esters, acids, phenols and alcohols, while three compounds remain un-identified (**Table 4**). Peak area was used as an indication of relative compound concentration between wine samples. Of the 30 compounds, 18 showed different concentrations in the hybrid yeast-made wines relative to the parent yeast-made wines, with thirteen compounds increasing in level and five compounds decreasing. Nine compounds displayed a two-fold (or more) increase; compounds contributing sweet attributes such as β-phenyl ethanol ('rose'), 9-decenoic acid ('fruity') and 3-hydroxy-4-phenyl-2-butanone ('caramel'), along with compounds contributing 'savoury' attributes; 3-methyl thiol propenol ('meat', 'potato' flavour) and ethyl-2-hydroxy-3-phenylpropanoate ('goaty', 'smokey').

The hybrids also produced some solvent-extractable volatile compounds at different levels to each other, but, as opposed to the targeted volatile compounds, hybrid strain CxM4 generally produced the higher levels.

Analyses of wine polyphenolics

Analysis of UV scan absorbance showed that both wines made with the hybrid strains contained higher levels of total phenolics, total hydroxycinnamic acids (HCA) and total flavonoid extracts, relative to the parent yeast-made wines (**Table 5**). Caffeic acid equivalents (CAE), a measure of non-flavonoid phenolics, was produced in higher amounts by both hybrid strains (110%). Hybrid strain CxM1 produced the highest level of catechin equivalents (CE), a measure of flavonoid phenolics, at 140%, with CxM4 producing 125% relative to the parent strain.

Discussion

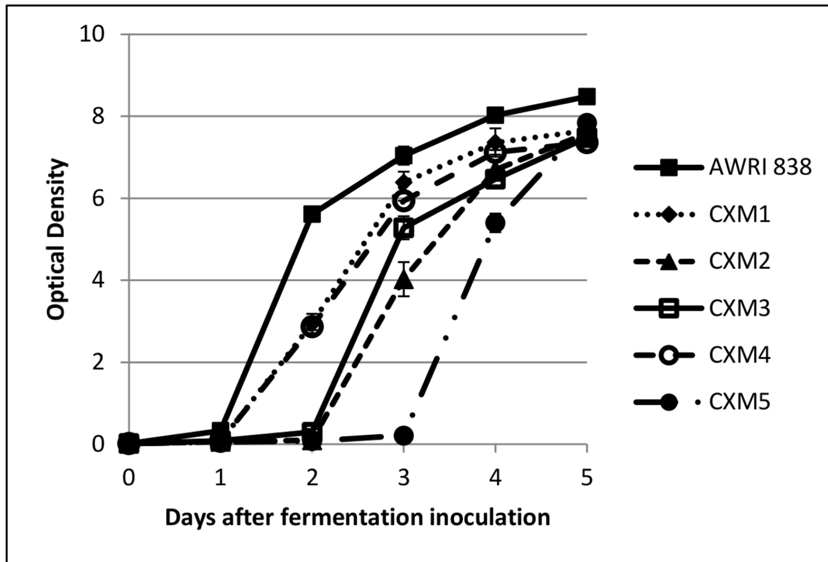
The current downturn in the global economy continues to have a large impact on wine markets around the world. As winemakers vie for a share of this market, the need for product differentiation plays an important role in winemaking practices. Many winemakers desire the sensorial characteristics of complex aroma and flavour profiles of spontaneous fermentations, but are reluctant to risk a quality product to spoilage. Studies of spontaneous fermentations have identified a genetically diverse range of yeast, (*Saccharomyces cerevisiae* being but one), populations of which wax and wane over the duration of a fermentation [25,26]. The metabolites produced by each yeast contribute to the myriad of flavours and aromas witnessed in the resultant wine [27]. Interspecific hybrid yeast have been shown to produce altered metabolite profiles relative to their *S. cerevisiae* wine yeast parent [17].

The use of a new robust *S. cerevisiae*-'style' wine yeast incorporating the genome of *S. cerevisiae* and a distant *Saccharomyces sensu stricto* species not associated with wine fermentation could potentially lead to wines with novel yeast-derived flavour-active metabolites. Indeed, traditional breeding techniques are used in the development of new yeast strains with altered phenotypic characteristics in brewing, breadmaking and winemaking industries [28,29,30,31]. However, this approach requires sporulation of the wine yeast parent strain with subsequent segregation of traits, potentially leading to loss of robust winemaking properties in progeny [32]. Thus, for the current work, rare mating [33] was

Table 2. Fermentation chemistry analysis of wines using HPLC.

	AWRI838	CxM1	CxM2	CxM3	CxM4	CxM5
Glucose *	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Fructose *	<0.1 c	<0.1 c	4.51±0.07 a	2.23±0.108 b	<0.1 c	<0.1 c
Ethanol ≠	16.3±0.06 a,b	16.4±0.02 a	16.1±0.09 b,c	15.8±0.06 c	16.1±0.02 a,b,c	16.4±0.01 a
Glycerol *	9.6±0.03 d	11.1±0.01 c	11.6±0.03 b	12.1±0.07 a	11.4±0.06 b,c	12.1±0.10 a
Acetic acid *	0.41±0.01 a	<0.1 c	<0.1 c	<0.1 c	0.22±0.04 b	<0.1 c
Succinic acid *	4.14±0.01d	4.59±0.01 c	4.53±0.01 c	4.75±0.04 b	4.59±0.03 c	4.85±0.01 a
Malic acid *	2.83±0.02 c	2.98±0.01 b	3.09±0.01 a	2.88±0.01 c	2.81±0.02 c,d	2.75±0.02 d
Lactic acid *	0.32±0.01 c	0.60±0.00 a	0.42±0.01 b	0.59±0.00 a	0.40±0.01 b	0.44±0.02 b
Tartaric acid *	3.12±0.01 a	2.61±0.01 b	2.62±0.01 b	2.71±0.01 b	2.68±0.01 b	2.94±0.08 a,b
Citric acid *	0.12±0.00 d	0.12±0.00 d	0.14±0.00 b	0.15±0.00 a	0.13±0.00 c	0.14±0.00 b

Detection Limit 0.1g/L * g/L, ≠ % v/v Levels not connected by same letter are significantly different ($p<0.05$).
doi:10.1371/journal.pone.0062053.t002



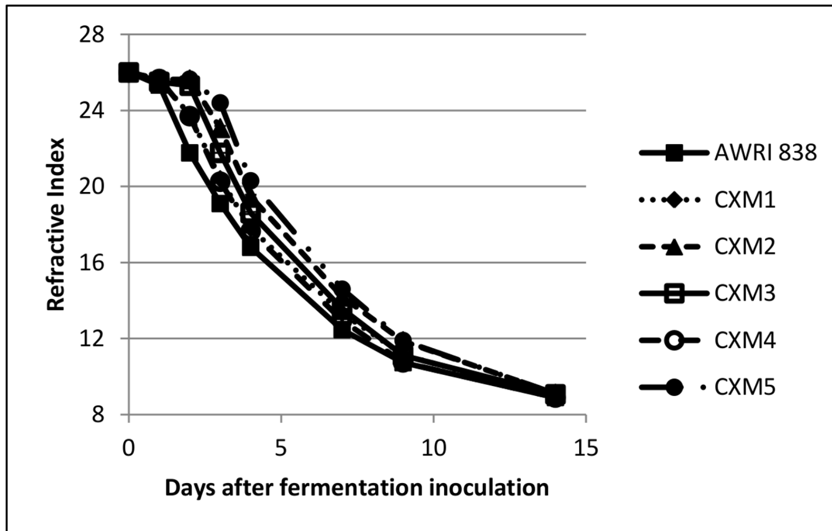


Figure 5. Grape juice fermentation profile of AWRI 838 and hybrid strains CxM1-CxM5. Figure 5a. (top) Cell growth during fermentation as determined by Optical Density. Data points are presented with error bars. Figure 5b. (bottom) Sugar utilisation during fermentation as determined by Refractive Index. Data points are presented with error bars.
doi:10.1371/journal.pone.0062053.g005

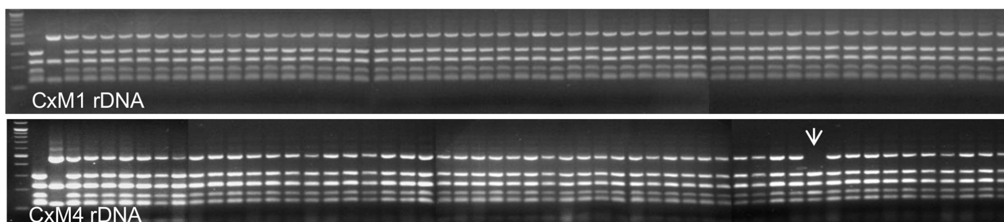


Figure 6. Genetic stability of *S. cerevisiae* x *S. mikatae* hybrids using rDNA ITS PCR-RFLP. Top gel, CxM1 fermentation isolates and bottom gel, CxM4 fermentation isolates. Lane 1 100 bp ladder, lane 2 AWRI838, lane 3 NCYC2888, lane 4 DNA from both parents, lane 5, Hybrid, lanes 6–55 isolates 1–50. Arrow points to isolate with loss of *S. mikatae* rDNA.
doi:10.1371/journal.pone.0062053.g006

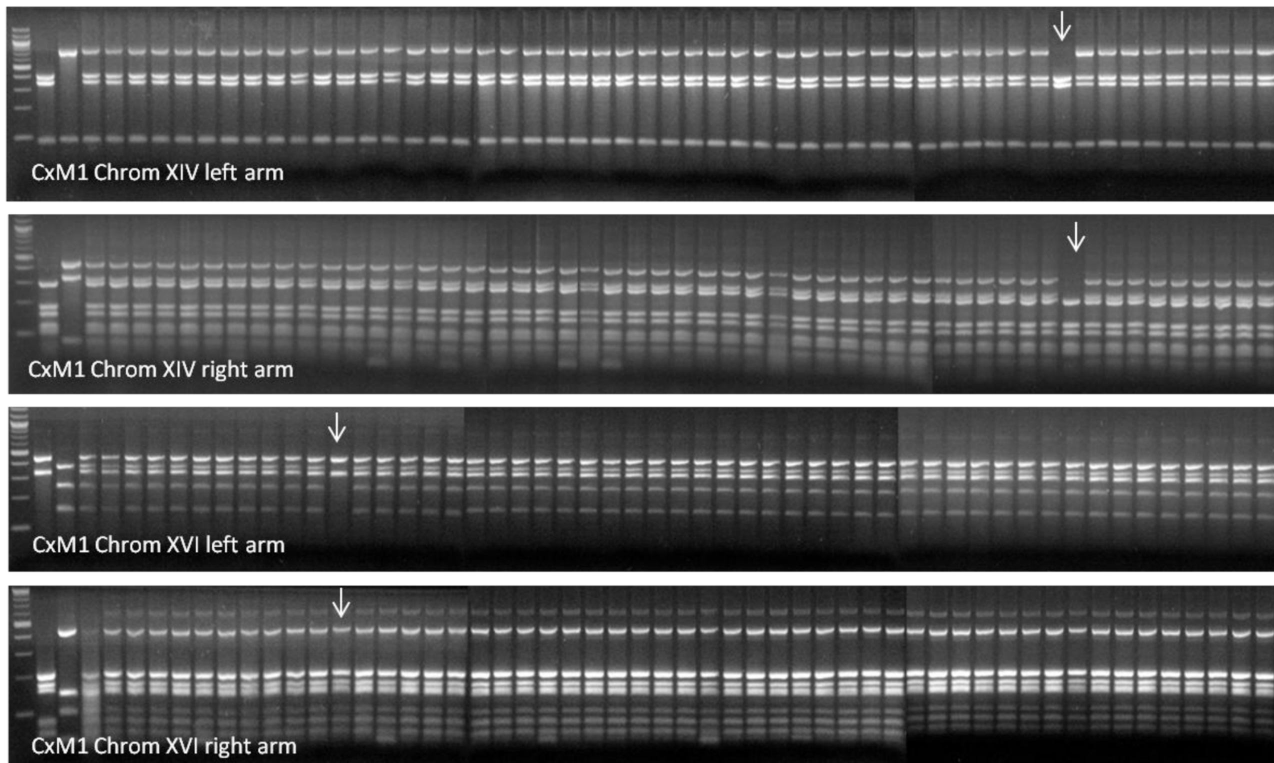


Figure 7. Genetic stability of CxM1 fermentation isolates using chromosomal targeted PCR-RFLP. First gel Chromosome XIV left arm, second gel Chromosome XIV right arm, third gel Chromosome XVI left arm and fourth gel Chromosome XVI right arm. Lane 1 100 bp ladder, lane 2 AWRI838, Lane 3 NCYC2888, lane 4 DNA from both parents, lane 5 Hybrid CxM1, lanes 6 to 55 isolates 1 to 50. Arrows point to isolates with altered chromosomal content.
doi:10.1371/journal.pone.0062053.g007

used to hybridise a diploid wine yeast with haploid spores of the non-wine yeast parent. (Previous studies have identified the triploid composition of natural, stable industrial/fermentation competent *Saccharomyces* hybrid yeast containing a diploid *S. cerevisiae* genome and a haploid non- *S. cerevisiae* genome [2,34], giving a precedent to the generation of triploid interspecific hybrid yeast for this study.)

Although mating between spores of *S. cerevisiae* and *S. mikatae* has previously been performed to determine species boundaries [9,35], no natural interspecific hybrids between these two species have been reported and no hybridisation events of diploid *S. cerevisiae* cells with *S. mikatae* spores have been reported previously.

Putative hybrids from successful rare mating events were confirmed using PCR-RFLP analysis of the ITS region within the rDNA tandem repeat on Chromosome XII. In addition, fluorescence flow cytometry analysis of CxM1 and CxM4 showed DNA fluorescent levels equivalent to a triploid genome, i.e. midway between levels displayed by the diploid and tetraploid control strains.

An assessment of parental phenotypic traits showed that all five hybrids from the *S. cerevisiae* x *S. mikatae* mating inherited traits from both parents: high temperature tolerance from the *S. cerevisiae* parent and low temperature tolerance from the *S. mikatae* parent. In addition, the hybrids also inherited from the *S. cerevisiae* wine yeast parent traits that are necessary for wine fermentation; the ability to grow on high sugar sources and tolerance to high ethanol levels. In fact, three of the five hybrid strains displayed transgressive phenotypes (hybrid vigor) with even stronger growth on high ethanol medium than their ethanol-tolerant *S. cerevisiae* parent.

The five hybrids differed in their abilities to tolerate stresses following inoculation into Chardonnay juice; there was an extended lag-phase prior to commencement of cell division for some hybrids. This is important because the practice of yeast inoculation of commercial wines requires the strain to quickly increase cell numbers in order outcompete indigenous, potentially spoilage, microorganisms. Yeast requiring an extended acclimatisation period in grape juice prior to the commencement of fermentation might compromise the quality of the resultant wine, hence hybrid strains showing this tendency are not suitable for commercial usage. On the other hand, two hybrid strains, (CxM1 and CxM4), showed a short lag-phase commensurate with the commercial wine yeast parent strain and were used for all subsequent in-depth wine fermentation analyses. The differences observed between individual hybrids, (growth in grape juice and wine chemical composition), may be attributable to heterozygosity of the *S. mikatae* diploid parent strain, sporulation of which would have led to spores carrying different combinations of alleles, resulting in triploid progeny containing identical *S. cerevisiae* genomes but differing *S. mikatae* alleleic content.

Basic fermentation chemistry analysis of the wines showed that all five hybrid strains were all able to convert sugars to ethanol, with resultant wines containing similar ethanol levels to the *S. cerevisiae* parent-made wines. Differences to note in the hybrid-made wines were, for all hybrids, an increase in glycerol production and a decrease in acetic acid production relative to the wine yeast parent. Glycerol is known to add to the sweetness of wine [36] and, due to its viscous nature, contributes to the smoothness and overall body of a wine [37,38], while acids greatly influence the taste of wines, contributing to the crispness of the

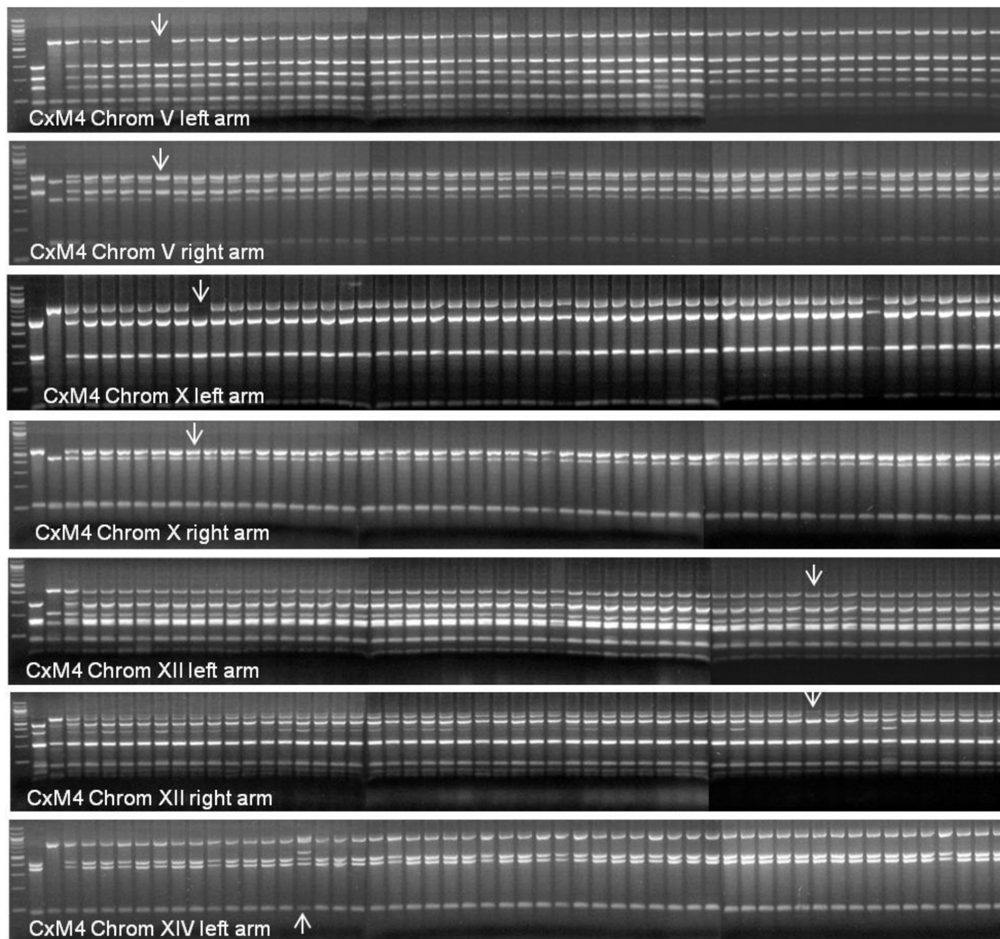


Figure 8. Genetic stability of CxM4 fermentation isolates using chromosomal targeted PCR-RFLP. First gel Chromosome XIV left arm, second gel Chromosome XIV right arm, third gel Chromosome XVI left arm and fourth gel Chromosome XVI right arm. Fifth gel Chromosome XII left arm, sixth gel Chromosome XII right arm, seventh gel Chromosome XIV left arm. Lane 1 100 bp ladder, lane 2 AWRI838, Lane 3 NCYC2888, lane 4 DNA from both parents, lane 5 Hybrid CxM4, lanes 6 to 55 isolates 1 to 50. Arrows point to isolates with altered chromosomal content.
doi:10.1371/journal.pone.0062053.g008

palate. However, acetic acid, with the non-desirable volatile and odorous aroma of 'vinegar' is of particular concern to winemakers. Wine yeast strains producing higher levels of glycerol while at the same time producing low, or undetectable, concentrations of acetic acid would greatly assist winemakers in improving the quality of their wines.

On the other hand, chemical analysis showed that some hybrid-made wines contained residual sugar in the form of fructose. The inactivation of sugar transport systems in yeast cells during alcoholic fermentation [39] and alterations to the glucose-fructose ratio of the fermenting must [40] often lead to sluggish or stuck fermentations, with the resultant wine having residual fructose. Yeast strains developed for the wine industry should to be free from potential fermentation problems, thus hybrid strains producing wines with residual fructose were considered to be unsuitable for further investigation.

The two hybrid strains, (CxM1 and CxM4), exhibiting problem-free, robust fermentation properties were chosen for further study. The chromosomal complement of hybrid strain CxM1 was investigated using a-CGH with results indicating that a complete set of chromosomes from each parent species exist in the hybrid genome. The hybrid showed lower fluorescence intensities of *S. mikatae* probes, relative to the *S. mikatae* parent, whereas intensities to *S. cerevisiae* probes were similar to that of the *S. cerevisiae* parent. This implies a haploid *S. mikatae* chromosomal content, which is in keeping with the flow cytometry results (triploid DNA content), indicating that the hybrid was formed when diploid *S. cerevisiae* cells mated with spores from *S. mikatae*.

The varied fluorescence intensity of bound *S. cerevisiae* probes in the microarray can be attributed to the polymorphic DNA sequence of the wine yeast parent strain, AWRI838 [41], resulting in diverse binding affinities to the probes designed to the S288c *S. cerevisiae* genome.

Initially, genetic stability of hybrid strains was assessed by the retention of ribosomal DNA from each parent. Plant studies have shown that changes in rDNA (loss or silencing of rDNA from one parental species) occurs at the incipient stages of evolution of interspecific hybrids [42,43,44]. The two hybrid strains chosen for further investigation (CxM1 and CxM4) had relatively stable genomes under the stressful fermentation conditions, (low pH and high sugar early in fermentation followed by high levels of ethanol in the later stages), with end-of-fermentation isolates revealing a loss of *S. mikatae* rDNA in only one of a total of 300 isolates analysed.

Subsequently, genomic analysis on end-of-fermentation isolates, targeting each of the sixteen chromosomes from both parental

Table 3. Target volatile fermentation products of AWRI 838, CXM1 and CXM4 in Chardonnay wines.

Ethyl esters (μg/L)	Aroma descriptor	AWRI838	CxM1	CxM4
Ethyl acetate	Nail Polish	25036±645 a	13181±4 b	10145±401c
Ethyl propanoate	Fruity	273±27 b	354±22 a	200±5 c
Ethyl 2-methyl propanoate	Fruity	42±6 a	47±4 a	28±1 b
Ethyl butanoate	Fruity	134±12 a	164±12 a	103±3 b
Ethyl 2-methyl butanoate	Sweet fruit	5.03±0.6 b	6.77 0±0.3 a	3.43±0.1 c
Ethyl 3-methyl butanoate	Berry	6.5±0.9 a	7.2±0.3 a	4.4±0.1 b
Ethyl hexanoate	Green apple	230±18 a	235±17 a	140±2 b
Acetates (μg/L)				
2-Methyl propyl acetate	Banana, fruity	16.8±2.1 a	17.6±1.8 a	11.7±0.3 b
2-Methyl butyl acetate	Banana, fruity	31.4±1.2 a	42.6±4.7 a	22.4±0.7 b
3-Methyl butyl acetate	Banana	577±56 a	657±62 a	450±16 b
2-Phenyl ethyl acetate	Floral	196±56 b	389±50 a	394±18 a
Hexyl acetate	Sweet perfume	10.1±0.4 b	13.8±1.4 a	8.0±0.4 c
Alcohols (μg/L)				
2-Methyl propanol	Fusel, spirituous	40871±556 a	41559±2927 a	42856±718 a
Butanol	Fusel, spirituous	1107±6 b	1321±52 a	1283±8 a
2-Methyl butanol	Nail polish	6525±153 b	8892±307 a	4344±340 c
3-Methyl butanol	Harsh, nail polish	8249±1126 a	7882±475 a	3587±98 b
Hexanol	Green, grass	3059±410 a	2594±84 a	2482±36 a

Levels not connected by same letter are significantly different ($p<0.05$).
doi:10.1371/journal.pone.0062053.t003

species was carried out. This was followed by phenotypic analysis to determine the retention of essential fermentation traits. A small number of isolates, (4% of CxM1 and 8% of CxM4), showed minor chromosomal alterations, with loss of one or both arms of a single chromosome from the *S. mikatae*-parent genome. In the first instance, primers were designed to a region towards the telomere of the long arm of each chromosome and if genomic loss was identified, then the short arm of the chromosome was investigated. No loss of *S. cerevisiae* chromosomal genome was detected in any isolate and fluorescence flow cytometry detected no loss of overall ploidy. However, there may be losses or duplications not detected by the methods used in this study. Importantly, the fermentation properties of tolerance to high sugar and ethanol levels were retained in all isolates, even those with partial loss of the *S. mikatae* genome. Studies have shown that genome instability can occur in tetraploid strains of *S. cerevisiae* [45] whereas polyploid *S. cerevisiae* interspecific hybrids have been shown to be more stable than polyploid *S. cerevisiae* intraspecific hybrids [46]. However, both studies involved yeast cell replication over a large number of generations and/or repeated re-pitching of cells into stressful environs. The modern winemaking practice of inoculation with an Active Dried Yeast preparation made from original stock cultures requires yeast to undergo only a maximum of seven to eight replication events during the course of fermentation, hence minimising the risk of large-scale instability impacting on fermentation performance and wine quality. Wine yeast are not re-pitched from one fermentation to the next.

Importantly, from a winemaking perspective, desirable transgressive phenotypes were apparent in CxM1 and CxM4 hybrids in the form of increased concentrations of secondary metabolites. Chardonnay wines produced using these hybrids showed differences in concentrations in a number of the target volatile metabolite compounds, relative to wine made using the parent *S. cerevisiae* wine yeast. Hybrid strain CxM1 produced higher concentrations in a number of compounds associated with flavours of 'fruity', 'banana', 'floral' and 'sweet perfume'. Increasing the concentration of a flavour or aroma compound can lead to an increased sensory impact of that particular compound, but may also lead to the masking of other flavours or aromas [47]. Conversely, although the second hybrid strain, (CxM4), produced wines with a greater number of compounds at different concentrations to what was present in the parent-made wine, all but one of the differences resulted in a decrease in concentration, with only 2-phenylethyl acetate ('floral' aroma) showing a two-fold increase. A positive side to the production of lower metabolite concentrations is that this yeast also produced lower levels of the three compounds analysed with the non-desirable aroma of nail polish. Lowering the concentration of a compound, particularly compounds with a negative sensory attribute, impacts not only on the compound concerned, but may also un-mask other flavours and aromas [47].

Chemical analysis of the solvent-extractable volatile portion of the wines also revealed differences in levels of flavour active metabolites. The hybrid yeast-made wines showed significantly higher levels of a number of compounds, including isobutyric acid ('sour', 'cheese'), 3-methyl thiol propanol ('meat', 'potato') and ethyl-2-hydroxy-3-phenylpropanoate ('goaty', 'smokey'), all which contribute savoury attributes that potentially add complexity to the overall flavour profile of these wines. Three solvent-extracted volatile compounds remain unidentified, two of which were produced at higher levels by the hybrid yeast and this may indicate that the *S. mikatae* parent is contributing novel metabolites, not previously recognised, to the wines. Of interest also, is that two identified compounds produced at higher levels by the *S. cerevisiae* x *S. mikatae* hybrids have been shown to be generated in wine in high levels by non-*Saccharomyces cerevisiae* species: isobutyric acid,

Table 4. Solvent-extractable volatile fermentation products of AWRI 838, CXM1 and CXM4 in Chardonnay wines.

			Peak Area X 10^4		
R.T.	**Compound Identity**	**Flavour Descriptor**	**AWRI838**	**CxM1**	**CxM4**
16.51	Ethyl octanoate	Sweet, soap	215±8 a	216±17 a	220±30 a
16.67	Acetic acid	Vinegar	655±9 a	127±10 c	283±55 b
19.08	2-Methyl-tetrahydrothiophen-3-one	Blackberry, fruit berry	34±3 a	16±1 b	17±2 b
19.54	2.3-Butanediol	Cashew, rubber	195±27 a	208±39 a	179±42 a
19.67	2,6-Dimethyl-4-heptanol	Yeasty, fermented	144±13 a	84±2 b	104±9 b
20.45	Isobutyric acid	Cheese, rancid, sour	48±5 b	73±9 a	74±4 a
20.61	1,3-Butanediol	Butter	83±5 a	74±5 a	81±8 a
21.90	1,2-Butanolide	Smokey, hot	630±3 a,b	590±3 b	678±4 a
22.20	Butanoic acid	Cheese, rancid, sweaty	36±2 a	42±4 a	42±5 a
22.60	Ethyl decanoate	Floral, soap	137±4 a	98±13b	106±14 b
23.41	2-Methyl butanoic acid	Cheese, sour, rancid	58±2 b	88±16 a	45±6 b
23.48	Diethyl succinate	Fruity	194±9 b	243±2 a	248±20 a
24.05	Ethyl-9-decanoate	Sweet, pleasant	20±6 c	45±4 b	60±7 a
24.56	3-Methyl thiol propanol	Savoury, meat, potato	45±4 b	110±16 a	99±8 a
27.03	Ethyl 4-hydroxybutanoate	Sweet, pleasant	610±18 a	502±10 c	546±12 b
27.27	β-Phenyl acetate	Sweet, solvent	50±2 b	96±10 a	98±12 a
28.20	Hexanoic acid	Vinegar, fermented	258±27 a	229±52 a	284±14 a
29.77	2-Phenyl ethyl alcohol	Floral, rose	9619±153 c	18194±244 a	17115±461 b
33.05	Diethyl malate	Green, fruity, caramel	26±2 a	26±2 a	29±1 a
33.16	Unidentified		52±2 b	58±3 b	74±4 a
33.62	Octanoic acid	Harsh, rancid	642±30 a	650±41 a	676±64 a
38.05	3-Hydroxy-4-phenyl-2-butanone	Fruity, sweet, caramel	22±2 c	47±3 b	60±5 a
38.36	Unidentified		20±5 c	46±1 b	78±9 a
38.46	Ethyl-2-hydroxy-3-phenylpropanoate	Goaty, smokey	34±5 b	69±3 a	77±17 a
38.69	Decanoic acid	Fatty	372±32 a	260±41 a	320 95 a
40.02	9-Decenoic acid	Fruity, waxy	60±4 c	147±3 b	193±11 a
40.77	4-Vinyl phenol	Pharmaceutical	173±1 a,b	168±7 b	187±9 a
41.15	Ethyl hydrogen succinate	Fruit (mild)	183±43 a	329±26 a	215±24 a
42.13	Unidentified		61±12 a	55±3 a	56±3 a
53.51	4-Hydroxybenzene ethanol	Sweet floral, fruity	580±28 c	910±33 a	741±13b

Levels not connected by same letter are significantly different ($p<0.05$).
doi:10.1371/journal.pone.0062053.t004

Torulaspora delbrueckii [48] and 2-phenyl ethyl alcohol, *Kluyveromyces lactis* [49].

Polyphenols contribute to sensory properties in wine. Grape and wine phenolic compounds can be divided into two groups; non-flavonoids and flavonoids. The primary class of non-flavonoids in white wine is the hydroxycinnamates (HCA), with esters of caffeic acid being the most abundant [50]. HCAs are potent antioxidants and have been shown to be involved in the prevention of browning of musts and wines [51] while catechins, a major class of flavonoids, are known for their bitterness [52]. In the current

Table 5. Polyphenolic analysis of Chardonnay wines made by AWRI 838, CxM1 and CxM4 using UV Scan data: an index of Phenolic content.

	Total Phenolics (a.u.)	**Total HCA (a.u.)**	**Flavonoid Extract (a.u.)**	**CAE (mg/L) (non-flavonoid)**	**CE (mg/L) (flavonoid)**
AWRI838	3.75±0.03 c	3.90±0.02 b	1.15±0.01 c	43.3±0.26 b	80.7±0.82 c
CxM1	4.46±0.04 a	4.24±0.04 a	1.64±0.01 a	47.2±0.5 a	114.7±0.6 a
CxM4	4.24±0.06 b	4.20±0.04 a	1.44±0.03 b	46.7±0.5 a	100.6± 2.0 b

Levels not connected by same letter are significantly different ($p<0.05$).
doi:10.1371/journal.pone.0062053.t005

work, polyphenolic content was assessed by spectral evaluation and estimations of non-flavonoid and flavonoid content were derived by using extinction co-efficients [23,24]. CxM1 and CxM4 produced wines with slightly higher levels of flavonoid and non-flavonoid content. It has been shown that differences in the concentrations of hydroxycinnamic derivatives constitute an important factor in browning, with the proportion of tartaric esters of caffeic acid, *p*-coumaric acid and ferulic acid playing important roles [51]. Both of the hybrid strains produced wines with higher concentrations of phenolics, (including total hydroxycinnamates), relative to wine produced by the wine yeast parent, potentially leading to different impacts on browning.

In conclusion, a new breed of interspecific wine yeast has been developed that incorporates the genomes of *S. cerevisiae* and *S. mikatae*, the latter of which has not previously been associated with wine fermentation. Whilst there are numerous natural *S. cerevisiae* x *Saccharomyces spp*. interspecific hybrids reported in the literature, no natural *S. cerevisiae* x *S. mikatae* hybrids have been isolated. The evolutionary distance between these two yeasts is considerable (they share only 73% of overall DNA sequence homology), therefore it was deemed to be a good candidate for the introduction of novel metabolic outputs to shape wine sensory characteristics. This proved to be the case; chemical analyses of wines made using *S. cerevisiae* x *S. mikatae* hybrids confirmed that the presence of a *S. mikatae* genome impacted favourably on the production of flavour-active volatile fermentation metabolites, potentially producing complex wines akin to spontaneous ferments. The safeguard of an inoculated ferment while providing complexity to their wines assists winemakers by providing additional tools to develop new wine styles.

