DELTA TYPING AND ANALYSIS OF ALCOHOL TOLERANCE GENES (*erg2, hsp104, sod2*) IN SIX LOCAL WINE STRAINS OF *Saccharomyces cerevisiae*

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The availability of reliable procedures to check if desirable wine strains of *Saccharomyces cerevisiae* are maintained is valuable for wine producers to ensure the quantity and quality of wines produced. Random Amplification of Polymorphic DNA (RAPD) in a previous study, generated Polymerase Chain Reaction (PCR) profiles that could distinguish *S. cerevisiae* wine strains (that exhibited growth in 10% and 15% ethanol) from a non-wine strain (that exhibited growth inhibition in 10 and 15% ethanol) and wine strains from one another. In this study, delta (δ) typing and sequence analysis of three specific genes (*erg2*, *hsp104* and *sod2*) implicated in alcohol tolerance were performed in search of a faster and cheaper procedure to distinguish strains of *S. cerevisiae*. The use of delta primer pair, δ_{12} / δ_2, resulted in a distinct profile for each of yeast strains studied. Compared to RAPD, which makes use of several random primers and several PCR runs, delta typing involves one PCR run and uses a single δ primer pair. Sequence analysis of three *S. cerevisiae* genes that code for proteins involved in major mechanisms proposed for alcohol tolerance of the species, revealed single (for the *erg2* and *hsp104* genes) to multiple (for the *sod2* gene) nucleotide variations. The nucleotide polymorphisms observed for the *erg2* and *sod2* genes are not expected to result in amino acid variation while the polymorphisms found in the amplified fragments of the *hsp104* gene resulted in amino acid variation at position 236. Nucleotide and protein polymorphisms in these genes, however, could not discriminate the yeast strains studied and the variation did not correlate with the reported strain differences in alcohol tolerance. Results of this study suggest that delta (δ) typing with primers δ_{12} and δ_2, is more discriminatory, faster, and less expensive than RAPD and is recommended to be the first procedure to perform in trying to discriminate wine strains of *S. cerevisiae*. Polymorphisms observed in the three genes, *erg2*, *hsp104* and *sod2*, cannot be used as a basis for designing PCR

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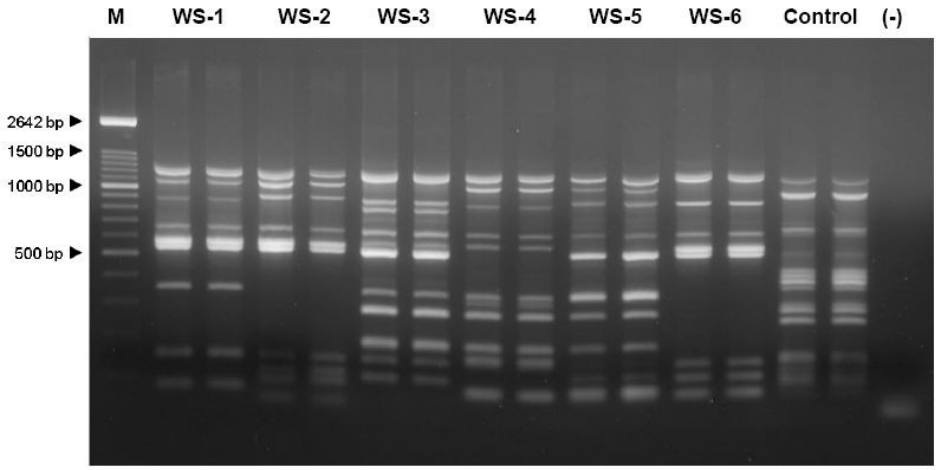

Figure 1. DNA profile of the wine strains (WS-1 to WS-6) and non-wine control strain of *S. cerevisiae* generated by δ typing using primers δ_{12} and δ_2.

