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Selection and Oenological Comparison of Taiwan Black Queen Grape Wine Yeasts

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ABSTRACT

More than hundreds of private wineries have been established since the Taiwan Tobacco and Alcohol Administration Act was enacted on April 19, 2000. It is then essential to screen local wine yeast in order to develop quality wine with unique flavor. Black Queen grape is a main ingredient for red wine brewing in Taiwan. In this study, ethanol productivity of yeasts isolated from spontaneous fermentation of Black Queen grape must (17 and 30 °Brix) was determined. Seven yeasts identified as *Saccharomyces cerevisiae* by Biolog System with higher ethanol productivity were individually inoculated into Black Queen grape must to undergo fermentation at 25°C for 14 days. The ethanol contents and volatile acidity in wine were above 15% (v/v) and below 0.7 g/L, respectively. Compared to commercial wine yeasts A, B and C, HP01 fermented red wine contained the highest total ester content, color intensity, taste grade, and overall preference value. On the other hand, HS13 fermented red wine contained the highest ethanol, higher alcohols, fatty acids and phenolic compounds content. The mtDNA restriction fragments profiles of these two wine yeast strains were different from those of other yeast strains. Due to their superiority in oenological characteristics, HP01 and HS13 can be applied in Black Queen red wine brewing to produce red wine with unique flavor.

Key words: wine yeasts, *Saccharomyces*, red wine, selection, Black Queen grapes

INTRODUCTION

Microflora of grapes and must are affected by many factors such as climates, geographical properties and cultivation methods[1-2]. *Candida, Hanseniaspora, Pichia, Torulaspora* and *Hansenula* are often found at the early stage of wine fermentation. However, these yeasts are inhibited by ethanol content which increases throughout the spontaneous fermentation. *Saccharomyces cerevisiae* therefore becomes predominant and completes the wine fermentation[3]. The acidity, ethanol content and sensory property of wine are thus heavily dependent on the fermentation properties of *S. cerevisiae*[4-6]. The existence of specific *S. cerevisiae* strains in different wine regions indicates that these wine yeasts exhibit at least some degree of geographic structure. Such diversity perhaps reflects their adaptation to specific winery environments such as temperature tolerance and substrate preference. Some oenologists suggest that the selection of wine yeasts with good oenological properties may improve the quality of wine[3-7].

Although wild yeasts in grapes or winery equipments can be used as natural inoculants of wine, consistent quality of wine is difficult to maintain. Therefore, some oenologists prefer using commercial yeast starters to control the quality of wine and reduce the fermentation time of wine production[8,9]. Several studies on wine yeast strains were conducted, for example, selection of the best wine yeast strain from Spain[4], evaluation of autolytic capacity and foam properties of yeasts for sparkling wine production[5], and isolation of yeast strains for premium quality South African brandy base[7]. Results from these reports indicate that yeast strains originated from wine making area usually produce wine with better quality.

More than hundreds of private wineries have been established since the Taiwan Tobacco and Alcohol Administration Act was enacted on April 19, 2000. It is then essential to screen local brewing yeast in order to develop quality wine with unique flavor. Black Queen grape is an important grape variety in Taiwan for red wine brewing. However, few reports are available on yeast strain selection for wine brewing in Taiwan. This study was conducted to select, identify and characterize valuable wine yeasts from spontaneous fermented Black Queen grape must and to compare the oenological properties and mitochondrial DNA restriction patterns with commercial wine yeasts.

MATERIALS AND METHODS

I. *Isolation and Storage of Yeasts*

Black Queen grapes were obtained from a farmhouse in Taichung County, Taiwan. After washing and stemming, grapes were homogenized by a Warring blender (blender 7010, Warring, CN, USA) into puree. Puree were divided into three groups: puree and puree supplemented with sucrose 17 and 30 °Brix, respectively. Then 100 mL puree of each treatment was filled into a

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flask and placed at 25°C for 3 to 6 days. Total time for fermentation depends upon individual's fermentation rate. Each fermented must was serially diluted with 0.85% NaCl solution. Afterwards, 0.1 mL of the appropriate diluent was spread onto YMPG agar containing 0.3% yeast extract, 0.3% malt extract, 0.5% Bacto peptone, 1% glucose, 1.5% agar, and 100 ppm chloramphenicol (Sigma, St. Louis, MO, USA), and incubated at 25°C for 24-48 hr. After the incubation, about 20 colonies were randomly taken from YMPG agar. Each colony (isolate) was further purified by streaking on YMPG plate at least 5 times before storage at -70°C.

II. *Microvinification*

Microvinification was carried out in glass jars (1.2 L) with 1 L of Black Queen grapes puree supplement with sucrose to 25 °Brix[10]. Freshly activated yeasts were inoculated with an initial cell number of 10^6 cells /mL puree. After fermentation at 25°C for 14 days, must was filtrated through a 60 mesh sieve to remove pomace and centrifuged at 5000 ×g for 15 min to remove precipitated yeast cells. The supernatant was assayed for its oenological properties including pH value, volatile acidity, ethanol content, titratable acidity and residual sugar. Wines prepared by commercial yeasts A, B (Dutch State Mines Food Specialties, Servian, France) and C (Gist-brocades, Seclin Cedex, France) under the same conditions were used as control.

III. *Identification of Yeast Isolates*

Yeast isolates were first streaked on Biolog Universal Yeast agar (Biolog Co., CA, USA) and incubated at 25°C for 48 hr. Several yeast colonies were pooled into 12-15 mL sterile water and suspended by a vortex. Then 100 μL of yeast suspension was added to each well of Biolog identification kits and incubated at 26°C for 24-72 hr. Yeast isolates were identified by a Biolog system (Biolog Co., CA, USA), base on their reaction for various carbon sources. In addition to identification by a Biolog system, the morphological and physiological characteristics of HP01 and HS13 were further determined by general method described by van der Walt and Yarrow[11].