Supporting Information

Figure S1 Genetic stability of fermentation isolates from CxM1 and CxM4 using chromosomal targeted PCR-RFLP. Figure S1a. CxM1 fermentation isolates. First gel Chromosome I left arm, second gel Chromosome II right arm, third gel Chromosome III right arm, fourth gel Chromosome IV right arm, fifth gel Chromosome V left arm, sixth gel Chromosome V right arm, seventh gel Chromosome VI left arm, eighth gel, Chromosome VII left arm and ninth gel Chromosome VIII left arm. In all gels; Lane 1 100 bp ladder, lane 2 AWRI838, Lane 3 NCYC2888, lane 4 DNA from both parents, lane 5 Hybrid CxM1, lanes 6 to 55 isolates 1 to 50. Figure S1b. CxM1 fermentation isolates continued. First gel Chromosome IX left arm, second gel Chromosome X left arm, third gel Chromosome X right arm, fourth gel Chromosome XI left arm, fifth gel Chromosome XII left arm, sixth gel Chromosome XII right arm, seventh gel Chromosome XIII right arm and eighth gel Chromosome XV left arm. In all gels; Lane 1 100 bp ladder, lane 2 AWRI838, Lane 3 NCYC2888, lane 4 DNA from both parents, lane 5 Hybrid CxM1, lanes 6 to 55 isolates 1 to 50. Figure S1c. CxM4 fermentation isolates. First gel Chromosome I left arm, second gel Chromosome II right arm, third gel Chromosome III right arm, fourth gel Chromosome IV right arm, fifth gel Chromosome VI left arm, sixth gel Chromosome VII left arm, seventh gel Chromosome VIII left arm, eighth gel and Chromosome IX right arm. In all gels; Lane 1 100 bp ladder, lane 2 AWRI838, Lane 3 NCYC2888, lane 4 DNA from both parents, lane 5 Hybrid CxM4, lanes 6 to 55 isolates 1 to 50. Figure S1d. CxM4 fermentation isolates. First gel Chromosome XI left arm, second gel Chromosome XIII right arm, third gel Chromosome XIV right arm, fourth gel Chromosome XV left arm, fifth gel Chromosome XVI left arm and sixth gel Chromosome XVI right arm. In all gels; Lane 1 100 bp ladder, lane 2 AWRI838, Lane 3 NCYC2888, lane 4 DNA from both parents, lane 5 Hybrid CxM4, lanes 6 to 55 isolates 1 to 50.
(PDF)

Figure S2 Fluorescence flow cytometry analysis of hybrid CxM1 post-fermentation isolates. Figure S2a. Row 1 Strains left to right; BY4742 (haploid), BY4743 (diploid), 53–7 (tetraploid), CxM1 (AWRI2526). CxM1 isolates left to right; Row 2 Isolate 1–5, Row 3 Isolate 5–10, Row 4 Isolate 11–15. Figure S2b. CxM1 isolates left to right; Row 1 Isolate 16–20, Row2, Isolate 21–25, Row 3 Isolate 26–30, Row 4 Isolate 31–35. Figure S2c. CxM1 isolates left to right; Row 1, Isolate 36–40, Row 2 Isolate 41–45, Row 3 Isolate 46–50.
(PDF)

Figure S3 Phenotypic assessment assay plates of CxM1 post-fermentation isolates. Figure S3a. Plates left to right; YEPD at temperature 22°C, YEP 25% glucose, YEPD 14% ethanol. Strains are plated in columns at 10 fold serial dilutions from top to bottom in two sections of the plate. Top section left to right; AWRI 838 (*Sc*), NCYC2888 (*Sm*), CxM1, CxM1 isolates 1–5. Bottom section left to right; CxM1 isolates 6–13. Figure S3b. Plates left to right; YEPD at temperature 22°C, YEP 25% glucose, YEPD 14% ethanol. Strains are plated in columns at 10 fold serial dilutions from top to bottom in two sections of the plate. Top section left to right; AWRI 838 (*Sc*), NCYC2888 (*Sm*), CxM1, CxM1 isolates 14–18. Bottom section left to right; CxM1 isolates 19–26. Figure S3c. Plates left to right; YEPD at temperature 22°C, YEP 25% glucose, YEPD 14% ethanol. Strains are plated in columns at 10 fold serial dilutions from top to bottom in two sections of the plate. Top section left to right; AWRI 838 (*Sc*), NCYC2888 (*Sm*), CxM1, CxM1 isolates 27–31. Bottom section left to right; CxM1 isolates 32–39. Figure S3d. Plates left to right; YEPD at temperature 22°C, YEP 25% glucose, YEPD 14% ethanol. Strains are plated in columns at 10 fold serial dilutions from top to bottom in two sections of the plate. Top section left to right; AWRI 838 (*Sc*), NCYC2888 (*Sm*), CxM1, CxM1 isolates 40–44. Bottom section left to right; CxM1 isolates 45–50.
(PDF)

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Author Contributions

Conceived and designed the experiments: JRB PJC. Performed the experiments: JRB FS DLC BLD. Analyzed the data: JRB DLC. Contributed reagents/materials/analysis tools: JRB FS DLC BLD. Wrote the paper: JRB FS DLC BLD PJC.

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Supplementary Figure Legends

Figure S1. Genetic stability of fermentation isolates from CxM1 and CxM4 using chromosomal targeted PCR-RFLP

Figure S1a. CxM1 fermentation isolates

First gel Chromosome I left arm, second gel Chromosome II right arm, third gel Chromosome III right arm, fourth gel Chromosome IV right arm, fifth gel Chromosome V left arm, sixth gel Chromosome V right arm, seventh gel Chromosome VI left arm, eighth gel, Chromosome VII left arm and ninth gel Chromosome VIII left arm.
In all gels; Lane 1 100 bp ladder, lane 2 AWRI838, Lane 3 NCYC2888, lane 4 DNA from both parents, lane 5 Hybrid CxM1, lanes 6 to 55 isolates 1 to 50.

Figure S1b. CxM1 fermentation isolates continued

First gel Chromosome IX left arm, second gel Chromosome X left arm, third gel Chromosome X right arm, fourth gel Chromosome XI left arm, fifth gel Chromosome XII left arm, sixth gel Chromosome XII right arm, seventh gel Chromosome XIII right arm and eighth gel Chromosome XV left arm.
In all gels; Lane 1 100 bp ladder, lane 2 AWRI838, Lane 3 NCYC2888, lane 4 DNA from both parents, lane 5 Hybrid CxM1, lanes 6 to 55 isolates 1 to 50.

Figure S1c. CxM4 fermentation isolates

First gel Chromosome I left arm, second gel Chromosome II right arm, third gel Chromosome III right arm, fourth gel Chromosome IV right arm, fifth gel Chromosome VI left arm, sixth gel Chromosome VII left arm, seventh gel Chromosome VIII left arm, eighth gel and Chromosome IX right arm.
In all gels; Lane 1 100 bp ladder, lane 2 AWRI838, Lane 3 NCYC2888, lane 4 DNA from both parents, lane 5 Hybrid CxM4, lanes 6 to 55 isolates 1 to 50.

Figure S1d. CxM4 fermentation isolates

First gel Chromosome XI left arm, second gel Chromosome XIII right arm, third gel Chromosome XIV right arm, fourth gel Chromosome XV left arm, fifth gel Chromosome XVI left arm and sixth gel Chromosome XVI right arm.
In all gels; Lane 1 100 bp ladder, lane 2 AWRI838, Lane 3 NCYC2888, lane 4 DNA from both parents, lane 5 Hybrid CxM4, lanes 6 to 55 isolates 1 to 50.

Figure S2. Fluorescence flow cytometry analysis of hybrid CxM1 post-fermentation isolates.

Figure S2a. Row 1 Strains left to right; BY4742 (haploid), BY4743 (diploid), 53-7 (tetraploid), CxM1 (AWRI2526). CxM1 isolates left to right; Row 2 Isolate 1-5, Row 3 Isolate 5-10, Row 4 Isolate 11-15.

Figure S2b. CxM1 isolates left to right; Row 1 Isolate 16-20, Row2, Isolate 21-25, Row 3 Isolate 26-30, Row 4 Isolate 31-35.

Figure S2c. CxM1 isolates left to right; Row 1, Isolate 36-40, Row 2 Isolate 41-45, Row 3 Isolate 46-50.

Figure S3. Phenotypic assessment assay plates of CxM1 post-fermentation isolates.

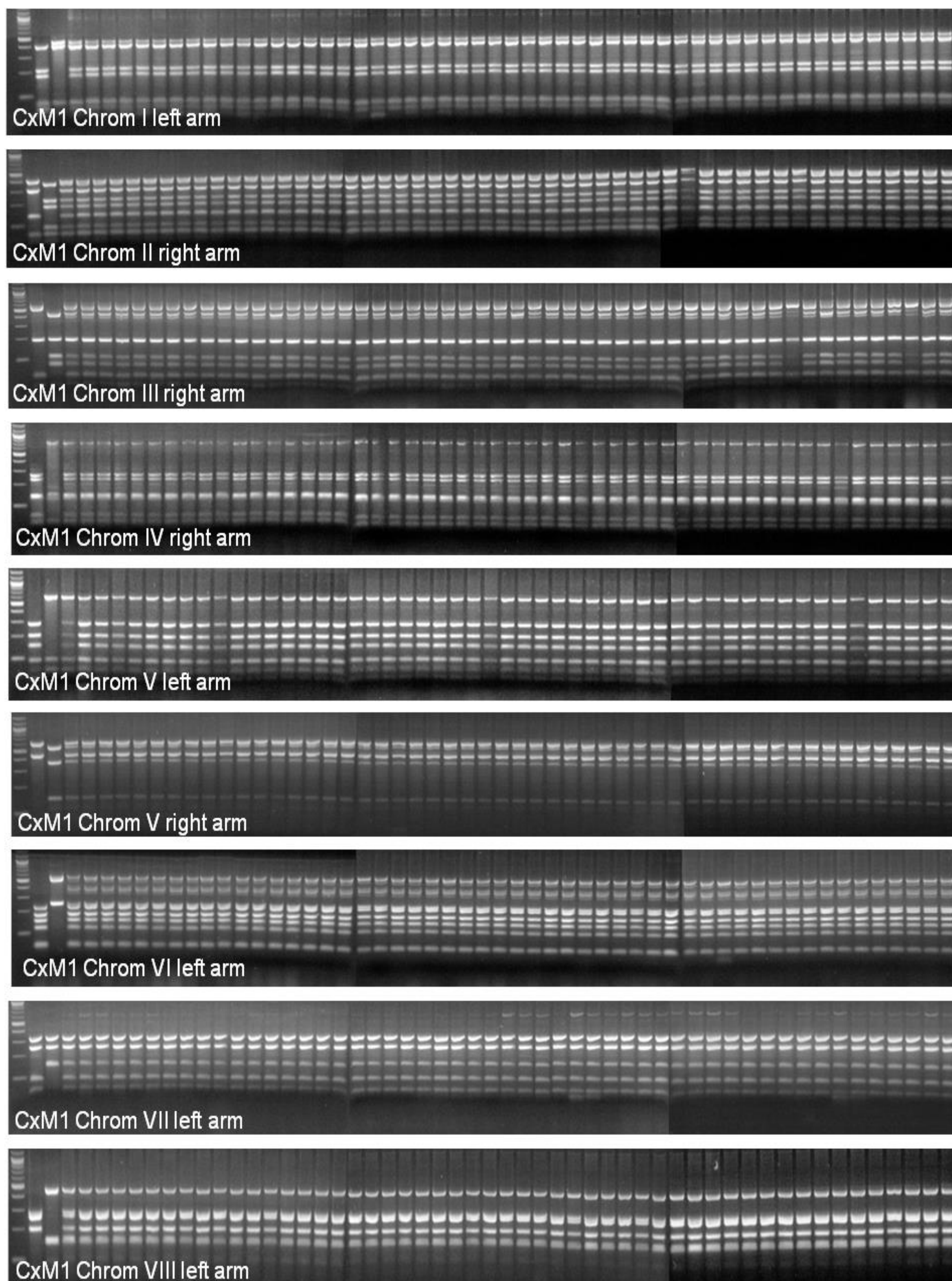
Figure S3a. Plates left to right; YEPD at temperature 22°C, YEP 25% glucose, YEPD 14% ethanol. Strains are plated in columns at 10 fold serial dilutions from top to bottom in two sections of the plate. Top section left to right; AWRI 838 (*Sc*), NCYC2888 (*Sm*), CxM1, CxM1 isolates 1-5. Bottom section left to right; CxM1 isolates 6-13.

Figure S3b. Plates left to right; YEPD at temperature 22°C, YEP 25% glucose, YEPD 14% ethanol. Strains are plated in columns at 10 fold serial dilutions from top to bottom in two sections of the plate. Top section left to right; AWRI 838 (*Sc*), NCYC2888 (*Sm*), CxM1, CxM1 isolates 14-18. Bottom section left to right; CxM1 isolates 19-26

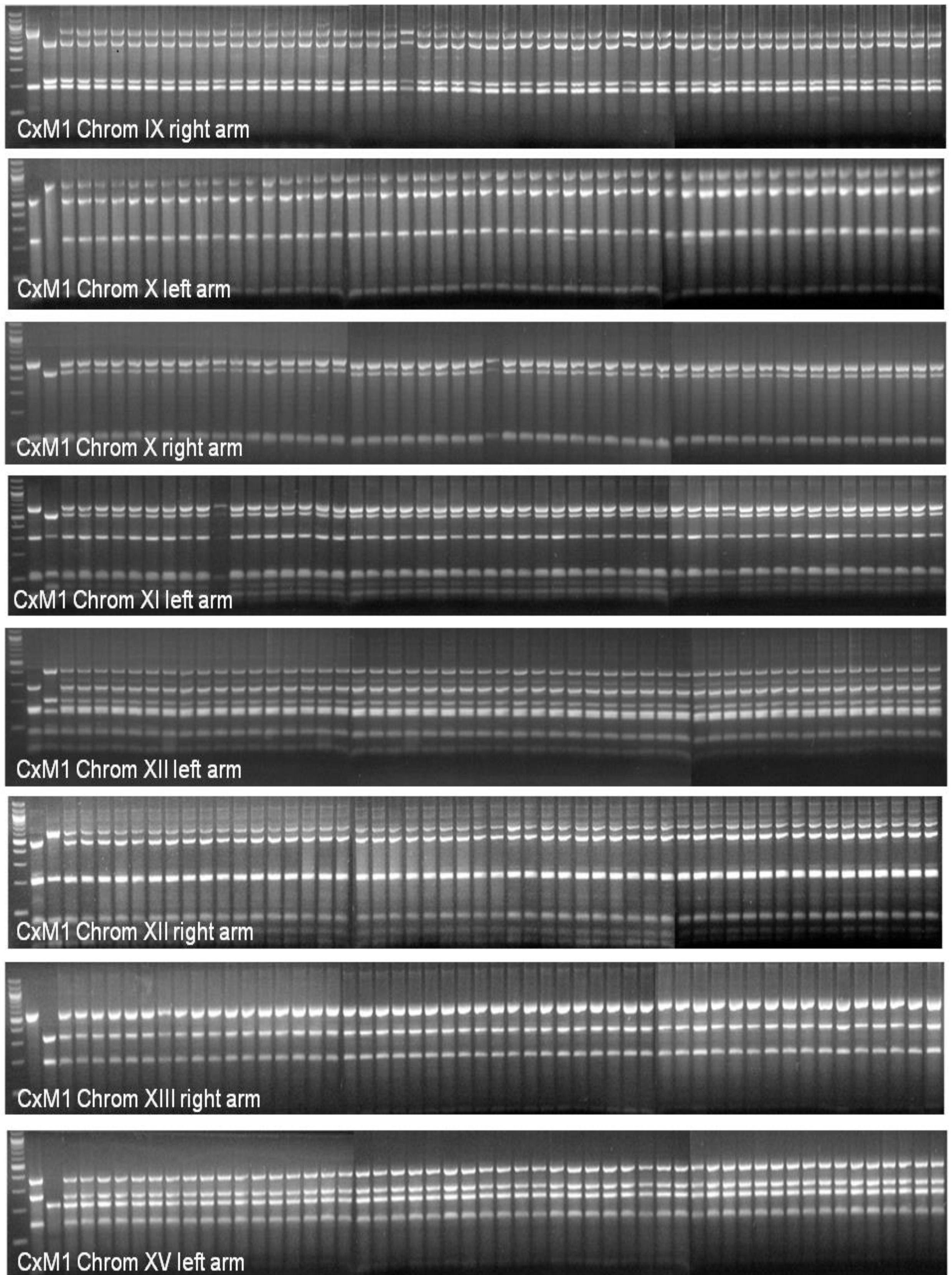
Figure S3c. Plates left to right; YEPD at temperature 22°C, YEP 25% glucose, YEPD 14% ethanol. Strains are plated in columns at 10 fold serial dilutions from top to bottom in two sections of the plate. Top section left to right; AWRI 838 (*Sc*), NCYC2888 (*Sm*), CxM1, CxM1 isolates 27-31. Bottom section left to right; CxM1 isolates 32-39.

Figure S3d. Plates left to right; YEPD at temperature 22°C, YEP 25% glucose, YEPD 14% ethanol. Strains are plated in columns at 10 fold serial dilutions from top to bottom in two sections of the plate. Top section left to right; AWRI 838 (*Sc*), NCYC2888 (*Sm*), CxM1, CxM1 isolates 40-44. Bottom section left to right; CxM1 isolates 45-50

Supporting Figure S1a



Supporting Figure S1b



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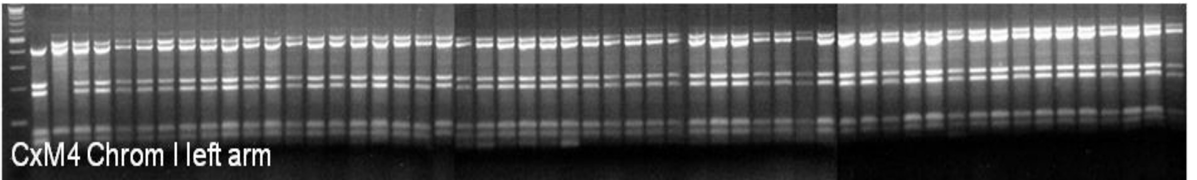
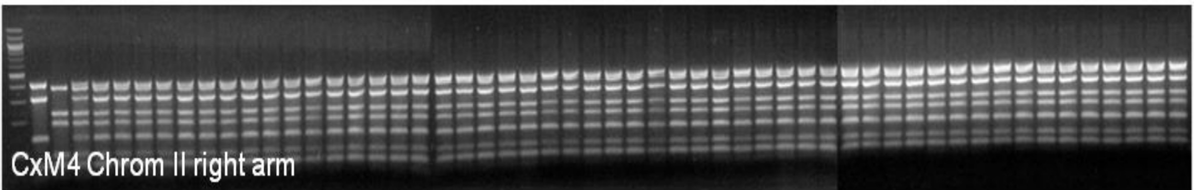
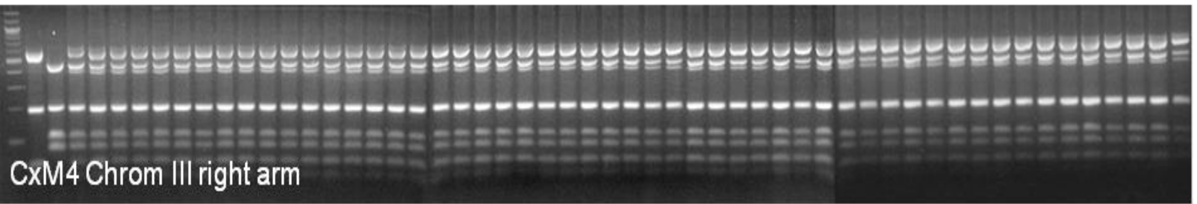
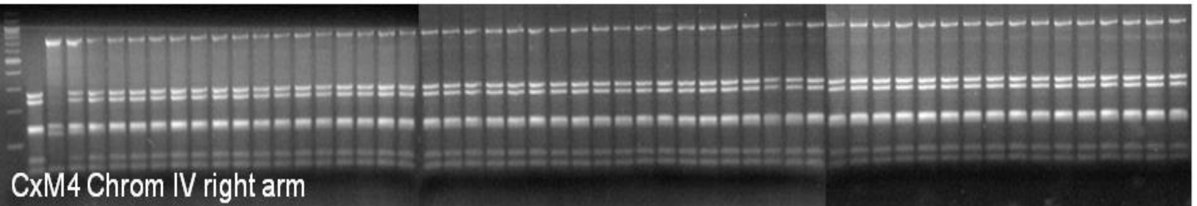
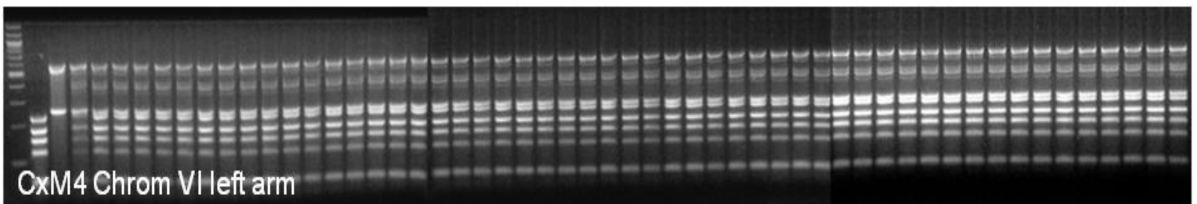
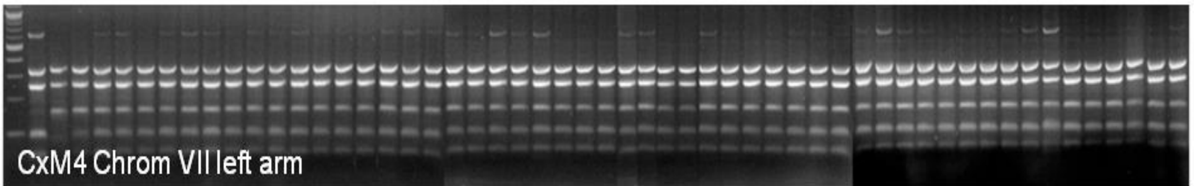


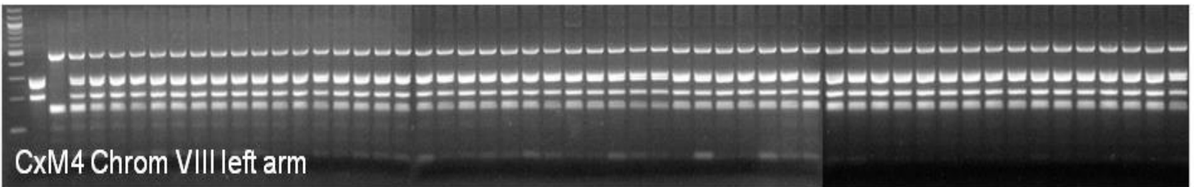


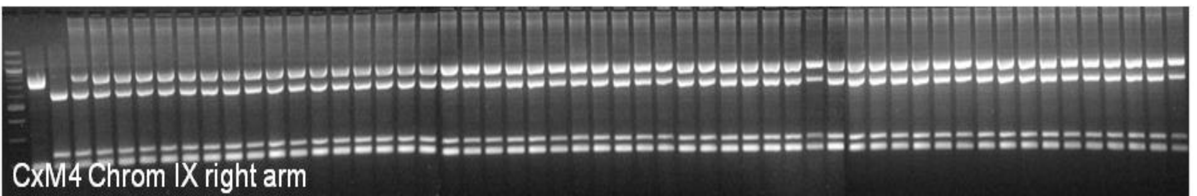




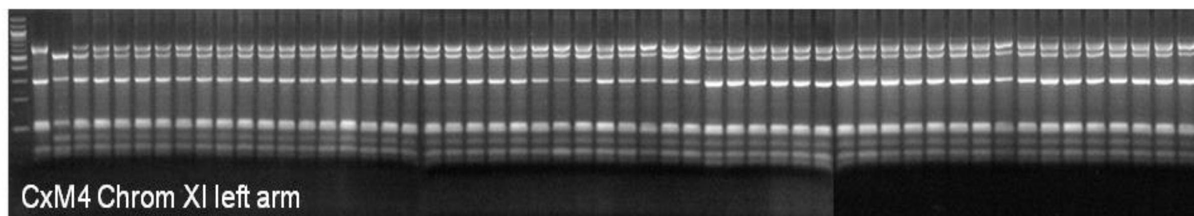


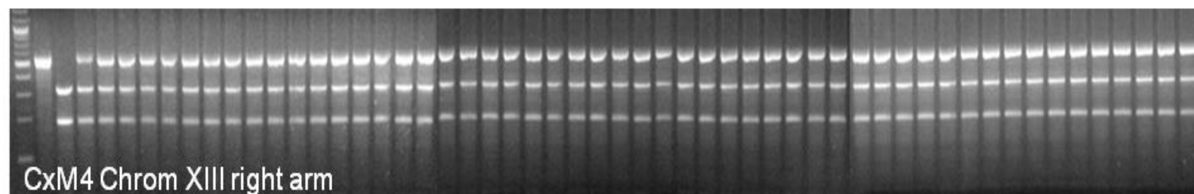


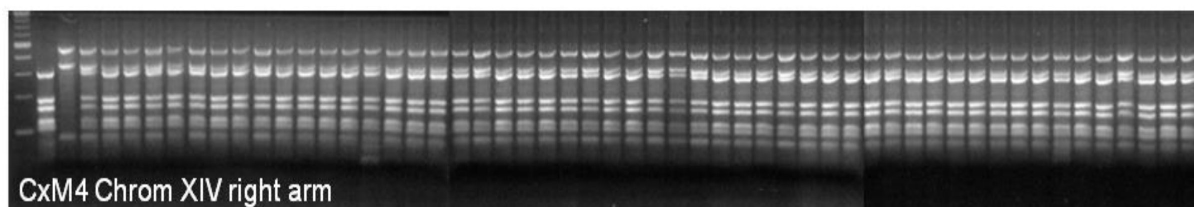


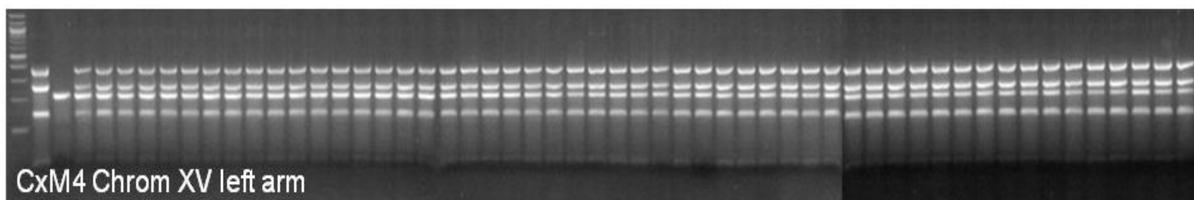


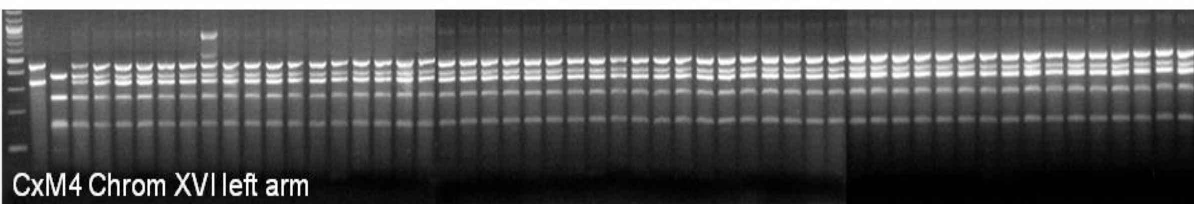
Supporting Figure S1d

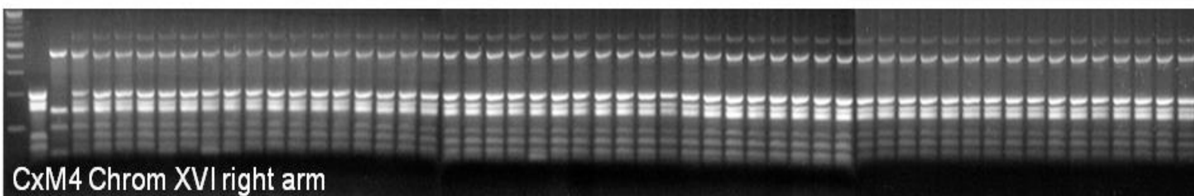




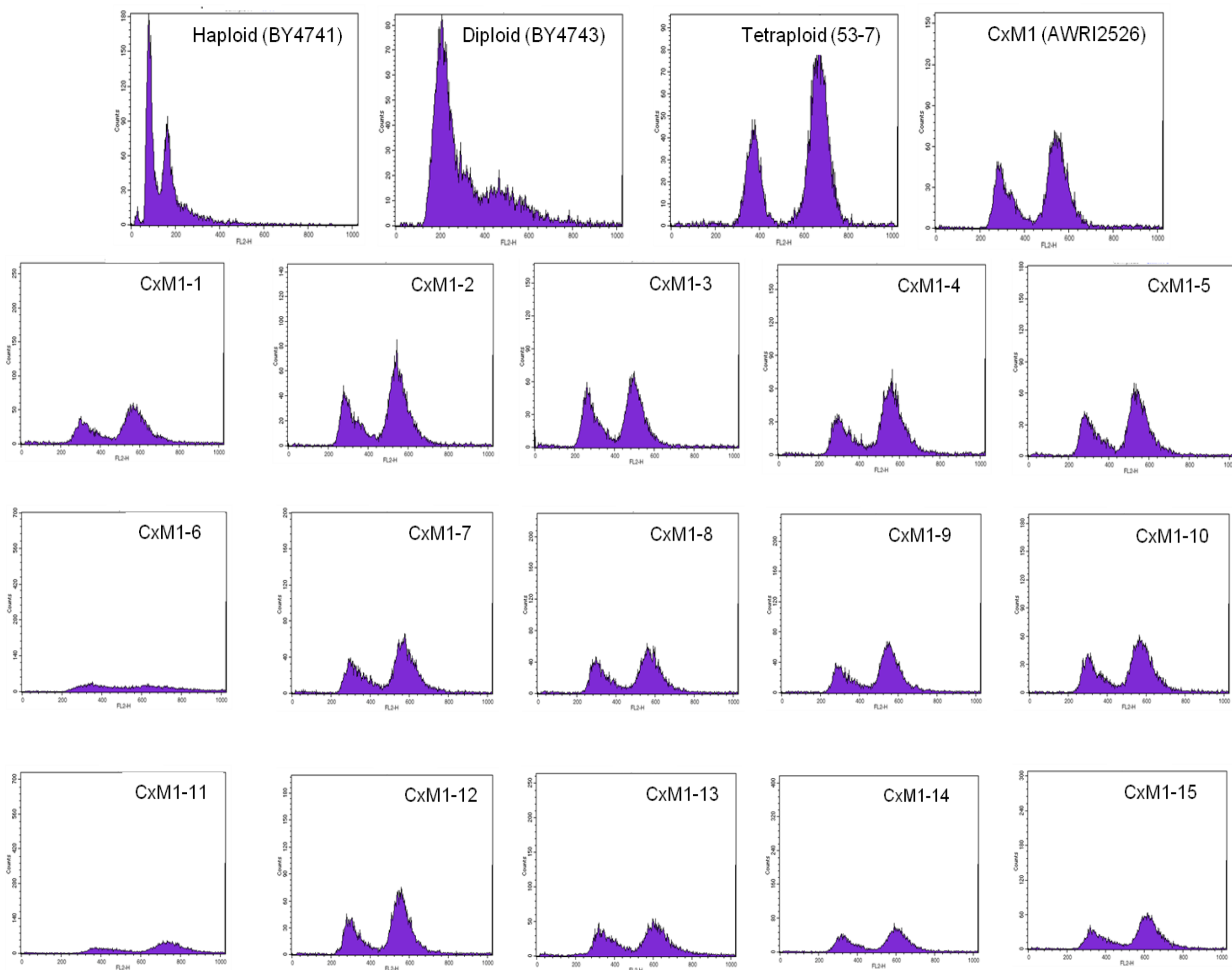








Supporting Figure S2a



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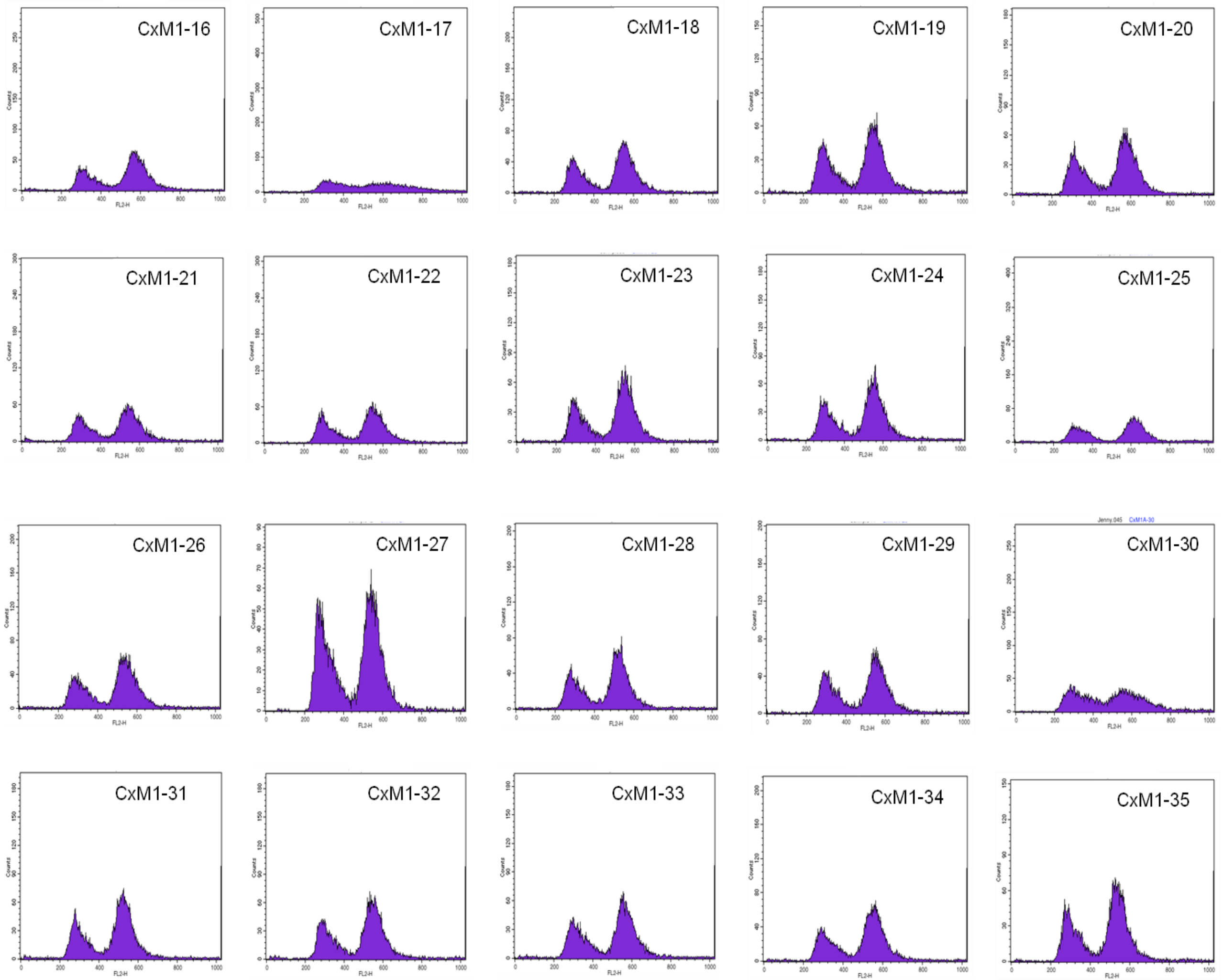