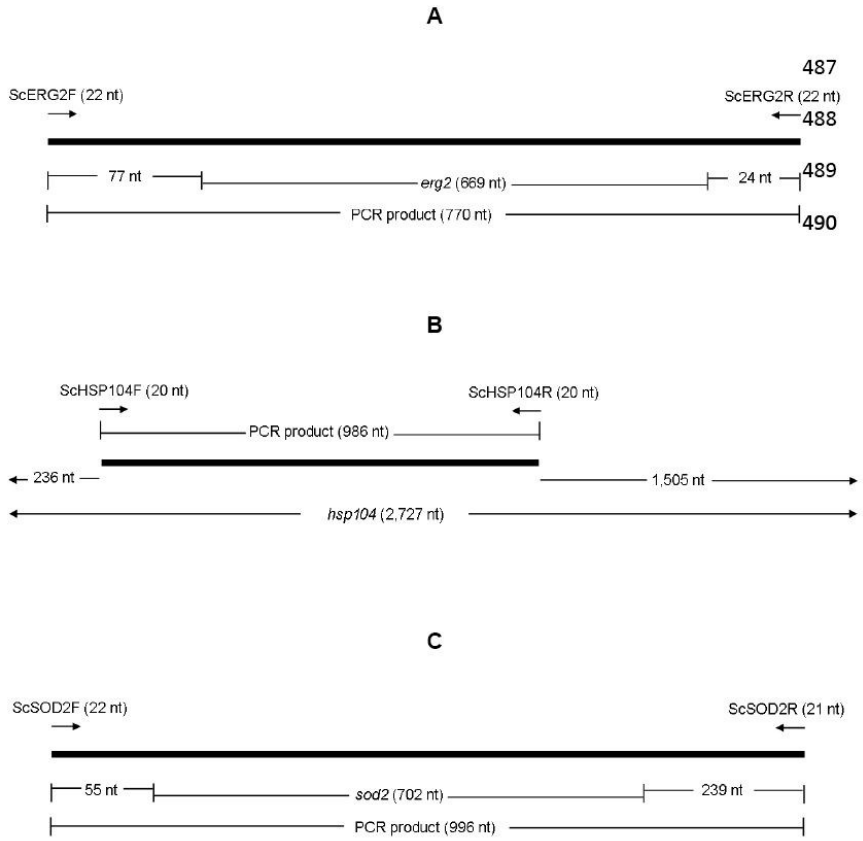

Figure 2. Schematic diagram of primer design for the amplification of genes *erg2* (A), *hsp104* (B) and *sod2* (C) implicated in alcohol tolerance.

primers that could generate discriminating profiles of wine strains versus the non-wine strain of yeast used. Sequence analysis of several other genes implicated in alcohol tolerance is recommended in order to identify a gene or a group of genes with nucleotide and amino acid sequence variation that could distinguish wine strains of *S. cerevisiae*.

KEYWORDS

wine strains, *Saccharomyces cerevisiae*, delta typing, *erg2, hsp104, sod2*

INTRODUCTION

Saccharomyces cerevisiae is the yeast used for alcohol production in winemaking, brewing, and in the biofuel industry due to higher tolerance of this species to ethanol compared to other yeasts and bacteria (Takahashi et al 2000). Since *S. cerevisiae* has been the yeast of choice to initiate alcohol fermentation for fruit wine production, the species is universally known as the "wine yeast" (Pretorius 2000). In general, wine yeast strains are selected based on certain established enological properties such as high tolerance to ethanol and SO_2, ability to ferment sugars and ability to produce high amounts of glycerol and low amounts of H_2S (Carrasco et al 2001). Wine strains of *S. cerevisiae* are usually not available for research. A few studies using wine strains, however, could be performed using strains purchased from companies producing selected strains for specific applications (Vaudano and Garcia-Moruno 2007), bought from culture collections like the China Center of Industrial Culture Collection (Institute of Industrial

Microorganisms, Shanghai, China), or donated by institutes or universities (e.g., Duke University, NC USA; the University of Missouri, MO, USA; and the National Research Institute of Brewing, Tokyo, Japan).

Commercial *S. cerevisiae* wine strains from different countries have been subjected to karyotype analysis, microsatellite typing, inter delta sequence analysis, restriction length polymorphism of mitochondrial DNA (Schuller et al 2004), and Random Amplification of Polymorphic DNA (RAPD) analysis (Capece et al 2004, Giusto et al 2006, Araujo et al 2007). A recent study (Guiamal and Hedreyda 2011) reported for the first time the RAPD analysis of six wine strains of *S. cerevisiae* from the Philippines (used in large scale home-made fruit wine production) that generated distinguishing profiles for each of the Philippine wine strains. Although RAPD is a rapid and reliable approach to distinguish the yeast wine strains, the procedure required the use of several primers and several PCR runs to produce enough discriminating profiles. The same Philippine wine strains were used in this study to test if the results of delta typing and gene sequence analysis of three genes implicated in alcohol tolerance could be the basis for developing a faster, cheaper and better discriminating procedure for wine strains of *S. cerevisiae*.

Delta typing is one of the recommended molecular typing tools for *S. cerevisiae* (Ness et al 1993, Pramateftaki et al 2000, Fernandez-Espinar et al 2001, Schuller et al 2004). The δ sequences are about 0.3 kb direct repeat elements that flank the Ty retrotransposons (Ty elements) in