IV. *Oenological Properties of Yeasts*

Oenological properties such as ethanol content, volatile acidity, titratable acidity, residual sugar, and color intensity were analyzed by HPLC[12], distillation[13], titration[13], phenol-sulfuric acid[14] and colorimetric method[15], respectively.

V. *Sensory Analysis*

Sensory properties of wine were analyzed by the method of Zarzoso *et al*.[4]. Fifteen trained panelists were asked for the hedonic preference of wines, including aroma, taste, and overall preference score range from 1 (extremely dislike) to 9 points (extremely like).

VI. *Extraction, Identification and Quantification of Wine Volatile Compounds*

Methods for extraction, identification, and quantification of wine volatile compounds were modified from Marielle *et al*.[16]. An aliquot of 200 mL wine sample, 400 mL of dichloromethane (Mallinckrodt Baker, NJ, USA), 30 g NaCl (Merck, Darmstadt, Germany) and 10 mg/L n-decanol (Sigma, St. Louis, MO, USA), an internal standard was mixed in a flask for 2 hr. The wine/dichloromethane emulsion formed during mixing was broken by centrifugation (9000 ×g, 20 min, 4°C). The wine and dichloromethane extracts were separated by a separator funnel, dried over anhydrous sulfate, and concentrated by a vacuum evaporator (30°C, 60 rpm, 30 cm-Hg) to 0.2 mL.

Volatile compounds identified and quantified by GC-Mass, were performed on a Hewlett-Packard 5890 Series II chromatograph coupled to a Hewlett-Packard 5890A MSD mass spectrometer. Assay conditions: Carbowax 20M column (30 m × 0.32 mm i.d., film thickness = 0.25 μm) (JandW Scientific Inc., Folsom, CA, USA); injector temperature: 220°C, detector temperature: 260°C, and the oven was programmed from 40°C to 230°C at a rate of increase 2°C/min. Carrier gas was helium at a flux of 0.8 mL/min. Compounds were identified by the comparison of GC-Mass spectra to their characteristic spectra of database.

VII. *mtDNA Restriction Analysis*

Yeast total DNA extraction and mtDNA restriction patterns analysis were performed by the method described by Querol *et al*.[17]. However, lyticase (Sigma, St. Louis, MO, USA) was used to substitute zymolyase 60 to digest the cell wall[18]. Yeast strains were cultured in 5 mL YEPD medium (1% yeast extract, 2% peptone, 2% glucose) at 25°C for 16-18 hr. Yeast cells were collected by centrifugation (2000 ×g, 5 min, 4°C) and resuspended in 0.4 mL of 1 M sorbitol, 0.1 M EDTA pH 7.5. Yeast cell suspension was transferred to a 1.5 mL microfuge tube and 0.1 mL lyticase solution (10000 U/mL) was added. Cell suspension mixture was then incubated at 30°C for 30-60 min to obtain the yeast spheroplasts. Spheroplasts were collected by centrifugation (2000 ×g, 5 min, 4°C) and resuspended in 0.5 mL of 50 mM Tris-HCl, 20 mM EDTA pH 7.4. After resuspension, 0.05 mL of 10% (w/v) SDS were added, and the mixture was incubated at 65°C for 30 min. Immediately, 0.2 mL of potassium acetate was added and the tube was placed on ice for 30 min and centrifuged at 12,000 ×g, 5 min, 4°C. The supernatant was transferred in a new microfuge tube and 1 volume of isopropanol was added to precipitate yeast total DNA. After incubation at room temperature for 5 min, yeast

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DNA was collected by centrifugation at 12,000 ×g, 5 min, 4°C. Yeast DNA was washed with 70% ethanol twice, dried at room temperature and dissolved in 50 μL TE pH 7.4. Yeast DNA (2 μL) was digested with Hinf I or Rsa I (final concentration: 500 U/mL, New England Biolabs, Beverly, USA) at 37°C for 3-6 hr and the mtDNA restriction patterns were analyzed in 1.2% agarose gels in 1× TBE buffer (89 mM Tris-borate-2 mM EDTA, pH 8.4) at 110V for 1.5 hr. Restriction fragments length polymorphism profiles of yeast strains HP01, HS13, commercial wine yeast A, E (Dutch State Mines Food Specialties, Servian, France), B, D, G (Lallemand, North Adelaide, Australia), C, F (Red Star, IN, USA), and *S. cerevisiae* BCRC 21450, 21599, 21812, 21992, 22013, 22049 and 22581 (Bioresources Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, ROC) were compared.

VIII. *Statistics*

Data were subjected to the analysis of variance (ANOVA). Significant difference ($p < 0.05$) between means was determined by Duncan's multiple range test using a Statistical Analysis System software package (SAS Institute, NC, USA).

RESULTS AND DISCUSSION

I. *Identification of Yeast Isolates by Biolog System*

Biolog system is a semi-automatic, computer-linked technology for yeast identification. It comprises a 96-well microtiter tray containing a range of dehydrated carbon sources to carry out assimilation and oxidation

