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Yeast species associated with wine grapes in China

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ABSTRACT

Having more information on the yeast ecology of grapes is important for wine-makers to produce wine with high quality and typical attributes. China is a significant wine-consuming country and is becoming a serious wine-producer, but little has been reported about the yeast ecology of local ecosystems. This study provides the first step towards the exploitation of the yeast wealth in China's vine-growing regions. The aim of this study was to investigate the yeast population density and diversity on three grape varieties cultivated in four representative vine-growing regions of China. Yeast species diversity was evaluated by using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) and sequence analysis of the 5.8S internal transcribed spacer (ITS) ribosomal DNA (rDNA) region of cultivable yeasts. The grapes harbored yeast populations at 10^2–10^6 CFU/mL, consisting mostly of non-*Saccharomyces* species. Seventeen different yeast species belonging to eight genera were detected on the grape samples tested, including *Hanseniaspora uvarum*, *Cryptococcus flavescens*, *Pichia fermentans*, *Candida zemplinina*, *Cryptococcus carnescens*, *Candida inconpicua, Zygosaccharomyces fermentati*, *Issatchenkia terricola*, *Candida quercitrusa*, *Hanseniaspora guilliermondii*, *Candida bombi*, *Zygosaccharomyces bailii*, *Sporidiobolus pararoseus, Cryptococcus magnus, Metschnikowia pulcherrima*, *Issatchenkia orientalis* and *Pichia guilliermondii*. *H. uvarum* and *C. flavescens* were the dominant species present on the grapes. For the first time *Sporidiobolus pararoseus* was discovered as an inhabitant of the grape ecosystem. The yeast community on grape berries was influenced by the grape chemical composition, vine-variety and vine-growing region. This study is the first to identify the yeast communities associated with grapes in China using molecular methods. The results enrich our knowledge of wine-related microorganisms, and can be used to promote the development of the local wine industry.

1. Introduction

Yeasts are the most important microorganisms in wine production, because they influence fermentation speed, wine flavor and other wine qualities (Fleet, 2003; Loureiro and Malfeito-Ferreira, 2003; Jolly et al., 2006). Grapes are a primary source of natural yeasts in wine production. The grape yeast flora can be either beneficial or detrimental to the quality of wine products. Consequently, having more information about the yeast communities present on grapes is important for wine-makers to produce wines with high quality and representative attributes. Much ecological research has been conducted on the yeast flora in different wine-producing regions (Pretorius et al., 1999; Sabaté et al., 2002; Combina et al., 2005; Raspor et al., 2006; Nisiotou and Nychas, 2007; González et al., 2007; Chavan et al., 2009). It has been found that freshly crushed grape juice harbors a diversity of yeast species, principally within the genera *Hanseniaspora* (anamorph *Kloeckera*), *Pichia*, *Candida*, *Metschnikowia*, and *Kluyveromyces*. Occasionally, species in other genera such as *Cryptococcus*, *Rhodotorula*, *Debaryomyces*, *Issatchenkia*, *Zygosaccharomyces*, *Saccharomycodes*, *Torulaspora*, *Dekkera*, *Schizosaccharomyces* and *Saccharomyces* have also been isolated from wine grapes of several wine-producing areas (Ribéreau-Gayon et al., 2000; Fleet et al., 2002; Fleet, 2003, 2008; Prakitchaiwattana et al., 2004; Jolly et al., 2006). These yeast species perform biochemical activities in the must, thus influencing positively or negatively the quality of the wine.

The population density and diversity of indigenous yeasts on grape berries are intricately linked to numerous factors, such as berry maturity, grape variety, geographic location, climatic condition, fungicide application, vineyard age and viticultural practices (Combina et al., 2005; Raspor et al., 2006; Nisiotou and Nychas, 2007; Chavan et al., 2009). However, insufficient quantitative data are available to be able to draw general conclusions about these influences. As wine-makers increasingly recognize that particular indigenous yeast species can contribute beneficially to or cause spoilage of wine, the

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need for deeper knowledge of the effects of these factors on the yeast ecology of grapes will become more apparent.