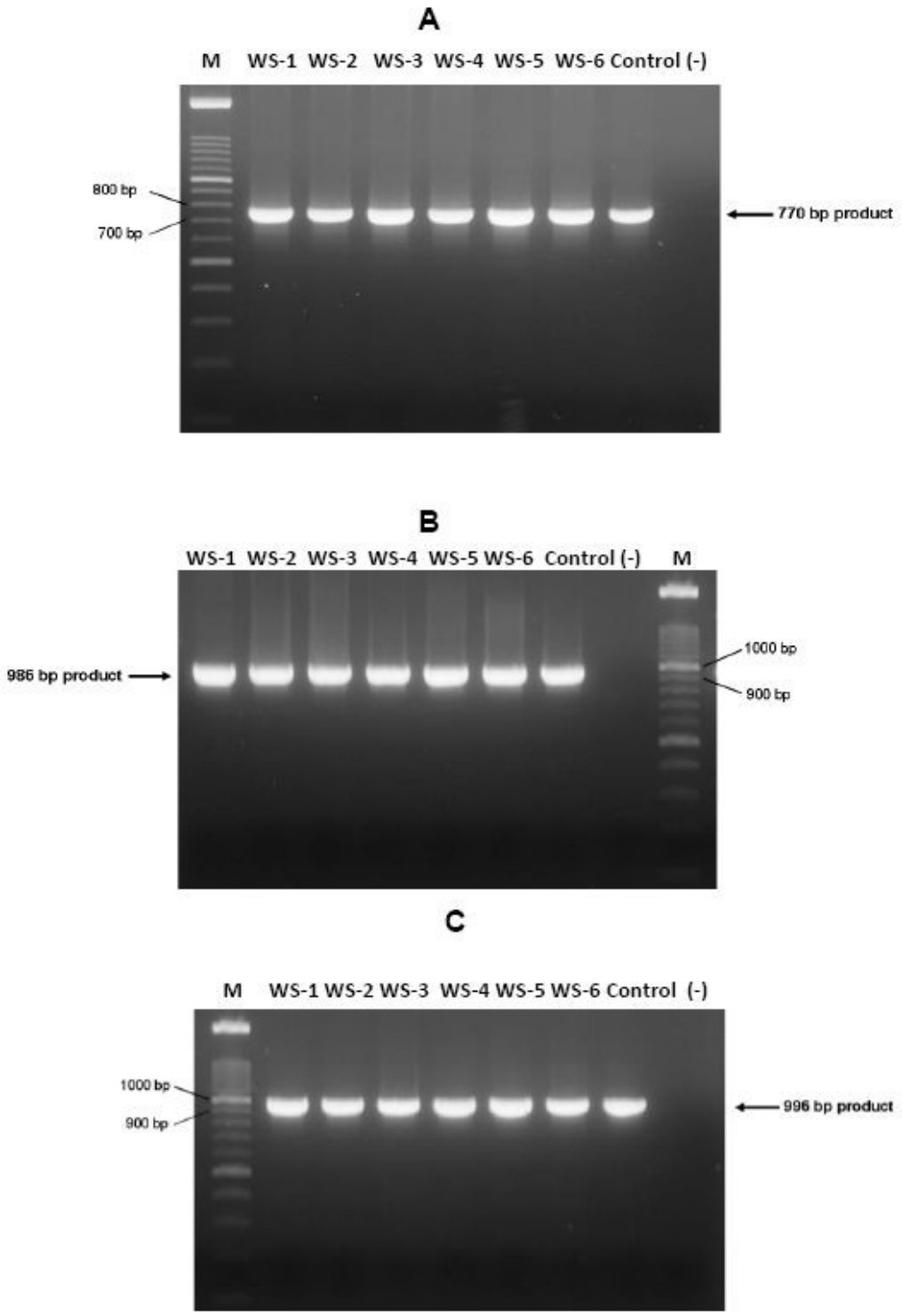

Figure 3. Amplified products of the *erg2* (A), *hsp104* (B) and *sod2* (C) gene targeted PCR from 6 wine strains and a non-wine control strain of *Saccharomyces cerevisiae*.

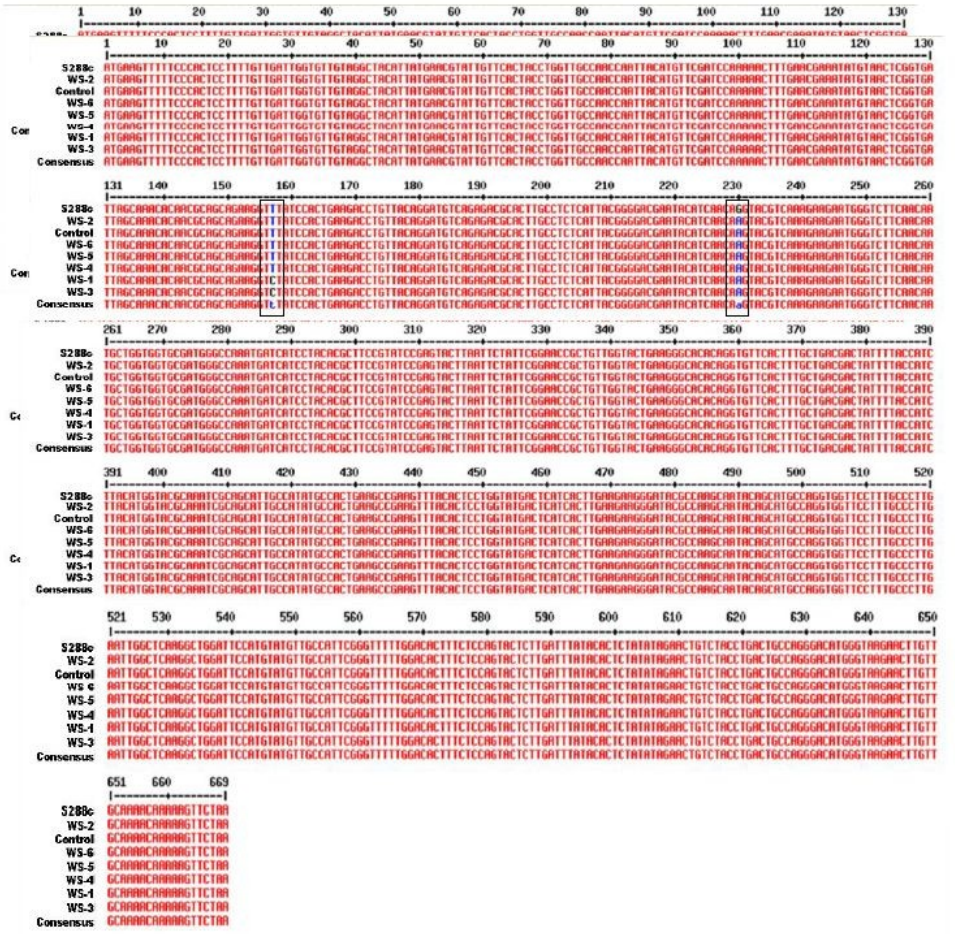

Figure 4. Sequence alignment of the *erg2* genes of the *S. cerevisiae* local wine strains, the non-wine control strain and the reference strain S288c (NC_001140.5).