Table 1. Oxidation, assimilation and identification of seven yeast isolates

Substrate	HP01	HP04	HP06	HP10	HP15	HP20	HS13
	Oxidation test						
Methyl succinate	-*	-	-	-	-	+	+
Gentibiose	-	-	-	-	-	+	+
Maltose	+**	+	+	+	+	+	+
Maltotriose	-	-	-	-	-	+	+
D-melezitose	-	-	-	-	-	+	+
D-raffinose	+	+	+	+	+	+	+
Stachyose	-	-	-	-	-	+	+
Sucrose	+	+	+	+	+	+	+
D-trehalose	+	+	+	+	+	+	+
Turanose	+	+	+	+	+	+	+
α-D-glucose	+	+	+	+	+	+	+
D-galactose	+	+	+	+	+	+	+
D-psicose	-	-	-	-	-	+	+
	Assimilation test						
Maltose	+	+	+	+	+	+	+
Maltotriose	+	+	+	+	+	+	+
Palatinose	-	-	-	-	-	-	-
D-raffinose	+	+	+	+	+	+	+
Stachyose	-	-	-	-	-	+	+
Sucrose	+	+	+	+	+	+	+
D-trehalose	+	+	+	+	+	+	+
Turanose	+	+	+	+	+	+	+
α-D-glucose	+	+	+	+	+	+	+
D-galactose	+	+	+	+	+	+	+
Amygdalin	-	-	-	-	-	+	+
Methyl succinate + D-xylose	-	-	-	-	-	+	+
D-galactose + D-xylose	+	+	+	+	+	+	+
Identification	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*	*S. cerevisiae*
Possibility (%)	99	97	97	97	97	95	99

* Negative.
** Positive.

tests. It is also known as "Metabolic Fingerprint" and used for identification of food and beverage yeasts[19]. Results of assimilation and oxidation tests of 7 yeast isolates are shown in Table 1.

According to the oxidation tests, all 7 yeasts could oxidize maltose, D-raffinose, sucrose, D-trehalose, turanose, α-D-glucose, and D-galactose. However, only HP20 and HS13 are capable of oxidizing methyl succinate, gentiobiose, maltotriose, D-melezitose, and D-psicose.

Assimilation test shows that all 7 yeasts could utilize maltose, maltotriose, raffinose, sucrose, D-trehalose, turanose,α-D-glucose, D-galactose, and D-galactose + D-xylose as sole carbon source. HP20 and HS13 could utilize stachyose, amygdalin and methyl succinate +

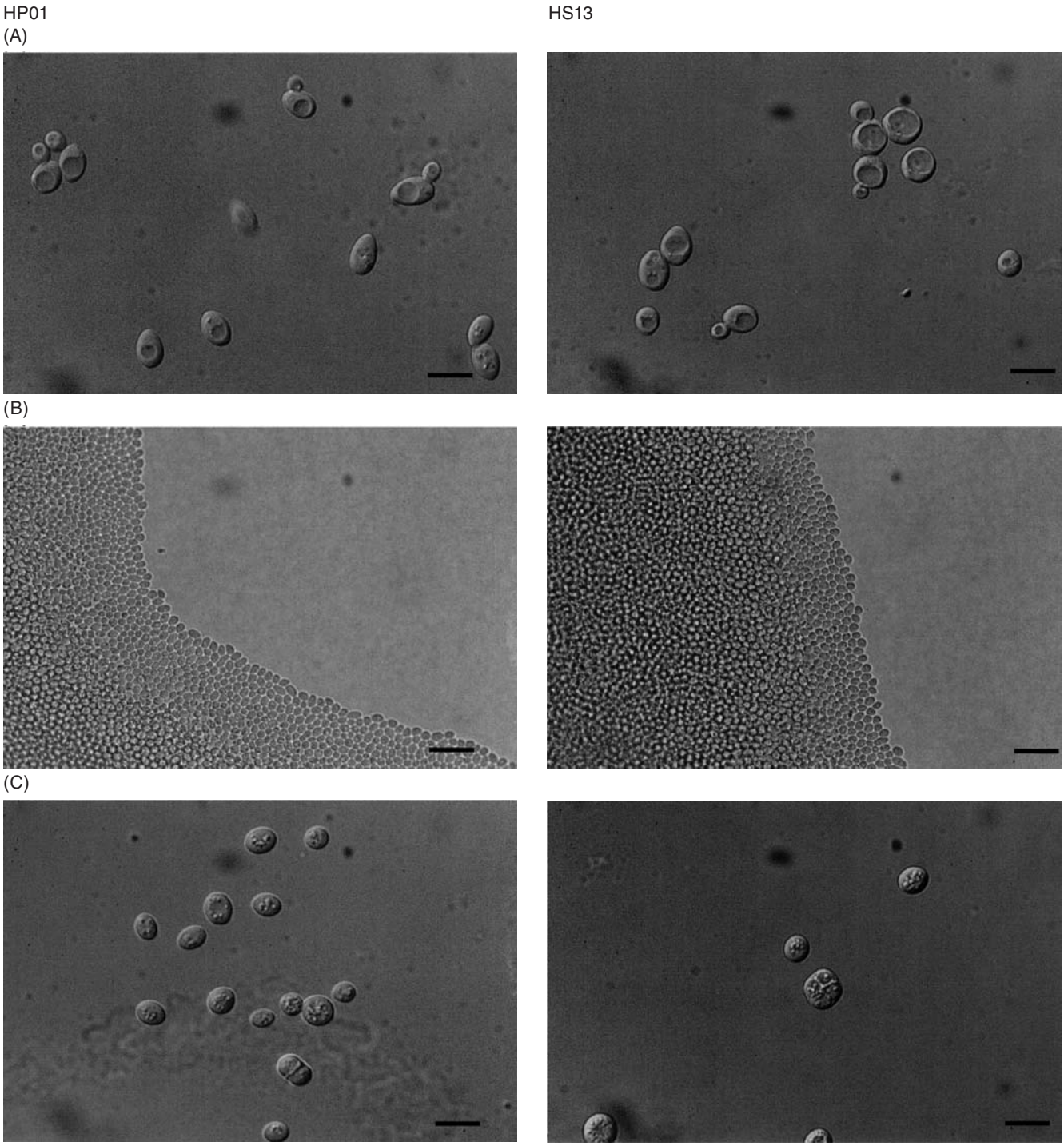

Figure 1. (A) Yeast cells of HP01 (left) and HS13 (right) after 3 days at 25°C in YPD broth. Bar = 10 μm (800×). (B) Dalmau plate culture of HP01 (left) and HS13 (right) after 7 days at 25°C on corn meal agar. Bar = 20 μm (400×). (C) Ascospores formation of HP01 (left) and HS13 (right) after 7 days at 25°C on Kleny's acetate agar. Bar = 10 μm (800×).