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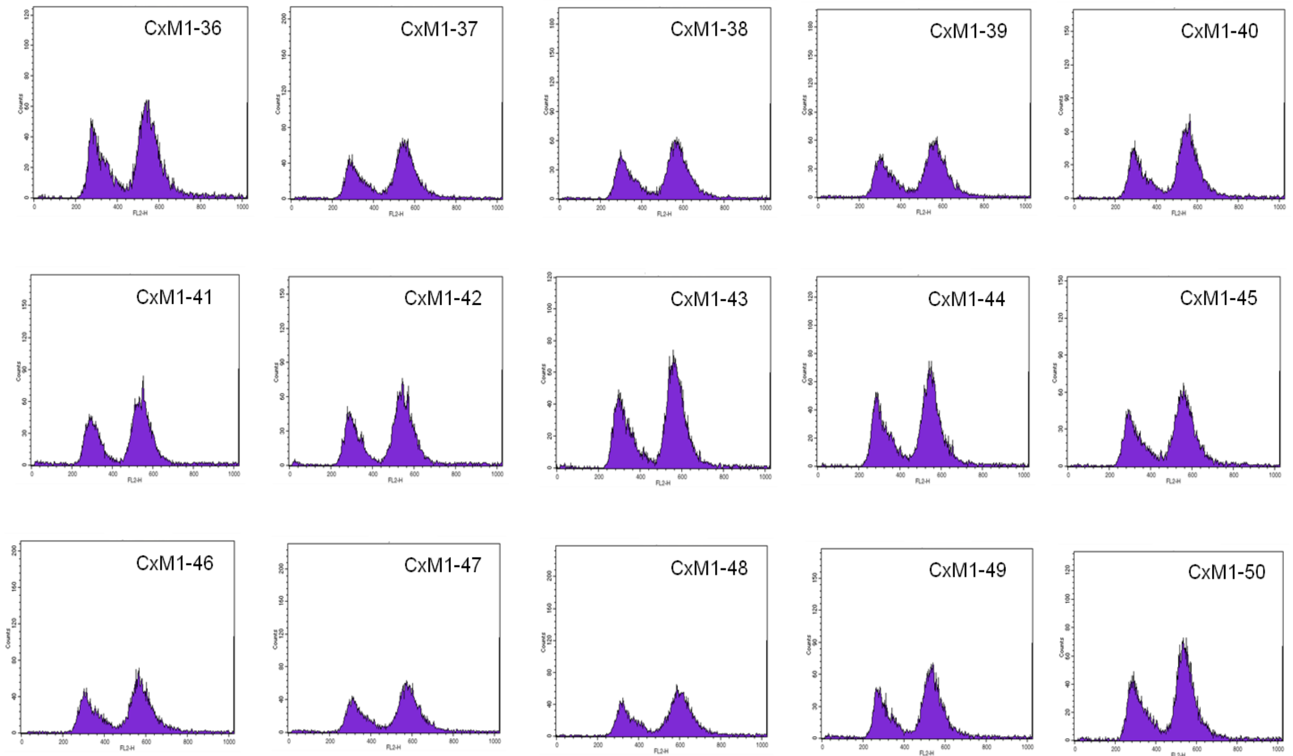

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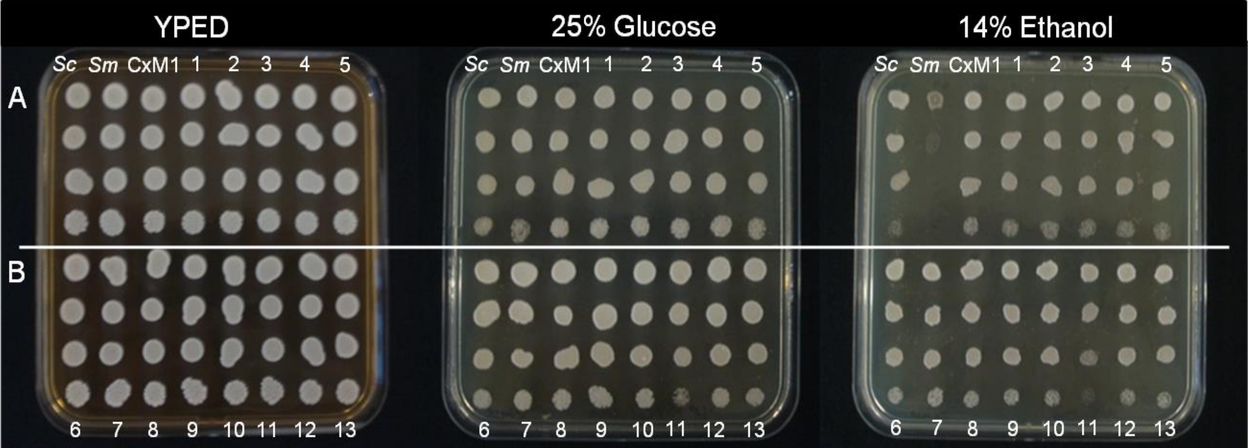

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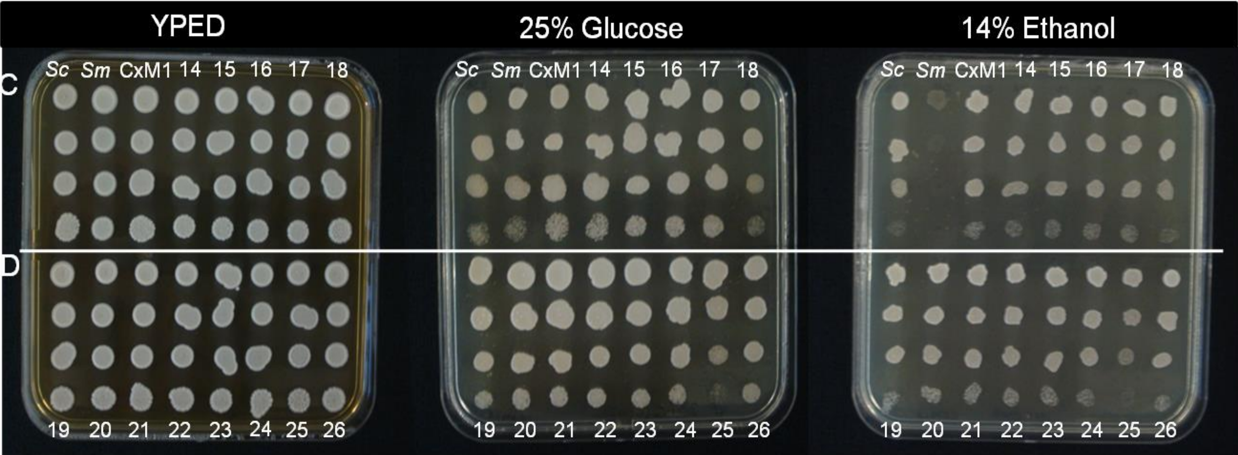

Supporting Figure S3c

Supporting Figure S3d

Statement of Authorship

Title of Paper	Designing and creating *Saccharomyces* interspecific hybrids for improved, industry relevant, phenotypes.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Jennifer R Bellon		
Contribution to the Paper	Generated and confirmed interspecific yeast hybrid strains, performed genetic stability analysis, fluorescence flow cytometry analysis, high-sugar Chardonnay fermentations, chemical analyses, interpreted data, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	16/11/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Fei Yang		
Contribution to the Paper	Performed icewine fermentations, chemical analysis, helped in data interpretation and manuscript evaluation.		
Signature		Date	Nov 13, 2017

Name of Co-Author	Martin P Day		
Contribution to the Paper	Performed wine colour analysis, assisted in data interpretation and helped to evaluate and edit the manuscript.		
Signature		Date	15/11/17

Name of Co-Author	Debra L Inglis		
Contribution to the Paper	Helped to evaluate and edit the manuscript		
Signature		Date	Nov 13/17

Name of Co-Author	Paul J Chambers		
Contribution to the Paper	Supervised development of work, helped to evaluate and edit the manuscript		
Signature		Date	16/11/17

Chapter 4

Designing and creating *Saccharomyces* interspecific hybrids for improved, industry relevant, phenotypes.

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In this manuscript, interspecific hybridisation was used in rational wine yeast development to introduce targeted outcomes designed to remediate specific winemaking problems common to high-sugar grape juice fermentations: elevated levels of volatile acidity and extended fermentation times due to inefficient fructose, uptake by yeast cells. The reported phenotype of *S. uvarum* strains to produce reduced levels of volatile acidity in wine fermentations and the knowledge that *S. uvarum* carries the *FSY1* gene (an active transporter with high affinity for fructose not found in *S. cerevisiae*) was applied to generate interspecific hybrids by rare mating between a robust *S. cerevisiae* wine yeast and spores of a *S. uvarum* strain. (At the time of publication this species was more commonly referred to as *S. bayanus*).

Hybrid progeny displayed suitability for high-sugar fermentation by utilising more fructose than their *S. cerevisiae* parent and producing wines with lower levels of acetic acid and ethyl acetate. Additionally, the hybrid yeast produced wines with novel aroma and flavour profiles and established that yeast strain choice can impact on wine colour.

Stability of hybrid genomes was confirmed following 200 mitotic generations; hybrids remained triploid and none of the 32 markers designed to monitor the presence of each arm of every parental chromosome were lost.

This work demonstrated that interspecific hybridisation can be used in rational wine yeast development to introduce targeted phenotypic outcomes. The novel interspecific hybrids generated produce wines with lower acetic acid levels relative to their *S. cerevisiae* parent and provide an opportunity for winemakers wishing to

minimize acetic acid levels in wine styles that are traditionally fraught with volatile acidity issues.

Whilst the interspecific hybrids generated in this work exhibited many desirable wine-relevant traits and was genetically stable in laboratory medium, fermentation efficiency and generally robustness was less than optimal for industrial application. In an attempt to make a commercially viable hybrid further work was conducted as described in chapter 5.

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APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Designing and creating *Saccharomyces* interspecific hybrids for improved, industry relevant, phenotypes

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Abstract To remain competitive in increasingly overcrowded markets, yeast strain development programmes are crucial for fermentation-based food and beverage industries. In a winemaking context, there are many yeast phenotypes that stand to be improved. For example, winemakers endeavouring to produce sweet dessert wines wrestle with fermentation challenges particular to fermenting high-sugar juices, which can lead to elevated volatile acidity levels and extended fermentation times. In the current study, we used natural yeast breeding techniques to generate *Saccharomyces* spp. interspecific hybrids as a non-genetically modified (GM) strategy to introduce targeted improvements in important, wine-relevant traits. The hybrids were generated by mating a robust wine strain of *Saccharomyces cerevisiae* with a wine isolate of *Saccharomyces bayanus*, a species previously reported to produce wines with low concentrations of acetic acid. Two hybrids generated from the cross showed robust fermentation properties in high-sugar grape juice and produced botrytised Riesling wines with much lower concentrations of acetic acid relative to the industrial wine yeast parent. The hybrids also displayed suitability for icewine production when bench-marked against an industry standard icewine yeast, by delivering icewines with lower levels of acetic acid. Additionally, the hybrid yeast produced wines with novel aroma and flavour profiles and established that choice of yeast strain impacts on wine colour. These new hybrid yeasts display the desired targeted fermentation phenotypes from both parents, robust fermentation in high-sugar juice and the production of wines with low volatile acidity, thus establishing their suitability for wine styles that are traditionally troubled by excessive volatile acidity levels.

Keywords *Saccharomyces* interspecific hybrids · Targeted wine yeast strain development · Non-genetically modified (non-GM) · High-sugar fermentation

Electronic supplementary material The online version of this article (doi:10.1007/s00253-015-6737-4) contains supplementary material, which is available to authorized users.

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Introduction

Developing improved strains of yeast is crucial for fermentation industries in the food and beverage sectors. There are many yeast phenotypes that stand to be improved (e.g., stress tolerance) and others that could be introduced (e.g., novel metabolic pathways for desirable flavour production) into existing strains. Whilst genetic engineering approaches provide a means of achieving this for a wide range of phenotypes, with the potential to deliver precise genetic changes and optimal quality assurance, there is reluctance by consumers in some market segments to accept genetically modified organisms (GMOs) in the human food chain. Thus, traditional approaches and variations thereof remain the only options in most food and beverage industries.

Fortunately, there are many non-GMO approaches that can be used for industrial yeast strain development. In recent years, our laboratory has used interspecific hybridization as a non-GMO strategy to produce novel desirable phenotypes in wine yeast. The hybrids were generated by mating a wine strain of *Saccharomyces cerevisiae* with *Saccharomyces paradoxus* (Bellon et al. 2011) and *Saccharomyces mikatae*

(Bellon et al. 2013). The *Saccharomyces* genus comprises several species of yeast that are evolutionarily closely related and have the same highly conserved mating system, enabling them to interbreed (see Morales and Dujon 2012 for a detailed review of interspecific hybridization in yeasts). Whilst the diploid progeny of *Saccharomyces* spp. interspecific hybrids is largely sterile, they can reproduce (and therefore grow) asexually (Naumov 1996). However, converting sterile diploid hybrids to allotetraploids restores fertility and allows the production of viable diploid spores (Greig et al. 2002). On the other hand, triploid interspecific hybrids have been shown to have poor spore viability (Sebastini et al. 2002), which is consistent with observations by Bellon et al. (2013) in which it was found that allotriploid interspecific hybrids were relatively stable.

The above hybrids were generated largely to see what was achievable in the context of their application to winemaking and to wine quality. For both hybrids, wine quality parameters were improved, but not in predictable ways. Building on this foundational work, the current study aimed to test interspecific hybridization as means of introducing targeted phenotypic changes to meet a particular challenge, namely the generation of a wine yeast strain capable of fermenting high-sugar must to make sweet dessert wines without excessive amounts of acetic acid or ethyl acetate.

Sweet dessert wines (e.g., icewine and botrytised wine) are made from grape juices with extremely high sugar content. When *S. cerevisiae* is in an environment with a high-sugar concentration, it produces increased levels of glycerol as a compatible solute. This process utilises NADH, which has to be regenerated to maintain redox balance. This is largely achieved through oxidation of acetaldehyde, leading to the production of acetic acid (Blomberg and Adler 1989).

Whilst acetic acid concentrations in Canadian commercial icewine range from 0.49 to 2.29 g/L (Nurgel et al. 2004), well below the sensory threshold of 3.185 g/L (Cliff and Pickering 2006), the production of high acetic acid levels during fermentation can lead to the esterification of acetic acid by ethanol to form another volatile metabolite, ethyl acetate, with a characteristic solvent or nail polish aroma. Nurgel et al. (2004) found that ethyl acetate concentrations in icewine ranged from 0.086 to 0.369 g/L, with some wines well above the sensory threshold of ethyl acetate of 0.198 g/L (Cliff and Pickering 2006).

In the case of botrytised wine, *Botrytis cinerea* not only concentrates sugar content in grapes, it also generates considerable amounts of acetic acid; as much as 1 g/L can be found in juice from infected grapes (Zoecklein et al. 1995a). Thus, there is a high level of acetic acid even before wine yeast begins fermentation.

In addition to the negative impacts of high osmolarity on acetic acid production, a high concentration of sugar also causes significant stress for yeast cells (Kontkanen et al. 2004) which can potentially lead to a suboptimal (stuck or sluggish) fermentation. When fermentation is compromised in this way, there is a disproportionate impact on the two major sugars in must. Grape juice contains approximately equal quantities of glucose and fructose. Uptake of these sugars by yeast is mediated by specific transporters, encoded by *HXT* genes. There are up to 20 *HXT* genes and these have varying substrate affinities (Wieczorke et al. 1999), but all have a higher affinity for glucose than fructose (Reifenberger et al. 1997). Consequently, as fermentation progresses, the ratio of fructose/glucose increases. At the same time, membrane transporters become compromised due to the increasing concentration of ethanol (Walker 1998). Extremely low glucose/fructose ratios can impact negatively on fermentation completion and result in slow or stuck fermentation (Gafner and Schütz 1996).

Unlike *S. cerevisiae*, *Saccharomyces bayanus* carries the *FSY1* gene, which encodes an active transporter with high affinity for fructose (Rodrigues de Sousa et al. 2004), thus offering the potential to reduce the risk of a suboptimal fermentation associated with accumulation of this sugar. In addition, studies have shown that some strains of *S. bayanus* contribute less acetic acid to wines than *S. cerevisiae* (Castellari et al. 1994) whilst contributing more savoury wine sensory attributes such as ‘cooked orange peel’, ‘honey’, ‘yeasty’, ‘nutty’ and ‘aldehydic’ (Eglinton et al. 2000). All-in-all, this *Saccharomyces* yeast has a great deal to offer in the context of high-sugar wine fermentations.

However, whilst phenotypic studies of *S. bayanus* grape juice isolates have shown reasonable sugar tolerance, this species has poor ethanol tolerance compared to *S. cerevisiae* wine strains (Belloch et al. 2008), which limits its usefulness in industrial wine production. Nonetheless, the combined traits of *S. bayanus* and wine strains of *S. cerevisiae* suggest progeny of a cross involving these yeasts would have the potential to efficiently ferment high-sugar juice and produce quality wine. However, the genetic basis of desirable winemaking properties in industrial yeasts is largely unknown. In this context, heterozygosities in the wine yeast parent used in this study may contribute to a wide range of wine-related phenotypes. Spore-spore hybridisations between these species have been undertaken by researchers previously (see for example: Zambonelli et al. 1997; Rainieri et al. 1998) and the assortment of chromosomes during meiosis led to the resultant hybrids displaying a diverse range of fermentation traits. For this reason, our approach has been to use diploid *S. cerevisiae* wine yeast for hybridisation relying upon a rare mating type switching event to produce mating-competent diploid cells.

In the current study, interspecific wine yeast hybrids were generated by rare mating a commercial *S. cerevisiae* wine yeast with a *S. bayanus* grape juice isolate. (The *S. bayanus* parent of the hybrids generated for this study has been molecularly typed as *S. bayanus* var *uvarum*; a subgroup of the *S. bayanus* species. Recent studies in other laboratories

indicate that this subgroup should constitute a separate species, *Saccharomyces uvarum* (Pérez-Través et al. 2014). At this time, however, there is not a single, agreed, classification so the authors have retained the existing name of *S. bayanus*.) Two progeny from the cross (AWRI 1571 and AWRI 1572) were investigated for their suitability to produce wines from high-sugar grape juices. The two strains were compared with their parents in a series of fermentations using Chardonnay juice with varying additional sugar supplementations and botrytised Riesling. Subsequently, the same two interspecific hybrid strains were assessed for icewine fermentation suitability by benchmarking against a *S. cerevisiae* industry standard yeast K1-V1116. Strains were evaluated for fermentation rates, sugar consumption patterns and production of ethanol, glycerol and acetic acid. Organic acid analyses were performed on the Chardonnay and botrytised Riesling wines, whilst ethyl acetate levels were quantified in botrytised Riesling and icewines. In addition, botrytised Riesling wines were analysed for targeted volatile flavour-active fermentation products and wine colour differences. Finally, in order to establish their potential for commercialisation, the hybrid strains were evaluated for genetic stability over 200 generations of mitotic growth.

Material and methods

Yeast strains

S. cerevisiae AWRI838 (an isolate of the commercial wine yeast strain EC1118), *S. bayanus* AWRI 1176 (isolated from fermenting grape juice) and standard commercial *S. cerevisiae* wine yeast K1-V1116 (supplied by Lallemand Inc. Montreal, QB, Canada) yeast strains were used. Control yeast strains for ploidy determinations using fluorescence flow cytometry analysis were BY4741 *MAT***a**, haploid and BY4743, diploid, (Euroscarf®, Frankfurt, Germany) and 53-7 tetraploid (Salmon 1997). AWRI strains are available from the Australian Wine Research Institute Microorganism Culture Collection (WDCM 22).

Generation of interspecific hybrid yeast

Rare mating was used for interspecific hybridizations between the diploid wine yeast *S. cerevisiae* AWRI 838 and haploid spores of a wine isolate of *S. bayanus* as described previously (Bellon et al. 2011).

PCR confirmation of hybrids

PCR-RFLP analysis on genomic DNA (Ausubel et al. 1994) of the rDNA internal transcribed spacer using the restriction enzyme *Hae*III (Esteve-Zarzoso et al. 1999) was undertaken to establish the presence of both parent species.

Genetic stability of interspecific hybrid isolates

To determine the genetic stability of interspecific hybrids over many rounds of mitotic growth, the hybrid strains were subcultured daily using nutritional liquid medium, YEPD (1 % *w/v* yeast extract, 2 %*w/v* peptone, 2 %*w/v* glucose) for 200 generations. Subsequently, 20 isolates from each hybrid were investigated using PCR-RFLP targeting each arm of the individual 16 chromosomes (Supplemental Table S1). Primer design and molecular analysis were performed as previously described (Bellon et al. 2013) using *S. cerevisiae* S288c and *S. bayanus* MCYC 623 sequences.

Fluorescence flow cytometry analysis to determine ploidy of interspecific hybrids

Ploidy analyses on newly formed hybrids were undertaken using the fluorescent dye propidium iodide as previously described (Bellon et al. 2013). Cells harvested following 200 generations of mitotic growth were analysed using a SYBR Green 1-based staining protocol which includes a protein removal step using 40 U/mL Proteinase K (Fortuna et al. 2001). SYBR Green 1-stained cells were detected at 530/30 nm (FL1) using BD FACSFlow™ (Becton Dickinson, Sydney, Australia) sheath fluid and fluorescence plotted to a linear scale. Twenty-five thousand cells per sample were analysed to obtain cell DNA intensities.

High-sugar Chardonnay fermentations

Fermentations were performed in filter sterilised Chardonnay juice: total sugars (glucose and fructose) 145 g/L, yeast assimilable nitrogen 269 mg/L, titratable acid 6.8 g/L, pH 3.01, acetic acid <0.05 g/L sourced from The Yalumba Wine Company (Angaston, South Australia) with the addition of 300 mg/L di-ammonium phosphate. High-sugar juices were prepared to 195, 250 and 355 g/L sugar levels by the addition of equal amounts of glucose and fructose.

All strains were initially grown in YEPD medium for 2 days and then acclimatised by 2 days growth in ½ X Chardonnay grape juice medium (diluted with sterile water), shaking (100 rpm), for 2 days, with the exception of the 355 g/L sugar fermentation that underwent a two-step acclimatisation over 4 days starting with a ¼ X Chardonnay grape juice medium for 2 days.

Triplicate 100 ml fermentations were carried out at 22 °C as described previously (Bellon et al. 2013) and sampled in duplicate for chemical analyses.

Botrytised Riesling fermentation

Riesling juice: total sugars (glucose and fructose) 315 g/L, yeast assimilable nitrogen 312 mg/L, titratable acid 10.5 g/L, pH 3.11 sourced from The Yalumba Wine Company (Angaston, South Australia), was filter sterilised, and fermentations were performed as previously described, using the two-step acclimitisation. Triplicate fermentations were sampled in duplicate for chemical analyses.

Wine chemical analysis

Concentrations of residual sugars (glucose and fructose), ethanol, glycerol, and acetic, succinic, lactic and citric acids, were determined by Agilent 1200 Series HPLC (Agilent, Melbourne, Australia) using a Bio-Rad HPX-87 column (Bio-Rad Laboratories, Sydney, Australia) (Nissen et al. 1997).

Targeted volatile fermentation products analysis

Stable isotope dilution combined with gas chromatography/mass spectroscopy (GC/MS) (Siebert et al. 2005) was used to analyse target compounds previously identified as important for wine flavour and aroma. Wine samples were prepared in two dilutions, 1/20 and 3/10, with model wine (11 % ethanol, 10 % potassium hydrogen tartrate, pH adjusted with tartaric acid to 3.1). Analysis was performed on an Agilent 7890A gas chromatograph equipped with Gerstel MPS2 multi-purpose sampler and coupled to an Agilent 5975C VL mass selective detector (Agilent, Melbourne, Australia). Instrument control and data analysis were performed with Agilent ChemStation software.

Wine colour

Wines were analysed spectrally to obtain the CIELab parameters L*, a*, b* (Bakker et al. 1986) by measuring the transmittance of the wine every 1 nm over the visible spectrum from 360 to 830 nm using a Varian Cary 300 spectrophotometer (Varian Australia, Melbourne, Australia) with a 10-mm quartz cuvette, a D65 illuminant and a 10° standard observer.

ΔE_{ab} values (colour difference) were determined by the Hunter-Scotfield equation (Damasceno et al. 2008)

$$\mathrm{CIELab}_{(1976)}\ \Delta E_{ab} = \left[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2\right]^{1/2}$$

Statistical analyses

A one-way analysis of variance (ANOVA) and Student's *t* test ($p<0.05$) were used to determine differences between wines produced by different yeast strains.

Icewine fermentation

Riesling icewine juice: sugars (glucose and fructose) 473 g/L, yeast assimilable nitrogen 359 mg/L, titratable acid 6.1 g/L, pH 3.48), was kindly provided by Niagara Vintage Harvesters Ltd (Virgil, ON, Canada) and filter sterilised. Three yeast strains (K1-V1116, AWRI 1571 and AWRI 1572) were inoculated from starter cultures in YEPD medium into ¼ X Riesling icewine juice (with the addition of 2 g/L di-ammonium phosphate) and grown aerobically at 25 °C with shaking at 130 rpm until cell density reached 2×10^8 cells per millilitre after which 25 ml were diluted with 25 ml of ½ X Riesling icewine juice, respectively, and held for 1 h without shaking at room temperature. A half volume (25 ml) of undiluted Riesling icewine juice was added into these 50-ml cultures, which were then held for 2 h without shaking at room temperature.

Following this acclimatisation procedure, each 75-ml starter cultures was inoculated into 425 ml of 1 X Riesling icewine juice to achieve a yeast inoculum rate of 1×10^7 cells per millitre in a final volume of 500 ml. Fermentations were carried out at 17 °C in triplicate and continued until the yeast stopped consuming sugar, signaled by no further change in sugar concentration for 3 days. Sugar concentration was measured by the Lane-Eynon method (Zoecklein et al. 1995b).

Yeast cell densities were determined by cell counting in a haemocytometer. Acetic acid, glycerol, ammonia nitrogen and primary amino nitrogen were determined using enzymatic kits (Megazyme International Ireland Ltd, Bray, Ireland). Ethanol and ethyl acetate were measured using gas chromatography (Agilent, Santa Clara, USA) using an Agilent 6890 system equipped with flame ionisation detector and DB Wax (30 m×0.23 mm×0.25 μm) column. The carrier gas was helium. For ethanol measurement, samples were diluted 10-fold and 1.0 μl was injected into the injection port heated to 225 °C. The column head pressure was set as 24.4 psig, and the flow rate of helium gas was 2.5 ml/min. The oven temperature was programmed to start at 60 °C, increase to 95 °C at 15 °C ml/min and then increase to 225 °C at 75 ml/min and hold for 1 min. The detector temperature was 225 °C, and 2 % 1-butanol was used as an internal standard. For ethyl acetate measurement, 1.0 μl of sample was injected and heated to 230 °C. The column head pressure was 15.4 psig, and helium flow rate was 1.5 ml/min. The oven temperature was hold at 35 °C for 2 min, and then increased to 230 °C at 10 ml/min and hold for 2 min. The detector temperature was 230 °C, and 5 % 4-methyl-2-pentanol (2 g/L) was used as an internal standard.

Differences between variables were determined by XLSTAT statistical software package released by Addinsoft (Version 7.1; Paris, France). Analysis of variance (ANOVA) with mean separation by Fisher's Least Significant Difference (LSD, $p<0.05$) was used for statistical analysis.

Results

Generation and genetic stability of interspecific hybrids

Two interspecific hybrid colonies (AWRI 1571 and AWRI 1572) were generated through the rare mating of the diploid *S. cerevisiae* wine yeast strain AWRI838 with spores of *S. bayanus* AWRI 1176. The existence of both parental genomes in these hybrids was confirmed by species-specific PCR-RFLP of target rDNA (Fig. 1).

Fluorescence flow cytometry analysis with linear plots of cell fluorescence was performed to determine ploidy levels of hybrid strains. Although cells were grown to late stationary phase, all cultures produced dual peaks of fluorescence and this could be attributed to some cells undergoing DNA synthesis, or perhaps more likely, it reflects cell pairs where mother-daughter cells have not yet completely separated. Diploid and tetraploid control strains gave non-dividing (G0/G1) fluorescent peaks in the region of double or quadruple fluorescent levels of the control haploid strain, respectively. Parental yeast strains, AWRI 838 and AWRI 1176, gave fluorescent peaks equivalent to the diploid control strain whilst both hybrid strains appear to have a triploid genome complement, with non-dividing peak levels approximately midway between diploid and tetraploid peaks (Fig. 2).

In addition, the genome of each hybrid strain was assessed for genetic stability after 200 mitotic generations. Species-specific PCR-RFLP markers to each arm of all 16 chromosomes revealed that the incipient hybrids were stable as no loss of chromosome from either parent species was identified in any of the twenty isolates from each hybrid investigated (Supplemental Data Fig. S1). Also, the hybrids remained stable triploids as no reduction in DNA fluorescence levels was observed in any 200-generation hybrid isolates (Supplemental data Fig. S2).

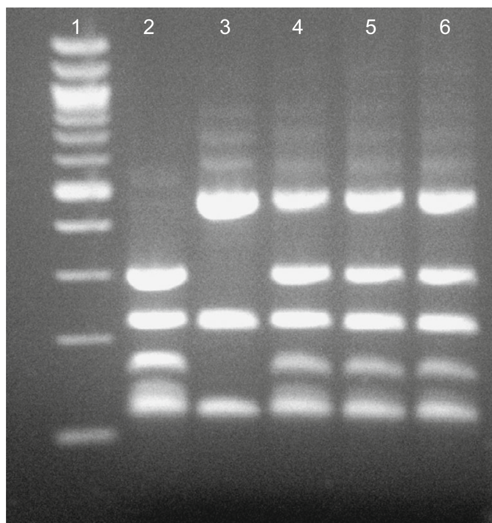

Fig. 1 Confirmation of interspecific hybridization by rDNA ITS PCR-RFLP. *Lane 1* 100 bp ladder, *lane 2* AWRI 838, *lane 3* AWRI 1176, *lane 4* DNA from both parents, *lane 5* AWRI 1571, *lane 6* AWRI 1572

Chardonnay wines

Four sets of replicate Chardonnay grape juice containing a range of reducing sugar concentrations were fermented with either a commercial *S. cerevisiae* strain AWRI 838, *S. bayanus* AWRI 1176 or their hybrid progeny AWRI 1571 or 1572. Analysis of the final wines revealed that all strains were capable of completing fermentation at the lowest sugar concentration of 145 mg/L and all were challenged, to varying degrees, by juices with 355 g/L reducing sugar (Fig. 3a, b).

Generally, the least robust yeast was the *bayanus* strain AWRI 1176, in most cases producing wines with higher levels of residual glucose (Fig. 3a) and fructose (Fig. 3b). AWRI1176 wines made from juice with 355 g/L had not only higher fructose concentrations, but more than double the concentration of residual glucose than wines made by *S. cerevisiae* AWRI 838 (57 g/L compared to 26 g/L). In contrast, hybrid strains AWRI 1571 and AWRI 1572 produced wines with the lowest residual sugars in all Chardonnay juices, but most notable in 355 g/L sugar Chardonnay juice (17.4 and 19.5 g/L glucose compared to 25.9 g/L produced by AWRI 838, and 65 and 67 g/L fructose compared to 78 g/L produced by AWRI 838).