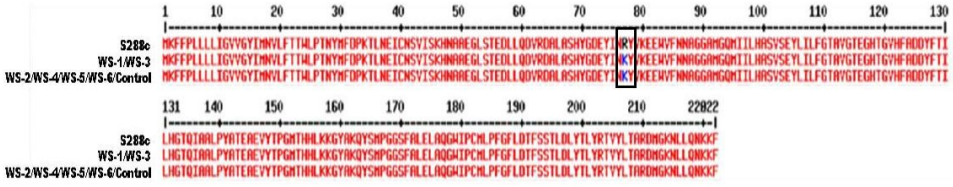

Figure 5. Alignment of the corresponding amino acid sequence of *erg2* protein (based on the *erg2* gene sequences) of the *S. cerevisiae* strains used in this study and the reference strain S288c (NC_001140.5).

the *S. cerevisiae* genome (Ness et al 1993, Lopez et al 2003). There are about a hundred copies of δ elements within the yeast genome present in different locations and are separated by sequences of variable lengths, resulting in discriminating profiles for different strains.

Sequence analysis of three genes (*erg2*, *hsp104*, and *sod2*), that code for proteins involved in three major mechanisms of ethanol tolerance of *S. cerevisiae,* was performed to determine if strains could be distinguished by gene sequence differences. The *erg2* gene codes for a protein involved in the synthesis of ergosterol (the predominant sterol in yeast membranes crucial for membrane stability in the presence of high alcohol concentration), *hsp104* is the gene for a heat shock protein (a protein that helps repair damage due to alcohol stress), and *sod2* is the gene for the antioxidant enzyme, mitochondrial manganese superoxide dismutase (an enzyme that can alter the effect of alcohol toxicity). Comparison of gene sequences could reveal if polymorphism exists and if the polymorphism correlates with differences in alcohol tolerance exhibited by the wine strains. Nucleotide sequence variation in a specific gene could be the basis for designing PCR primers that could be used for the detection of nucleotide variations associated with each strain and its alcohol tolerance phenotype.

MATERIALS AND METHODS

Yeast Strains

Six strains of *S. cerevisiae* (designated WS-1, WS-2, WS-3, WS-4, WS-5 and WS-6) that have been used in local fruit wine production, were kindly provided by Dr. Priscilla C. Sanchez (Retired Professor, Institute of Food Science and Technology, UP Los Baños). A

control non-wine strain of *S. cerevisiae* var. *ellipsoideus* (UPCC 2115) was obtained from the Natural Sciences Research Institute, UP Diliman. These strains of *S. cerevisiae* were grown at 30°C and maintained at room temperature in YEPD agar (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) slants.

DNA Isolation

Genomic DNA from all the yeast strains used in this study was isolated using ZR Fungal/Bacterial DNA Kit™ (Zymo Research, USA), following the protocol described by the manufacturer.

Delta Typing

PCR amplification of δ sequences was performed in 10-µL reactions containing 1X Titanium PCR buffer (Clontech, USA), 0.4 mM dNTPs (Promega, USA), 0.5 µM of each primer [δ_{12} - TCA ACA ATG GAA TCC CAA C and δ_2 - GTG GAT TTT TAT TCC AAC (Schuller *et al* 2004)], 0.25X Titanium *Taq* polymerase (Clontech, USA) and 1 µL yeast genomic DNA extract. Reactions were prepared in replicate.

Thermocycler conditions used were as follows: initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 30 sec, annealing at 45°C for 1 min and extension at 72°C for 1 min; and final extension at 72°C for 10 min (Multigene, Labnet International, Inc., USA).

Primer Design and Amplification of Selected Ethanol Tolerance Genes

Primers to amplify the genes for the heat shock protein (Hsp104), the C-8 sterol isomerase and mitochondrial manganese superoxide dismutase (MnSOD) were designed based on the gene sequences of *S. cerevisiae* S288c strain (ATCC 204508) (NCBI Sequence Reference No. NC_001145.2, NC_001144.4 and NC_001140.5 for