D-xylose but not palatinose as carbon source.

Oxidation and assimilation results of 7 yeast isolates were compared with the Biolog database. All yeast isolates were identified as *Saccharomyces cerevisiae* with reliability of 95-99%. *S. cerevisiae* is a common starter used in the production of wine and alcoholic beverage[8,9,13].

Morphological characteristics of HP01 and HS13 are shown in Figure 1. Vegetative cells of HP01 and HS13 were globose or ellipsoidal, single or in pairs. Both of asexual reproduction types of HP01 and HS13 were multilateral budding. Cell sizes of HP01 and HS13 were 3.2-8.0 × 4.8-10.8 μm and 3.2-8.0 × 5.6-10.8 μm, respectively (Figure 1A). No mycelium or pseudomycelium was found in Dalmau culture of HP01 and HS13 (Figure 1B). Two-four globose ascospores were found in asci of HP01 and HS13 (Figure 1C). It was more credible to identify HP01 and HS13 as *S. cerevisiae* by comparing with the morphology and metabolic data of *S. cerevisiae*[20,21].

II. *Fermentation Properties of Yeasts in Black Queen Grape Must*

Seven *S. cerevisiae* strains with high ethanol productivity were isolated from Black Queen grapes must or sucrose supplemented 17 and 30 °Brix must. These yeasts were used as starters for oenological and sensory analysis.

Black Queen grape is an important variety cultivation in Taiwan for red wine brewing[22]. Oenological properties of red wine fermented by the 7 yeasts are shown in Table 2. In general, the ethanol contents of commercial red wines range from 10% to 15% (v/v)[2]. The ethanol contents of red wine made by 7 yeast isolates were 15.0-15.9%. The pH values of desirable wine are in the range of 3.0-4.0[2, 22]. The pH values of Black Queen grape must before and after fermentation were 3.2-3.3 and 3.4-3.5, respectively. Changes of pH values in grape substrate were not significant. Because many conjugated acid and base pairs, such as tartarate, malate, and citrate are present in grapes, the buffer capacity of these organic acids resists the changes in pH value.

In general, the titratable acidity of red wine should be less than 8 g/L[13]. Table 2 also shows that the titratable acidity of red wine was 12.9-14.4 g/L. The volatile acidities of all wine samples were 0.3 to 0.6 g/L, which were less than the upper limits of red wine (1.2 g/L). An inverse relationship between ethanol (15.0-15.9%) and residual sugar content (5.9-9.8 g/L) is exhibited in Table 2.

Table 2 also shows that wine made by HP01 and HS13 were more preferred among the selected yeasts. Therefore, these two novel yeasts could be considered as starters with potential to improve red wine brewing in Taiwan. In previous reports, oenologists found that quality wine requires good yeast starters from microarea where wines were produced[4]. Therefore, the selection of suitable wine yeasts for specific wine making should not be overlooked.

III. *Comparison of Oenological Properties and Sensory Attributes of Wine Fermented by HP01, HS13 and Commercial Red Wine Yeast A, B and C*

HP01 and HS13 possess the highest sensory attributes and better fermentation properties among yeasts selected from Black Queen grapes in Taiwan. We further compared the oenological properties and sensory attributes of HP01 and HS13 with three commercial red wine yeasts A, B and C. The results are shown in Table 3. The ethanol yield of HP01 and HS13 was higher than that of 3 commercial yeast strains A, B and C. HS13 had the highest ethanol yield of 13.8% (v/v). The pH value and titratable acidity among these yeast strains were not significantly different. The volatile acidity of HP01 and HS13 was slightly higher than that of the 3 commercial strains. However, the volatile acidity of all strains was less than the limitation for red wine (1.2 g/L).

Total polyphenolic content of HP01, HS13, A, B

Table 2. Fermentation properties and sensory attributes* of red wine made by 7 yeast isolates

Yeast strains	Fermentation properties					Sensory attributes		
	Ethanol (%, v/v)	pH	Titratable acidity (g tartarate/L)	Volatile acidity (g acetate/L)	Residual sugar (g/L)	Aroma	Taste	Overall
HP01	15.0 ± 0.25	3.4 ± 0.00	14.4 ± 0.20	0.3 ± 0.02	9.8 ± 0.34	6.1 ± 1.5^{a}**	6.1 ± 0.9^{a}	5.9 ± 0.5^{a}
HP04	15.2 ± 0.32	3.4 ± 0.00	14.8 ± 0.28	0.4 ± 0.03	8.5 ± 0.12	5.7 ± 1.0^{a}	5.4 ± 1.1^{b}	4.7 ± 1.0^{b}
HP06	15.2 ± 0.15	3.2 ± 0.00	13.2 ± 0.10	0.4 ± 0.01	7.3 ± 0.22	5.9 ± 1.1^{a}	5.7 ± 1.0^{a}	5.7 ± 1.2^{a}
HP10	15.9 ± 0.20	3.5 ± 0.00	12.9 ± 0.26	0.3 ± 0.02	6.8 ± 0.25	5.6 ± 1.0^{a}	5.1 ± 1.5^{b}	4.6 ± 1.3^{b}
HP15	15.2 ± 0.51	3.4 ± 0.00	15.0 ± 0.15	0.3 ± 0.03	5.9 ± 0.17	6.1 ± 1.1^{a}	5.5 ± 1.6^{b}	4.8 ± 1.2^{b}
HP20	15.0 ± 0.24	3.5 ± 0.00	14.0 ± 0.45	0.4 ± 0.05	6.9 ± 0.25	6.0 ± 1.0^{a}	5.7 ± 1.7^{a}	5.3 ± 1.2^{b}
HS13	15.0 ± 0.10	3.5 ± 0.00	13.7 ± 0.37	0.3 ± 0.02	7.7 ± 0.10	5.9 ± 1.0^{a}	5.9 ± 1.0^{a}	5.8 ± 1.0^{a}

*Rate by a 15 member panel, 9 = like extremely; 5 = neither like nor dislike; 1 = dislike extremely.