From Fig. 3c, it is evident that at lower levels of reducing sugar (145 and 195 g/L), *S. cerevisiae* AWRI 838 produced wines with more than double the amount of acetic acid than *S. bayanus* AWRI 1176 and hybrids AWRI 1571 and 1572. However, whilst *S. cerevisiae* AWRI 838 still produced almost double the amount of acetic acid relative to the hybrid strains in fermentations with higher concentrations of reducing sugar (0.38 g/L compared to 0.15 g/L for hybrid strains in 250 g/L reducing sugar and 1.11 g/L compared to 0.65 g/L for hybrid strains in 355 g/L reducing sugar), *S. bayanus* AWRI 1176 produced wines with excessively high concentrations of acetic acid, 0.61 and 1.42 g/L in 250 g/L reducing sugar and 355 g/L reducing sugar, respectively.

Glycerol content in final wines is shown in Fig. 3d. Whereas *S. bayanus* AWRI 1176 generally produced wines with glycerol concentration 2 g/L higher than *S. cerevisiae* AWRI 838 in each juice, at lower concentrations of reducing sugar the hybrid strains produced wines with glycerol levels similar to their *S. cerevisiae* parent, AWRI 838, but in 355 g/ L reducing sugar the production of glycerol by the hybrid strains increased dramatically to levels higher than their *S. bayanus* parent, reaching 17.8 and 15.7 g/L for AWRI 1571 and 1572, respectively, compared to 14.8 g/L for AWRI 1176 and 12.7 g/ L for AWRI 838.

Little difference was observed in ethanol production between *S. cerevisiae* AWRI 838 and hybrid strains AWRI

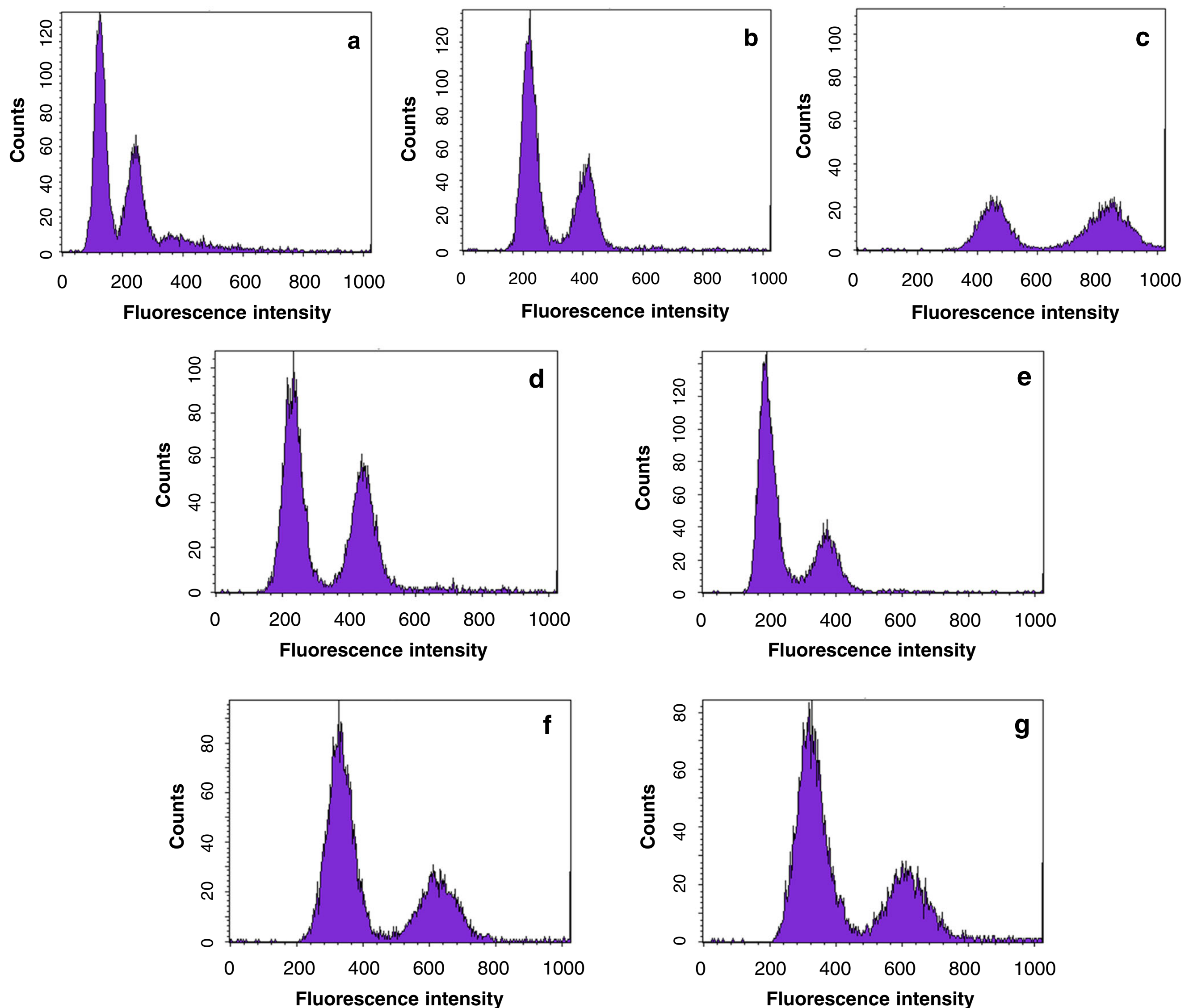

Fig. 2 Fluorescence flow cytometry analysis. **a** Control ploidy strain BY4741 (haploid). **b** Control strain BY4743 (diploid). **c** Control strain 53–7 (tetraploid). **d** Parent strain AWRI 838. **e** Parent strain AWRI 1176. **f** Hybrid strain AWRI 1571. **g** Hybrid strain AWRI 1572

1571 and 1572 (Fig. 3e). *S. bayanus* AWRI 1176 made wine with reduced concentrations of ethanol in each juice, particularly in 250 and 355 g/L reducing sugar, but this result is attributable to the much higher residual sugar levels in the final wines.

Measurements of cell growth (optical density) and sugar utilisation (refractive index) during all Chardonnay fermentations are provided in supplementary data (Supplemental data Fig. S3 and S4 respectively). *S. cerevisiae* strain AWRI 838 showed the most robust fermentation properties of all strains, evident for cell growth and final cell density following fermentation of the juice with the highest sugar concentration (Supplemental data Fig. S3d). Although little difference in sugar utilisation was observed between strains at lower concentrations of juice sugar, *S. bayanus* strain AWRI 1176 displayed a slower rate of sugar utilisation at higher juice sugar levels (Supplemental data Fig. S4c and S4d).

Botrytised Riesling wines

All four yeast strains started well in botrytised Riesling juice. However, after day 2, *S. bayanus* strain AWRI 1176 grew at a slower rate than the other three strains, which had similar increases in cell densities (Fig. 4a) and sugar utilisation profiles (Fig. 4b). The lower cell density of AWRI 1176 was reflected in a reduced level of sugar utilisation during fermentation with the finished wine having higher residual sugars, glucose 69 g/L and fructose 139 g/L compared to glucose concentrations of 47–52 g/L and fructose concentrations of 119–124 g/L in *S. cerevisiae* and hybrid-made wines (Table 1).

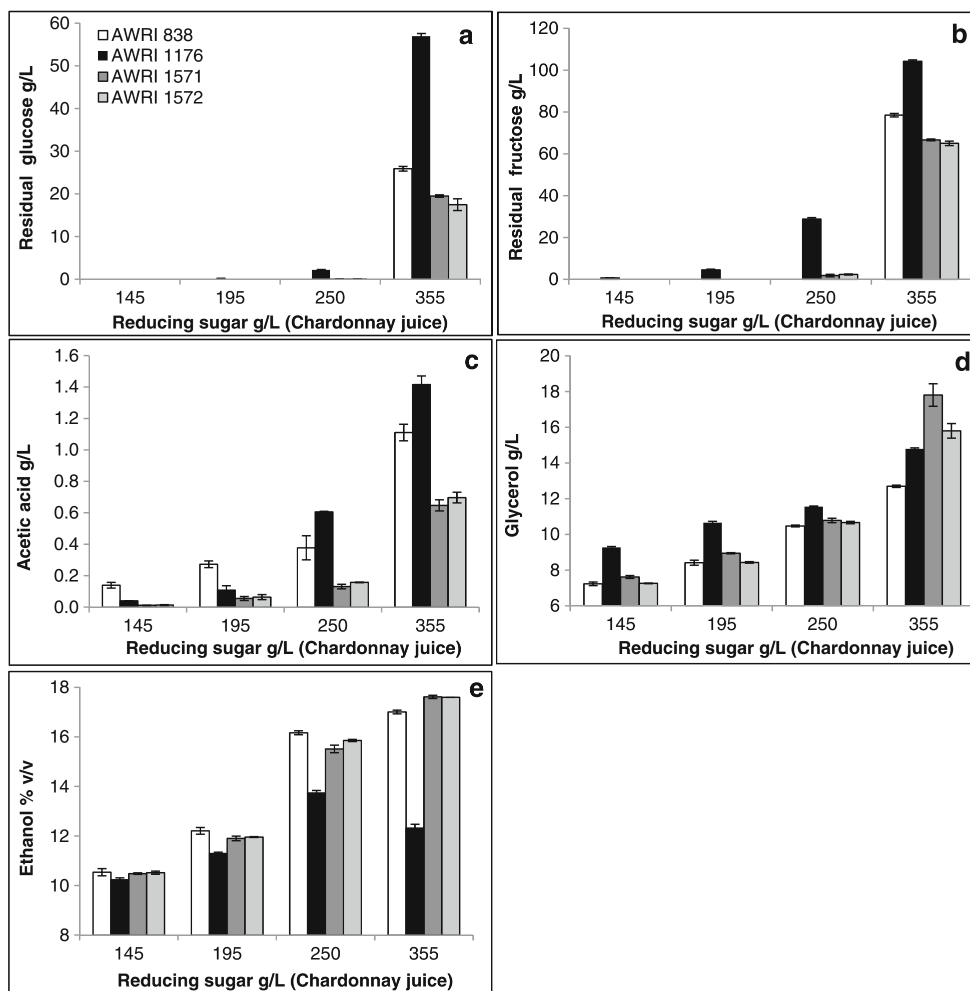

Fig. 3 Basic chemical composition of Chardonnay wines following fermentation of juices with varying sugar concentrations. Data points are presented with *error bars*. **a** Residual glucose. **b** Residual fructose. **c** Acetic acid concentrations. **d** Glycerol concentrations. **e** Ethanol concentrations

AWRI 1176 also produced wines with a lower ethanol concentration, 9.5 %*v/v* compared to 12.1–12.5 %*v/v* for *S. cerevisiae* and hybrid-made wines. All strains produced wines with similar levels of glycerol (23.4 to 24.5 g/L) and little difference was observed in succinic, lactic and citric acids levels. However, acetic acid concentrations varied considerably between wines (Table 1), with AWRI 1176 (*S. bayanus*) and AWRI 838 (*S. cerevisiae*) producing considerably more than the hybrid strains AWRI 1571 and AWRI 1572, (1.1 and 0.9 g/L compared to 0.55 and 0.67 g/L, respectively).

Analysis of volatile fermentation products (Table 2) revealed that there were clear differences between the two 'parental wines' (there were significant differences for 13 of the 16 compounds analysed), and these differed from the hybrid-made wines. In general, hybrid AWRI 1571 produced wines

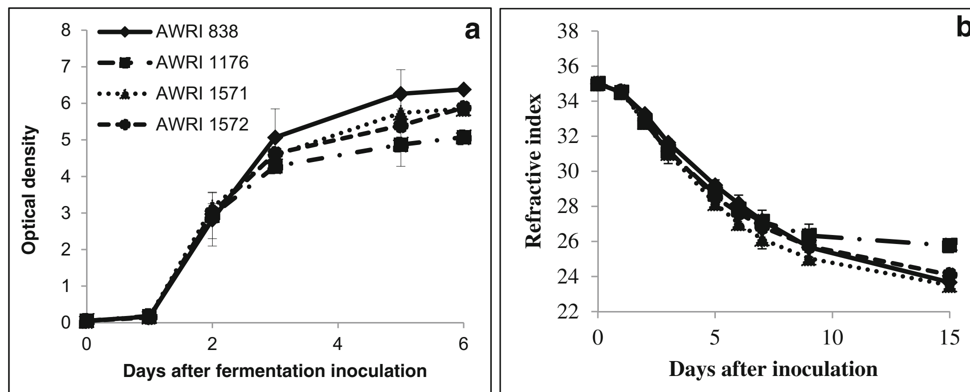

Fig. 4 Botrytised Riesling fermentations, data points are presented with *error bars*. **a** Cell growth as determined by optical density. **b** Sugar utilisation as determined by refractive index

Table 1 Fermentation products in botrytised Riesling wines

Compound (g/L)	AWRI838	AWRI1176	AWRI1571	AWRI1572
Glucose	52.1±0.01 b	69.0±2.8 a	47.7±0.4 c	49.7±0.5 b
Fructose	123±1 b	139±2 a	119±1 c	125±1 b
Glycerol	23.3±0.2 a	23.4±0.1 a	24.5±0.5 a	24.4±0.3 a
Ethanol[a]	12.1±0.2 a	9.5±0.1 b	12.5±0.1 a	12.4±0.1 a
Acetic acid	0.91±0.05 a	1.1±0.06 a	0.55±0.01 b	0.67±0.03 b
Succinic acid	8.92±0.04 b	9.60±0.09 a	9.61±0.22 ab	9.30±0.19 ab
Lactic acid	3.92±0.04 a	3.81±0.04 a	3.83±0.08 a	3.80±0.19 a
Citric acid	2.55±0.01 a	1.98±0.01 c	2.21±0.02 b	2.22±0.01 b

Within a row, values connected by same letter are not significantly different ($p<0.05$)

[a] % (*v/v*)

with volatile fermentation product concentrations similar to that of the *S. cerevisiae* parent, including similar levels of ethyl acetate. Only two compounds followed solely the *S. bayanus* parental profile, 2-methyl propyl acetate (banana) and 2-methyl propanol (fusel), whilst two compounds were produced at an intermediate level, ethyl 3-methyl butanoate (berry) and 2-phenyl ethyl acetate. Ethyl 2-methyl butanoate (sweet fruit) was produced at a higher concentration than for either parent.

Wines produced by hybrid AWRI 1572 were less similar to *S. cerevisiae* parent-made wine, with only seven of the sixteen compounds analysed following the *S. cerevisiae* parent wine profile. Four compounds were produced in much lower concentrations than either parent, ethyl acetate (nail polish), ethyl 2-methyl propanoate (fruity), 2-methyl propyl acetate (banana) and 2-methyl propanol (fusel). Conversely, butanol (fusel) was produced in much higher concentrations than either parent, 721 μg/L compared to 630 μg/L for AWRI 838 (*S. cerevisiae*) and 445 μg/L AWRI 1176 (*S. bayanus*).

Wine colour was analysed spectrally by measuring absorbance across the visible range of the spectrum from 360 to 830 nm, using CIELab parameters, L* (a measure of intensity, the higher the value the lighter the colour), a* (positive values relate to redness, negative values to greenness) and b* (positive values relate to yellowness, negative values to blueness). Browness of wines is attributed to absorbance at 420 nm.

Botrytised Riesling wines made by *S. bayanus* AWRI 1176 had the strongest browness (A_{420} 58.36) with *S. cerevisiae* AWRI 838 having the weakest (A_{420} 42.91), whilst wines made by hybrids AWRI 1571 and 1572 showed an intermediate level of brownness (A_{420} 53.09 and A_{420} 48.77,

Table 2 Volatile fermentation products in botrytised Riesling wines

Compound (μg/L)	Aroma descriptor	AWRI838	AWRI1176	AWRI1571	AWRI1572
Ethyl acetate	Nail polish	20971±1480 a	21014±2882 a	16800±650 a	13746±810 b
Ethyl propanoate	Fruity	136±19 ab	93±12 c	143±4 a	113±5 bc
Ethyl 2-methyl propanoate	Fruity	103±16 a	115±22 a	103±5 a	62±1 b
Ethyl butanoate	Fruity	74±17 a	27±7 b	79±3 a	68±1 a
Ethyl 2-methyl butanoate	Sweet fruit	2.65±0.2 b	n.d. c	3.75±0.05 a	3.15±0.2 b
Ethyl 3-methyl butanoate	Berry	3.25±0.2 a	2.05±0.2 c	2.8±0.1 ab	2.55±0.2 bc
Ethyl hexanoate	Green apple	170±36 a	67±17 b	190±16 a	168±4 a
2-Methyl propyl acetate	Banana, fruity	49.9±9.1 a	36.9±7.1 ab	30.7±1.8 b	21.1±0.3 c
2-Methyl butyl acetate	Banana, fruity	20.9±3.1 a	11.9±1.2 c	15.8±1.8 abc	14.1±1.1 bc
3-Methyl butyl acetate	Banana	304±61 a	164±30 b	212±13 b	165±1 b
2-Phenyl ethyl acetate	Floral	n.d. c	342±44 a	95±15 b	85±18 b
2-Methyl propanol	Fusel, spirituous	98810±880 a	76620±630 b	79590±405 b	57060±240 c
Butanol	Fusel, spirituous	630±30 b	445±9 c	643±2 b	721±14 a
2-Methyl butanol	Nail polish	4648±610 a	n.d. b	4427±45 a	4472±290 a
3-Methyl butanol	Harsh, nail polish	6873±510 a	3193±536 b	6898±804 a	6085±757 a
Hexanol	Green, grass	1850±862 a	1238±16 a	1357±162 a	1370±177 a

Within a row, values connected by same letter are not significantly different ($p<0.05$)

n.d. not detected

respectively) (Table 3). The CIELab L* parameter revealed that the intensity of colour was in the reverse order to browness, AWRI 1176 wines being lightest and AWRI 838 wines being darkest. The hybrid-made wines also showed intermediate levels of red/green and yellow/blue hues, AWRI 838-produced wines with both higher a* values, (less negative value relates to less green colour) and higher b* values (more yellow) whilst AWRI 1176 produced wines with the greatest green colour and the least yellow colour. The obvious visual differences in colour intensity and hue between the wines was confirmed with calculated ΔE_{ab} values of 11.1 (AWRI 838/AWRI 1176), 8.6 (AWRI 838/AWRI 1571) and 6.0 (AWRI 838/AWRI 1572). Note that ΔE_{ab} >1 indicates that samples are just readily perceived visually as different to each other in colour.

Icewines

Riesling icewine fermentations were conducted with three yeast strains; commercial *S. cerevisiae* icewine standard yeast K1-V1116, and two *S. cerevisiae* x *S. bayanus* hybrid strains AWRI 1571 and AWRI 1572. Although growth appeared slower and reached a lower cell density for hybrid strains AWRI 1571 and 1572 relative to the commercial strain K1-V1116 (Supplemental data Fig. S5), all three strains showed similar rates of sugar consumption throughout fermentation (Fig. 5a) with final residual sugar values being virtually identical (ranging from 263 to 267 g/L) (Table 4)

Commercial wine yeast K1-V1116 showed the highest production of acetic acid during the fermentation (Fig. 5b) with a final wine concentration of 2.13 g/L, whilst hybrid strains AWRI 1571 and 1572 produced significantly lower levels at 1.72 and 1.57 g/L, respectively (Table 4).

Interestingly, analysis of ethanol, glycerol and ethyl acetate concentrations of wines made by standard yeast K1-V1116 and wines made by hybrids AWRI 1571 and AWRI 1572 showed no significant differences with average concentrations of 10.5 % *v/w* ethanol, 10.1 g/L glycerol and 73.7 mg/L ethyl acetate (Table 4).

Table 3 Spectral colour measurements of botrytised Riesling wines including CIELab parameters

	A_{420}	L*	a*	b*	Δ E_{ab} (cf AWRI 838)
AWRI 838	42.91	92.72	−0.0369	25.73	
AWRI 1176	58.36	96.12	−1.0496	58.66	11.1 ab
AWRI 1571	53.09	95.06	−0.92005	17.54	8.6 bc
AWRI 1572	48.77	94.61	−0.6475	20.05	6.0 c

ΔE_{ab} >1 indicates that samples are just visually readily perceived as different to each other in colour. Within a row, values connected by same letter are not significantly different ($p<0.05$)

Discussion

Microbial strain development for the food and beverage sector is hindered by consumer reluctance to accept genetically modified organisms (GMOs) in the human food chain. This has led researchers to return to traditional approaches including targeted breeding. Whilst such strategies lack the precision of genetically modified (GM) techniques and quality assurance is more of a challenge, there are many non-GMO methods available, particularly when working with highly tractable microbes such as *Saccharomyces* spp.

Building on prior research and development that tested the feasibility of using interspecific breeding of yeasts in the *Saccharomyces* genus to generate novel phenotypes for application in winemaking (Bellon et al. 2011, 2013), the current manuscript describes a proof of concept trial to generate interspecific *Saccharomyces* spp. hybrids to introduce targeted improvements in important, wine-relevant traits. Specifically, the aim was to generate novel wine yeast that can tolerate the many challenges of growing in and fermenting high-sugar grape juice without generating excessive volatile acidity in the form of acetic acid and ethyl acetate.

Winemakers endeavouring to produce sweet dessert wines from high-sugar juices require wine yeasts that can conduct efficient fermentation in reasonable time whilst reaching a target ethanol concentration (10 and 13 % *v/v* ethanol for Canadian icewine and French Sauternes, respectively, for instance). However, fermentations involving high-sugar juices commonly suffer from elevated volatile acidity levels and extended fermentation times.

S. bayanus has been reported to produce reduced levels of volatile acidity in wine fermentations (Eglinton et al. 2000), and has some potential advantages in dealing one of the problems associated with suboptimal (slow or sluggish) fermentation, namely the production of heightened ratios of fructose/glucose in the latter stage of fermentation. This becomes particularly pronounced in suboptimal fermentations and is due to *S. cerevisiae* lacking a high affinity fructose transporter. An active fructose-specific transport system (*FSY1*) has been identified in *S. bayanus* (Rodrigues de Sousa et al. 2004),

Whilst *S. bayanus* may seem like a good option to trial in high-sugar fermentations, it is not sufficiently robust to deal with the harsh conditions of grape must fermentation; it is highly affected by high glucose content and high ethanol content in culture media (Belloch et al. 2008). However, it's potential when mated with a robust *S. cerevisiae* wine yeast is clearly worth exploring.

In the current study, laboratory-scale fermentations in high-sugar juice (Chardonnay with sugar additions, botrytised Riesling and Riesling icewine) were used to investigate the suitability of two *S. cerevisiae*×*S. bayanus* hybrid strains (AWRI 1571 and AWRI 1572) for high-sugar grape juice wine production. The strategy of rare mating (where a diploid

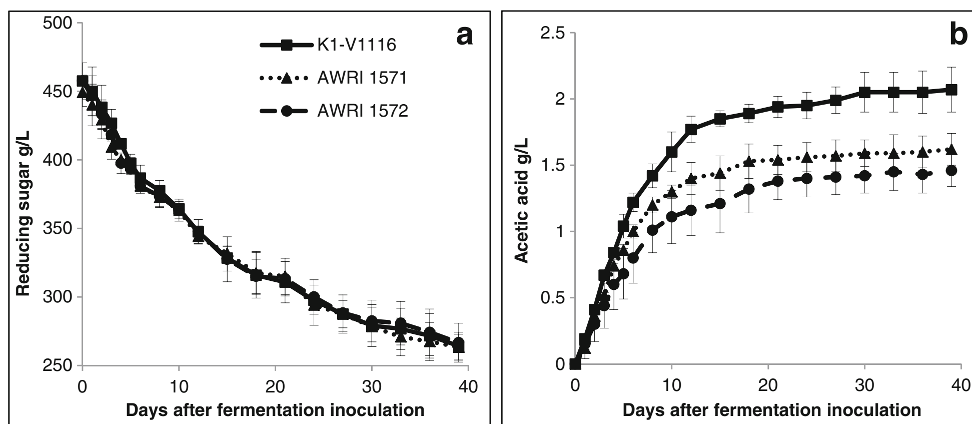

Fig. 5 Icewine fermentations, data points are presented with *error bars*. **a** Sugar utilisation. **b** Acetic acid production

cell becomes homozygous for mating type and can mate without sporulation) was adopted to ensure that no loss of important fermentation traits from the wine yeast parent would occur due to the assortment of chromosomes that would occur if it was sporulated (Zambonelli et al. 1997). Previous reports have identified stable natural allotriploid yeast from the wine industry including the wine yeast VIN7 (Borneman et al. 2012) and the spoilage yeast *Brettanomyces bruxellensis* (Curtin et al. 2012).

The putative hybrids from successful rare mating events between a diploid *S. cerevisiae* commercial wine yeast AWRI 838 and haploid spores of a *S. bayanus* Australian grape juice isolate, AWRI 1176, were confirmed using PCR-RFLP analysis of the ITS region within the rDNA tandem repeat on chromosome XII. Fluorescence flow cytometry analysis showed that hybrid ploidy content was consistent with fluorescent levels of a triploid genome with peaks midway between that of the diploid and tetraploid genome control strains. Genome instability and ploidy reduction have previously been reported in *S. cerevisiae* polyploids (Mayer and Aguilera 1990) and *Saccharomyces* interspecific hybrids (Kunicka-Styczyńska and Rajkowska 2011; Kumaran et al. 2013). Thus it was important to evaluate stability of the hybrids generated for the current study. Thirty-two species-specific genetic markers were designed to monitor the presence of each arm of every parental chromosome. None of these markers were lost from either hybrid after two hundred mitotic generations. Furthermore, fluorescence flow cytometry analyses confirmed that each hybrid remained triploid. In addition, it is important to note that wine yeast is not re-pitched as in the brewing industry. Instead, inocula are generated fresh from stock cultures each vintage and go through no more than 8–10 generations during a wine fermentation. Thus, it is unlikely that genomic instability will prove to be an issue in the application of the yeast strains generated in the current study.

An assessment of fermentation ability showed that, generally, the interspecific hybrids fermented the various high-sugar juices at least as well as the *S. cerevisiae* wine yeast parent, with similar levels of residual sugar, similar growth curves and similar rates of sugar utilisation. In contrast, and as expected, the *S. bayanus* parent performed poorly except at the lowest concentrations of sugar in the Chardonnay juice fermentations. In addition, the hybrid yeasts wines had reduced levels of volatile acidity for all three musts.

Other wine quality parameters (glycerol, ethanol, succinate, etc. concentrations) were favourable, largely being similar to AWRI 838 wine yeast or falling somewhere between the two parents. In the Chardonnay juice fermentations at high-sugar levels, the two hybrid strains outperformed the *S. cerevisiae* wine yeast in sugar utilisation and were similar to this wine yeast in all other respects apart from acetic acid and glycerol production. Acetic acid levels for both hybrids were about 60 % of what was found in the *S. cerevisiae*-made wines and glycerol levels were slightly greater. Interestingly, whilst the *S. bayanus* parent, as expected, produced wines with lower levels of acetic acid compared to the *S. cerevisiae* parent at lower sugar levels, this was reversed in juices with 250 g/L and above. Whilst the reason for this is unknown, the phenotype did not carry over into the hybrids.

Botrytised Riesling wines produced by hybrid yeast strains showed differences in concentrations in a number of the volatile secondary metabolites relative to wines made by *S. cerevisiae* parent AWRI 838. Both hybrids produced acetic

Table 4 Fermentation products in icewines

Compound (g/L)	K1-V1116	AWRI 1571	AWRI 1572
Sugar	264±10 a	263±10 a	267±14 a
Ethanol [a]	10.8±1.4 a	10.5±0.9 a	10.3±1.2 a
Glycerol	9.57±0.39 a	10.22±0.43 a	10.46±0.95 a
Acetic acid	2.13±0.16 a	1.72±0.10 b	1.57±0.19 b
Ethyl acetate[b]	79±31 a	79±22 a	63±21 a

[a] % (*v/v*)

[b] mg/L

Within a row, values connected by same letter are not significantly different ($p<0.05$)

acid at levels at about 65 % of the *S. cerevisiae* parent but in the case of ethyl acetate only AWRI 1572 produced significantly lower levels. Nonetheless, ethyl acetate has been shown to have a suppressive effect on the formation of other fruity-aroma compounds, even at concentrations below the sensory threshold for this compound (Etiévant 1991). In addition, ethyl acetate levels below the sensory threshold can impart an added richness and sweetness, whereas levels above convey a characteristic solvent or nail polish remover aroma. Importantly, both hybrids produced less volatile acidity overall than the *S. cerevisiae* parent.

Desirable (from a winemaking perspective) flavour-active compounds were produced in higher concentrations by one or both hybrid strains relative to their *S. cerevisiae* wine yeast parent; ethyl 2-methyl butanoate ('sweet fruit') and 2-phenyl ethyl acetate ('floral'). Increasing the concentration of a flavour or aroma compound can lead to an increased sensory impact of that particular compound, and can also lead to the masking of other (potentially non-desirable) flavours or aromas (Saison et al. 2009).

Production of volatiles in the hybrids was moderated relative to the *S. cerevisiae* parent: a number of metabolites were produced in mid-range concentrations by both hybrids relative to the parental strains. Generally, wine made by hybrid AWRI 1571 followed the highest metabolite concentration produced by *S. cerevisiae* parent AWRI 838 more often than hybrid AWRI 1572, although, on a small number of occasions, metabolites were produced at lower concentrations similar to *S. bayanus* parent yeast AWRI 1176. Interestingly, transgressive phenotypes were more apparent in hybrid AWRI 1572 than hybrid AWRI 1571, in the form of both increased and decreased concentrations in a number of secondary metabolites. A positive aspect to the production of lower metabolite concentrations is that this hybrid yeast produced much lower concentrations of two compounds with negative sensory attributes, ethyl acetate ('nail polish') and 2-methyl propanol ('fusel').

It is important to note that differences between the phenoypes of the two hybrids are not unexpected. Whilst they have the same *S. cerevisiae* genomic inputs, the *S. bayanus* parent was sporulated prior to mating to produce haploids. The meiotic events in this process would have generated genetic variants with differing phenotypic traits (Zambonelli et al. 1997).

Colour is one of the first wine sensory properties evaluated in the glass and invariably one of the first descriptors used in assessing a botrytised wine, with colour terms ranging from 'glowing yellow-green' to 'pale gold', 'deep gold' and 'amber'. The easily visually discernible colour differences between the botrytised wines were confirmed by high ΔE^*_{ab} values recorded for each assessment of *S. cerevisiae* wines relative to *S. bayanus* and hybrid wines; 11.1 (*S. cerevisiae* AWRI 838/*S. bayanus* AWRI 1176), 8.6 (*S. cerevisiae* AWRI 838/hybrid AWRI 1571) and 6.0 (*S. cerevisiae* AWRI 838/hybrid AWRI 1572).

B. cinerea-infected vines have a grey, powdery appearance with the berries developing a light brown colour in white cultivars, resulting in a distinctly brown-coloured grape juice. *B. cinerea* produces a powerful oxidative enzyme (laccase) that can oxidise hydroxycinnamic acids (grape juice phenolic compounds caftaric and coutaric acid) to caftaric acid *o*-quinone. Condensation reactions of cafteric acid *o*-quinone generate brown polymeric pigments (Salgues et al. 1986). Glutathione can interfere with this process by trapping caftaric acid quinones in the form of 2-S-glutathionyl caftaric acid, also referred to as grape reaction product (GRP). The formation of GRP is believed to limit juice browning (Cheynier et al. 1986). As all botrytised Riesling fermentations were conducted in the same juice and under the same conditions, the differences in wine colour can be attributed to differences in yeast metabolism between the different strains. Studies have shown that the ratio of grape hydroxycinnamic acids to glutathione molar ratio alone does not correlate well with oxidative browning and that the presence of other compounds capable of trapping free *o*-quinones may be involved (Cheynier et al. 1990). Both hybrid strains produced wine colour attributes intermediate to the individual parent-made wines, indicating that genetic material inherited from both parents impact on yeast fermentation metabolites involved in the development of wine colour. Descriptor marketing plays an important role for wine companies wanting an edge in tight economical times and yeast that can deliver colour variations to the commonly industry-used *S. cerevisiae* strains could assist winemakers in developing novel wine styles.

The hybrid strains also displayed suitability for icewine production. Whilst the hybrid strains consumed equivalent amounts of sugar as *S. cerevisiae* industry standard yeast K1-V1116 during fermentation, their wines contained acetic acid levels of approximately 75 % relative to the *S. cerevisiae*-made wine. No difference in ethanol, glycerol and ethyl acetate concentrations were seen between hybrid-made wines and *S. cerevisiae*-made wine. Growth curves for the three strains in icewine indicate that the hybrids had a lower growth rate and reached a lower final cell number than K1-V1116. This however appears not to have impacted on their fermentation performance.

In conclusion, this manuscript demonstrates that interspecific hybridization can be used in rational wine yeast development to introduce targeted phenotypic outcomes. The introduction of genetic material from non-*cerevisiae* *Saccharomyces* species to traditional *S. cerevisiae* wine yeast can impact positively on wine yeast metabolite production during fermentation of high-sugar musts to deliver wines with low volatile acidity. Novel interspecific hybrids generated from a cross between a robust *S. cerevisiae* wine yeast and a *S. bayanus* grape juice isolate produce botrytised Riesling

wines with much lower concentrations of acetic acid relative to the industrial wine yeast parent and lower levels of acetic acid when benchmarked against an industry standard icewine yeast. Additionally, the hybrid yeast produce wines with novel aroma and flavour profiles and establish that yeast strain choice can impact on wine colour. These new wine yeast provide an opportunity for winemakers wishing to minimise acetic acid levels in wine styles that are traditionally fraught with volatile acidity issues.