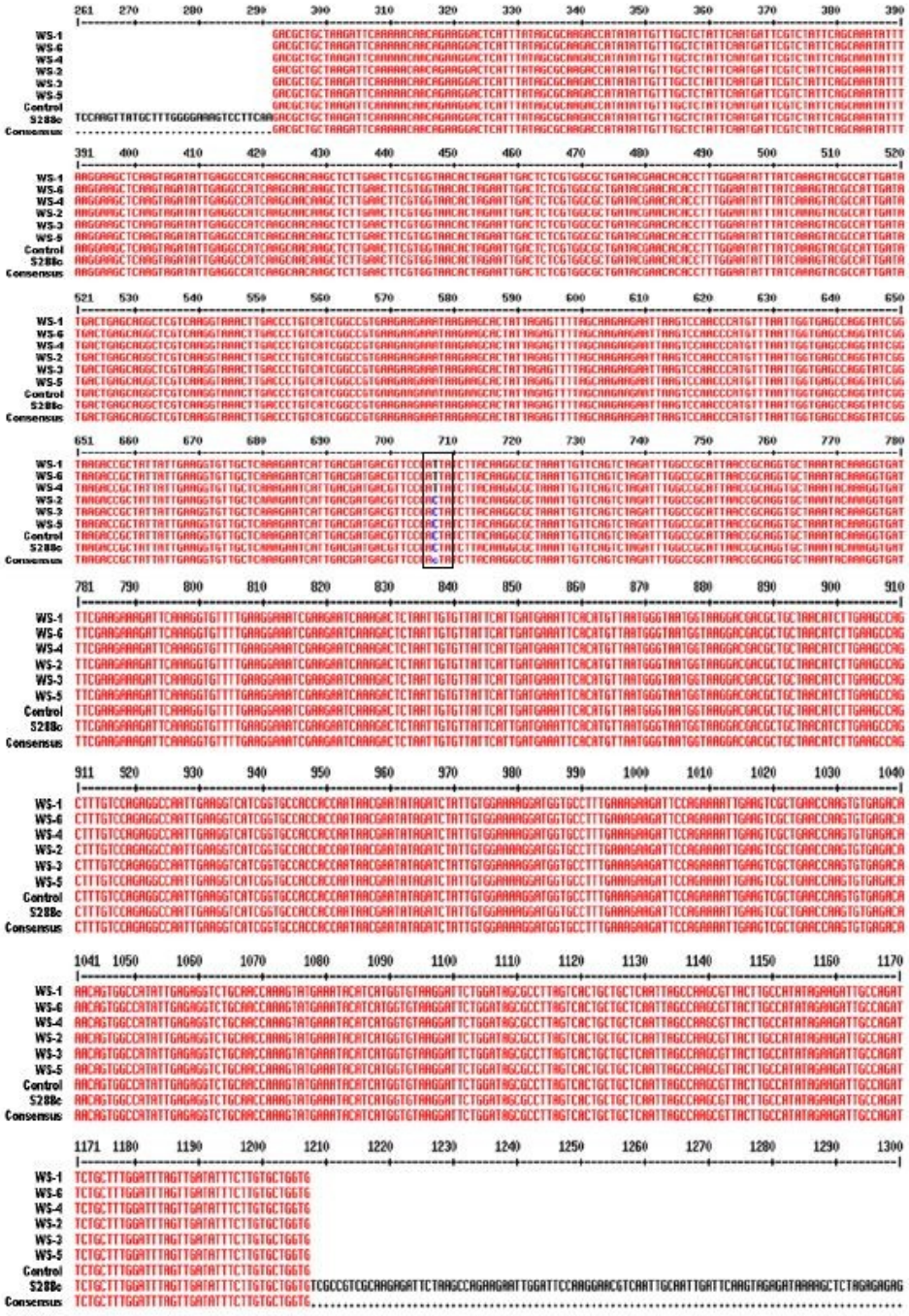

Figure 6. Sequence alignment of the *hsp104* genes of the *S. cerevisiae* local wine strains, the non-wine control strain and the reference strain S288c (NC_001140.5).

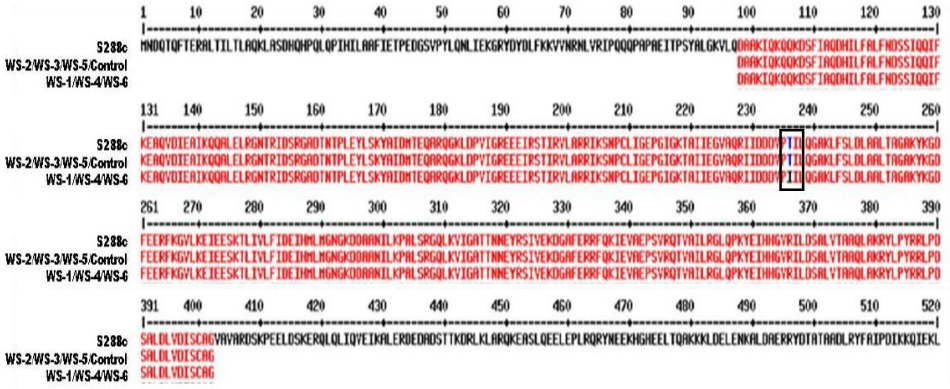

Figure 7. Alignment of the corresponding amino acid sequence of *hsp104* protein (based on the *hsp104* gene sequences) of the *S. cerevisiae* strains used in this study and the reference strain S288c (NC_001140.5).