**Means with different superscript letters (a, b) are significantly different ($p < 0.05$).

Table 3. Fermentation properties and sensory attributes* of red wine made by yeast isolates HP01, HS13 and 3 commercial yeast starters A, B and C

	Yeast strains				
	HP01	HS13	A	B	C
	Fermentation properties				
Ethanol (%,v/v)	13.3 ± 0.46^{ab}**	13.8 ± 0.25^{a}	12.9 ± 0.12^{b}	12.6 ± 0.45^{b}	13.0 ± 0.00^{b}
pH	3.5 ± 0.00	3.5 ± 0.00	3.6 ± 0.00	3.5 ± 0.00	3.5 ± 0.00
Titratable acidity (g tartarate/L)	10.5 ± 0.06	10.4 ± 0.25	10.2 ± 0.23	10.6 ± 0.1	10.0 ± 0.00
Volatile acidity (g acetate/L)	0.47 ± 0.03^{ab}	0.49 ± 0.01^{a}	0.34 ± 0.01^{b}	0.46 ± 0.09^{ab}	0.39 ± 0.03^{ab}
Total polyphenol (g/L)	4.2 ± 0.19^{ab}	4.4 ± 0.10^{a}	3.6 ± 0.11^{c}	3.9 ± 0.10^{b}	3.9 ± 0.14^{b}
OD 620nm	2.6 ± 0.11^{a}	2.6 ± 0.11^{a}	2.3 ± 0.13^{b}	2.3 ± 0.12^{b}	2.6 ± 0.06^{a}
OD 520nm	12.5 ± 0.12^{a}	12.4 ± 0.12^{a}	10.9 ± 0.20^{c}	11.3 ± 0.23^{b}	12.3 ± 0.09^{a}
OD 420nm	6.5 ± 0.09^{a}	6.5 ± 0.11^{a}	5.7 ± 0.12^{c}	6.2 ± 0.15^{b}	6.5 ± 0.07^{a}
Colour intensity	21.6 ± 0.29^{a}	21.5 ± 0.38^{a}	19.0 ± 0.46^{c}	19.8 ± 0.49^{ab}	21.3 ± 0.23^{b}
Tint	52.4 ± 0.27^{c}	52.8 ± 0.44^{bc}	52.6 ± 0.31^{c}	54.9 ± 0.29^{a}	53.1 ± 0.22^{b}
	Sensory attributes				
Color	6.4 ± 1.3^{ab}	6.4 ± 1.3^{ab}	6.0 ± 1.2^{b}	6.8 ± 1.1^{ab}	6.9 ± 1.1^{a}
Aroma	6.2 ± 1.0^{a}	6.1 ± 1.3^{a}	5.4 ± 1.7^{b}	5.9 ± 1.4^{ab}	6.4 ± 1.0^{a}
Taste	5.7 ± 1.6^{a}	5.0 ± 1.4^{a,b}	5.3 ± 1.6^{a,b}	4.6 ± 1.3^{b}	3.9 ± 1.4^{c}
Overall	5.9 ± 1.2^{a}	5.6 ± 1.1^{a}	5.3 ± 1.4^{a}	5.4 ± 1.2^{ab}	4.5 ± 1.5^{c}

*Rate by a 15 member panel, 9 = like extremely; 5 = neither like nor dislike; 1 = dislike extremely.
**Means with different superscript letters (a, b) are significantly different ($p < 0.05$).

and C was 4.2, 4.4, 3.6, 3.9 and 3.9 g/L, respectively. HP01 and HS13 had higher polyphenolic content than three commercial red wine yeasts. Phenolic compounds, including flavanoids, volatile phenols, anthocyanins, tannins and others, contributed to wine characters, such as color, astringency, bitterness and flavor. Wine yeasts change the phenolic content through different ways, such as absorption of anthocyanins by yeast cell wall[23], stabilization of pigment by yeast metabolites pyruvic acid[24] and acetaldehyde[25], and pigment hydrolysis by yeast enzyme such as anthocyanin-β-D-glucosidase[26]. Therefore, we consider that high total phenol content is helpful for wine quality.

Wine color is an obvious property of wine quality. Anthocyanins, a group of polyphenol, are the main pigment in red grapes and wines. Parameters to evaluate color quality of red wines include color intensity, tint, and total polyphenol index, etc[15]. Using certain yeast and enzyme in the vinification process can improve wine color properties[27]. OD620nm, OD520nm and OD420nm are referred as blue, red and yellow color, respectively. Color intensity is the sum of OD620nm, OD520nm and OD420nm. Color intensity values of HP01, HS13, A, B and C were 21.6, 21.5, 19.0, 19.8 and 21.3, respectively. HP01 and HS13 fermented wine exhibited higher color intensity than commercial red wine yeasts A, B and C, and with significantly higher color intensity than that of A and B ($p < 0.05$).