This study was performed at laboratory-scale as 'proof of concept'. Future industry-scale winemaking trials will determine the commercial potential of these strains for application in the wine industry.

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Conflict of interest The authors state that they have no conflicts of interest to disclose.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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Designing and creating *Saccharomyces* interspecific hybrids for improved, industry relevant, phenotypes

Applied Microbiology and Biotechnology

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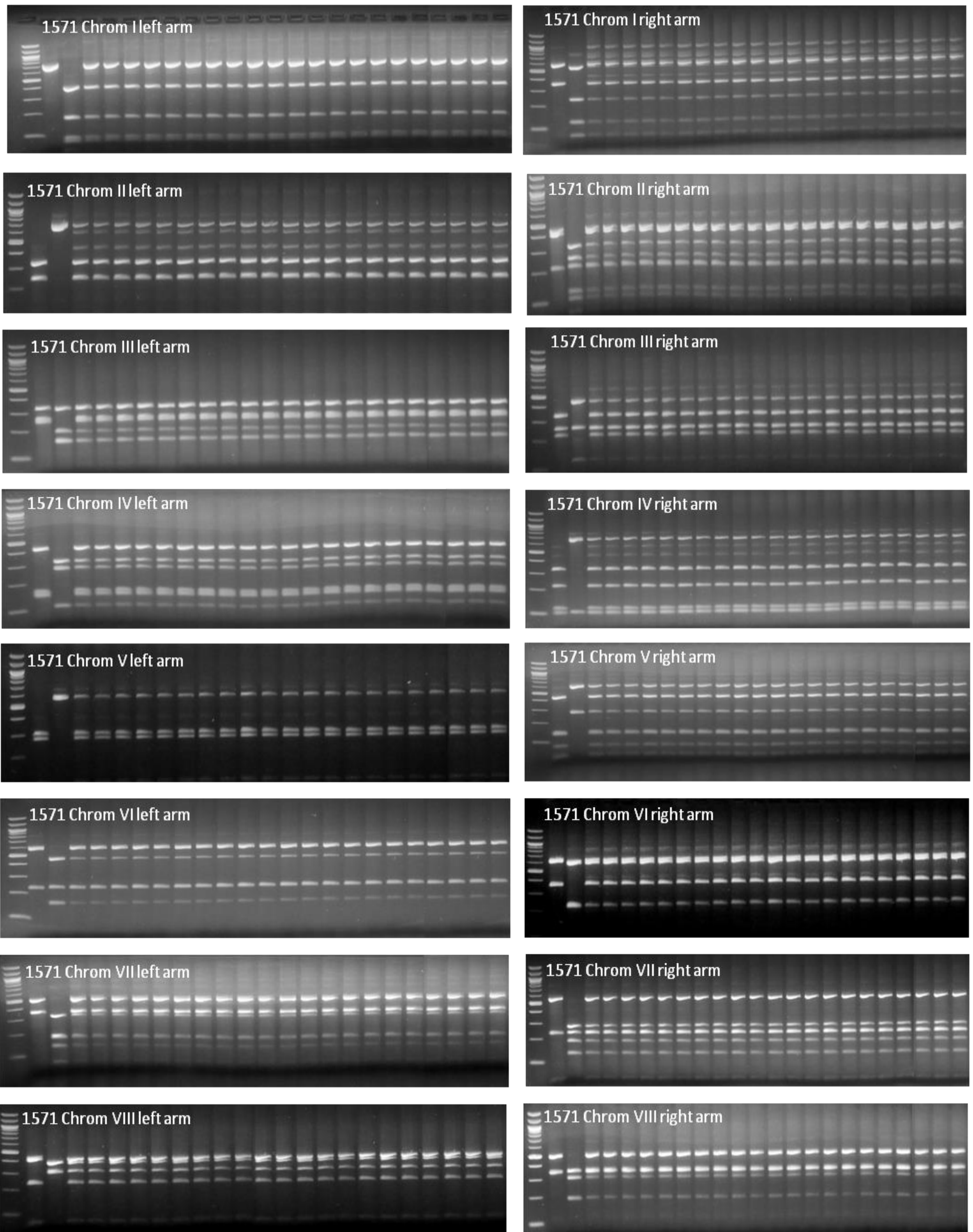
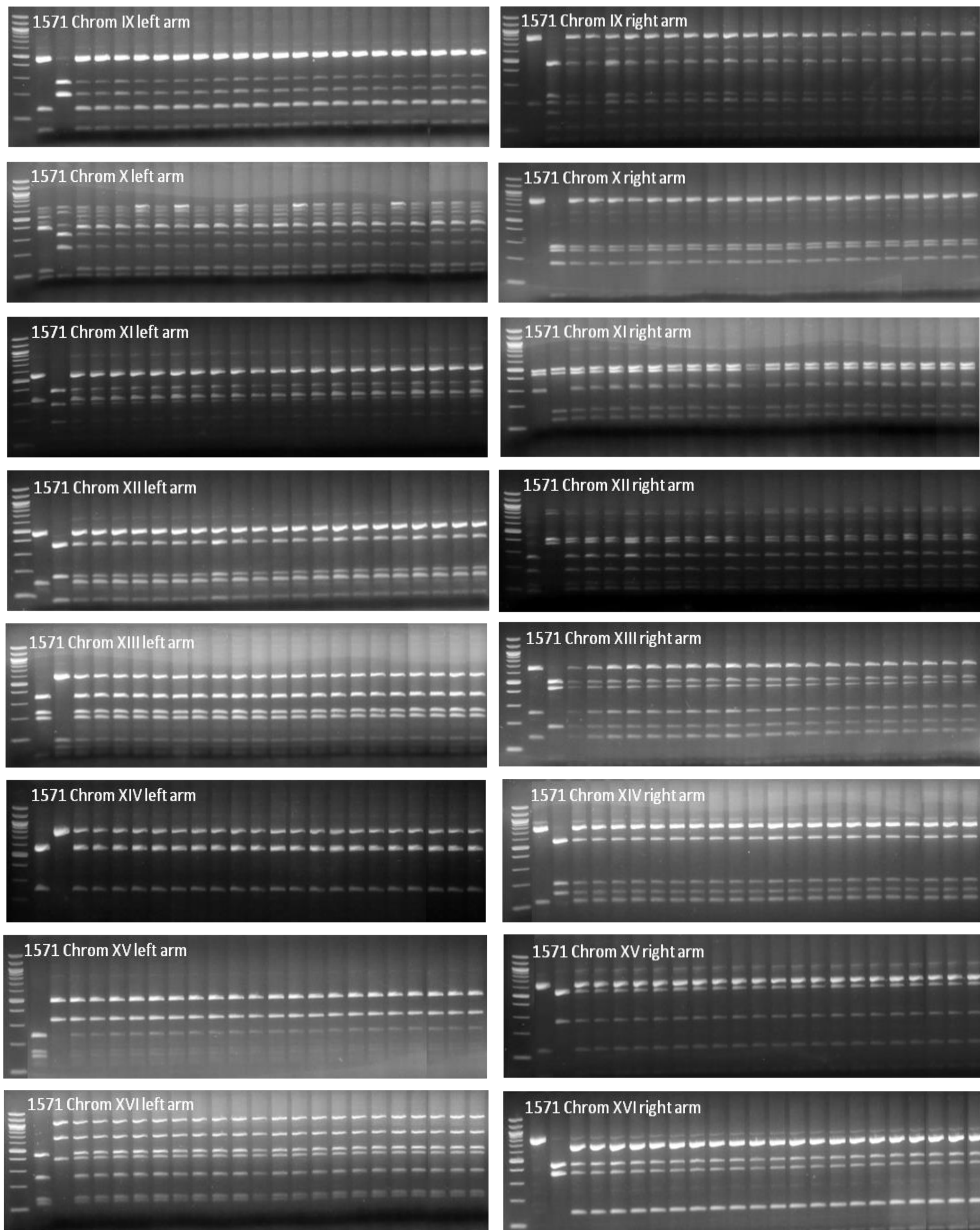
Supplemental data Fig. S1a Genetic stability analyses of hybrid AWRI 1571 following 200 mitotic generations using PCR-RFLP genomic markers to each arm of chromosomes I to VIII

In each gel; lane 1 100bp ladder, lane 2 AWRI 838, lane 3 AWRI 1176, lane 4 AWRI 1571, lanes 5 to 24 hybrid isolates of AWRI 1571 following 200 generations.



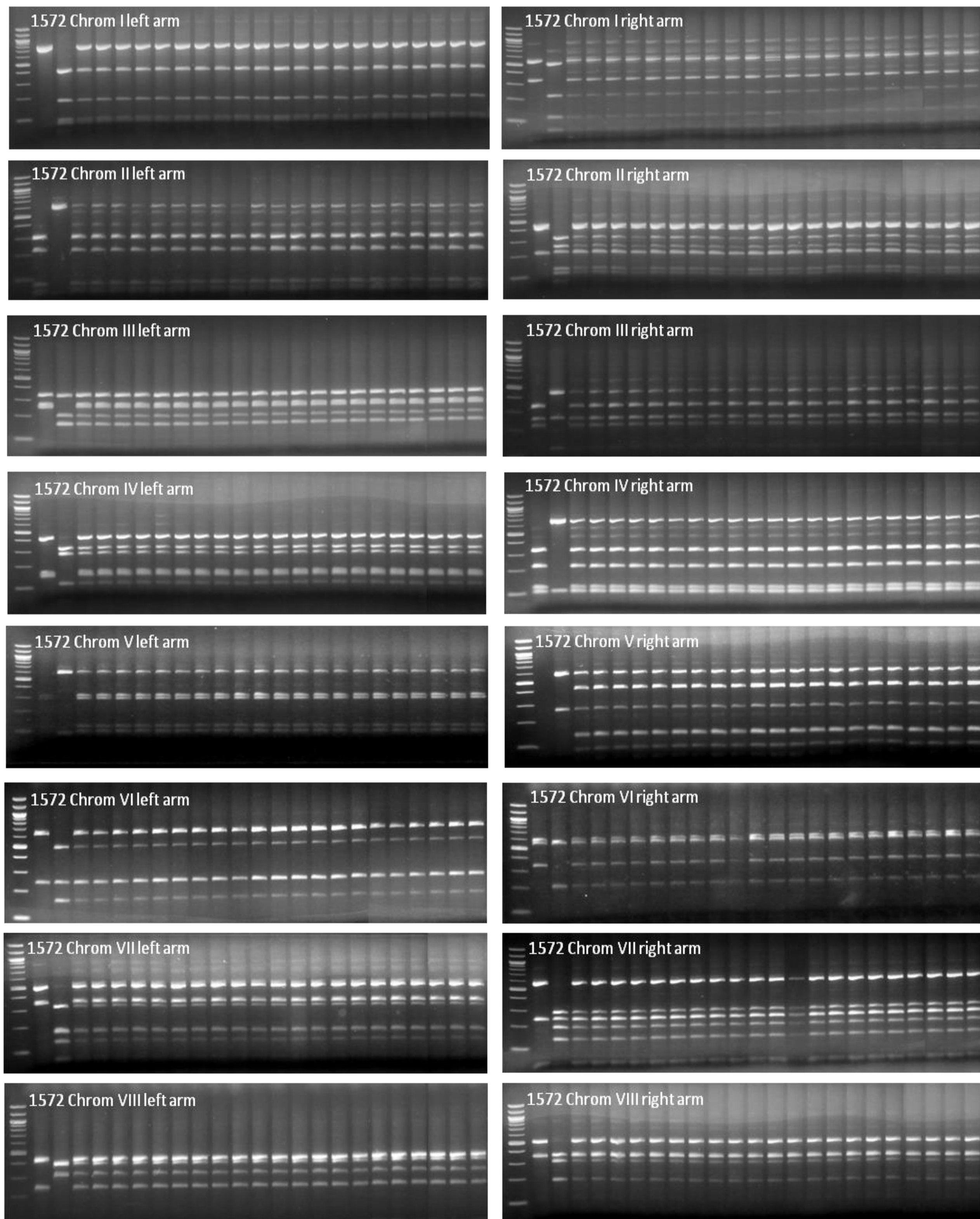
Supplemental data Fig. S1b Genetic stability analyses of hybrid AWRI 1571 following 200 mitotic generations using PCR-RFLP genomic markers to each arm of chromosomes IX to XVI

In each gel; lane 1 100bp ladder, lane 2 AWRI 838, lane 3 AWRI 1176, lane 4 AWRI 1571, lanes 5 to 24 hybrid isolates of AWRI 1571 following 200 generations



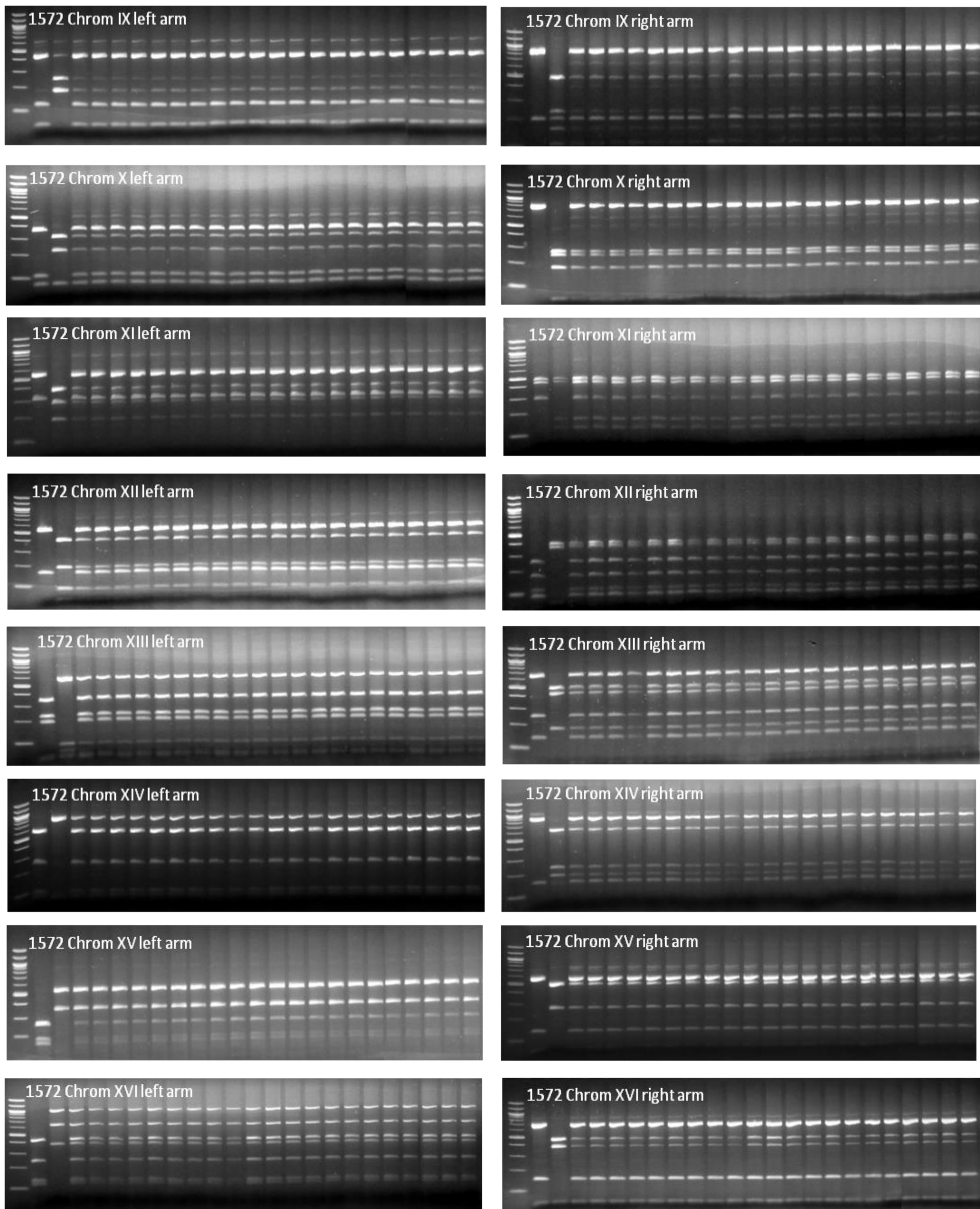
Supplemental data Fig. S1c Genetic stability analyses of hybrid AWRI 1572 following 200 mitotic generations using PCR-RFLP genomic markers to each arm of chromosomes I to VIII

In each gel; lane 1 100bp ladder, lane 2 AWRI 838, lane 3 AWRI 1176, lane 4 AWRI 1572, lanes 5 to 24 hybrid isolates of AWRI 1572 following 200 generations.



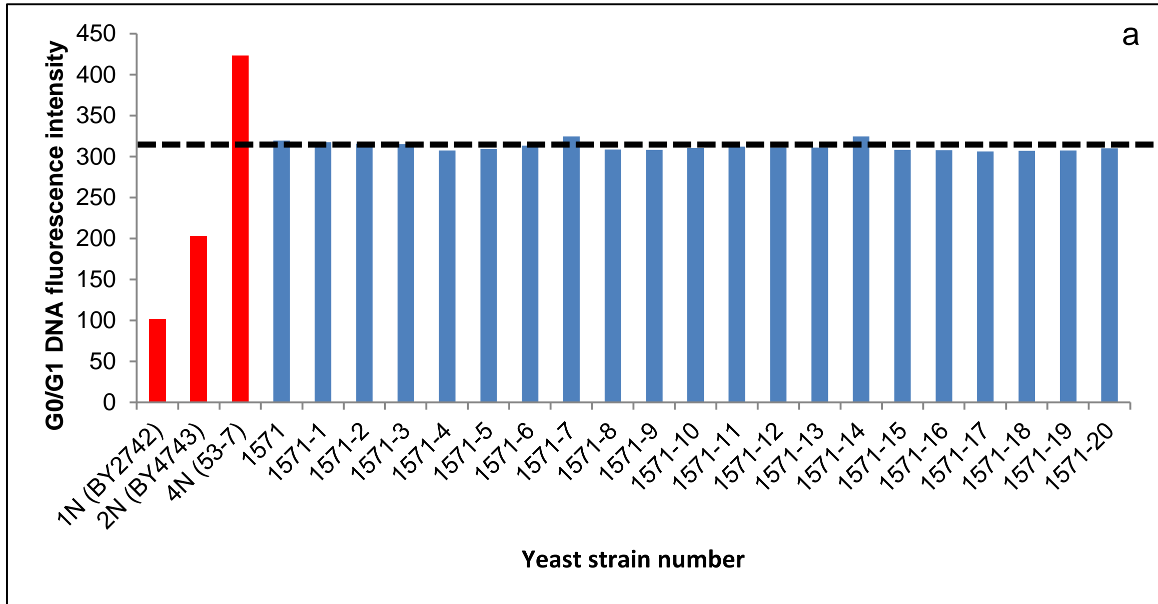
Supplemental data Fig. S1d Genetic stability analyses of hybrid AWRI 1572 following 200 mitotic generations using PCR-RFLP genomic markers to each arm of chromosomes IX to XVI

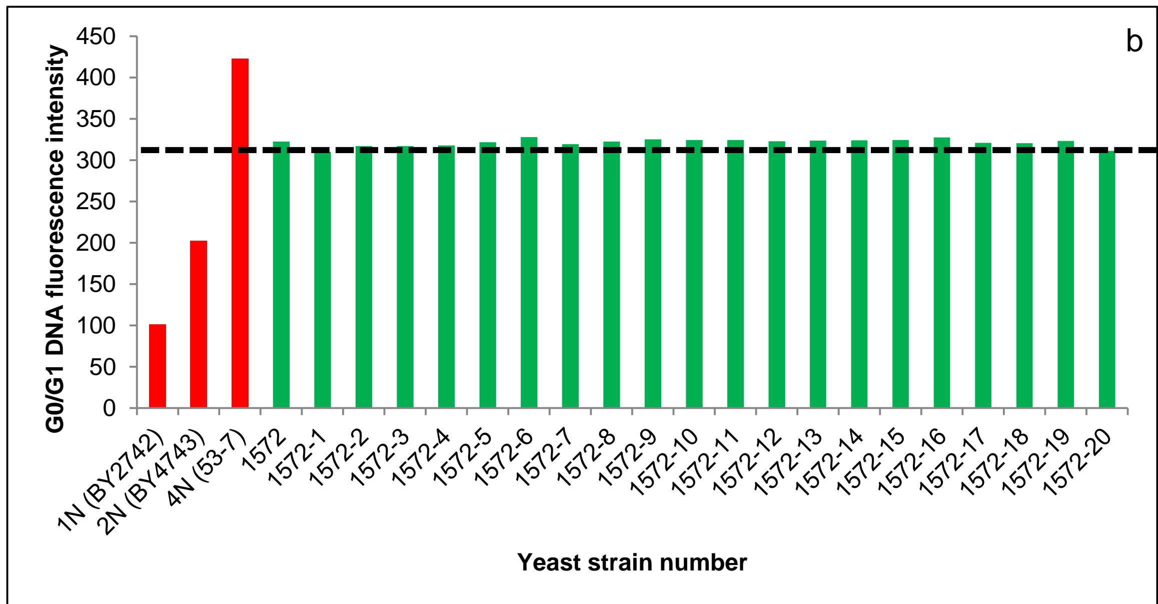
In each gel; lane 1 100bp ladder, lane 2 AWRI 838, lane 3 AWRI 1176, lane 4 AWRI 1572, lanes 5 to 24 hybrid isolates of AWRI 1572 following 200 generations.



Supplemental data Fig. S2 Fluorescence flow cytometry analysis of 200 generation isolates from hybrids AWRI 1571 and AWRI 1572 with dashed line showing fluorescence intensity of original hybrid strain : a. AWRI 1571; b. AWRI 1572

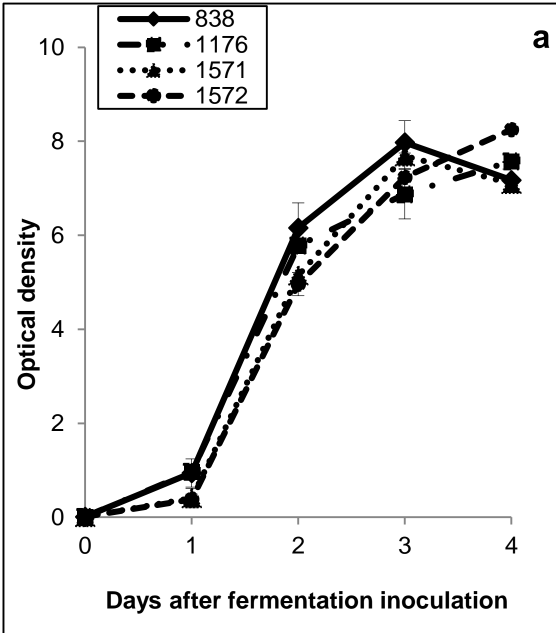
In both figures; strain #1 haploid control, strain #2 diploid control; strain #3 tetraploid control, strain #4 original hybrid, strains #5 to #24 hybrid isolates following 200 generations of mitotic growth

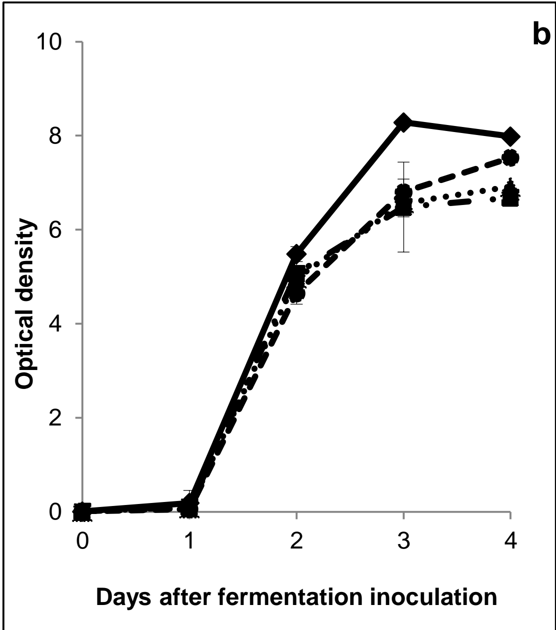


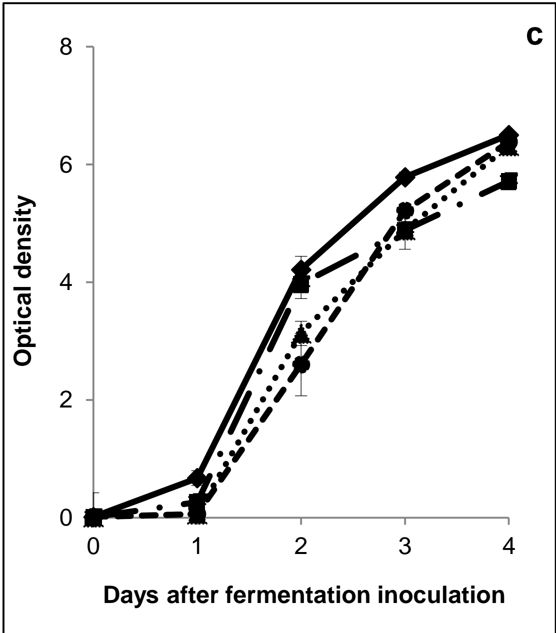


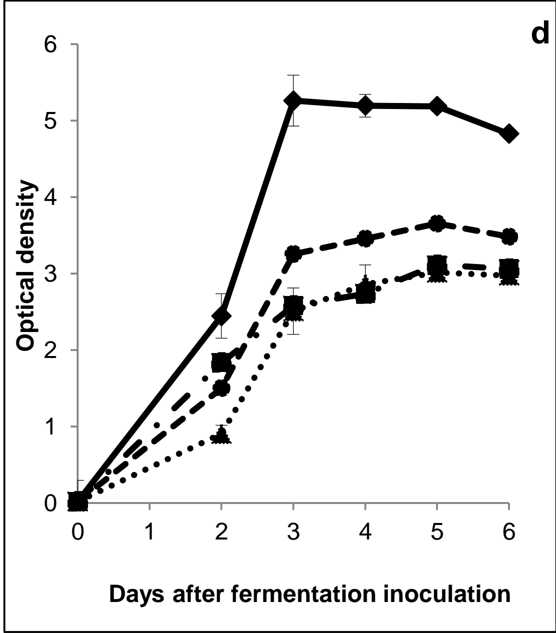
Supplemental data Fig. S3 Growth of parental and interspecific hybrid strains (as determined by optical density) in Chardonnay juice containing;

a. 145 g/L sugar; b. 195 g/L sugar; c. 250 g/L sugar; d. 355 g/L sugar



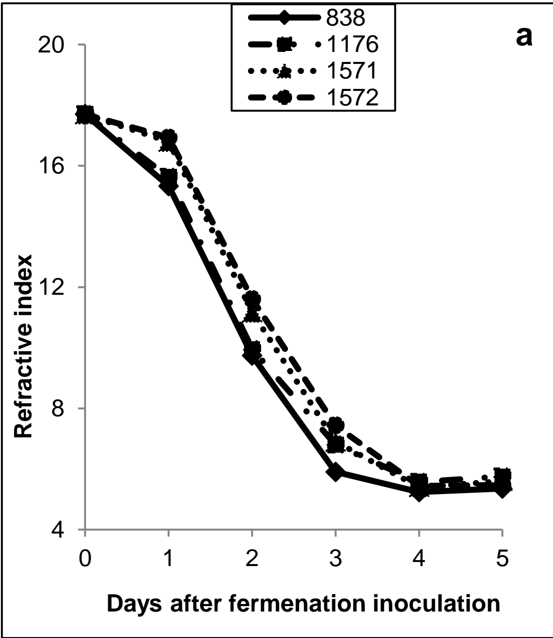


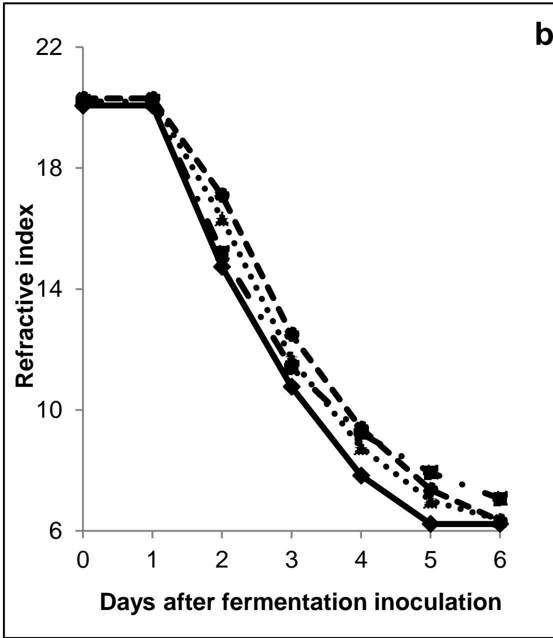


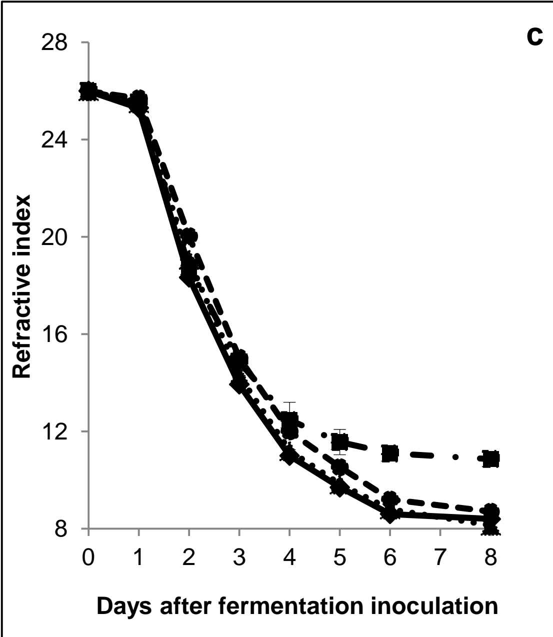


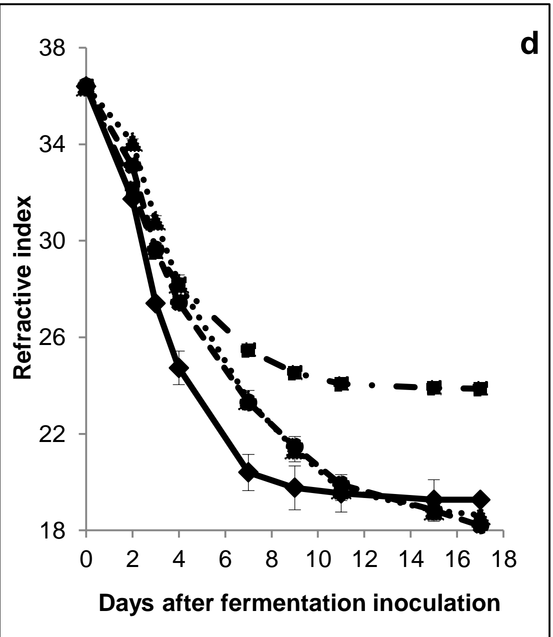
Supplemental data Fig. S4 Sugar utilisation of parental and interspecific hybrid strains (as determined by refractive index) in Chardonnay juice containing;

a. 145 g/L sugar; b. 195 g/L sugar; c. 250 g/L sugar; d. 355 g/L sugar

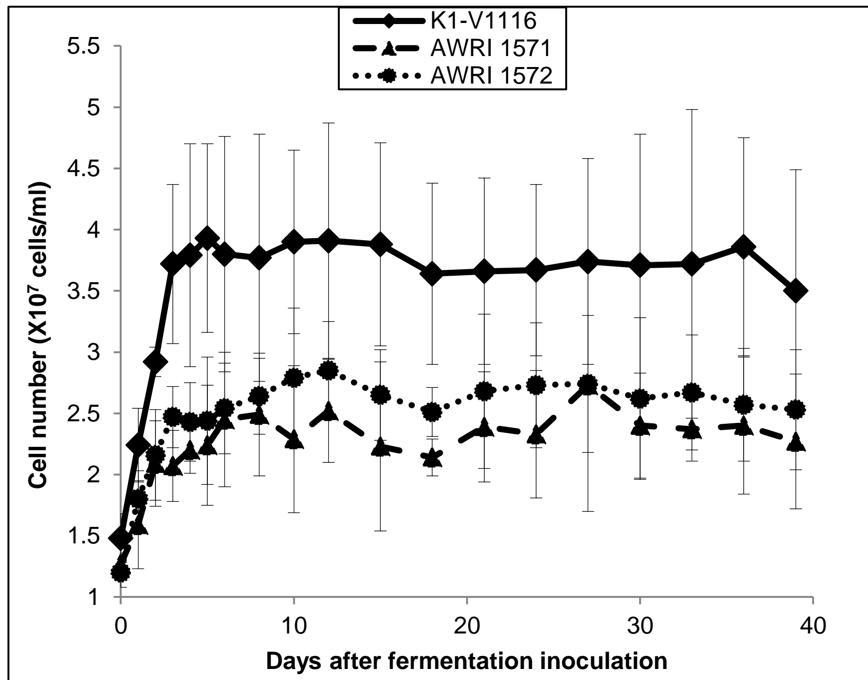








Supplementary Fig. S5 Cell growth during icewine fermentation



Supplemental data Table S1 Genetic stability study primer sequences and restriction enzymes for 32 species specific genomic markers