China is one of the six biggest wine-consuming countries in the world and has a great potential for developing viticulture and its wine industry. China has over ten major viticultural regions, each with distinct ecological characters. Nevertheless, very little has been known about the yeast ecology involved in local ecosystems. Development of knowledge about the yeast community of local ecosystems is essential to produce wines with regional attributes and to improve the wine-making practice. This yeast ecological study is an essential step towards the preservation and exploitation of the hidden oenological potential of indigenous yeast wealth in China. Therefore, this study has an important significance for the local wine industry.

In recent years, two molecular techniques, polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) and sequence analyses of the ribosomal DNA (rDNA) region including 5.8S internal transcribed spacer (ITS) region, have proved to be useful for the rapid identification of wine yeast species (Clemente-Jimenez et al., 2004; Combina et al., 2005; Nisiotou and Nychas, 2007; Zott et al., 2008). In our study, yeast species diversity was evaluated by PCR-RFLP and sequence analyses of the 5.8S-ITS rDNA.

The aim of this work was to study population density and diversity of the indigenous yeasts on wine grapes in China's main viticultural regions. Furthermore, the impact of grape chemical composition, vine-variety and geographic location on the yeast distribution was evaluated.

2. Materials and methods

2.1. Grape sampling

Grape samples were collected during harvest (between September and October) in 2006 vintage. The three grape varieties analyzed were: Cabernet Sauvignon, Merlot and Chardonnay. Grapes were sampled from four representative vineyards in China: (A) Shacheng of Hebei province (annual active accumulated temperature, 3500–3564 °C; annual average rainfall, 413 mm), (B) the coastal region, Changli of Hebei province (annual active accumulated temperature, 3756–4174 °C; annual average rainfall 638 mm), (C) the coastal region, Penglai of Shandong province (annual active accumulated temperature, 3800–4200 °C; annual average rainfall, 592 mm), and (D) Wuwei of Gansu province (annual active accumulated temperature, 2800–3000 °C; annual average rainfall, 110 mm). These districts are separated by distances as great as 1911 km.

For each grape variety from each selected vineyard, around 1–2 kg of each sample was randomly and aseptically removed from at least five different vines within the vineyard. Only healthy and undamaged grapes were collected from the selected vineyards and stored at 4 °C. Samples were transported to the laboratory in Beijing by road or air and analyzed within 24 h of harvest from the vine.

2.2. Chemical analysis

Reducing sugar content, titratable acidity and pH of the grape samples were determined according to the National Standard of the People's Republic of China (GB15038-2005). All experiments were performed in triplicate and the results were expressed as the mean value ± the standard deviation. Data were subjected to one-way analysis of variance (ANOVA) at 5% level of significance, and the means were separated by Student–Newman–Keuls test using SPSS statistical software (version 11.0, SPSS Ins., USA) for Windows.

2.3. Yeast enumeration and isolation

About 500 g of each grape sample was randomly mixed and aseptically homogenized for 30 s at normal intensity (HAODE SG-280A4, Foshan, China). Homogenates were serially diluted with the sterile physiological saline (0.85% NaCl). For the enumeration of yeasts, 0.1 mL of each dilution was spread in triplicate on three different media: Wallerstein Laboratory nutrient agar (WL; Oxoid, England), lysine medium (LM; Oxoid, England) and ethanol sulfite agar (ESA; containing 1% yeast extract, 2% bacteriological peptone, 2% dextrose, 12% ethanol, 0.015% sodium metabisulfite and 2% agar). All media were supplemented with 100 mg/L chloramphenicol (Sigma, USA) to inhibit bacterial growth. Plates were incubated at 28 °C for 3 days. ESA was used to detect the native populations of *Saccharomyces* species, because non-*Saccharomyces* yeasts have lower tolerance of ethanol and sulfur dioxide (Kish et al., 1983). LM was used to monitor the presence of non-*Saccharomyces* species effectively, since it is a medium with L-lysine as the sole nitrogen source and *Saccharomyces* spp. are unable to grow on this medium (Angelo and Siebert, 1987). According to Pallmann et al. (2001), the WL medium is useful for the wine industry to quantify and identify wine microorganisms, since it can discriminate between the yeast genus and species by colony morphology and color.

The various colony types on WL medium were counted. Based on colony morphology and frequency, 15 representative colonies on WL medium were proportionally isolated from each grape sample. The representative isolates were purified by repetitive streaking on yeast extract peptone dextrose agar (YEPD; containing 1% yeast extract, 2% bacteriological peptone, 2% dextrose and 2% agar) and then stored at 4 °C on YEPD slants for future identification.