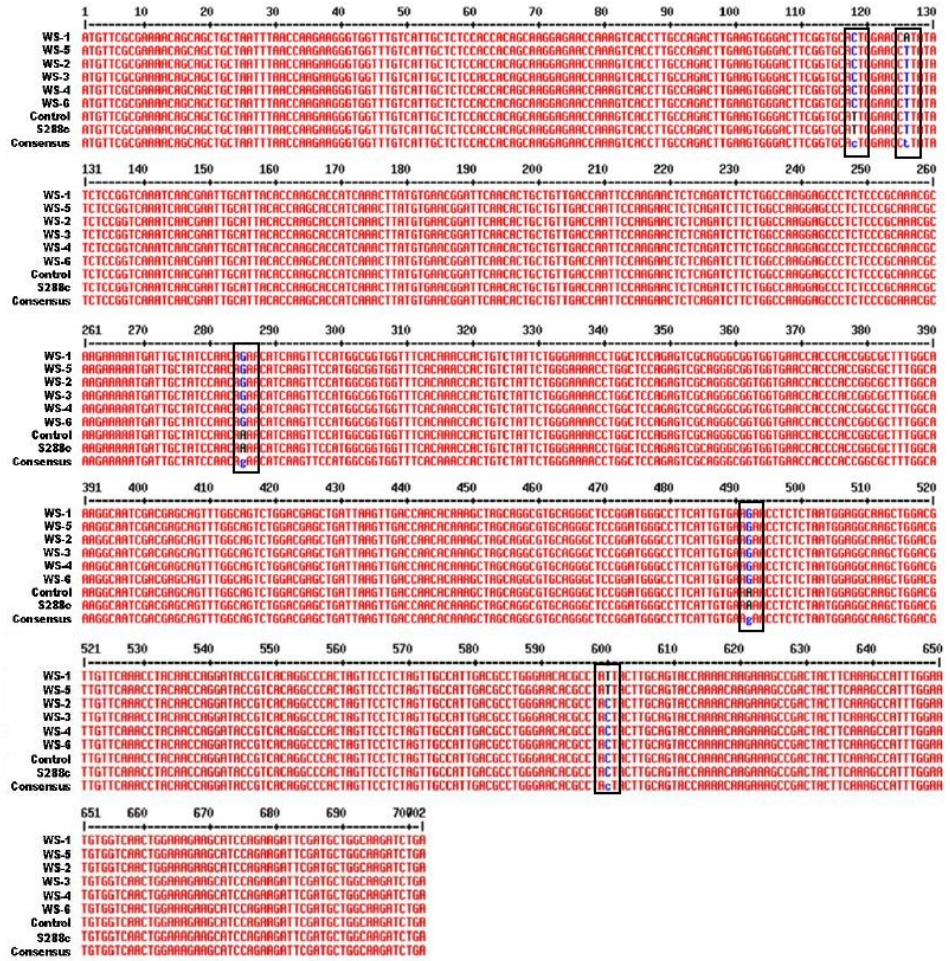

Figure 8. Sequence alignment of the *sod2* genes of the *S. cerevisiae* local wine strains, the non-wine control strain, and the reference strain S288c (NC_001140.5).

genes *erg2*, *hsp104* and *sod2*, respectively).

Amplifications of the target DNA sequences were performed according to the specifications below (Tables 1 and 2). Amplified products were directly sent out for sequencing (First Base Laboratories, Malaysia).

Agarose Gel Electrophoresis

PCR reaction mixture (4µL) mixed with 1 µL gel loading buffer was subjected to agarose gel electrophoresis (1.5% agarose) in 1X TAE buffer at 100V (Gel XL Ultra V-2, Labnet International, Inc., USA). After electrophoresis, the gel was stained with ethidium bromide and DNA was visualized under UV light and photographed using Alpha DigiDoc Pro (Alpha Innotech, USA).

Sequence Analysis of the Ethanol Tolerance Genes

Nucleotide sequences were translated into amino acid sequences using ExPASy Translate Tool (Gasteiger et al 2003). Nucleotide and expected amino acid sequences of the *hsp104* gene fragments, and the *erg2* and *sod2* genes of the *S. cerevisiae* strains were aligned using the Multalin sequence alignment program (Corpet 1988).

RESULTS AND DISCUSSION

Delta typing

The delta primer pair, δ_{12} and δ_2, was used in this study because the use of this primer pair in earlier studies resulted in highly polymorphic PCR profiles (Schuller et al 2004). A unique PCR profile was observed for each strain of *S. cerevisiae*

studied (Fig. 1) when primers, δ_{12} and δ_2, were used to amplify the δ sequences in the yeast genome. Profile differences in terms of number of bands or type of banding patterns allow the non-wine strain to be distinguished from the wine strains and the six wine strains to be distinguished from one another.

Primer design for the amplification of genes implicated in alcohol tolerance

The primers were designed based on available sequences in the database following the schematic diagram below (Fig. 2). The sets of primer sequences and expected size of PCR products are given in Table 3. The products of PCR amplification using these primers are expected to contain the complete *erg2* and *sod2* genes and a fragment (36%) of the 2,727–bp *hsp104* gene.

Amplification of target genes

Amplification products of expected size were produced for each yeast strain using gene-targeted PCR primers (Fig. 3). Amplicons of about 770 bp were produced from the DNA of the *S. cerevisiae* strains using the primers ScERG2F and ScERG2R, suggesting successful amplification of the complete *erg2* gene (Fig. 3A). The 986-bp PCR product corresponding to 36% of the 2,727-bp *hsp104* gene (Fig. 3B) was amplified from the DNA of the *S. cerevisiae* strains using the primers ScHSP104F and ScHSP104R. The 996-bp amplicon that contains the complete 702-bp *sod2* gene was also amplified from all the *S. cerevisiae* strains in PCR using primers ScSOD2F and ScSOD2R (Fig. 3C).

Table 1. Content of PCR reaction mixture for the amplification of target genes ***erg2***, ***hsp104*** **and** ***sod2***

PCR COMPONENT	TARGET GENE		
	erg2	***hsp104***	***sod2***
Titanium PCR buffer (Clontech, USA)	1X	1X	1X
dNTPs (Promega, USA)	0.2 mM	0.2 mM	0.2 mM
Primers	0.3 µM each of ScERG2F and ScERG2R	0.3 µM each of ScHSP104F and ScHSP104R	0.2 µM each of ScSOD2F and ScSOD2R
Titanium *Taq* polymerase (Clontech, USA)	0.25X	0.25X	0.25X
DNA template	150ng	150ng	150ng

Table 2. PCR conditions for the amplification of target genes ***erg2***, ***hsp104*** **and** ***sod2***