Results from the sensory analysis of Black Queen red wine fermented by 5 yeasts are shown in Table 3. Color attributes of HP01, HS13, A, B and C were 6.4, 6.4, 6.0, 6.8 and 6.9, respectively. Yeast A had the lowest color attributes, but the color attributes among HP01, HS13, B and C showed no significant difference ($p > 0.05$). Aroma attributes of HP01, HS13, A, B and C were 6.2, 6.1, 5.4, 5.9 and 6.4, respectively. C had the highest aroma score but there were no significant difference between HP01 and HS13 ($p > 0.05$). Taste attributes of HP01, HS13, A, B and C were 5.7, 5.0, 5.3, 4.6 and 3.9, respectively. HP01 had the highest taste attribute among 5 yeasts and significantly different from B and C ($p < 0.05$). C had the highest aroma attributes with the lowest taste attribute. The overall attributes of Black Queen red wines made by HP01 and HS13 (5.9 and 5.6, respectively) were higher than the 3 commercial wine yeasts A, B and C (5.3, 5.4 and 4.5, respectively).

Based on these results, HP01 and HS13 fermented Black Queen red wines contain higher ethanol, total polyphenol content, color intensity and sensory attributes. We considered that HP01 and HS13 are more suitable for Black Queen red wine vinification.

IV. *Identification, Quantification of Volatile Compounds from Wine Made by HP01, HS13 and Commercial Red Wine Yeast A, B and C*

Hundreds of volatile compounds are referred as wine flavor compounds of wines such as higher alcohol,

aldehydes, esters, fatty acids, ketones, lactones, monoterpenes, volatile phenol, and sulfur compounds, etc[28,29]. The volatile compounds production of wine fermentation are influenced by many factors such as variety, maturity and sugar content of grape, yeast strain, fermentation temperature and vinification methods etc[30]. Many aroma compounds are released to the must as secondary metabolites of yeasts or formation by enzymes of yeasts such as esterase, glycosidase, alcohol acetyl-transferase and alcohol acyl-transferase[28,30-32]. In this study, we compared the volatile compounds content of wine fermented with HP01, HS13 and three commercial red wine yeasts A, B and C.

The concentration, threshold and descriptor of volatile compounds of red wine made by HP01, HS13 and commercial red wine yeast A, B and C are shown in Table 4. Many esters formed during the alcoholic fermentation mostly have pleasant smell and wine aroma. Isoamyl acetate was the most abundant ester in wine made by HP01, HS13 and B, 1.35, 0.97 and 0.20 mg/L. All wine samples had a "banana" odor above perception threshold of 0.03 mg/L[32]. Ethyl octanoate

Table 4. Volatile compounds of red wine made by yeast isolates HP01, HS13 and 3 commercial yeast strains A, B and C

Compounds (mg/L)	Yeast strains						
	HP01	HS13	A	B	C	Threshold***	Odor descriptor
Esters							
Isoamyl acetate	1.35 ± 0.044	0.97 ± 0.040	0.40 ± 0.003	0.20 ± 0.002	0.50 ± 0.001	0.03	Banana[32]
Ethyl octanoate	0.43 ± 0.010	0.60 ± 0.020	0.27 ± 0.010	-	0.82 ± 0.001	0.005	Ripe fruits, pear, sweety[29]
Di-ethyl succinate	0.70 ± 0.087	0.65 ± 0.009	0.42 ± 0.034	0.07 ± 0.002	0.58 ± 0.005	6.0	Wine[30]
Ethyl oleate	0.43 ± 0.046	-	-	-	-		
Total esters	2.91 ± 0.066^{a}*	2.22 ± 0.050^{ab}	1.09 ± 0.015^{c}	0.27 ± 0.000^{d}	1.90 ± 0.006^{bc}		
Higher alcohols							
Isoamyl alcohol	30.60 ± 0.566	36.35 ± 0.061	20.59 ± 0.079	12.05 ± 0.051	23.66 ± 0.062	30.0	Fusel, rancid, cheese[28]
1-Hexanol	0.33 ± 0.006	-	0.25 ± 0.025	0.22 ± 0.002	0.41 ± 0.003	8.0	Green, grass[30]
Phenyl methanol	0.12 ± 0.010	0.08 ± 0.032	0.09 ± 0.010	0.05 ± 0.002	0.13 ± 0.001		
2-Phenylethanol	14.77 ± 0.167	15.81 ± 0.117	8.21 ± 0.101	5.95 ± 0.004	11.84 ± 0.055	14.0	Roses, sweet[28]
Total higher alcohols	45.82 ± 0.615^{ab}	52.24 ± 0.098^{a}	29.14 ± 0.101^{c}	18.27 ± 0.046^{d}	36.04 ± 0.108^{bc}		
Fatty acids							
Hexanoic acid	1.19 ± 0.035	1.63 ± 0.026	1.39 ± 0.020	0.53 ± 0.002	1.53 ±0.042	0.42	Fatty acid, cheese[27]
Octanoic acid	1.30 ± 0.050	2.09 ± 0.043	1.50 ± 0.050	0.52 ± 0.000	1.85 ± 0.010	0.5	Fatty acid, rancid[27]
Decanoic acid	0.63 ± 0.026	0.88 ± 0.049	-	-	-	1.0	Fatty acid, rancid, soap[27]
Myristic acid	-**	0.06 ± 0.017	-	-	-		
Palmitic acid	-	0.92 ± 0.005	0.56 ± 0.052	0.41 ± 0.010	0.67 ± 0.000		
Stearic acid	-	-	0.11 ± 0.032	-	-		
Totals fatty acids	3.12 ± 0.061^{bc}	5.58 ± 0.072^{a}	3.56 ± 0.121^{b}	1.46 ± 0.011^{c}	4.05 ± 0.039^{ab}		
Lactones							
γ-Butyrolactone	1.07 ± 0.068	0.85 ± 0.010	0.98 ± 0.017	0.34 ± 0.001	0.82 ± 0.010		Sweety, buttery[28]
γ-Decalactone	0.16 ± 0.020	0.33 ± 0.057	0.22 ± 0.038	0.10 ± 0.015	0.18 ± 0.001		Coconut[34]
Total lactones	1.23 ± 0.048^{a}	1.18 ± 0.067^{a}	1.20 ± 0.021^{a}	0.44 ± 0.016^{c}	1.00 ± 0.010^{b}		
Sulfur compounds							
Methionol	0.25 ± 0.012	0.24 ± 0.011	0.12 ± 0.010	0.06 ± 0.006	0.14 ± 0.010		Cooked cabbage[35]