2.4. Yeast identification

A total of 180 yeast isolates from 12 grape samples were submitted to identification using molecular methods as described below.

2.4.1. 5.8S-ITS rDNA PCR-RFLP analysis

The genomic DNA of each representative yeast strain was extracted with the yeast genomic DNA extraction kit (Biofuture, China) according to manufacturer's instructions. The primers used for amplification of 5.8S-ITS rDNA region were ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). The DNA amplifications were carried out in a final volume of 50 μL containing 1 U *Taq* DNA polymerase (Biofuture, China), 1 × PCR reaction buffer, 0.2 mM of each dNTP, 2 mM $MgCl_2$, 0.1 μM of each primer and 5–25 ng template DNA. The PCR reaction was performed on a Mastercycler ep gradient (Eppendorf, Germany). The PCR conditions were as follows: initial denaturation at 95 °C for 5 min; 35 cycles of denaturing at 94 °C for 1 min; annealing at 55.5 °C for 2 min, an extension at 72 °C for 2 min; and a final extension step of 10 min at 72 °C. The PCR products were subjected to restriction analysis with the restriction endonucleases *Hin6*I, *Bsu*RI, and *Hinf*I (Fermentas, USA), following the manufacturer's instructions. Amplified products and their restriction fragments were separated on 1.4% or 3% (w/v) agrose gels at 100 V constant voltage for 1 h. Electrophoresis gels were stained with ethidium bromide (0.5 μg/mL) and photographed under UV light (Xiaoyuan, China). A 100-bp DNA ladder marker (Biofuture, China) was used as size standard.

2.4.2. 5.8S-ITS rDNA sequence analysis

Three randomly selected isolates of each distinct PCR-RFLP profile were identified by 5.8S-ITS rDNA region sequencing. PCR products of the 5.8S ITS rDNA region were sequenced using ITS1 or/and ITS4 primers after purification by a PCR purification kit (Biofuture, China). The ITS1-5.8S-ITS2 sequences obtained were compared with sequences available in GenBank database available at the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST) available at http://www.ncbi.nlm.nih.gov/blast/. Sequences with 98% nucleotide identity or higher in the 5.8S-ITS rDNA region were considered to represent the same species

(Zott et al., 2008). When all the yeast isolates were identified, the species-specific partial 5.8S ITS region sequences were submitted to the GenBank database under accession numbers: GU237044 *Zygosaccharomyces fermentati*, GU237045 *Candida quercitrusa*, GU237046 *Issatchenkia orientalis*, GU237047 *Candida bombi*, GU237048 *Pichia fermentans*, GU237049 *Sporidiobolus pararoseus*, GU237050 *Hanseniaspora uvarum*, GU237051 *Cryptococcus carnescens*, GU237052 *Cryptococcus magnus*, GU237053 *Pichia guilliermondii*, GU237054 *Candida inconpicua*, GU237055 *Issatchenkia terricola*, GU237056 *Cryptococcus flavescens*, GU237057 *Candida zemplinina*, GU237058 *Zygosaccharomyces bailii*, GU237059 *Metschnikowia pulcherrima*, and GU237060 *Hanseniaspora guilliermondii*.