Primer name	Primer sequence	Primer name	Primer sequence	Restriction enzyme
ScSu1Lf	AGCACTCAAGCACATCGCCT	ScSu1Lr	AATATTCGCCACCTTGAGGG	*Taqa*I
ScSu1Rf	AGTGCTCCATCTCATGCTCCA	ScSu1Rr	TATTTGTCTCGATGGGGTGGT	*Mse*I
ScSu2Lf	GCATCTTTTTTCCTCCCAACT	ScSu2Lr	ACGCTGCCTGAAATCATGTAT	*Mse*I
ScSu2Rf	AACCATCCAACAAGACAGCA	ScSu2Rr	GCGACCAATTCCCAACAAA	*Taqa*I
ScSu3Lf	ACCGCAGCATATACTGACACC	ScSu3Lr	ACTTTTTCACCCAGCGAGAT	*Taqa*I
ScSu3Rf	CGCCATGTGGATAGATGATGA	ScSu3Rr	TGTGGATTCTGTGGTTGAACA	*Taqa*I
ScSu4Lf	CGCCCATGAACCAGAACTACT	ScSu4Lr	GCCATAAGCGAAGGTTGTAA	*Mse*I
ScSu4Rf	GATTGCCGATTTTGGTTTGTC	ScSu4Rr	TGATCCATGAAGGGTGATTG	*Rsa*I
ScSu5Lf	TTTCAAGTCACTGACGTGGCA	ScSu5Lr	CATCTGCGATTTCTTGGCAA	*Mse*I
ScSu5Rf	TAGAAAACGAGCCAACACTGG	ScSu5Rr	CTCAATCCCAATCCCGTATT	*Mse*I
ScSu6Lf	TTGTCATGTGGATGACATCGA	ScSu6Lr	GGTGTGGGCAACTGATAAAA	*Hae*III
ScSu6Rf	CCGTATCTGGAAAAGCATTG	ScSu6Rr	TAACTGGTTGCTTTGGAGATG	*Taqa*I
ScSu7Lf	TCGTTTCCCACCTGAACCTT	ScSu7Lr	AAAGCCCAGATCAAGTTCCA	*Mse*I
ScSu7Rf	GGTGATATGCAGTTGATTTGC	ScSu7Rr	GATATATTACCTCCGTGCCCA	*Hae*III
ScSu8Lf	CGCCCTCTATCTTGTCTTTGT	ScSu8Lr	TGCCATCGTGAAATTTCTGC	*Taqa*I
ScSu8Rf	ATCTTTGATGCCAGGTGGTT	ScSu8Rr	TTGGCTGGCAATCTTTCAGA	*Rsa*I
ScSu9Lf	TACAACAACACGAGTGGGTTT	ScSu9Lr	GAAAACTTGCGGACCAAAGA	*Rsa*I
ScSu9Rf	CAGAGACTTGAAACCGGTTGA	ScSu9Rr	ATACATAGAGCCATTGCCACA	*Taqa*I
ScSu10Lf	ATGAAATTGCCACAGGCAC	ScSu10Lr	TCATCAACAATTGGTAACGGA	*Mse*I
ScSu10Rf	CAACTGTAAGGTTCAGAGGCA	ScSu10Rr	TTCTGGGTTCATTTCACCGT	*Hae*III
ScSu11Lf	TGGGTAAGGAAGCAATGTTGA	ScSu11Lr	CACCTCTTGCCTGATAGGAAA	*Hae*III
ScSu11Rf	TCCGCACCATTCCAAAACTA	ScSu11Rr	GCCAAACCAGTGAATAACCA	*Hae*III
ScSu12Lf	GAAGCTTTGGAAATGGCCAA	ScSu12Lr	TGTGTGCGTTTTTTATTTCGA	*Hae*III
ScSu12Rf	TTAAGCGCCGACACTTCGT	ScSu12Rr	CCATATGCTTCGCATTATTCC	*Mse*I
ScSu13Lf	CATCAATACCTCATGAGCGTC	ScSu13Lr	CGTCCAAAGTCCCGCTTATAT	*Taqa*I
ScSu13Rf	ACCACAACTCCTTGGGCGAT	ScSu13Rr	TCAACGTAAAGGTCAGGCAA	*Hae*III
ScSu14Lf	AGCCTGTGCGTACAAAGAACA	ScSu14Lr	AATGGATTTCTACCGCCAA	*Hae*III
ScSu14Rf	TGGACGAATGTTTAGAAAGGC	ScSu14Rr	TTAGAAGCAGATCTGGCTTGG	*Hae*III
ScSu15Lf	TCGATTTCACCGCAGGTATT	ScSu15Lr	CCAGCGGTAATGATCAAAAG	*Hae*III
ScSu15Rf	GGCAAATCTCCATGTGAAATG	ScSu15Rr	AATCTCATGATGCAGGCCAA	*Hae*III
ScSu16Lf	CCGCTTTGCTAATCGGTTTT	ScSu16Lr	TCCTTGAGCTTTCAAAGCCA	*Hae*III
ScSu16Rf	ACAAAATGAAAGCACCGCTGA	ScSu16Rr	CAAACAAGAGATCCATCGCA	*Hae*III

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Jennifer R Bellon
Contribution to the Paper	Designed and undertook all the experiments, interpreted the data, wrote manuscript and acted as corresponding author.
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 14/02/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 5

A novel approach to isolating industrial *Saccharomyces* interspecific wine yeasts using chromosomal mutations as a marker for increased fitness.

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Work described in this chapter was designed to improve the fitness of the strain developed in the work described in Chapter 4. An adaptive evolution approach was used to select strains that were more reproductively successful than the parent hybrid. However, laboratory-based adaptive evolution as a strategy for improving fitness has some serious limitations. Traditional approaches to adaptive evolution utilise end-point sampling after hundreds of generations, carrying the risk of introducing collateral mutations that shape phenotypes in addition to that which is targeted. Identifying a mutant with increased fitness from an early timepoint in the evolutionary process reduces this risk. This was achieved by an approach to screening the rationale for which was as follows: Research from several laboratories has shown that, over time, *Saccharomyces* interspecific hybrids from industrial sources have lost chromosomal material from one or both parental lineages. This loss presumably leads to greater fitness for the hybrid in the industrial environment they were isolated from. Thus, chromosomal mutations have the potential to act as markers for increased fitness in evolving populations.

Multiple rounds of consecutive grape juice batch fermentations passaged yeast cells from one hostile environment to another (high sugar concentration of grape juice at inoculation followed by high ethanol levels and nutrient deprivation at the end of fermentation).

A set of 32 PCR-RFLP chromosomal markers were used to assess presence/absence of *S. cerevisiae* and *S. uvarum* chromosomes in cells isolated from different stages of an evolving interspecific hybrid population.

Changes occurred in a number of different chromosomes, however, the overriding alteration was a loss of *S. uvarum* Chromosome 14. Competitive growth in Chardonnay juice between the original hybrid and an evolved isolate with loss of

S. uvarum Chromosome 14 but no other detectable changes in karyotype demonstrated that the evolved strain had increased fitness. Fermentation kinetics showed that the evolved strain also had increased fermentation performance relative to the original hybrid and retained the desirable fermentation traits of its parent.

The evolved *S. cerevisiae* x *S. uvarum* interspecific hybrid has the potential to maximise the fermentation capabilities of a wine yeast previously developed to deliver low volatile acidity in wines produced from high-sugar juices.

A novel approach to isolating improved industrial interspecific wine yeasts using chromosomal mutations as a marker for increased fitness.

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Keywords

interspecific wine yeast hybrids, evolving populations, chromosomal mutations, increased fitness

Abstract

Wine yeast breeding programs utilising interspecific hybridisation deliver cost-effective tools to winemakers looking to differentiate their wines through the development of new wine styles. The addition of a non-*S. cerevisiae* genome to a commercial wine yeast can generate novel phenotypes ranging from wine flavour and aroma diversity to improvements in targeted fermentation traits. In the current study we utilised a novel approach to screen isolates from an evolving population for increased fitness in a *S. cerevisiae* x *S. uvarum* interspecific hybrid previously generated to incorporate the targeted phenotype of lower volatile acidity production. Sequential grape-juice fermentations provided a selective environment from which to screen isolates. Chromosomal markers were used in a novel approach to identify isolates with potential increased fitness. A strain with increased fitness relative to its parents was isolated from an early timepoint in the evolving population, thereby minimising the risk of introducing collateral mutations and potentially undesirable phenotypes. The evolved strain retained the desirable fermentation trait of reduced volatile acidity production, along with other winemaking traits of importance while exhibiting improved fermentation kinetics.

Introduction

An increasingly competitive global market requires winemakers to minimise production costs and target market niches by differentiating their wines through, for example, development of novel wine styles. One way of achieving these

ends is to generate new yeast strains with improved fermentation traits and/or novel phenotypes that shape wine flavour and aroma. Such yeasts provide winemakers with tools that are readily and easily introduced into the winery without incurring additional costs or requiring processing interventions.

There are various ways to generate new yeast strains, including breeding programs. Traditionally this would involve mating strains of the same species, which, in the context of wine yeast, is *Saccharomyces cerevisiae*. However, more recently the potential to bring a higher level of phenotypic diversity into wine yeast by hybridisation with non-*cerevisiae* species of the *Saccharomyces* clade has been realised. This has led to the generation of interspecific hybrids for use in a range of beverage industries, particularly brewing (Krogerus et al. 2015; Krogerus et al. 2017) and winemaking (Rainieri et al. 1999; Bellon et al. 2011; Perez-Traves et al. 2012). Interspecific hybrids have, for example, incorporated phenotypes from the non- *S. cerevisiae* parent that are either not present in wine yeast, or for which wine yeast has a reduced capacity (e.g. respectively: growth at low temperatures (Libkind et al. 2011) and increased glycerol production (Gonzalez et al. 2007; Rainieri et al. 1999)).

Our laboratory previously reported the ability of laboratory-generated interspecific yeast hybrids to introduce flavour and aroma diversity to wines by incorporating the genome of a closely-related *Saccharomyces* species (*Saccharomyces paradoxus* and *Saccharomyces kudriavzevii*) with a commercial *S. cerevisiae* wine yeast strain (Bellon et al. 2011). Utilising a more divergent *Saccharomyces* species (*Saccharomyces mikatae*) as a genetic contributor generated hybrid strains capable of producing novel, not previously recognised, flavour-active metabolites in wines (Bellon et al. 2013). The most recent publication from this work described the targeted improvement of reduced volatile acidity production in high-sugar fermentation by the generation of *S. cerevisiae* x *S. bayanus* (var. *S. uvarum*) interspecific hybrids (Bellon et al. 2015). One hybrid from this work (AWRI 1572) showed exceptional promise in terms of what it could bring to wine quality (Bellon et al. 2015) and was chosen for further development.

In research from other laboratories the genetic composition of a number of naturally occurring interspecific hybrid strains isolated from different fermentation sources has been evaluated revealing substantial loss of chromosomal material from one or both parental lineages (Dunn and Sherlock 2008; Borneman et al. 2011; Peris et al. 2012; Borneman et al. 2016). This loss may have been due to genome incompatibilities (Scannell et al. 2006), and presumably led to greater fitness for the yeast in the industrial environment they were isolated from.

With this in mind, it was decided to passage the aforementioned laboratory-generated interspecific hybrid wine yeast, AWRI 1572, through a series of successive grape must fermentations with the aim of selecting for spontaneous

mutants with increased fitness in a winemaking context. The rationale was to screen for chromosomal mutations in isolates from the evolving passaged populations. Any isolates with chromosomal mutations that became highly represented in the populations were candidates for strain development and were subsequently tested for their fitness compared to the AWRI 1572 and for retention of desirable traits of the parent. The potential of this novel approach of screening for highly represented chromosomal mutations as a marker for increased fitness was realised: A chromosomal marker that increased in frequency in the evolving population was identified. This enabled isolation from an early stage of the evolutionary process progeny with increased fitness relative to its parent. This novel hybrid retained the previously reported desirable 'low acetic acid' phenotype, along with other winemaking traits of importance.

Material and Methods

Yeast strains

Saccharomyces spp. interspecific hybrid strain AWRI 1572, generated using rare-mating hybridisation between *S. cerevisiae* diploid strain (AWRI 838) and spores from *Saccharomyces uvarum* strain AWRI 1176 as described in Bellon et al. 2015; hybrid strain AWRI 2530 (an evolved strain of AWRI 1572 generated in this study) and control yeast strains for haploid, diploid and tetraploid DNA intensity determinations BY4741 *MAT***a**, BY4743 (Euroscarf ®, Frankfurt, Germany) and 53-7 (Salmon 1997) respectively.

The *S. uvarum* parent of the hybrid AWRI 1572 had been molecularly typed as *Saccharomyces bayanus* var *uvarum*; a sub group of the *S. bayanus* species. Recent studies in other laboratories indicate that this sub group should constitute a separate species *Saccharomyces uvarum* (Libkind et al. 2011; Nguyen et al. 2011) thus we refer to AWRI 1176 as *S. uvarum* in this manuscript.

Selection for increased fitness in a passaged, evolving population of wine yeast hybrid AWRI 1572

Selection was performed in filter-sterilised Chardonnay juice fermentations (juice sourced from a vineyard in Blewitt Springs, South Australia): total sugars (glucose and fructose) 225 g/L, yeast assimilable nitrogen 226 mg/L, pH 3.39. This juice was supplemented with the 300 mg/L di-ammonium phosphate. Yeast strain AWRI 1572 was pre-cultured in YEPD medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) for two days with an incubation temperature of 22°C and agitation rate of 150 rpm. The cells were then acclimatised to the higher sugar concentration of grape juice by two days of

growth in ½ X Chardonnay juice (diluted with sterile water) before being inoculated from a cell density of 2 x 10^8 cells per ml into 100 ml of Chardonnay juice to a final cell density of 2 x 10^6 cells per ml. Triplicate fermentations were carried out under conditions described previously (Bellon et al. 2013). At completion of fermentation, cells were isolated and fresh Chardonnay juice was then inoculated with 1 ml of 2 x 10^8 cells per ml from the previous ferment. This serial transfer procedure was repeated a further three times until five batch fermentations were completed (**Figure 1**).

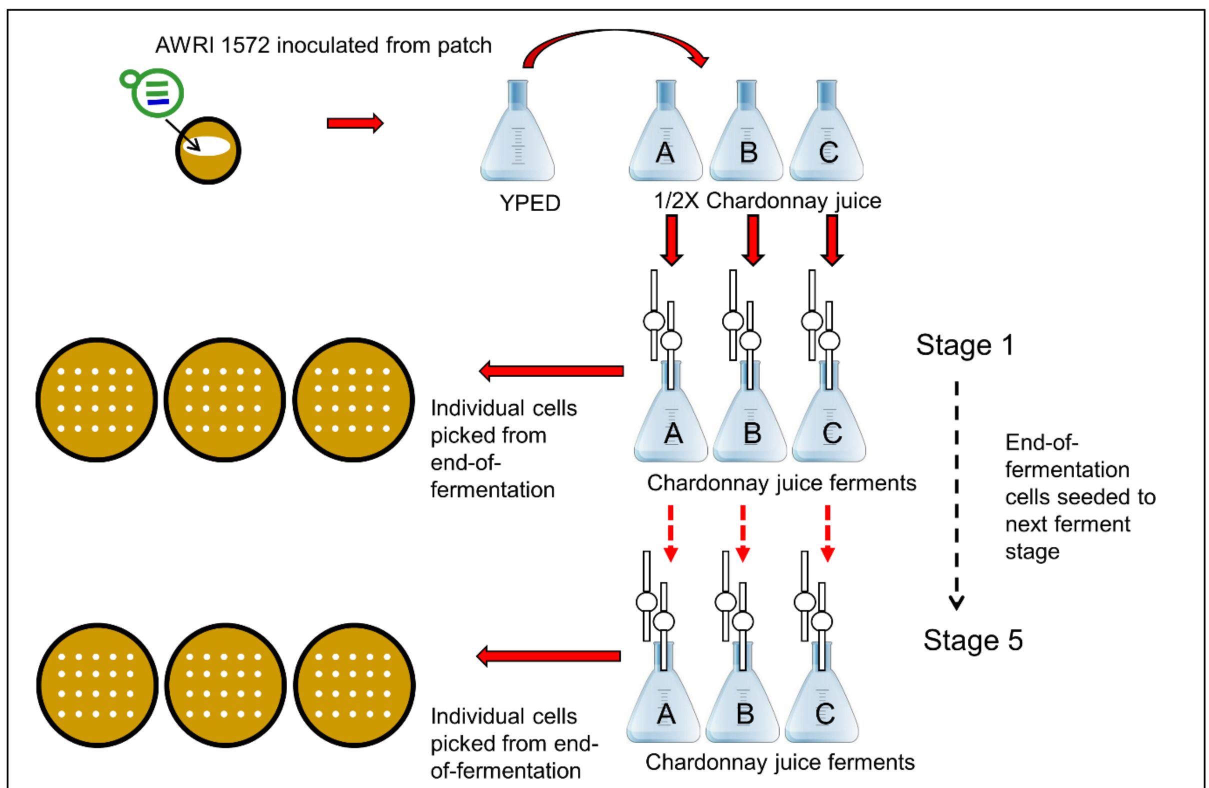

Figure 1. Design of adaptive evolution experiment to generate mutants with increased fitness in a winemaking context. Serial batch passaging of *S. cerevisiae* x *S. uvarum* interspecific hybrid AWRI1572 in Chardonnay juice.

Chromosomal analysis of isolates from passaged populations

At fermentation completion of each passage a Singer® micromanipulator was used to pick twenty individual cells from each replicate fermentation culture onto YEPD plates, which were then incubated at 22°C until clonal colonies had formed. PCR-RFLP analysis on genomic DNA was performed using species-specific markers targeting each arm of the 16 *Saccharomyces spp.* chromosomes as previously described (Bellon et al. 2015) with additional markers for Chromosome 14 (**Table 1**).

Table 1. Loci amplified, primer sequences and Restriction Enzymes for species-specific chromosomal markers

Primer	Amplified loci in *S. cerevisiae*	Sequence	Restriction enzyme
1L	*OAF1* 48685-49530 bp	AGCACTCAAGCACATCGCCT	*Taq*aI
		AATATTCGCCACCTTGAGGG	
1R	*SWH1* 192768-193723 bp	AGTGCTCCATCTCATGCTCCA	*Mse*I
		TATTTGTCTCGATGGGGTGGT	
2L	*CDC27* 67260-68152 bp	GCATCTTTTTTCCTCCCAACT	*Taq*aI
		ACGCTGCCTGAAATCATGTAT	
2R	*CHS2* 312499-313610 bp	AACCATCCAACAAGACAGCA	*TaqaI*
		GCGACCAATTCCCAACAAA	
3L	*FUS1* 72313-73191 bp	ACCGCAGCATATACTGACACC	*TaqaI*
		ACTTTTTCACCCAGCGAGAT	
3R	*AGT15* 237694-238530 bp	CGCCATGTGGATAGATGATGA	*TaqaI*
		TGTGGATTCTGTGGTTGAACA	
4L	*UGA3* 156723-157490 bp	CGCCCATGAACCAGAACTACT	*Rsa*I
		GCCATAAGCGAAGGTTGTAA	
4R	*SNF1* 1412947-1413758 bp	GATTGCCGATTTTGGTTTGTC	*Mse*I
		TGATCCATGAAGGGTGATTG	
5L	*AFG1* 57189-58004 bp	TTTCAAGTCACTGACGTGGCA	*Rsa*I
		CATCTGCGATTTCTTGGCAA	
5R	*BCK2* 519304-520266 bp	TAGAAAACGAGCCAACACTGG	*Rsa*I
		CTCAATCCCAATCCCGTATT	
6L	*STE2* 82779-83727 bp	TTGTCATGTGGATGACATCGA	*Hae*III
		GGTGTGGGCAACTGATAAAA	
6R	*MET10* 214927-215981 bp	CCGTATCTGGAAAAGCATTG	*Taq*aI
		TAACTGGTTGCTTTGGAGATG	
7L	*GUS1* 3960-40528 bp	TCGTTTCCCACCTGAACCTT	*Taq*aI
		AAAGCCCAGATCAAGTTCCA	
7R	*GND2* 1005189-1006087 bp	GGTGATATGCAGTTGATTTGC	*Hae*III
		GATATATTACCTCCGTGCCCA	
8L	*OCA5* 46666-47424 bp	CGCCCTCTATCTTGTCTTTGT	*Taq*aI
		TGCCATCGTGAAATTTCTGC	
8R	*GND1* 471369-472285 bp	ATCTTTGATGCCAGGTGGTT	*Rsa*I
		TTGGCTGGCAATCTTTCAGA	
9L	*SUC2* 37698-38383 bp	TACAACAACACGAGTGGGTTT	*Rsa*I
		GAAAACTTGCGGACCAAAGA	
9R	*DAL4* 408763-409845 bp	CAGAGACTTGAAACCGGTTGA	*Taq*aI
		ATACATAGAGCCATTGCCACA	
10L	*ECM25* 54547-55202 bp	ATGAAATTGCCACAGGCAC	*Rsa*I
		TCATCAACAATTGGTAACGGA	
10R	*PMT4* 698900-699726 bp	CAACTGTAAGGTTCAGAGGCA	*Hae*III
		TTCTGGGTTCATTTCACCGT	
11L	*UBA1* 39239-40239 bp	TGGGTAAGGAAGCAATGTTGA	*Hae*III
		CACCTCTTGCCTGATAGGAAA	
11R	*PTR2* 616024-617343 bp	TCCGCACCATTCCAAAACTA	*Hae*III
		GCCAAACCAGTGAATAACCA	
12L	*FRA1* 82096-82864 bp	GAAGCTTTGGAAATGGCCAA	*Hae*III
		TGTGTGCGTTTTTTATTTCGA	
12R	*LEU3* 1037035-1037930 bp	TTAAGCGCCGACACTTCGT	*Mse*I
		CCATATGCTTCGCATTATTCC	
13L	*BUL2* 47651-48521 bp	CATCAATACCTCATGAGCGTC	*Taq*aI
		CGTCCAAAGTCCCGCTTATAT	
13R	*TDA1* 852727-853919 bp	ACCACAACTCCTTGGGCGAT	*Hae*III
		TCAACGTAAAGGTCAGGCAA	
14L	*LEM3* 32074-33077 bp	AGCCTGTGCGTACAAAGAACA	*Hae*III
		AATGGATTTCTACCGCCAA	
14LM	*CBK1* 333495-334599 bp	CGCCATTGAAAGAAATGAAAG	*Hae*III
		TTCATCTGCACCACCATGTCT	
14LC	*NOP2* 510540-511741 bp	TCATAAGAACAAGCAAGCCG	*Taq*aI
		TGTTGGTACAGCCTAGACGGT	
14RC	*LRO1* 640952-641994 bp	AAAGCTGGGGAGTTATTGGA	*Rsa*I
		TGGGTTGTTCACCCCGTATAT	
14R	*PPG1* 686010-687116 bp	TGGACGAATGTTTAGAAAGGC	*Hae*III
		TTAGAAGCAGATCTGGCTTGG	
15L	*GRE2* 43709-44440 bp	GTTCATTGCCCAACACATTG	*Hae*III
		AGCCTTTGCAACATCACGAA	
15R	*RDR1* 1051369-1052163 bp	GGCAAATCTCCATGTGAAATG	*Hae*III
		AATCTCATGATGCAGGCCAA	
16L	*SAM3* 23260-24344 bp	CCGCTTTGCTAATCGGTTTT	*Hae*III
		TCCTTGAGCTTTCAAAGCCA	
16R	*PRP4* 892389-893300 bp	ACAAAATGAAAGCACCGCTGA	*Hae*III
		CAAACAAGAGATCCATCGCA	

Ploidy determination using Fluorescence flow cytometry analysis

Colonies from YEPD plates were inoculated into liquid YEPD medium. Cells were harvested after 5 days of growth at 22°C and prepared in triplicate using a propidium iodide staining protocol for FACs analysis as described in Bellon et al. (2013). Cells were analysed using a Guava® easyCyte 12HT Sampling Flow Cytometer (Merck, Germany) instrument equipped with a 150 milliwatt DPSS laser emitting at 488 nm. Cells were detected at 583/26 nm using a Yellow B PMT filter with a flow rate of 7 μl/minute and fluorescently plotted to a linear scale. Five thousand cells per sample were analysed to obtain cell DNA intensities. Analysis was undertaken on 6 biological replicates of control ploidy strains and ancestral hybrid AWRI 1572 and 60 isolates from each fermentation series. Duplicate fluorescent readings were taken of all samples.

Evaluation of yeast performance

Fermentations were conducted in two Chardonnay juices: the original Chardonnay juice containing 225 g/L reducing sugars, and the same juice supplemented with an addition of glucose and fructose, increasing the total reducing sugars concentration to 350 g/L. Fermentations were carried out in triplicate under conditions previously described (Bellon et al. 2015) with a single pre-conditioning step of growth in ½ X grape juice implemented for the 225 g/L sugar ferments, while an addition growth step in 1X chardonnay juice was used to pre-condition cells before inoculation into the high-sugar Chardonnay juice. Fermentation rates were determined by weight loss (OHRUS Adventurer^{TM} weighing meter) as a measure of CO_2 egress from fermentation vessels.

Ethanol tolerance of yeast strains was determined by plating onto YEPD agar medium containing a range of ethanol concentrations (12%, 14% and 16%). Plates were prepared when YEPD (plus 2% agar) medium was cooled to 50°C by the addition of a requisite volume of absolute ethanol. Strains were pre-cultured to a cell density of 2 x 10^8 cells per ml in liquid YEPD for 2 days at 22°C and 5 μl of 10-fold serial dilutions spotted to plates and then further incubated at 22°C until sufficient colony growth could be observed.

Analysis of wines

Wines were analysed using HPLC to determine concentrations of organic acids (malic, succinic and acetic acids), residual sugars, glycerol and ethanol as described previously (Bellon et al. 2013).

Statistical analyses

A one-way analysis of variance (ANOVA) and Student's t-test (*p<0.5*) were used to determine differences between wines produced by different yeasts.

Results

Chromosomal analysis of isolates from populations of passaged yeast hybrid AWRI 1572

In order to generate an evolved hybrid strain with increased fitness in a fermentation context, consecutive grape juice batch fermentations were undertaken. Triplicate fermentations of Chardonnay grape juice inoculated with AWRI 1572 were serially passaged four times with cells harvested from each end-of-fermentation seeded into the next ferment at a rate of 2×10^6 cells/ml (**Figure 1**). At the end of each fermentation single cells were harvested. Chromosomal compositions determined by PCR-RFLP revealed that loss of *S. uvarum* Chromosome 14 was the overriding chromosomal alteration occurring during the fermentation series (**Figure 2**).

Isolates harvested from the first stage ferment showed a stable genome with sporadic chromosomal loss detected in only 4 of the 60 isolates; a single marker in 3 isolates and two markers in the 4th isolate (**Supplementary Figure S1**).

Analysis of cells harvested from Stage 2 fermentation series revealed a level of *S. uvarum* Chromosome 14 instability with whole or partial loss in some isolates from all replicate ferments (three of twenty from replicate A, sixteen of twenty from Replicate B and all twenty isolates from Replicate C) (**Figure 3a**). Additional markers for Chromosome 14; 14ML (midway along the left arm), 14LC (proximal to the centromere on the left arm) and 14RC (proximal to the centromere on the right arm) (**Table 1**) were designed and used to confirm loss along the entire *S. uvarum* chromosome.

Figure 2a.

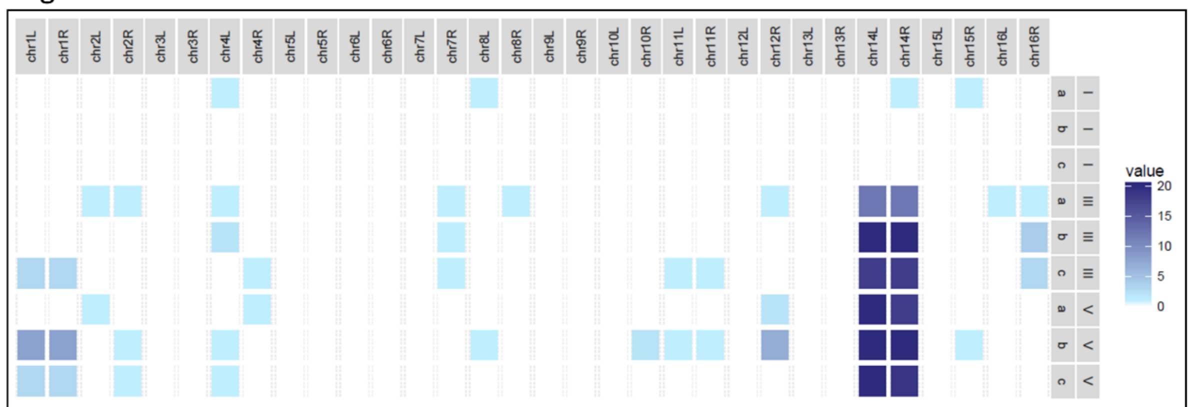

Figure 2b.

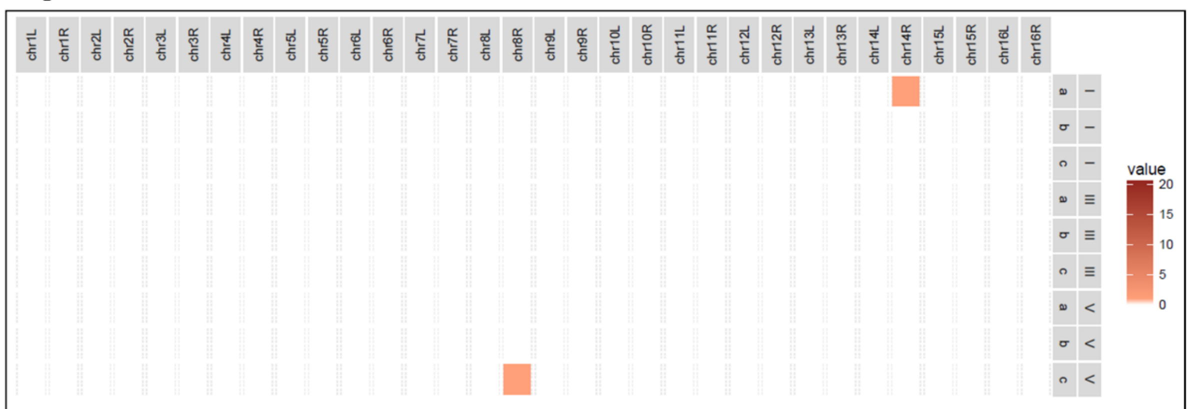

Figure 2. Heat map depicting AWRI1572 chromosomal loss in adaptive evolution experiment: a. Blue box displays *S. uvarum* chromosomal loss with darkness of hue linked to increased frequency; b. Red box displays *S. cerevisiae* chromosomal loss with darkness of hue linked to increased frequency

The frequency of loss continued to increase in the Stage 3 ferments with 12 isolates of twenty from Replicate A, all twenty from Replicate B and eighteen of twenty from Replicate C having lost the complete *S. uvarum* Chromosome 14 (**Figure 3b**), culminating at Stage 5 with complete *S. uvarum* Chromosome 14 loss in 57 isolates while the remaining three isolates (two from Replicate A and one from Replicate C) showed partial loss, retaining the right arm (**Figure 3c**).

Ploidy determination of AWRI 1572 cells undergoing serial Chardonnay fermentations using Fluorescence flow cytometry analysis

Fluorescence flow cytometry analysis was used to confirm that fitness improvements of hybrid isolates were not due to a major change in ploidy. While this analysis is not sufficiently sensitive to distinguish differences in DNA content arising from loss of a single chromosome in a triploid background, it can be used to determine larger scale changes.

