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ORIGINAL ARTICLE

# Analysis of culturable yeast diversity in spontaneously fermented orange wine, orange peel and orangery soil of a Ponkan plantation in China

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**Abstract** A culturable diversity analysis of yeast species in spontaneous fermentation orange wine, orange peel and orangery soil of a Ponkan plantation in China was followed in this study. A total of 160 isolated yeast strains were identified at the species level through 5.8S-ITS-RFLP and sequence analysis of the 26S rDNA D1/D2 domain. The ten identified species were *Barnettozyma californica*, *Candida humilis*, *Candida tropicalis*, *Clavispora lusitaniae*, *Hanseniaspora occidentalis*, *Hanseniaspora opuntiae*, *Hanseniaspora uvarum*, *Pichia kudriavzevii*, *Pichia terricola* and *Torulaspora delbrueckii*. *Barnettozyma californica* and *H. occidentalis* were the most abundant species in orangery soil. *Pichia terricola* and *H. opuntiae* were the prevailing species in orange peel. Additionally, *P. kudriavzevii*, *C. lusitaniae and T. delbrueckii* were the dominant species during the beginning, middle and final stages of fermentation, respectively. As a first step in exploring untapped yeast resources of the region, it is important to investigate the yeast microbiota in spontaneous fermentations and screen indigenous yeast which will produce orange wine with regional characteristics.

**Keywords** Non-*Saccharomyces* · Orange wine · Yeast culturable diversity · Spontaneous fermentation

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## Introduction

Oranges have been one of the largest world crops for a long time, grown throughout the world in tropical and subtropical areas. China is the third top producer of oranges with 3.276 million-tons yielded in 2013. Among orange varieties, Ponkan is a native orange variety of *Citrus reticulata* and is produced on a large scale in the Wuhan district of the Hubei province in central China. Oranges can be processed into orange juice, beverages or wine. Orange juice is the most popular juice in the world for its flavor, but orange wine is not as popular due to its poor flavor after fermentation with a commercial *Saccharomyces cerevisiae* strain (Kelebek et al. 2009).

It is generally accepted that spontaneous fermentation can improve wine flavor through synergistic interaction among different *S. cerevisiae* strains or *S. cerevisiae* with isolated non-*Saccharomyces* strains from spontaneous fermentation wine (Ciani et al. 2010; Ciani and Comitini 2011; Sadoudi et al. 2012). The isolated non-*Saccharomyces* strains has been used to improve the flavor of many kinds of fruit wine, such as wine, tequila wine, cider, mango wine, cherry wine (Satora and Tuszynski 2009; de Arruda Moura Pietrowski et al. 2012; Sadineni 2012; Amaya-Delgado et al. 2013; Duarte et al. 2013; Sun et al. 2014). Heras-Vazquez et al. (2003) isolated and identified nine yeast species from spontaneously fermented orange juice. However, yeast diversity may vary among planting areas, climatic conditions, orchard age and orange variety (Maro et al. 2007). A clearer investigation of the yeast microbiota during the fermentation process will provide an approach to produce fruit wine with a unique regional character (Fleet 2008). There was no report on the yeast diversity in spontaneously fermented orange juice, orange peel and orangery soil of a Ponkan plantation in China.

The aim of this study is to investigate culturable yeast diversity in spontaneously fermented orange wine, orange peel

and orangery soil of a Ponkan plantation in China by using 5.8S-ITS-RFLP and 26S rDNA D1/D2 sequencing.

## Materials and methods

### Sampling and yeast isolation

The orangery soil and the orange samples were collected October 2013 from a Ponkan orangery in Wuhan. Twelve soil samples (10 g/sample) were taken from four positions about 10 cm beneath the soil surface (pH 4.2–6.0) (Kurtzman 2011). Soil samples and three orange peel samples (10 g/samples) were put into an aseptic flask with 200 mL YPD (1 % yeast extract, 2 % peptone, 2 % glucose), and shaken at 150 rpm for 30 min (Bezerra-Bussoli et al. 2013). One-liter orange juice samples (Brix value 11°, 3.8 g/L total acid, pH 3.36) in duplicate were obtained from the orange pulp by using a centrifugal juice extractor, and fermented spontaneously at 28 °C after adding 40 mg/L $H_2SO_3$ into the juice. Samples in triplicate were taken at the beginning (0 days), middle (5 days) and end of orange wine fermentation (12 days). Samples were then diluted with sterile $ddH_2O_2$, and 0.1-mL serially diluted samples were spread onto potato dextrose agar (PDA; potato juice 2 %, peptone 2 %, glucose 2 %, agar 2 %) supplemented with 100 ng/μL streptomycin sulphate and incubated at 28 °C for 2 days. Yeast colonies were randomly isolated from the PDA plate according to morphological characteristics. Pure yeast culture was preserved on a PDA slant at 4 °C and in glycerol stock (20 %) at −80 °C for future use.