*Means with different superscript letters (a, b, c, d) at the same row are significantly different ($p < 0.05$).
**Not detectable.
***Odor descriptors and thresholds reported in the literature (27-30, 32, 34, 35).

also was found in wine made by HP01, HS13, A and C, 0.43, 0.60, 0.27 and 0.82 mg/L, but was not found in B. The odor of ethyl octanoate was characterized as "sweet, fruity and fresh" and ethyl octanoate concentration of 4 wine samples was above the perception threshold of 0.25 mg/L[29]. The concentrations of diethyl succinate of wine made by HP01, HS13, A, B and C was 0.70, 0.65, 0.42, 0.07 and 0.58 mg/L, respectively, and all below the diethyl succinate threshold 6 mg/L[28]. However, the total esters concentrations of wine made by HP01 (2.91 mg/L) and HS13 (2.22 mg/L) were significantly higher than commercial red wine yeasts A and B ($p < 0.05$).

Higher alcohols were synthesized by yeasts in two ways: anabolic pathway from glucose, or catabolic pathway from their corresponding amino acids such as valine, leucine, isoleucine and phenylalanine[33]. Isoamyl alcohol was the most abundant higher alcohols among wine made by 5 yeasts. Concentration of HP01, HS13, A, B and C was 30.60, 36.35, 20.59, 12.05 and 23.66 mg/L, respectively. The threshold of isoamyl alcohol was 30 mg/L and was characterized by an "alcohol, fusel" odor. The threshold of 1-hexanol was 8 mg/L and gave a "green, grass" odor. 1-Hexanol concentration of HP01, A, B and C was 0.33, 0.25, 20.59, 0.22 and 0.41 mg/L, respectively and was not above the thresholds[29]. Another abundant higher alcohol was 2-phenylethanol and only HP01 and HS13 had higher concentration (14.77 and 15.81 mg/L) than their thresholds (14 mg/L). The odor of 2-phenylethanol was described as "rosy and sweet"[28]. Higher alcohols impart aroma and body of wine; they also offer desirable complexity of wine when the contents were less than 300 mg/L[30]. Total higher alcohol content of HP01, HS13, A, B and C was 45.82, 52.24, 29.14, 18.27 and 36.04 mg/L, respectively. Black Queen red wine made by HP01 and HS13 had more abundant higher alcohol to enhance wine body and aroma complexity.

Within the fatty acids, haxanoic and octanoic acids were notable for their high concentrations in Black Queen red wine made by 5 yeasts. Wine made by HS13 had the highest concentration of numerous kinds of fatty acids among 5 yeasts. The threshold value of hexanoic acid, octanoic acid and decanoic acid was 0.42, 0.5 and 1.0 mg/L, respectively[27]. Although fatty acids exhibit vinegar, pungent, and unpleasant flavor at the concentrations above their thresholds, they are precursors of many esters[30].

Two lactones, γ-butyrolactone and γ-decalactone were found in Black Queen red wine made by 5 yeasts. These compounds were formed by self esterization of the corresponding γ-hydroxycarboxylic acid. The content of lactones in wine varied with yeast strain and ageing[29]. The odor of γ-butyrolactone and γ-decalactone was described as "sweet, buttery" and "coconut"[28,34]. Higher lactones content could enhance aroma and bouquet of wine[29]. Total lactone contents of wine made by HP01, HS13, A, B and C were 1.23, 1.18, 1.20, 0.44 and 1.00 mg/L, respectively. HP01, HS13 and strain A had higher total lactones contents than strain B and C.

The occurrence of 3-(mithylthio)-1-propanol (methionol) in wine was related to transamination and decarboxylation of methionine by yeast. Methionol had a "cooked cabbage" odor and was related as off-flavor to wine in high concentration (> 0.5 mg/L)[35]. Methionol concentration of wine made by HP01, HS13, A, B and C was 0.25, 0.24, 0.12, 0.06 and 0.14 mg/L, respectively. The concentrations of methionol in all wines were below 0.5 mg/L, which thus might have no negative effect on Black Queen aroma.

Red wine made by HP01 had the highest total ester content, color intensity, taste index, and overall sensory preference, whereas red wine made by HS13 had the highest ethanol, higher alcohols, fatty acids and phenolic compounds contents. Therefore, HP01 and HS13 should be more suitable for brewing Taiwan Black Queen red wine.

V. *mtDNA Restriction Pattern of HP01, HS13 and Other Yeast Strains*

Although we identified and classified HP01 and HS13 as *S. cerevisiae* group by Biolog system, we could not differentiate among these yeast strains. DNA polymorphisms of yeast strains were useful for distinguishing HP01 and HS13 from other yeast strains. A rapid and simple method wine yeast mtDNA restriction analysis had been developed[17]. Several reports characterized *S. cerevisiae* by mtDNA restriction fragments length polymorphism[2,17,36,37]. Querol *et al.*[16]. reported yeast total DNA digestion with specific restriction endonucleases that recognize 4 bp (*Rsa* I etc.) or 5 bp (*Hinf* I etc.). These enzymes recognize many sites in yeast nuclear DNA, but few sites in the mtDNA. Hence, mtDNA restriction fragments are easily distinguished by agarose gel electrophoresis. The mtDNA restriction analysis allows the differentiation of the four species of *Saccharomyces* 'sensu stricto' (*S. bayanus*, *S. cerevisiae*, *S. paradoxus* and *S. pastorianus*)[37]. The mtDNA restriction patterns digested by *Hinf* I and *Rsa* I of *Saccharomyces* spp. are shown in Figure 2. mtDNA restriction patterns of HP01 and HS13 digested by Rsa I were similar to commercial yeast E (Lane 7), but different by Hinf I. Therefore, HP01 and HS13 had special patterns from other yeast strains. Different mtDNA restriction patterns may conduct grape varieties, climatic condition, cultivate area and composition of nutrients in must, but the meaning of the heterogeneity in mtDNA restriction pattern is not clear[18,37]. In Figure 2, we confirm that HP01 and HS13 are specific wine yeast strains selected form Black Queen grape. But HP01 and HS13 have similar mtDNA patterns. These may be attributed to selection from the same grape varieties in the same area. We will use other molecular techniques such as electrophoretic karyotyping or PCR amplification to distinguish HP01 and HS13 in the future.