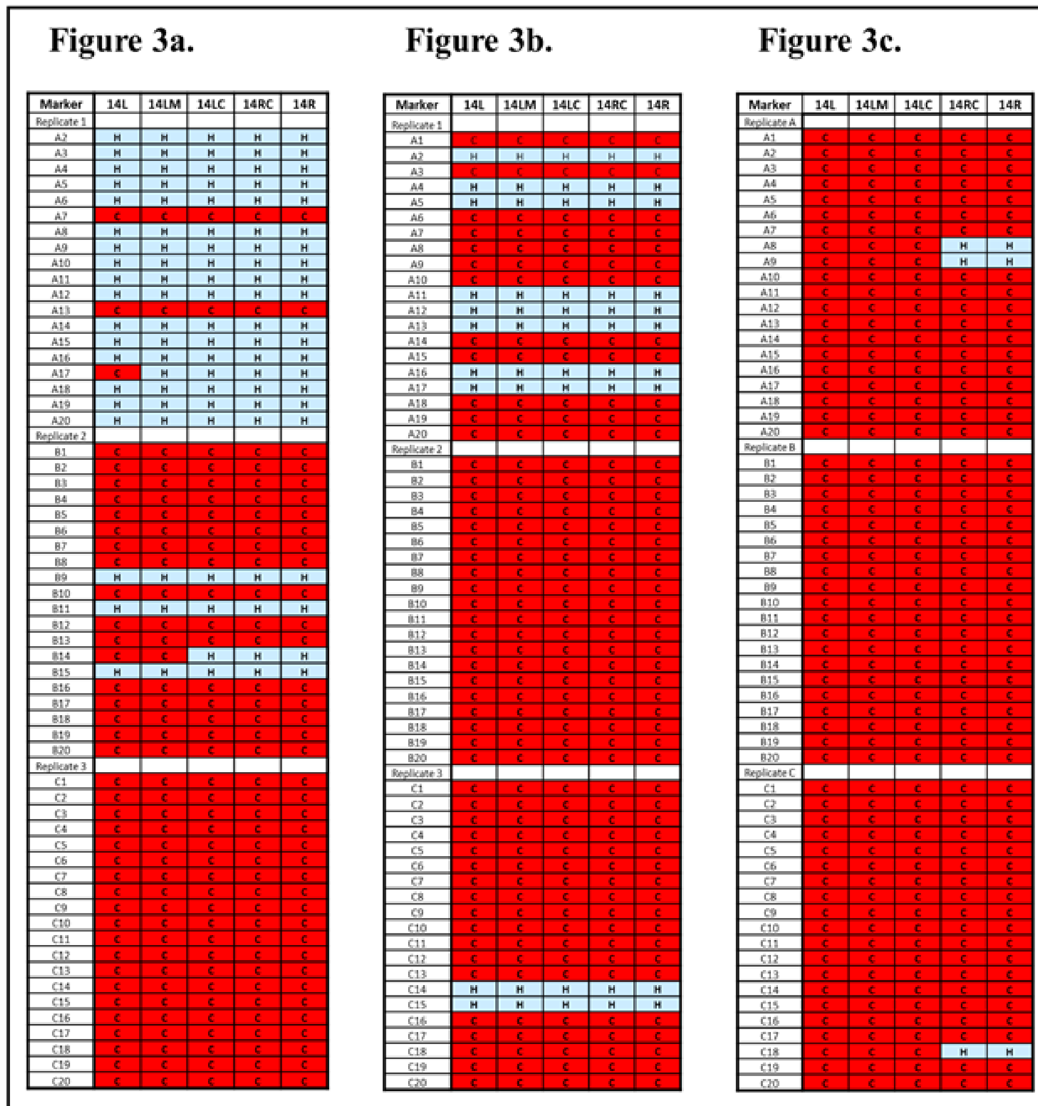

Figure 3a.

Marker	14L	14LM	14LC	14RC	14R
Replicate 1					
A2	H	H	H	H	H
A3	H	H	H	H	H
A4	H	H	H	H	H
A5	H	H	H	H	H
A6	H	H	H	H	H
A7	C	C	C	C	C
A8	H	H	H	H	H
A9	H	H	H	H	H
A10	H	H	H	H	H
A11	H	H	H	H	H
A12	H	H	H	H	H
A13	C	C	C	C	C
A14	H	H	H	H	H
A15	H	H	H	H	H
A16	H	H	H	H	H
A17	C	H	H	H	H
A18	H	H	H	H	H
A19	H	H	H	H	H
A20	H	H	H	H	H
Replicate 2					
B1	C	C	C	C	C
B2	C	C	C	C	C
B3	C	C	C	C	C
B4	C	C	C	C	C
B5	C	C	C	C	C
B6	C	C	C	C	C
B7	C	C	C	C	C
B8	C	C	C	C	C
B9	H	H	H	H	H
B10	C	C	C	C	C
B11	H	H	H	H	H
B12	C	C	C	C	C
B13	C	C	C	C	C
B14	C	C	H	H	H
B15	H	H	H	H	H
B16	C	C	C	C	C
B17	C	C	C	C	C
B18	C	C	C	C	C
B19	C	C	C	C	C
B20	C	C	C	C	C
Replicate 3					
C1	C	C	C	C	C
C2	C	C	C	C	C
C3	C	C	C	C	C
C4	C	C	C	C	C
C5	C	C	C	C	C
C6	C	C	C	C	C
C7	C	C	C	C	C
C8	C	C	C	C	C
C9	C	C	C	C	C
C10	C	C	C	C	C
C11	C	C	C	C	C
C12	C	C	C	C	C
C13	C	C	C	C	C
C14	C	C	C	C	C
C15	C	C	C	C	C
C16	C	C	C	C	C
C17	C	C	C	C	C
C18	C	C	C	C	C
C19	C	C	C	C	C
C20	C	C	C	C	C

Figure 3b.

Marker	14L	14LM	14LC	14RC	14R
Replicate 1					
A1	C	C	C	C	C
A2	H	H	H	H	H
A3	C	C	C	C	C
A4	H	H	H	H	H
A5	H	H	H	H	H
A6	C	C	C	C	C
A7	C	C	C	C	C
A8	C	C	C	C	C
A9	C	C	C	C	C
A10	C	C	C	C	C
A11	H	H	H	H	H
A12	H	H	H	H	H
A13	H	H	H	H	H
A14	C	C	C	C	C
A15	C	C	C	C	C
A16	H	H	H	H	H
A17	H	H	H	H	H
A18	C	C	C	C	C
A19	C	C	C	C	C
A20	C	C	C	C	C
Replicate 2					
B1	C	C	C	C	C
B2	C	C	C	C	C
B3	C	C	C	C	C
B4	C	C	C	C	C
B5	C	C	C	C	C
B6	C	C	C	C	C
B7	C	C	C	C	C
B8	C	C	C	C	C
B9	C	C	C	C	C
B10	C	C	C	C	C
B11	C	C	C	C	C
B12	C	C	C	C	C
B13	C	C	C	C	C
B14	C	C	C	C	C
B15	C	C	C	C	C
B16	C	C	C	C	C
B17	C	C	C	C	C
B18	C	C	C	C	C
B19	C	C	C	C	C
B20	C	C	C	C	C
Replicate 3					
C1	C	C	C	C	C
C2	C	C	C	C	C
C3	C	C	C	C	C
C4	C	C	C	C	C
C5	C	C	C	C	C
C6	C	C	C	C	C
C7	C	C	C	C	C
C8	C	C	C	C	C
C9	C	C	C	C	C
C10	C	C	C	C	C
C11	C	C	C	C	C
C12	C	C	C	C	C
C13	C	C	C	C	C
C14	H	H	H	H	H
C15	H	H	H	H	H
C16	C	C	C	C	C
C17	C	C	C	C	C
C18	C	C	C	C	C
C19	C	C	C	C	C
C20	C	C	C	C	C

Figure 3c.

Marker	14L	14LM	14LC	14RC	14R
Replicate A					
A1	C	C	C	C	C
A2	C	C	C	C	C
A3	C	C	C	C	C
A4	C	C	C	C	C
A5	C	C	C	C	C
A6	C	C	C	C	C
A7	C	C	C	C	C
A8	C	C	C	H	H
A9	C	C	C	H	H
A10	C	C	C	C	C
A11	C	C	C	C	C
A12	C	C	C	C	C
A13	C	C	C	C	C
A14	C	C	C	C	C
A15	C	C	C	C	C
A16	C	C	C	C	C
A17	C	C	C	C	C
A18	C	C	C	C	C
A19	C	C	C	C	C
A20	C	C	C	C	C
Replicate B					
B1	C	C	C	C	C
B2	C	C	C	C	C
B3	C	C	C	C	C
B4	C	C	C	C	C
B5	C	C	C	C	C
B6	C	C	C	C	C
B7	C	C	C	C	C
B8	C	C	C	C	C
B9	C	C	C	C	C
B10	C	C	C	C	C
B11	C	C	C	C	C
B12	C	C	C	C	C
B13	C	C	C	C	C
B14	C	C	C	C	C
B15	C	C	C	C	C
B16	C	C	C	C	C
B17	C	C	C	C	C
B18	C	C	C	C	C
B19	C	C	C	C	C
B20	C	C	C	C	C
Replicate C					
C1	C	C	C	C	C
C2	C	C	C	C	C
C3	C	C	C	C	C
C4	C	C	C	C	C
C5	C	C	C	C	C
C6	C	C	C	C	C
C7	C	C	C	C	C
C8	C	C	C	C	C
C9	C	C	C	C	C
C10	C	C	C	C	C
C11	C	C	C	C	C
C12	C	C	C	C	C
C13	C	C	C	C	C
C14	C	C	C	C	C
C15	C	C	C	C	C
C16	C	C	C	C	C
C17	C	C	C	C	C
C18	C	C	C	H	H
C19	C	C	C	C	C
C20	C	C	C	C	C

Figure 3. *S. uvarum* Chromosome 14 loss from AWRI1572 during adaptive evolution experiment. Red 'C' box depicts only *S. cerevisiae* chromosome retained. Blue 'H' box depicts both *S. cerevisiae* and *S. uvarum* chromosome retained: a. Series 2 fermentation; b. Series 3 fermentation; c. Series 5 fermentation.

Fluorescence peak intensities for non-dividing G_0 peaks showed diploid and tetraploid strain fluorescent levels approximately double or quadruple that of the control haploid strain respectively, while hybrid strain AWRI 1572 gave a G_0 fluorescent peak level midway between diploid and tetraploid intensities.

Fluorescence cell flow cytometry showed no discernible difference in ploidy status between the triploid ancestral hybrid strain AWRI 1572 and isolates (60 in total) from the stage one ferment (**Figure 4**) and while isolates from Stage 3 and Stage 5 ferments showed greater diversity of DNA intensities, no isolates showed a gain or loss of complete ploidy level.

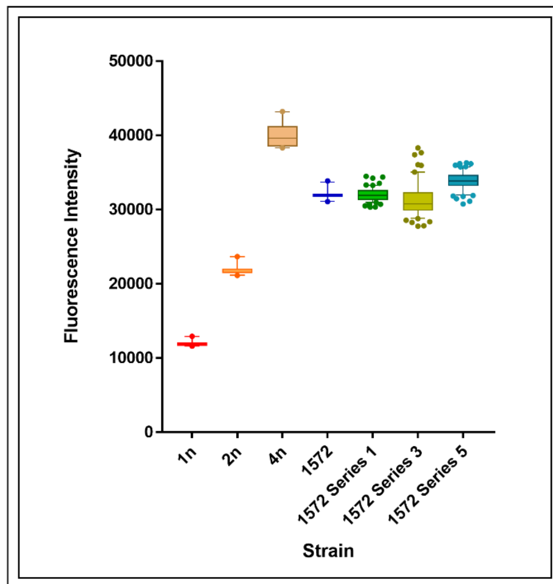

Figure 4. Ploidy levels of AWRI1572 during adaptive evolution experiment. Analyses of 60 isolates from each ferment series with mean ploidy values and whiskers at 10-90 percentile.

Comparison of AWRI 1572 fermentation rates and products vs evolved hybrid strain

Fermentations in Chardonnay juice were undertaken to establish that the loss of *S. uvarum* Chromosome 14 in the evolved hybrid strain had not compromised fermentation proficiency. One isolate, AWRI 2530, was chosen from Stage 3 that showed only loss of *S. uvarum* Chromosome 14 with an apparent 3n ploidy equivalent to the original hybrid strain.

The fermentation properties of parent strains of original hybrid (*S. cerevisiae* AWRI 838, *S. uvarum* AWRI 1176) and hybrid strains (ancestral AWRI 1572 and evolved AWRI 2530) were evaluated in two juices: the original Chardonnay juice containing 225 g/L reducing sugars, and a high-sugar juice fermentation with an addition of glucose and fructose to the Chardonnay juice increasing the total reducing sugars concentration to 350 g/L. The evolved hybrid strain (AWRI 2530) fermented at much faster rate than the original hybrid strain (AWRI 1572) in both fermentations with only a slightly reduced rate relative to the *S. cerevisiae* wine yeast parent strain, AWRI 838, in the 225 g/L sugar juice (**Figure 5a**) while matching the fermentation kinetics of the *S. cerevisiae* parent strain in the high-sugar ferment (**Figure 5b**). The *S. uvarum* parent strain (AWRI 1176) showed the slowest fermentation rate in both fermentations and chemical analysis of the final wines established that this yeast was unable to complete fermentation in the 225 g/L sugar Chardonnay juice with a residual fructose concentration of 30g/L fermentation. Similar residual sugar levels were

observed for the *S. cerevisiae* wine yeast parent strain and both hybrid strains in the high-sugar (350 g/L) ferments, but again, the *S. uvarum* parent strain was unable to utilise sugars to the same degree

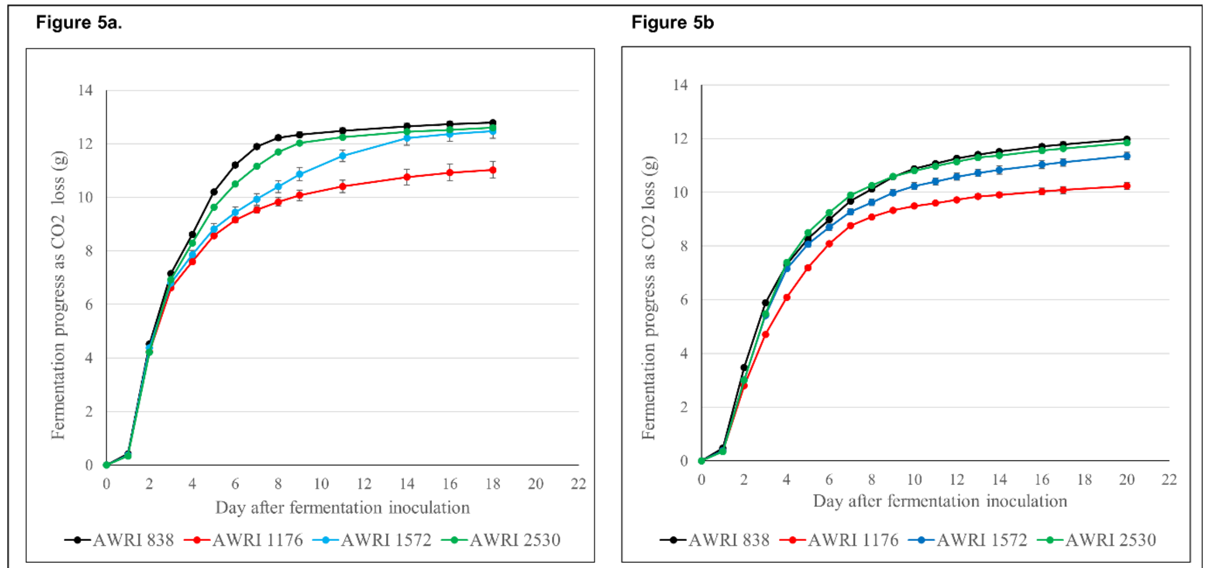

Figure 5. Chardonnay juice fermentation progression as determined by weight loss in grams of CO_2. Data points are represented with error bars: a. 225g/L sugar Chardonnay juice; b. 350g/L sugar Chardonnay juice.

Similar residual sugar levels were observed for the *S. cerevisiae* wine yeast parent strain and both hybrid strains in the high-sugar (350 g/L) ferments, but again, the *S. uvarum* parent strain was unable to utilise sugars to the same degree. Importantly, chemical analysis of the wines confirmed that the evolved strain AWRI 2530 had retained the desirable low-acetic acid production phenotype for which the ancestral hybrid strain was generated. (**Table 2**). No difference in organic acids, glycerol and ethanol concentrations was discernible between wines made by the evolved hybrid strain relative to the ancestral hybrid strain.

Table 2. Secondary fermentation products present in Chardonnay wine produced using different yeast strains.

Compound (g/L)	AWRI838	AWRI1176	AWRI1572	AWRI2530
Chardonnay juice (225 g/L sugar)				
Glucose	0.00	0.00	0.00	0.00
Fructose	0.00 ±0.0 b	30.69 ±3.3 a	0.53 ±0.1 b	0.14 ±0.1 b
Glycerol	8.98 ±0.1 c	12.27 ±0.2 a	10.35 ±0.1 b	10.58 ±0.1 b
Ethanol*	15.1 ±0.1 a	12.8 ±0.2 b	14.9 ±0.1 a	15.1 ±0.1 a
Acetic acid	0.20 ±0.01 b	0.35 ±0.01 a	0.04 ±0.01 c	0.03 ±0.03 c
Succinic acid	3.66 ±0.07 c	9.87 ±0.08 a	4.25 ±0.02 b	4.45 ±0.04 b
Malic acid	3.66 ±0.03 b	3.35 ±0.05 b	4.33 ±0.04 a	4.46 ±0.03 a
Chardonnay juice (350 g/L sugar)				
Glucose	36.06 ±0.25 b	43.37 ±0.57 a	35.22 ±2.22 b	31.75 ±1.28 b
Fructose	92.29 ±0.82 b	99.63 ±2.10 a	88.59 ±2.38 b	86.57 ±3.43 b
Glycerol	10.22 ±0.48 b	13.77 ±0.51 a	14.12 ±0.82 a	14.65 ±0.35 a
Ethanol*	14.82 ±0.21 a	11.77 ±0.51 b	14.83 ±0.25 a	14.99 ±0.31 a
Acetic acid	0.91 ±0.09 a	1.29 ±0.14 a	0.30 ±0.02 b	0.27 ±0.05 b
Succinic acid	2.68 ±0.18 b	5.87 ±0.24 a	3.49 ±0.18 b	3.40 ±0.12 b
Malic acid	1.80 ±0.05 a	2.09 ±0.09 a	2.06 ±0.11 a	1.99 ±0.12 a

* % (v/v)

Within a row, values connected by same letter are not significantly different ($p<0.05$)

Ethanol tolerance

To verify that the important fermentation trait of high ethanol tolerance was retained in the evolved hybrid strain, growth on high ethanol medium was performed. Assay plating confirmed that the evolved strain AWRI 2530 retained the high ethanol tolerance trait of ancestral hybrid strain AWRI 1572, with tolerance slightly higher than the original *S. cerevisiae* grandparent wine yeast strain AWRI 838, visualised as denser cell growth at 14% and 16% ethanol. (**Figure 6**)

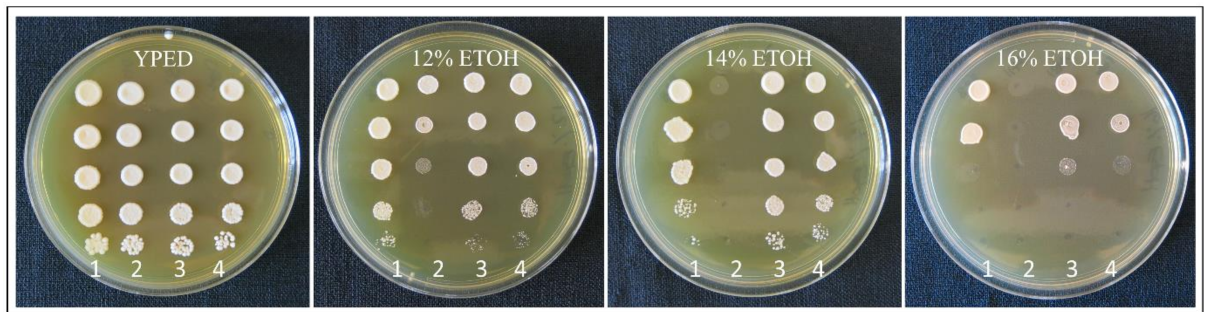

Figure 6. Ethanol tolerance assay plates. Plates left to right; YEPD, YEPD with 12% ethanol, 14% ethanol or 16% ethanol. Strains were plated in columns at 10 fold serial dilutions from top to bottom; column1 AWRI 838 (*S. cerevisiae*), column 2 AWRI 1176 (*S. uvarum*), column 3 AWRI 1572 (ancestral hybrid), column 4 AWRI 2530 (evolved hybrid).

Competitive growth between parent hybrid (AWRI 1572) and the evolved isolate (AWRI 2530):

A competitive growth assay was carried out in Chardonnay juice to validate the improved fitness status of the evolved isolate relative to the ancestral hybrid strain in a fermentation context.

A co-fermentation of AWRI 1572 and AWRI 2530 was conducted in triplicate using Chardonnay juice. Both strains were subcultured individually in YEPD for 2 days and then acclimatised to Chardonnay grape juice by growth in 1/2X juice for 2 days. Equal numbers of cells (1 x 10^6 cells/ml) of AWRI 1572 and AWRI 2530 were then co-inoculated into triplicate, full strength Chardonnay juice. At fermentation completion, cells were then passaged into a second stage ferment at 2 x 10^6 cells/ml.

Cells were harvested at end-of-fermentation from the first ferment, and early stationary in the Stage 2 ferment (day 3). One hundred cells from each fermentation medium were picked to YPD plates using a Singer manipulator.

Colonies were identified through PCR-RFLP species specific marker (14L) using primers targeting the left arm of Chromosome 14 which was missing in the evolved hybrid (AWRI 2530). Analysis of cells harvested from the final stage of the competition fermentation revealed that AWRI 2530 had out-competed the ancestral strain and cell numbers had risen to 90% of the population (**Figure 7**) with replicate ferments having AWRI 2530 populations ranging from 86%-94%. However, only a slight growth advantage was observed during first ferment as AWRI 2530 cell numbers increased to a final proportion of only 55% from the initial inoculum of 50%.

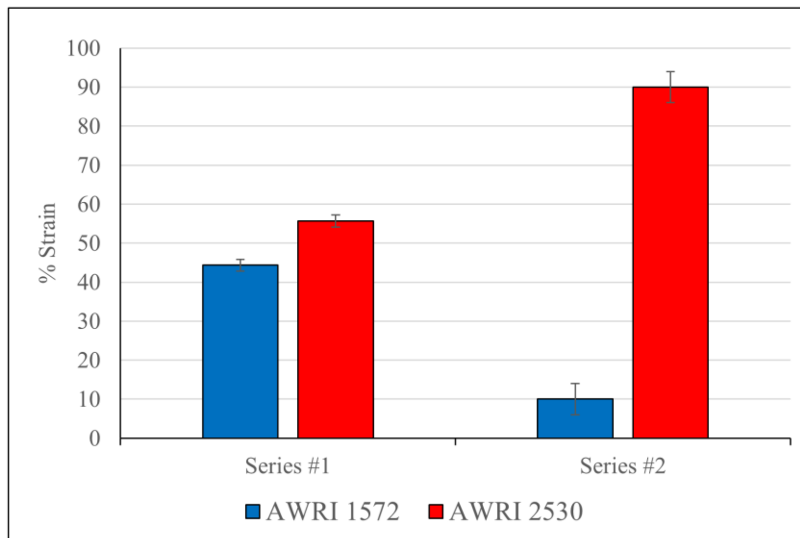

Figure 7. Competition assay of AWRI1572 vs AWRI2530. Data points are represented with error bars. 100 isolates analysed for presence/absence of *S. uvarum* Chromosome 14 from each triplicate fermentation.

Twenty AWRI 2530-identified cells from each series 2 replicate fermentation were analysed using the 32 PCR-RFLP marker system to confirm no further genomic instability in the evolved strain. Of the 60 clones evaluated only two showed any chromosomal mutations; both were lacking *S. uvarum* Chromosome 4L marker (**Supplementary Figure S2**) while Fluorescence flow cytometry confirmed that the overall DNA content of this strain remained stable (**Supplementary Figure S3**).

In order to establish that no loss of *S. uvarum* chromosome 14 occurred in the parent hybrid strain (AWRI 1572) during the experiment, separate ferments where undertaken in parallel in which this strain was the sole yeast inoculated into the grape must. PCR-RFLP analysis of 100 clones from each triplicate AWRI 1572-solo ferment confirmed that the ancestral strain AWRI 1572 was stable with respect to *S. uvarum* Chromosome 14 in the context of these two sequential Chardonnay juice fermentations (**Supplementary Figure S4**).

Discussion

The research described in the current manuscript builds upon work previously reported from our laboratory on the design and generation of *S. cerevisiae* x *S. uvarum* interspecific yeast hybrids targeted to the phenotype of reduced production of acetic acid in grape juice fermentation. Here we describe the selection of an improved hybrid with increased fitness in grape juice fermentation using serial, grape juice fermentations as summarised in Fig. 1. Identification of a mutant with an improved phenotype was made possible by the introduction of a novel approach to screening for candidate strains with

increased fitness from evolving populations: a set 32 PCR/RFLP primer and restriction enzyme pairs designed to target each arm of the 16 *Saccharomyces* chromosomes was used to confirm presence/absence of *S. cerevisiae* and *S. uvarum* chromosomes in cells isolated from different stages of the evolving population. This enabled retrospective identification of cells that had the most represented endpoint chromosomal complement, but from an early timepoint.

Traditional approaches to adaptive evolution in yeast strains utilise end-point sampling from multi-batch culture growths (McBryde et al. 2006; Cadière et al. 2011), or steady state growth conditions using chemostats with population sampling following hundreds of cell generations (Hansche et al. 1978: Gresham et al. 2008; Kvitek and Sherlock 2011; Kutyna et al. 2012). This carries the risk of selecting for mutations that shape phenotypes over and above that which is targeted. Identifying a mutant with the desired phenotype from an early timepoint in the evolutionary process would reduce this risk.

Hybrid isolates recovered from the final (5th) round ferment revealed that chromosomal mutations in *S. uvarum* Chromosome 14 occurred in all isolates analysed: 95% of isolates lost the entire chromosome while 5% still retained the right arm.

While minor Chromosome 14 instability was identified in isolates from the first stage fermentation, by the completion of the second fermentation stage it was evident that *S. uvarum* Chromosome 14 was preferentially lost (67% of isolates showed partial or whole chromosome loss) and the frequency of this karyotype increased to 100% over subsequent passaging steps. A possible reason for this was that loss of *S. uvarum* Chromosome 14 leads to increased fitness in this interspecific hybrid.

Loss of other chromosomal markers during later stages of the fermentation series was also identified (10-15% of clones showed an alteration in Chromosomes 1, 12 or 16 during stages 3 to 5) but none of these showed a frequency increase in all replicates over the course of the experiment. It is unlikely that these changes, at least in the genetic background of AWRI 1572, and in Chardonnay juice led to increased fitness.

An isolate (AWRI 2530) with loss of *S. uvarum* Chromosome 14 but no other detectable changes in karyotype was chosen for further characterisation. Serial growth competition in Chardonnay juice between the original hybrid (AWRI 1572) and the above isolate demonstrated that loss of *S. uvarum* Chromosome 14 is likely to contribute to the observed increase in fitness.

Chromosomal assessment of AWRI 2530 isolates from the competition assay showed a stable hybrid karyotype. Whilst some slight differences in DNA fluorescence levels were detected during the two-series fermentation, no overall change in ploidy was observed; all isolates seemingly retained their triploid status (although differences in chromosomal aneuploidy levels cannot

be discerned by our methods). Interestingly, other studies describing the stabilisation of synthetic polyploid interspecific hybrids have reported ploidy stabilisation to triploid levels; a tetraploid *S. cerevisiae* x *S. kudriavzevii* hybrid showed a loss of DNA content, stabilising at a level similar to triploid when undergoing fermentation stresses (Perez-Traves 2014), while in another study diploid *S. cerevisae* x *S. bayanus* hybrids increased their DNA content by 60% (approximating triploid ploidy levels) after 50-80 vegetative generations (Kunicka et al. 2011).

Fermentation kinetics showed that the evolved strain had an increased fermentation performance relative to the original hybrid as it was able to metabolise sugars at a faster rate and complete fermentation in a shorter time-frame. Ethanol tolerance was not diminished as both hybrid strains displayed slightly higher tolerance than the wine yeast parent, which was evident at a concentration of 14% ethanol.

Analysis of the resultant wines indicated that the desirable winemaking traits of the original hybrid AWRI 1572 had not been compromised in the evolved strain as no difference in secondary fermentation products and ethanol production was seen and the evolved strain retained the low volatile acidity production trait of the original hybrid strain.

Genome stability and the maintenance of appropriate gene regulation is essential for normal functioning and cell viability. However, a certain amount of genome plasticity can be an advantage when organisms encounter challenging environs, potentially enabling acclimatisation to changing conditions. Kingdoms that utilise a sexual cycle generate variability by recombination and chromosomal assortment. Interspecific hybridisation (mating between closely related species) brings even greater novelty to an organism, enhancing genetic and biochemical flexibility relative to the parents. On the other hand, plant studies have shown that interaction between different genomes with inherent incompatibilities can lead to genomic instability with alterations such as chromosomal losses, translocations, gene repetitions and silencing (Comai et al. 2000; Adams and Wendel 2005)

In addition, mating that results in polyploidy provides redundancy that can accelerate genomic change (Selmecki et al. 2015) and function divergence. Polyploidy has been a very important factor in plant evolution (Wendel 2000) and many flowering plants and common crop plants (i.e. wheat, rice, coffee and banana) have polyploid derivation. While the cereal species of wheat and rice are evolutionary hybrids and their genomic stabilisation may have been the result of eons of minor genomic changes, studies of incipient *Brassica* interspecific hybrids have shown that rapid and extensive genomic changes can occur within five generations of hybridisation and that a relationship exists between frequency of change and divergence of parental genomes (Song et.al 1995).

Genome instability on Chromosome 14 in *S. cerevisiae* x *S. uvarum* diploid hybrids exposed to nitrogen limiting conditions has been reported earlier by Dunn et al. in 2013. In their study, reciprocal translocations between *MEP2* (a high-affinity ammonium permease) occurred, resulted in chimeric chromosomes each carrying a MEP2 fusion gene. This chromosomal rearrangement also increased hybrid fitness, allowing evolved hybrid strains to grow faster under nitrogen-limitation than ancestral hybrids. An evolutionary study involving a different alloploid interspecific hybrid strain, *Saccharomyces pastorianus*, also showed instability in Chromosome 14 with loss of *S. cerevisiae* right arm copy number (Brickwedde et al. 2017).

Recently, genomic sequencing of a small number of natural wine yeast hybrids was reported (Borneman et al. 2016) and, while the only *S. cerevisiae* x *S. uvarum* hybrid sequenced (Lalvin S6U) had retained the *S. uvarum* Chromosome 14, two *S. cerevisiae* x *S. kudriavzevii* natural wine yeast hybrids (Enoferm Assmunshansen and Maurivin EP2) lost the non- *S. cerevisiae* Chromosome 14 (however, both these hybrids also sustained large losses of *S. kudriavzevii* genome and have *S. kudriavzevii* contributions from only six chromosomes).

In the current study, little evidence of partial loss of *S. uvarum* Chromosome 14 was identified by the marker system used, with only six isolates from a total of 240 revealing partial alteration. This could mean that partial loss of Chromosome 14 is rare, fundamentally unstable, or leads to decreased fitness.

The cause of increased fitness in the evolved strain remains to be determined. Loss of the *S. uvarum* Chromosome 14 may, for example, have impacted on acclimatisation to fresh medium leading to a decreased lag phase, faster growth in exponential phase, increased cell population at stationary phase, the ability to tolerate stresses such high sugar and high ethanol concentrations or the ability to uptake and metabolise sugars at a faster rate. While there is high DNA sequence divergence between *S. cerevisiae* and *S. uvarum* (similar to that between human and mouse with 62% nucleotide identity in aligned positions (Kellis et al. 2003)) *S. cerevisiae* and *S. uvarum* Chromosomes 14 are co-linear with no translocations reported (Fischer et al. 2000) and there is potential for a number of genes on this chromosome to impact on cell fitness in a fermentation context: roles in stress tolerance e.g. *FIG4*, *WSC2*, *HCH1*, *SKO1*, *CRZ1*, *PDR18*; roles in glucose metabolism e.g. *HXT14*, *GCR2*, *YCK2*, *SSN8*; roles in cell growth e.g. *IES2*, *YGP1*.

In conclusion, we report the successful generation of an evolved interspecific wine yeast hybrid with increased fitness in a fermentation context relative to the ancestral hybrid strain. This was achieved using a novel screening approach that utilised chromosomal mutations as markers for the trait of interest. The evolved hybrid strain retained the targeted fermentation trait of reduced volatile acidity production while exhibiting improved fermentation kinetics. The

chromosomal marker system employed allowed the pattern of genomic plasticity that arose during the evolution of the interspecific hybrid to be exposed and future work on individual isolates from the evolution study may reveal information about genomic alterations that lead to interspecific yeast hybrid stabilisation.

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Author contributions

JB designed and undertook all the experimental work, interpreted the data and wrote the manuscript. CF, AB, and PC assisted in experimental design, supervised experimental work, helped to evaluate and edit the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

A novel approach to isolating improved industrial interspecific wine yeasts using chromosomal mutations as a marker for increased fitness.

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Supplementary Figure 1. Chromosomal marker loss from AWRI 1176 following first series of adaptive evolution experiment.
Red 'C' box depicts S. cerevisiae marker only retained, yellow 'U' box depicts *S. uvarum* marker only retained, blue 'H' box depicts both *S. cerevisiae* and *S. uvarum* markers retained. Chromosomal markers are shown along the top horizontal of the figure and isolates are numbered 1-20 in triplicates A, B & C along the left vertical.

Supplementary Figure 2. Chromosomal marker 4L loss from AWRI 2530 isolates following 2nd series competition experiment.
Left gel 'A' replicate AWRI 2530 isolates and right gel 'B' replicate AWRI 2530 isolates with 4L marker (top) and 4R marker (bottom). In each gel; lane 1 100bp ladder, lane 2 AWRI 838, lane 3 AWRI 1176, lane 4 AWRI 2530, lanes 5 to 24 isolates of AWRI 2530. White arrows point to marker loss.

Supplementary Figure 3. Ploidy levels of AWRI2530 following 2nd series competition experiment.
Analyses of 60 isolates from each 2nd series replicate ferment with mean ploidy values and whiskers at 10-90 percentile.

Supplementary Figure 4. PCR-RFLP 14L chromosomal marker from ancestral hybrid AWRI 1572 isolates following 2nd series competition fermentation.
1st row Replicate 'A' isolates 1-50, 2nd row Replicate 'A' isolates 51-100, 3rd row Replicate 'B' isolates 1-50, 4th row Replicate 'B' isolates 51-100, 5th row Replicate 'C' isolates 1-50, 6th row Replicate 'C' isolates 51-100. In each gel; lane 1 100bp ladder, lane 2 AWRI 838, lane 3 AWRI 1176, lane 4 AWRI 1572, lanes 5 to 54 hybrid isolates of AWRI 1572 following 2nd series competition fermentation.