PCR cycle			
Denaturation	***erg2***	***hsp104***	***sod2***
	94°C, 5 min	94°C, 5 min	94°C, 5 min
Denaturation Annealing Extension	30 cycles of: 94°C, 30 sec 63°C, 30 sec 72°C, 45 sec	30 cycles of: 94°C, 30 sec 66°C, 30 sec 72°C, 1 min	30 cycles of: 94°C, 30 sec 66°C, 30 sec 72°C, 1 min
Final Extension	72°C, 10 min	72°C, 10 min	72°C, 10 min

Table 3. Sequence of primers for the amplification of the *erg2, hsp104* and *sod2* genes and expected size of target amplicons

PRIMER NAME	PRIMER SEQUENCE	PRODUCT SIZE (bp)	NCBI SEQUENCE REF. NO.
ScERG2F	AGC GGT AAC GTT TGA CAC TGG G	770	NC_001145.2
ScERG2R	ACT GAT TTC GTG AGG TCG GGC A		
ScHSP104F	GCA ACC TGC ACC TGC GGA GA	986	NC_001144.4
ScHSP104R	CTC TTG CGA CGG CGA CAC CA		
ScSOD2F	GCT GCT ACA GGA ACG AAA CCC C	996	NC_001140.5
ScSOD2R	GCG CGA GTA ACG TAG GAA GCG		

Alignment of *erg2* gene sequences and corresponding amino sequences

Alignment of the amplified *erg2* gene sequences of the *S. cerevisiae* strains in this study and the reported gene sequence of a reference strain S288c (NC_001145.2) using the Multalin sequence alignment program (Fig. 4) revealed one polymorphism at position 157. Thymine (T) is present in strains WS-2, WS-4, WS-5, WS-6 and the control non-wine strain, while cytosine (C) is present in strains WS-1 and WS-3. All strains have adenine (A) at position 230 while the reference strain S288c has guanine (G); but no variation in amino acid sequence of the protein was observed for all the strains studied. A single amino acid difference at position 77 was observed between the protein sequence of the reference strain S288c and all the strains under study. Arginine (R) is present in the reference strain while lysine (K) is present in all wine strains as well as the control non-wine strain (Fig. 5).

Alignment of *hsp104* gene sequences and corresponding amino sequences

Multiple sequence alignment of the amplified *hsp104* gene fragments of all the *S. cerevisiae* strains in this study and the reference strain S288c (NC_001144.4) revealed a single nucleotide polymorphism at position 707 (Fig. 6). While cytosine (C) is present in strains WS-2, WS-3, WS-5 and the control non-wine strain at this position, thymine (T) is present in strains WS-1, WS-4 and WS-6. The nucleotide polymorphism is expected to result in amino acid variation among the yeast strains at position 236 (Fig. 7), where threonine was expected to be present in the Hsp104 proteins of strains WS-2, WS-3, WS-5 and the control non-wine strain, while isoleucine was expected to be present in strains WS-1, WS-4 and WS-6.

Alignment of *sod2* gene sequences and corresponding amino sequences

Alignment of the *sod2* genes of the *S. cerevisiae* strains under study and the reference strain S288c (NC_001140.5) revealed five nucleotide polymorphisms at positions 118, 126, 285, 492 and 600 (Fig. 8). Three of these single nucleotide variations (at positions 118, 285 and 492) could differentiate the control non-wine strain from the wine strains. These nucleotide variations, however, are not expected to result in a change in the amino acid sequence of the protein.

Earlier studies reported the successful use of molecular typing procedures, particularly RAPD, to distinguish wine strains of *S. cerevisiae* from one another (Schuller et al 2004, Capece et al 2004, Giusto et al 2006, Araujo et al 2007). Molecular typing, done for the first time with wine strains used for fruit wine production in the Philippines, revealed that PCR using several RAPD primers was effective in generating profiles that could differentiate the alcohol tolerant wine strains from a non-tolerant control strain (Guiamal and Hedreyda 2011). In the present study, the same wine strains from the Philippines were subjected to PCR using the primer pair, δ_{12}/δ_2, which amplifies delta sequences, and primers that target the three genes coding for proteins involved in alcohol tolerance of *S. cerevisiae*. The experiments were performed in order to gain information that could help identify, or develop, an additional procedure that is faster and less expensive than RAPD, but equally reliable in discriminating among wine strains of *S. cerevisiae*.

Previous delta typing performed by Schuller et al (2004) demonstrated that using the delta primer pair, δ_2/δ_{12}, resulted in distinct profiles for 21 out of the 23 *S. cerevisiae* strains studied. Results of PCR using the delta primer pair, δ_1/δ_2, generated only 10 different patterns among the 23 strains; thus, the primer pair, δ_2/δ_{12}, was used in this study. Although other molecular typing techniques such as RAPD were able to differentiate the wine strains from the non-wine control strain in earlier studies, several sets of RAPD primers were necessary to obtain a unique profile for each of the wine yeast strains. In this study, PCR using a single delta typing primer pair, δ_{12} and δ_2, generated a unique profile for each of the six wine strains and the non-wine control strain of *S. cerevisiae* (Fig. 1). The delta typing procedure does not only have better discriminating power, it is also faster and less expensive than RAPD because it involves the use of only one primer pair and a single PCR experiment compared to several sets of primers and PCR runs needed for RAPD. It is recommended that delta typing be the first choice among techniques to be performed to discriminate wine strains of *S. cerevisiae.* Other molecular typing tools will be used if desired discriminating profiles are not achieved using a single pair (δ_{12} and δ_2) of primers. Furthermore, it could be assumed that whatever procedure will work for discriminating wine strains of the *S. cerevisiae* may also work in discriminating other strains of *S. cerevisiae*, including alcohol-tolerant strains used in the biofuel production.