### Reference strains

Reference strains were purchased from China General Microbiological Culture Collection Center (CGMCC2.898: *Candida humilis*; CGMCC2.2735: *Candida tropicalis*; CGMCC2.1596: *Clavispora lusitaniae*; CGMCC2.3266: *Hanseniaspora opuntiae*; CGMCC2.3213: *Hanseniaspora uvarum*; CGMCC2.3216: *Issatchenkia terricola*; CGMC C2.454: *Pichia kudriavzevii*; CGMCC2.1602: *Torulaspora delbrueckii*; CGMCC2.4315: *Zygowilliopsis californica*) and Centraalbureau voor Schimmelcultures (CBS $2592^T$: *Hanseniaspora occidentalis*).

### DNA extraction

Yeast DNA was extracted as described by Wang and Liu (2013).

### PCR and RFLPs of ITS1-5.8S-ITS2 rDNA

The 5.8S-ITS rDNA region was amplified by using the primers ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3'. The components of the PCR reaction solution were the following: 10 ng of genomic yeast DNA, 20 pmol of each primer, 100 μmol/L of each dNTP, 10×PCR buffer with $Mg^{2+}$ and 1 U of DNA polymerase (Finnzymes Oy, Finland) in 50 μL reaction solution. The PCR program was set as: 95 °C for 5 min, 35 cycles at 95 °C for 1 min, 55 °C for 2 min and 72 °C for 2 min, followed by final extension at 72 °C for 10 min. PCR amplicons were verified through gel electrophoresis in 1.0 % (w/v) agarose. The amplified 5.8S-ITS rDNA PCR amplicons were digested with the restriction endonucleases *Hae*III, *Hinf*I, and *Hha*I (Takara, Japan) at 37 °C for 1 h. Restriction fragments were separated by gel electrophoresis in 2 % (w/v) agarose and quantitatively analyzed with Quantityone 4.6.2. Each distinct 5.8S-ITS rDNA profile was compared with that of described species available in the yeast ID database (http://www.yeast-id.com/).

### PCR and sequencing of D1/D2 26S rDNA

Yeast strains representative of each profile were submitted for sequence analysis. The D1/D2 26S rDNA fragment was amplified with NL1: 5'-GCATATCAATAAGCGGAGGAAAAG-3' and NL4: 5'-GGTCCGTGTTTCAAGACGG-3' (Kurtzman and Robnett 1998). The PCR program was: 95 °C for 5 min, 36 cycles at 94 °C for 1 min, 52 °C for 1 min and 72 °C for 2 min, followed by final extension at 72 °C for 10 min. D1/D2 26S rDNA amplicons were purified and sequenced by Sangon Biotech (Shanghai) Co. Ltd. The obtained sequences were compared with those of described species available in the GenBank database (http://www.ncbi.nlm.nih.gov/).

## Results and discussion

### Isolation and identification of yeast species

A total of 160 colonies were isolated in the present study; 55 strains were isolated from orangery soil, 38 strains were isolated from orange peel and 67 strains were isolated from spontaneously fermented orange wine. 5.8S-ITS rDNA fragments were amplified from the isolated yeast strains with primers ITS1 and ITS4 and digested with restriction endonucleases *Hae*III, *Hinf*I and *Hha*I. The results in Fig. 1A and Table 1 indicated that the 5.8-ITS rDNA PCR amplicons had different sizes, ranging from 370 bp to 800 bp. The restriction profiles of 5.8S-ITS rDNA fragments were different when they were digested with *Hae*III, *Hinf*I and *Hha*I (Fig. 1B, C, D and Table 1). Sequences of the D1/D2 26S rDNA region from strains representative of each ITS-5.8S rDNA RFLP profile were compared with those of described species available in the GenBank database (Table 1).

**Table 1** Identification results of isolated yeast according to 5.8S-ITS-RFLP and 26S rDNA D1/D2 sequencing

| Strain number | Amp (bp) | Restriction fragments (bp) | | | Identity | GenBank accession number | Species |
|---|---|---|---|---|---|---|---|
| | | *Hae*III | *Hinf*I | *Hha*I | | | |
| I | 550 | 450,90 | 270 | 285,250 | 100 % | EU543670.1 | *Candida tropicalis* |
| II | 640 | 600 | 250 | 320,270 | 100 % | EF550276.1 | *Barnettozyma californica* |
| III | 750 | 750 | 350,200,180 | 320,105 | 99.8 % | EU386730.1 | *Hanseniaspora uvarum* |
| IV | 450 | 295,125 | 240,105 | 130,100 | 100 % | HQ149318.1 | *Pichia terricola* |
| V | 750 | 750 | 340,190,170 | 320,120 | 99.8 % | KC111446.1 | *Hanseniaspora opuntiae* |
| VI | 670 | 400,230 | 350,280 | 265,160,140 | 100 % | GU138487.1 | *Candida humilis* |
| VII | 750 | 640,110 | 250,170,110 | 330,100 | 99.8 % | EU268638.1 | *Hanseniaspora occidentalis* var. *occidentalis* |
| VIII | 450 | 300,70 | 200,130 | 220,190 | 99.8 % | AY529499.1 | *Pichia kudriavzevii* |
| IX | 370 | 370 | 180,160 | 210,90 | 99.2 % | KC442253.1 | *Clavispora lusitaniae* |
| XX | 800 | 800 | 410,380 | 330,220,150,100 | 99.5 % | JQ965838.1 | *Torulaspora delbrueckii* |