Supplementary Figure 1.

Marker	1L	1R	2L	2R	3L	3R	4L	4R	5L	5R	6L	6R	7L	7R	8L	8R	9L	9R	10L	10R	11L	11R	12L	12R	13L	13R	14L	14R	15L	15R	16L	16R
Replicate A																																
A1	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
A2	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
A3	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
A4	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	U	H	H	H	H
A5	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
A6	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
A7	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
A8	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
A9	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
A10	H	H	H	H	H	H	C	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
A11	H	H	H	H	H	H	H	H	H	H	H	H	H	H	C	H	H	H	H	H	H	H	H	H	H	H	H	H	H	C	H	H
A12	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	C	H	H	H	H
A13	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
A14	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
A15	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
A16	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
A17	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
A18	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
A19	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
A20	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
Replicate B																																
B1	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
B2	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
B3	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
B4	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
B5	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
B6	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
B7	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
B8	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
B9	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
B10	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
B11	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
B12	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
B13	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
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B19	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
B20	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
Replicate C																																
C1	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
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C3	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
C4	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
C5	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
C6	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
C7	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
C8	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
C9	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
C10	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
C11	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
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C13	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
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C15	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
C16	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
C17	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
C18	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
C19	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
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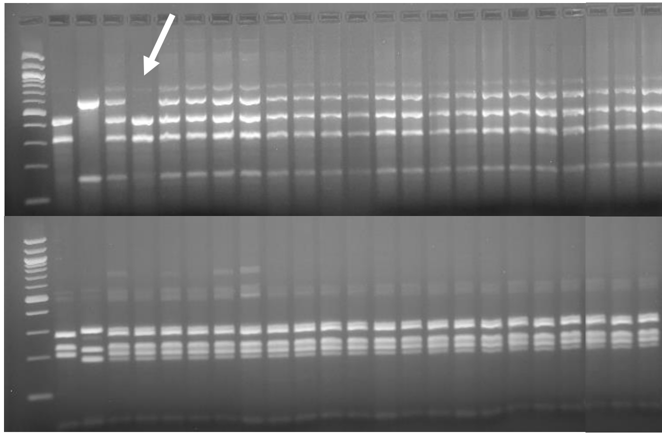
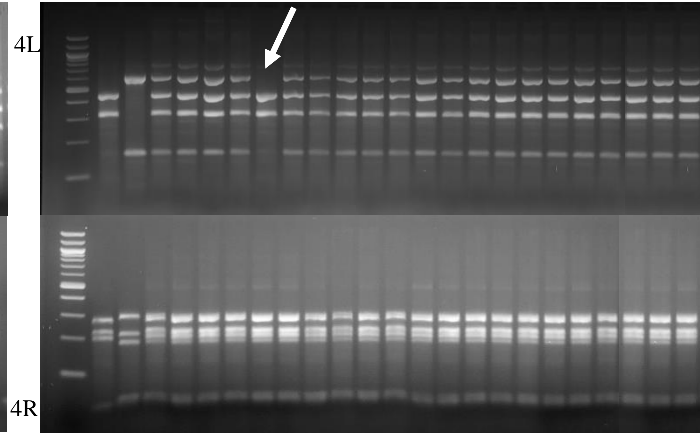
Supplementary Figure 2.

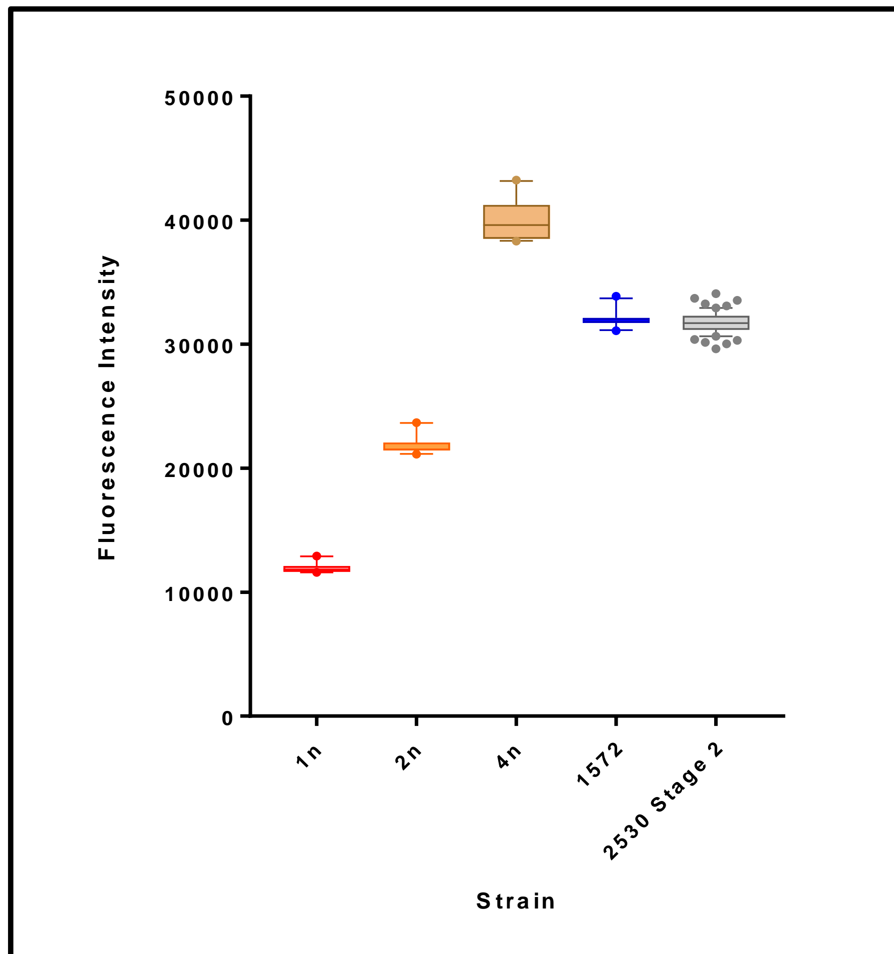
Series #2 A replicate



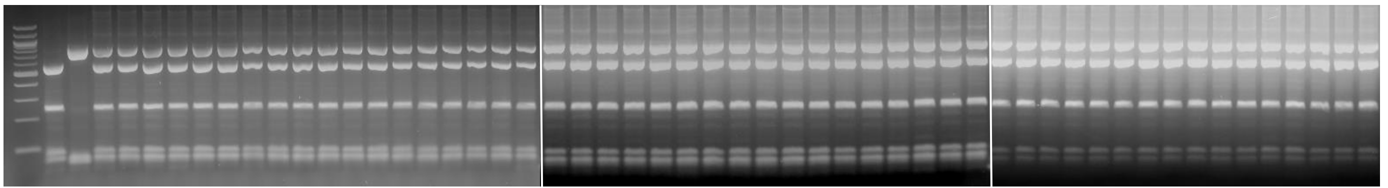
Series #2 B replicate



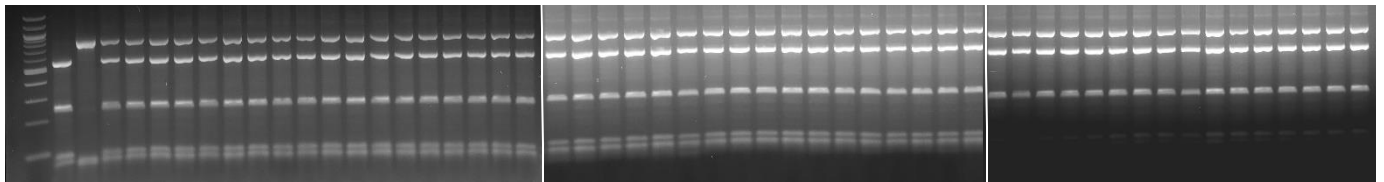
Supplementary Figure 3.



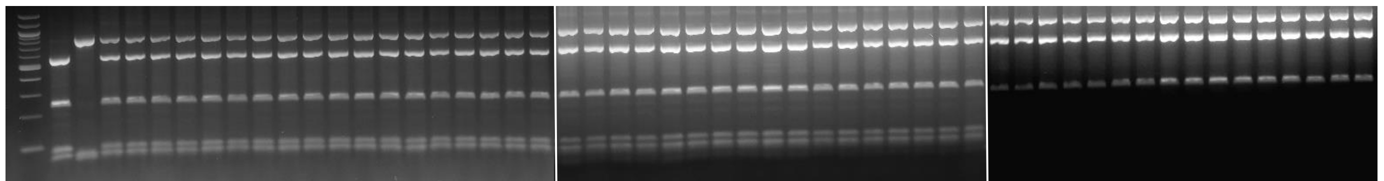
Supplementary Figure 4



Series 2A AWRI 1572 isolates #51-100



Series 2B AWRI 1572 isolates #1-50



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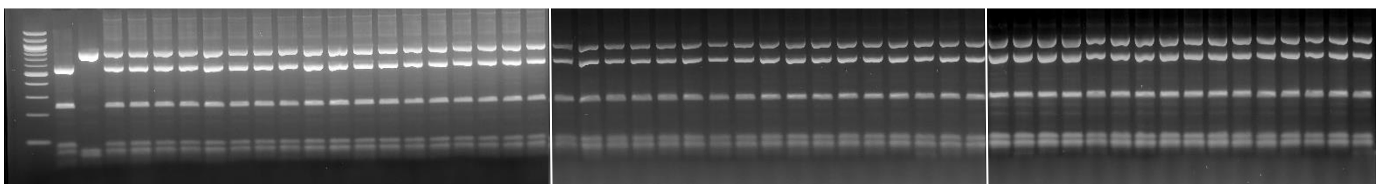
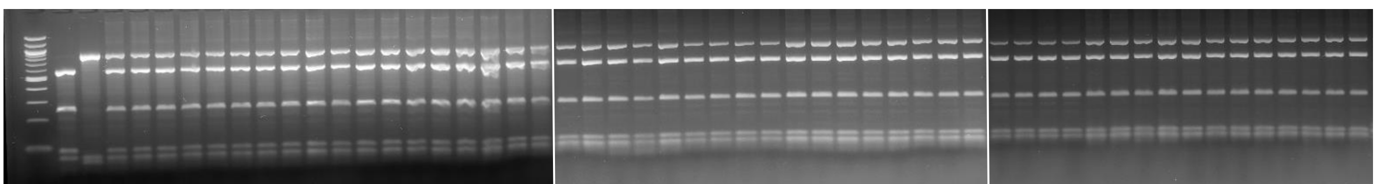
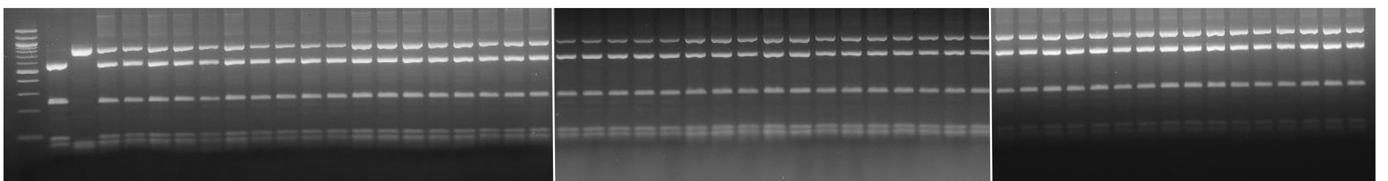


Series 2C AWRI 1572 isolates #1-50



Series 2C AWRI 1572 isolates #51-100



Chapter 6

Concluding remarks

The research discussed in this thesis was undertaken to develop new yeast strains with diverse wine flavour and aroma profiles and/or improved fermentation traits. Such yeasts have the potential to deliver tools to the winemaker for differentiating wine styles readily, and with little additional costs. While there is an array of commercial wine yeast strains already available to the winemaker, there is a view in the industry that greater sensory complexity is achieved when species of yeast other than *S. cerevisiae* contribute to the fermentation as happens in spontaneous fermentations which rely on the indigenous microorganism population of vineyard.

However, spontaneous fermentations can be unreliable and problematic with off flavours generated and ferments being slow or sluggish. Many winemakers would like to increase complexity in their wines, but don’t want the associated risks.

As demonstrated from work described in this thesis interspecific wine yeast hybrids can enhance complexity thus negating the need to adapt risky spontaneous fermentations.

The work presented shows the progression of yeast strain development from initial random interspecific hybrid generation with closely-related species (Chapter 2) to the inclusion of more divergent genetic material from a more distantly-related species (Chapter 3), leading to a targeted hybridisation approach utilising specific phenotypes in the non-*S. cerevisiae* parent (Chapter 4) and concludes with employing an evolutionary selection of hybrid progeny to increase fermentation fitness (Chapter 5).

The yeast hybridisation process undertaken utilised the mating cycle of *Saccharomyces* species. However, this would normally require a meiotic division leading to production of haploid spores. Reducing diploid wine yeast to the haploid state might result in the loss of important winemaking traits. For our experiments we exploited the natural, but infrequent, ‘mating type switching’ that allows diploid wine yeast to mate with haploid strains to form triploid interspecific hybrids.

Initially, mating experiments centred on crossing strains from members of closely related species to *S. cerevisiae*; *S. paradoxus* and *S. kudriavzevii* (Chapter 2).

Chemical analysis of yeast metabolites produced by a *S. cerevisiae* x *S. paradoxus* hybrid in defined medium confirmed that the addition of the *S. paradoxus* genome had an impact on the parental wine yeast metabolome and hence, had potential for delivering novel yeast metabolite profiles to wines.

Subsequently, yeast volatile fermentation metabolites that have previously been identified as important contributors to flavour and aroma in wines were assessed in *S. cerevisiae* x *S. paradoxus* and *S. cerevisiae* x *S. kudriavzevii* hybrid yeast-made wines. Chemical analyses showed concentrations of compounds that were greater or

less than those produced by the wine yeast parent. Interestingly, the magnitude of differences varied between the two interspecific hybrids, highlighting the potential for different hybrid strains to tailor wines towards different consumer groups. The compounds that were present at altered levels in the hybrid-made wines contribute flavours such as fruits (banana, strawberry and green apple), perfumes and flowers, and compounds with the more pungent attributes of blue cheese, rancid cheese and fusel (Chapter 2).

The introduction of genetic material from closely-related *Saccharomyces* species into a *S. cerevisiae* wine yeast background impacted favourably on the wine flavour and aroma profiles of a commercial wine yeast. Additionally, some flavour-active metabolites were produced at concentrations not predicted by their parental metabolite profiles. Thus, interspecific hybridisation could lead to the generation of new yeast strains capable of creating unique wine styles from conventional grape varieties.

In an endeavour to deliver wines with novel non-*S. cerevisiae*-like yeast derived flavour-active metabolites, a distant *Saccharomyces* species not associated with wine fermentation (*S. mikatae*) was used to generate a new breed of wine yeast (Chapter 3). The evolutionary distance between *S. cerevisiae* and *S. mikatae* is considerable (they share only 73% of overall DNA sequence homology), therefore it was deemed to be a good candidate for the introduction of novel metabolic outputs to shape wine sensory characteristics.

Although mating between spores of *S. cerevisiae* and *S. mikatae* had previously been performed to determine species boundaries, no natural interspecific hybrids between these species had been reported and no rare mating events of diploid *S. cerevisiae* with *S. mikatae* spores had been reported previously. Putative hybrids between a robust *S. cerevisiae* wine yeast and *S. mikatae* were confirmed using species-specific fingerprints generated from PCR-RFLP analysis of rDNA while fluorescence flow cytometry analysis showed DNA content fluorescence levels equivalent to a triploid genome.

The chromosomal content of a single hybrid strain was investigated using a microarray approach (array-Comparative Genome Hybridisation) with results indicating that a complete set of chromosomes from each parent species exist in the hybrid genome. (Chapter 3). An assessment of parental phenotypes showed that hybrids inherited traits from both parents: high temperature tolerance from the *S. cerevisiae* parent and low temperature tolerance from the *S. mikatae* parent. In addition, some of the hybrids displayed transgressive phenotypes (hybrid vigor) with even stronger growth on high ethanol medium than their ethanol-tolerant *S. cerevisiae* parent (Chapter 3). The hybrids also differed in their ability to tolerate the stresses involved in growth in Chardonnay juice with some hybrids exhibiting an extended lag-phase prior to commencement of cell division (Chapter 3).

Two hybrid strains chosen for further investigation showed robust fermentation properties in Chardonnay juice and end-of-ferment isolates were assessed for genomic stability. Markers for each of the sixteen chromosomes from both parental species were appraised. While no loss of *S. cerevisiae* chromosomal genome was detected a small number of isolates (~6%) showed minor chromosomal alterations in the *S. mikatae*-parent genome, with loss of one or both arms from a single chromosome. Importantly, phenotypic analysis of essential fermentation traits confirmed that tolerance to high sugar and ethanol levels were retained in all isolates, even those with partial loss of *S. mikatae* genome (Chapter 3). As wine yeast are not re-pitched from one fermentation to the next, a minor level of genomic instability occurring at the end of fermentation will have little effect on fermentation performance and wine quality and each vintage there is a return to the mother culture to prepare new product for winemakers.

Notably, from a winemaking perspective, desirable transgressive phenotypes were apparent in the *S. cerevisiae* x *S. mikatae* hybrids in the form of increased concentrations of secondary metabolites. Chemical analyses of wines made using the hybrids confirmed that the presence of a *S. mikatae* genome impacted favourably on the production of flavour-active volatile fermentation metabolites. A non-targeted chemical analysis approach to the solvent-extractable portion of the wines revealed that the hybrid-made wines produced significantly higher levels of a number of compounds that contribute savoury attributes (‘cheese’, ‘meat’, potato’, ‘smokey’) and potentially add complexity to the overall flavour profile of these wines. Two identified compounds produced at higher levels by the *S. cerevisiae* x *S. mikatae* hybrids have been shown to be generated in wine in high levels by non-*Saccharomyces* species: isobutyric acid, commonly associated with *Torulaspora delbrueckii*, and 2-phenyl ethyl alcohol, commonly associated with *Kluyveromyces lactis*. Of interest also, is that two compounds produced at higher levels by the hybrid yeast remained unidentified and this may indicate that the *S. mikatae* parent is contributing novel metabolites, not previously recognised, to the wines (Chapter 3).

Hybridisation of a robust *S. cerevisiae* wine yeast with a more evolutionary-distant *Saccharomyces* species (*S. mikatae*) generated interspecific hybrid progeny capable of producing novel metabolite outputs, shaping wine-sensory characteristics towards more complex wines.

Interspecific hybridisation was next used as a strategy to introduce the targeted improvement of an important, wine-relevant trait: volatile acidity in the form of acetic acid (Chapter 4). Winemakers wrestle with challenges particular to fermenting high-sugar juice when producing sweet dessert wines as this style of wine can frequently lead to excessive volatile acidity levels. When *S. cerevisiae* is in an environment with a high sugar concentration, it produces increased levels of glycerol as a compatible solute. This process utilises NADH, and, in order to maintain redox balance NADH is regenerated primarily through conversion of acetic acid from acetaldehyde. Studies had shown that some strains of *S. bayanus* (*S. bayanus* var *uvarum* now re-classified

as *S. uvarum* in literature) contribute less acetic acid to wines than *S. cerevisiae*. However, whilst phenotypic studies of *S. uvarum* strains have shown reasonable sugar tolerance, this species has poor ethanol tolerance when compared to *S. cerevisiae* thus limiting its usefulness in commercial winemaking. Interspecific progeny from crosses between these two species could potentially deliver strains capable of efficient fermentation of high-sugar juices while producing wines without elevated acetic acid levels.

Interspecific hybrids were generated by rare mating a robust *S. cerevisiae* wine yeast with spores of a *S. uvarum* strain previously reported to produce low concentrations of acetic acid (Chapter 4). Two hybrid progeny were investigated for high-sugar (>300 g/L) grape juice fermentations in three different media: Chardonnay juice with sugar additions, botrytized Riesling and Riesling icewine. An assessment of fermentation ability showed that, in general, the hybrids fermented the various high-sugar juices at least as well as the *S. cerevisiae* wine yeast parent, with similar levels of residual sugar, similar growth curves and similar rates of sugar utilisation. As expected, the *S. uvarum* parent performed poorly except at the lowest sugar concentration in Chardonnay juice. Importantly, the hybrid yeast produced wines with reduced levels of acetic acid in all three musts (Chapter 4).

Reports from other laboratories had described genetic instability in their laboratory-generated interspecific hybrids and thus it was important to evaluate stability of the hybrids generated in this study. Fluorescence flow cytometry analysis was used to establish ploidy status while 32 PCR-RFLP species-specific markers were designed to monitor the presence of each arm of every parental chromosome. The genomes of both hybrid strains were shown to be genetically stable following 200 mitotic generations in laboratory nutrient liquid medium as no loss of chromosome from either parent was identified in any of the twenty isolates from each hybrid investigated and no change in the triploid DNA fluorescence levels was observed (Chapter 4).

This work confirmed that interspecific hybridisation can be used to introduce targeted phenotypic changes to meet a particular challenge, namely the generation of wine yeast capable of making sweet dessert wines from high sugar juice without the production of excessive amounts of acetic acid and ethyl acetate. Whilst the *S. cerevisiae* x *S. uvarum* hybrids displayed strong fermentation kinetics, further investigations showed that these hybrids did not display as robust growth characteristics in grape juice as their *S. cerevisiae* parent.

An evolutionary approach using mitotic cell division with a selective pressure was employed to generate an interspecific yeast hybrid with improved fitness. A *S. cerevisiae* x *S. uvarum* interspecific hybrid strain previously designed to produce low levels of acetic from high-sugar juices (see Chapter 4) was subjected to repeated exposure of fermentation stresses through a series of Chardonnay juice batch fermentations in an endeavour to isolate spontaneous mutants with increased fitness in a fermentation context (Chapter 5). Traditional approaches to adaptive evolution of

yeast strains in the laboratory utilise end-point sampling following hundreds of cell generations, carrying the risk of selecting for mutations that shape phenotypes over and above that which is targeted. Identifying a mutant with increased fitness from an early timepoint in the evolutionary process reduces this risk. Research from other laboratories has shown that during their evolution industrial *Saccharomyces* interspecific hybrids have lost chromosomal material from one or both parental lineages. This loss, presumably, led to greater fitness in the fermentation environs that they were isolated from. Hence, chromosomal mutations have the potential to be used as markers for increased fitness in evolving populations. With this in mind, the evolving passaged populations were screened for chromosomal mutations using a 32 species-specific chromosomal marker system. This allowed for the identification of different mutants that developed during the time course, revealing that under these conditions the hybrid *S. uvarum* genome is very plastic and considerably more unstable (and chromosomes preferentially lost) than the *S. cerevisiae* genome.

While a number of different *S. uvarum* chromosomes showed instability in the hybrid genome over the series, only loss of Chromosome 14 became fixed in the population (Chapter 5). An evolved hybrid strain with loss of *S. uvarum* Chromosome 14 but no other detectable changes in karyotype was chosen for further investigation. Cell fitness evaluated by competitive growth in Chardonnay juice confirmed the improved fitness status of the evolved hybrid relative to the original hybrid as cell numbers reached 90% of the total population over two fermentation series (Chapter 5). Fermentation kinetics in Chardonnay juice showed that the evolved strain had an increased fermentation performance relative to the original hybrid and was able to metabolise sugars at a faster rate and complete fermentation in a shorter time-frame. The evolved strain retained the targeted fermentation trait of reduced volatile acidity production while exhibiting improved fermentation kinetics (Chapter 5).

The approach of using chromosomal markers as reliable indicators of increased fitness in evolving interspecific hybrid yeast populations was used successfully to identify increased fitness candidate strains from an early stage of evolutionary progression.

The research presented in this thesis demonstrates that the addition of non-*S. cerevisiae* genomic content into an existing robust *S. cerevisiae* wine yeast can impact favourably on wine flavour and aroma profiles. *Saccharomyces* interspecific hybrids are shown to produce more complex wines driven from a wider and more varied spectrum of yeast flavour-active metabolites. In addition, interspecific hybridisation by natural mating methods can be used to incorporate important fermentation-relevant phenotypes derived from the non-*S. cerevisiae* parent to improve the industrial application of an existing commercial wine yeast strain. Finally, screening individual early isolates from an evolving population under fermentative stress can deliver interspecific hybrid mutants with improved fermentation kinetics.

Highlights of work described in this thesis and relevance to industry

To the best of my knowledge, the manuscript published as Chapter 2 is the first report of wine flavour-active metabolite concentrations produced by wine yeast interspecific hybrids of different *Saccharomyces* species generated in the laboratory with the same wine yeast parent background.

The manuscript published as Chapter 3 gives the first report of triploid *S. cerevisiae* x *S. mikatae* wine yeast hybrids generated in the laboratory and the first documentation of the potential for *S. cerevisiae* x *S. mikatae* wine yeast hybrids to impact on wine style by producing novel, non-*S. cerevisiae*-like flavour-active metabolites in wines. From a personal perspective, work reported in this manuscript gave me an important research experience as I was able to travel to the USA and perform the CGH analysis at Stanford University (Department of Genetics, Associate Professor Gavin Sherlock's laboratory) under the guidance of Dr Barbara Dunn. (https://web.stanford.edu/group/sherlocklab/people.shtml see section titled Visiting Graduate Students)

The wine industry community has shown interest in the interspecific wine yeast hybrids generated by the research described in this thesis. Commercial-scale vintage trials have been undertaken by wineries in Australia (Yalumba, SA; Oliver's Taranga Vineyards, SA; Barwick Estate, WA; Lerida Estate, NSW) in an array of different grape varietals such as Chardonnay, Viognier, Fino, Pinot Grigio and Pinot Noir. Wines from these trials have been showcased in workshops that I convened at national wine conferences in Australia; 2010, 2013 and 2016 Australian Wine Industry Technical Conference.

Winemakers have also been enthusiastic to perform vintage trials using a hybrid strain for specialised wine styles. Successful trials with AWRI 1572 *(S. cerevisiae* x *S. uvarum* hybrid) in high-sugar juice ferments with late-picked Viognier grapes (4,500L tank ferments, Wolfe Blass, SA) and botrytized Riesling (200L barrel ferments at Yalumba, SA) were performed in 2017. Chemical analyses of wines from both trials showed acetic acid concentrations of wines produced by AWRI 1572 were less than 50% than that of the *S. cerevisiae* parent-made wines.

In addition, trials to produce sparkling wines were performed internationally with UK wineries (Gusbourne Estate, Bolney Wine Estate and Nyetimber) using AWRI 1572 (*S. cerevisiae* x *S. uvarum*) and AWRI 2526 (*S. cerevisiae* x *S. mikatae*). Sparkling wines from this trial were showcased in a workshop that I convened at the 2016 International Cool Climate Wine Symposium held in Brighton, UK. One of the delegates attending the workshop (Sally Easton, Master of Wine) posted her account of the workshop online on June 20th, 2016. Her account can be found at:

http://www.winewisdom.com/articles/saccharomyces-interspecific-hybrids-a-new-tool-for-sparkling-winemaking/

An excerpt from Sally's post: 'The research proved chemical differences exist. It then set out to identify if sensory differences exist. Which led in to the symposium demonstration tasting … which was fascinating in its exposure of how organoleptically important and different are different yeast species.'

Responses such as Sally's demonstrate that taking research out into the wider wine community is very valuable in terms of research exposure and translating research results into applied outcomes.

Wine yeast manufacturers have begun to take an interest in the *Saccharomyces* wine yeast hybrids developed in this research. Wine yeast hybrid AWRI 1503 (*S. cerevisiae* x *S. kudriavzevii*) reported in Chapter 2 has been commercialised by AB Biotek (a business division of AB MAURI) and is available to winemakers internationally. Pilot-scale manufacture of active dried yeast products and industrial application testing for another two interspecific hybrid yeasts generated from this research is also underway.

Future Perspectives

The research described in this thesis has shown that interspecific hybridisation between a *S. cerevisiae* wine yeast and other members of the *Saccharomyces* clade can produce a new breed of wine yeast capable of delivering novel flavour-active yeast metabolites and improved winemaking attributes.

Interspecific hybridisation and the targeted incorporation of novel wine-relevant phenotypes would enhance the winemaking properties of current commercial wine yeast.

New species have been added to the *Saccharomyces* genus in recent years (*Saccharomyces eubayanus*, Libkind D et al. 2011; *Saccharomyces jurei*, Naseeb et al. 2017) and inclusion of genetic material from these species could impact positively in wine-relevant traits. However, whilst *S. cerevisiae* yeast strains are the backbone of fermentation industries, few other *Saccharomyces* species are found in fermentation environs and their potential for useful winemaking phenotypes remains undetermined.

Future work would entail phenotypic characterisation of non-*S. cerevisiae* species to prospect for traits that could prove useful in a winemaking context. Traits of interest could include: robust growth at temperatures lower than the optimum minimum fermentation temperature for *S. cerevisiae* strains (ie lower than 15°C); the ability to metabolise malic acid into a compound that has a positive effect on wine quality (currently, a secondary fermentation by lactic acid bacteria is required for the tart malic acid to be converted to the softer lactic acid); and the production of proteins that protect against haze formation in white wines.

In addition, the potential of interspecific hybrids to influence parameters particular to red winemaking is yet to be investigated. Release of polysaccharides by yeast has been shown to influence colour stability and wine astringency in red wines (Escot et al. 2001) while colour intensity and polyphenol composition of wines has also been shown to be influenced by yeast strain choice (Caridi et al. 2004).

Increasing the ratio of non-*S. cerevisiae* to *S. cerevisiae* genomic content in an interspecific hybrid could increase the impact of non-*S. cerevisiae* traits

The wine yeast interspecific hybrids reported in this research are triploids and have been generated by rare mating a diploid *S. cerevisiae* wine yeast with spores from another species of the *Saccharomyces* clade. In order to increase the impact on the non-*S. cerevisiae* genome, crosses could be made with a diploid non-*S. cerevisiae* strain and spores from the *S. cerevisiae* wine yeast. Chromosomal assortment during the sporulation process could result in progeny that have not inherited all of the important fermentation traits from the wine yeast parent and intensive screening of fermentation kinetics will be necessary of all interspecific hybrids generated.

To avoid this issue, interspecific hybrids could be generated by using a stable haploid wine yeast strain already confirmed to have robust winemaking traits. However, this pathway is difficult to achieve using the current industrial yeast strains at our disposal, as wine yeast are homothallic: single mating type haploid cells germinated from spores are able to switch mating type and mate to other cells of the opposite mating type within the population. This event changes a multi heterozygote into completely homozygous diploids (Mortimer et al. 1994). To date, generation of haploid non-mating type-switching strains has been achieved by genetically modified means but GMO strains are not currently used in Australian wine production. However, new technologies such as the CRISPR-Cas systems (DiCarlo et al. 2013) that can re-engineer yeast genomes without leaving residual heterologous genomic material may be approved for use on microorganisms intended for the food and beverage industries in the future.

Another way to alter interspecific hybrid genomes in *Saccharomyces spp*. is to use meiotic chromosomal assortment on laboratory-generated strains. Although interspecific hybrid yeasts have low fertility, mass plating of sporulated hybrid cells will give rise to F1 progeny that have varying combinations of each parental input. Screening of F1 strains using species-specific chromosomal markers (as described in Chapter 5) will allow for the identification of hybrid yeast that have lost *S. cerevisiae* chromosomes. By this means, interspecific hybrids could be generated that have only non-*S. cerevisiae* contributions for some individual chromosomes, thus potentially increasing the impact of the non-*S. cerevisiae* genome. This could be quite advantageous with regards to producing wines with higher concentrations of non-*S. cerevisiae* generated flavour-active metabolites, but could come at a cost as the robust wine fermentation traits are derived from the *S. cerevisiae* parent and valuable winemaking properties may be compromised. However, the subsequent application of directed evolution (as described in Chapter 5) should enable the selection of novel hybrids with increased fitness.

Gene regulation and 'cross talk' in yeast interspecific hybrids

The interspecific wine yeast hybrids generated in the research described in this thesis can be used as a tool to elucidate insights into evolution and speciation arising from interspecific hybridisation. A large proportion of genes differ in their expression patterns between closely related species, and this divergence is thought to be an important driver of phenotypic evolution. However, little is known about the genetic basis of this divergence.

A small number of studies by other researchers have begun to address these questions:

- Borneman et al. (2007) investigated the binding sites for two Transcription Factors Ste12 and Tec 1 showed that there were extensive binding site differences between *S. cerevisiae*, *S. mikatae* and *S. bayanus*. The authors suggested that gene regulation resulting from transcription factor binding is likely to be a major cause of divergence between related species.
- Tirosh et al. (2009) used microarrays to measure allele-specific expression in a *S. cerevisiae* x *S. paradoxus* hybrid to examine the possible connection between expression rewiring and the emergence of novel hybrid phenotypes.
- Khan et al. (2012) studied the relative contribution of *cis*-acting and *trans*-acting regulatory differences in protein expression to inform on divergence between yeast species in a *S. cerevisiae* x *S. uvarum* hybrid.
- Dunn et al. (2013) found evidence of rapid introgression had occurred in incipient *S. cerevisiae* x *S. uvarum* hybrids when interspecific genome rearrangements led to increased fitness in evolved populations.

Future research could utilise ChIP-seq analysis (Park 2009) to investigate alterations of Transcription Factor binding sites in the stable of different species hybrids generated through my research. This investigation might inform on gene regulatory differences that lead to phenotypic evolution and speciation.

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