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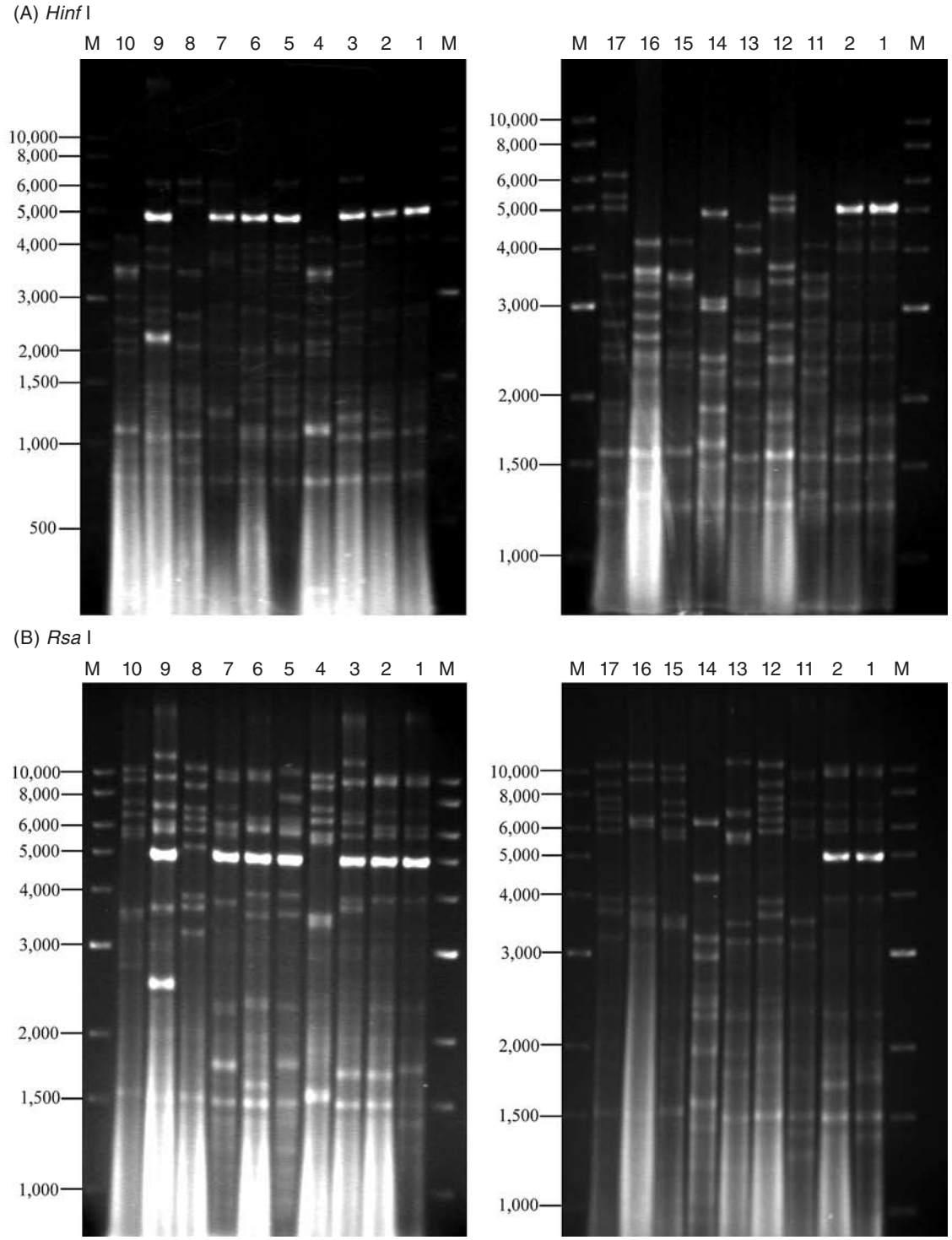

Figure 2. Different patterns of mtDNA restriction analysis with (A) Hinf I and (B) Rsa I endonucleases of HP01, HS13 and other *Saccharomyces* spp. obtained from commercial wine yeast and BCRC. Lanes M correspond to a 1 Kb ladder marker obtained from New England Biolabs, Beverly, USA. 1: HP01 and 2: HS13, 3-10: commercial wine yeast A-H and 11-17: *S. cerevisiae* BCRC 21450, 21599, 21812, 21992, 22013, 22049 and 22581, respectively.

CONCLUSIONS

This study proposes a protocol for the isolation, selection, and identification of suitable wine yeasts for brewing Black Queen red wine in Taiwan. Seven yeasts exhibited high ethanol production by an ethanol productivity test. With microvinification tests, red wine made by HP01 contained the highest total ester content, color

intensity, taste grade, and overall preference value, whereas red wine made by HS13 contained highest ethanol, higher alcohols, fatty acids and phenolic compounds content. This study showed that HP01 and HS13 provided good oenological properties and high volatile compounds productivity. Therefore, HP01 and HS13 are valuable for red wine making in Taiwan.

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