The species *H. occidentalis* was recently split into two varieties, *Hanseniaspora occidentalis* M.Th. Smith var. *occidentalis* (2006) and *Hanseniaspora occidentalis* M.Th. Smith var. *citrica* Cadez, Raspor & M.Th. Smith (2006) which could be distinguished by habitat preference. The variety *occidentalis* was mostly isolated from soil, whereas the variety *citrica* was isolated from orange and its processed products (Cadez et al. 2006). Compared with reference strain CBS 2592$^{T}$, all *H. occidentalis* strains isolated in the present study were classified into the *occidentalis* variety.

## Distribution of yeast species isolated from orangery

The distribution of yeast species in orangery soil, orange peel and different stages of spontaneous orange wine fermentation were shown in Table 2. The results indicated that among the seven species isolated from orangery soil, *B. californica* and *H. occidentalis* were the most abundant species with the highest isolates (17, 21 respectively), followed by *C. tropicalis* (6), *P. terricola* (4), *H. uvarum* (3), *H. opuntiae* (3) and *P. kudriavzevii* (1). On orange peel, *P. terricola* and *H. opuntiae* were the prevailing yeast species with 20 and 17

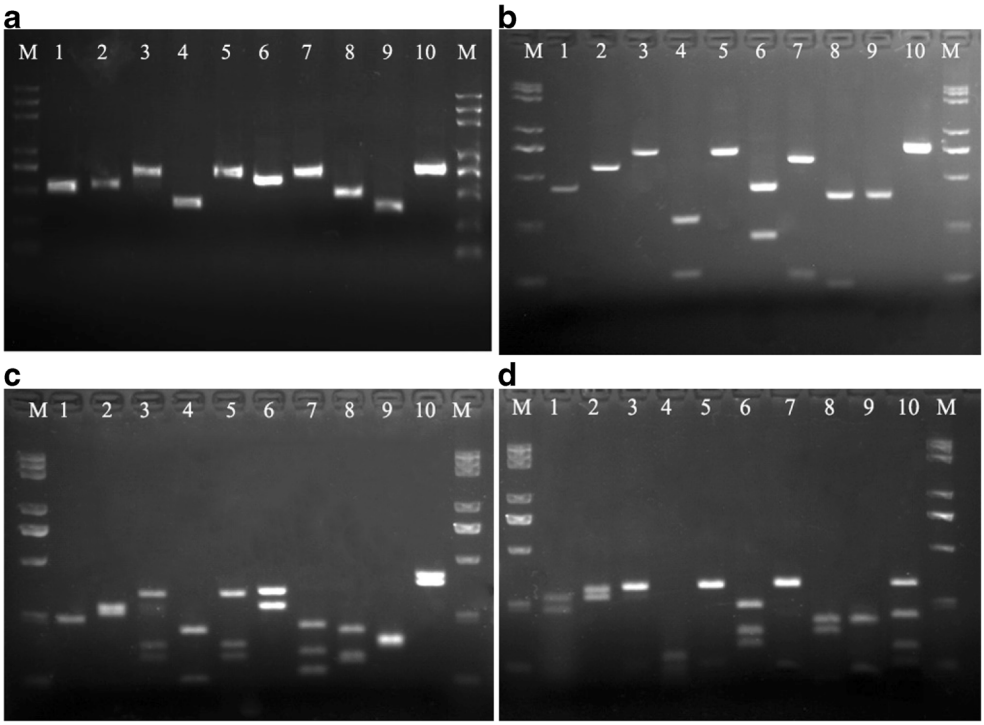


**Fig. 1** Electrophoretograms of 5.8S-ITS rDNA amplificons from isolated yeast and their restriction profiles. **a** 5.8S-ITS rDNA amplificons from the isolated yeast; **b c d** Restriction fragments of 5.8S- ITS rDNA digested with *Hae*III, *Hinf*I and *Hha*I, respectively