Another focus of this study was the sequence analysis of genes implicated in alcohol tolerance, which was based on the hypothesis that sequence variation in *S. cerevisiae* genes for proteins implicated in ethanol tolerance may be observed between wine strains (that were reported to be more alcohol tolerant) and the non-wine control strain. The alcohol-tolerant strains of *S. cerevisiae* may have acquired mutations in these genes that will result in protein function enhancement towards increased tolerance to alcohol. Design of primers that will target such polymorphisms was perceived to be an alternative approach to detecting alcohol-tolerant wine strains of the species. The three genes *erg2*, *hsp104* and *sod2,* were selected for sequence analysis in this study because those genes code for proteins involved in three different mechanisms of ethanol tolerance proposed for *S. cerevisiae*.

The ERG2 protein (coded by the *erg2* gene) is a C-8 sterol isomerase that plays a role in the synthesis of ergosterol (NCBI, 2011). Ergosterol, the predominant sterol in yeast cells (Laurie et

al 1980), is highly implicated in ethanol tolerance because of its ability to modulate plasma membrane fluidity (Chi & Arneborg 2000). It has been demonstrated that mutations in the *erg2* gene resulted in ethanol sensitivity of *S. cerevisiae* cells. Hsp104, the gene product of *hsp104* gene, is a heat shock protein (Piper 1995, NCBI 2011) that acts as a chaperone (Pereira et al 2001) and repairs denatured proteins by resolubilizing insoluble protein aggregates. The *sod2* gene product, the SOD2 protein, is the antioxidant enzyme mitochondrial manganese superoxide dismutase (MnSOD) (NCBI 2011). This enzyme is involved in altering the effect of ethanol toxicity through the conversion of superoxide anion to dioxygen and hydrogen peroxide (H_2O_2), which is further degraded by catalase or peroxidases (Costa et al 1993, Costa 1997, Pereira et al 2001).

The *erg2* gene nucleotide at position 157 is thymine in four wine strains, in a non-wine strain and in the reference strain S288c (NC_001145.2) of *S. cerevisiae*; in two wine strains, cytosine is present (Fig.4). This single polymorphism has not been reported in other strains of *S. cerevisiae* in the database, although the nucleotide change is not expected to result in amino acid change and could not have affected protein function related to alcohol tolerance. All strains studied have a lysine (K) at position 77 of the ERG2 protein (Fig. 5), compared to the reference strain which has arginine (R). Polymorphisms at both locations could not be used to distinguish the wine strains from the non-wine strain, nor the wine strains from one another.

The nucleotide polymorphism found in the amplified fragments of the *hsp104* gene (Fig. 6) translates into an amino acid variation in the Hsp104 protein at position 236. A polar amino acid (threonine) is expected in the Hsp104 proteins of three wine strains and the control non-wine strain, while a non-polar amino acid (isoleucine) is expected in three wine strains. Hsp104 is a chaperone protein that is involved in the unfolding and subsequent degradation of misfolded or damaged polypeptides, as well as in the resolubilization of protein aggregates via an ATP-dependent mechanism (Bosl et al 2005). The amino acid residue at position 236 is part of the highly conserved ATPase-type nucleotide-binding domain 1 (NBD1) of the *S. cerevisiae* Hsp104 which plays an essential role in the interaction of the polypeptide substrates with the chaperone protein (Bosl et al 2005, EMBL-EBI 2011). It is not known how the amino acid variation can affect the protein function, but the variation does not correlate with the reported alcohol tolerance phenotype of the strains studied.

Five nucleotide polymorphisms in the *sod2* gene of the *S. cerevisiae* strains in this study have not been reported in other *S. cerevisiae* strains in the database. The nucleotide variations in *sod2*, like the polymorphism in the *erg2* gene, are not expected to result in amino acid sequence variation in the protein product. Consequently, the gene polymorphisms are not expected to affect protein function relevant to alcohol tolerance.

CONCLUSION

This study confirmed the usefulness of using the delta typing primer pair, δ_2/δ_{12}, in generating distinguishing PCR profiles from different wine strains of *S. cerevisiae.* Delta typing using a single primer pair in a single PCR experiment is more rapid and less expensive than RAPD, but equally reliable in generating profiles that could distinguish wine strains from non wine strains of *S. cerevisiae* and wine strains from one another. The use of this technique as the first procedure in discriminating among wine strains of *S. cerevisiae* is recommended.

The three genes (*erg2*, *hsp104* and *sod2*) coding for proteins implicated in alcohol tolerance that were evaluated in this study did not exhibit polymorphism that could be the basis for distinguishing wine strains from the non-wine strain. About 250 genes have been implicated in ethanol tolerance of wine yeasts (Hu et al 2007). It is recommended that more genes from that list be evaluated in order to identify polymorphisms in one or more genes that could be used to distinguish among alcohol-tolerant wine strains of *S. cerevisiae*.

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CONFLICT OF INTEREST STATEMENT

There is no conflict of interest in the conduct of this study and the preparation of this manuscript.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Dr. Cynthia T. Hedreyda is the project leader who conceptualized the project, supervised the conduct of research and prepared the manuscript. Ms. Zahara Joy Guiamal performed literature and database search, conducted the experiments and helped in finalizing the manuscript.

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