**Table 2** Distribution number of yeast species in orangery soil, orange peel and orange wine

| Yeast species | Colony number | | | | |
|---|---|---|---|---|---|
| | Orangery soil | Orange peel | Spontaneous fermentation period | | |
| | | | BF [a] | MF [b] | EF [c] |
| *Candida tropicalis* | 6 | | | | |
| *Barnettozyma californica* | 17 | | | | |
| *Hanseniaspora uvarum* | 3 | 1 | | | |
| *Pichia terricola* | 4 | 20 | | | |
| *Hanseniaspora opuntiae* | 3 | 11 | 9 | | |
| *Candida humilis* | | | 2 | | |
| *Hanseniaspora occidentalis* var. *occidentalis* | 21 | | | | |
| *Pichia kudriavzevii* | 1 | | 31 | 3 | |
| *Clavispora lusitaniae* | | | | 12 | |
| *Torulaspora delbrueckii* | | | | | 10 |

[a] beginning of fermentation

[b] middle of fermentation

[c] end of fermentation

isolates, respectively, followed by *H. uvarum* (1). The results indicated that yeast species isolated in orangery soil exceeded those on orange peel, and some yeast species isolated from orange peel also existed in orangery soil. The phenomenon was also found in other research (Botha 2011).

In spontaneously fermented orange wine, *P. kudriavzevii* and *C. humilis* were the prevailing yeast species with 31 and 9 isolates, respectively, followed by *H. opuntiae* (2) at the beginning stage of fermentation. *Clavispora lusitaniae* and *P. kudriavzevii* were the dominant yeast species with 12 and 3 isolates, respectively, at the middle stage of fermentation. The number of yeast species decreased with fermentation progressing and *T. delbrueckii* was the only species at the final stage of fermentation. These results were consistent with the fact that some non-*Saccharomyces* species had similar ethanol tolerance with *S. cerevisiae* and a stronger resistance to fermentation conditions than *S. cerevisiae* (Xufre et al. 2006; Hong and Park 2013). The *P. kudriavzevii* number decreased significantly from day 0 to the middle stage of fermentation. Lopes (2002) reported that *P. kudriavzevii* was sensitive to $SO_2$, so the decrease of *P. kudriavzevii* number may be related to the addition of $SO_2$. Ethanol in wine may also play an important role in inhibiting the growth of *P. kudriavzevii*.

Among the five yeast species isolated from orange wine, *C. lusitaniae* was also detected in spontaneously fermenting oranges and orange juice in Spain (Heras-Vazquez et al. 2003). *Pichia kudriavzevii* and *T. delbrueckii* isolated from spontaneously fermented orange wine in this research was also detected in pasteurized and subsequently recontaminated single-strength orange juice (Arias et al. 2002). However, *C. humilis* and *H. opuntiae*, which have never been reported to be isolated from oranges, were found in this study. Different yeast microbiota in orange wine from different regions might be explained by varying regional climates. The Wuhan orangery is located in a subtropical monsoon climate area with abundant precipitation (1205 mm per year), full sunshine and four distinctive seasons, and the main soil type is red clay. Temperature and rainfall obviously vary seasonally, with an annual mean value of 17 °C and 1205 mm, respectively. Nevertheless, it was not easy to find a direct relationship between the microbiota and the analytical parameters, geographical areas, climate indexes and orange varieties. This was due to the high number of factors influencing the microbiota (Pretorius 2000) and more investigative work should be done in the future.

It is generally recognized that the non-*Saccharomyces* are the dominant species during the early stage of fermentation, and *Saccharomyces* will become the dominant species with an increased ethanol concentration in spontaneously fermented wine (Wang and Liu 2013). It was worth noting that no *S. cerevisiae* was isolated from these three resources. This might be due to the absence of *Saccharomyces* in orangery soil, the brewing environment and in orange pulp. Furthermore, *Saccharomyces* in wineries primarily comes from alcoholic products or contaminated winery equipment rather than from the vineyard (Mortimer and Polsinelli 1999; Querol et al. 2003). *Saccharomyces* is also rarely found on the surfaces of berries (Barata et al. 2012).

Many non-*Saccharomyces* species isolated from a vineyard can be used to produce improved flavor and an aromatic profile during wine brewing. The yeast strain *T. delbrueckii* has been marketed for sequential fermentation or mixed cultures to reduce the volatile acidity and enhance the aromatic profile

of wine (Renault et al. 2009). *Hanseniaspora uvarum* can increase the isoamyl acetate in mixed fermentation with *S. cerevisiae* (Moreira et al. 2008). *Pichia terricola* can enhance the aroma of white Muscat wine (González et al. 2011). Kim et al. (2008) reported that *P. kudriavzevii* KMBL 5774 can degrade malic acid and ferment wine with a high sensory evaluation score when it was used to ferment wine with *S. cerevisiae* W-3. The brewing characteristics of the non-*Saccharomyces* isolated from orangery soil and their influence on the flavor profiles of orange wine will be studied further.

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