3. Results and discussion

3.1. Population of yeasts on wine grapes

The grape samples were crushed and the must were used for enumeration and isolation of different yeast species as described in Section 2. The reducing sugar content of 12 samples collected from four regions in China varied from 142.3 to 250.5 g/L. Titratable acidity of the grapes ranged between 3.38 to 8.44 g/L at harvest, with pH levels from 2.99 to as high as 3.66. We evaluated the total yeast count, non-*Saccharomyces* and *Saccharomyces* populations by the spread plate method on non-selective culture medium (WL medium) and selective culture media (LM and ESA medium). The yeast populations on wine grapes are given in Table 1. Similar CFU counts (10^3–10^5 CFU/mL) were obtained on WL medium and LM, indicating that the non-*Saccharomyces* yeasts represented most of the total yeast population on grapes. This observation is similar to previous reports (Sabaté et al., 2002; Raspor et al., 2006; Mercado et al., 2007; Nisiotou and Nychas 2007). The yeast population on healthy and ripe Malbec grapes from Argentina obtained by crushing grape berries and plating on malt extract agar (MEA) was in the order of 10^5–10^6 CFU/g, which is higher than found in our research (Combina et al., 2005). *Saccharomyces* on the grapes, as determined by plating on ESA medium, occurred in extremely low populations (less than 10^2 CFU/mL) or was not even detected (Table 1), which is in agreement with previous reports (Mortimer and Polsinelli, 1999; Fleet, 2003; Sabaté et al., 2002; Mercado et al., 2007). Due to the extremely low occurrence of *Saccharomyces* by direct plating from healthy undamaged grapes, there is significant controversy as to their natural origin in wine production and some authors have postulated a “winery origin” for them (Martini, 1993; Martini et al., 1996; Mercado et al., 2007). However *Saccharomyces* were not totally absent in our study and were found on the samples with higher sugar content and lower pH (Table 1). Mortimer and Polsinelli (1999) found that damaged grape berries were rich depositories of microorganisms including *Saccharomyces*. These results reflected that the specific must environment, i.e. low pH, high sugar content (high osmotic pressure), played a role in determining which species of yeasts can survive and grow.

3.2. Molecular identification of yeast isolates on wine grapes

WL medium has proved to be extremely useful in the isolation and initial presumptive identification of different wine yeast species (Pallmann et al., 2001; Tofalo et al., 2009). After counting, a total of 180 representative yeast colonies, 15 for each grape variety from each region, were isolated from WL plates for further molecular identification. It has previously been demonstrated that the 5.8S-ITS rDNA analysis is a reliable routine technique for the differentiation of wine yeasts at species level (Clemente-Jimenez et al., 2004; Combina et al., 2005; Nisiotou and Nychas, 2007; Zott et al., 2008). To determine the yeast diversity present on grapes, we used a PCR-RFLP analysis of the 5.8S-ITS rDNA region to identify 180 representative yeast isolates. Seventeen different restriction patterns were generated by using three restriction enzymes *Hin6*I, *Bsu*RI, and *Hin*fI. The sizes of the PCR product and restriction fragments were visually estimated by comparison with a 100-bp DNA ladder. During the analysis we discovered that DNA bands smaller than 80 bp were difficult to visualize and subsequently excluded these bands from this analysis. Nine of the 17 restriction patterns were directly identified after comparing the molecular mass of the restriction products with those previously described (Esteve-Zarzoso et al., 1999; Combina et al., 2005; Nisiotou and Nychas, 2007; Nisiotou et al., 2007). These patterns corresponded to *H. uvarum*, *P. fermentans*, *P. guilliermondii*, *M. pulcherrima*, *Z. bailii*, *C. zemplinina*, *C. bombi*, *I. terricola*, and *I. orientalis*. An additional, eight species were not identified by this method because their 5.8S-ITS patterns have not been previously described. In order to find the identities of other eight unknown restriction patterns and to reconfirm the identities of the nine patterns previously identified by restriction analysis of the 5.8S-ITS rDNA region, the PCR products from the 17 groups were sequenced and compared with the available DNA sequence databases. Based on identification using sequence analysis of the 5.8S-ITS rDNA region, the eight new 5.8-ITS patterns corresponded to the species *C. carnescens*, *C. flavescens*, *C. inconpicua*, *C. quercitrusa*, *C. maqnus*, *S. pararoseus*, *Z. fermentati* and *H. guilliermondii* (Table 2).

3.3. Yeast diversity on wine grapes

The yeast species associated with wine grapes of the most popular varieties from four different vineyards in China are presented in

Table 1
Chemical characteristics and yeast counts for wine grape samples in China.

Vine-growing region	Grape variety	Reducing sugar content (g/L as glucose) *	Titratable acidity (g/L as tartaric acid) *	pH *	Yeast counts (log CFU/mL) *		
					TY#	NS	S
Shacheng	Cabernet Sauvignon	214.29 ± 1.41^{a}	6.29 ± 0.13^{ad}	3.06 ± 0.00^{a}	4.75 ± 0.16^{a}	4.72 ± 0.03^{ab}	1.49 ± 0.20^{a}
	Merlot	181.21 ± 1.70^{b}	6.34 ± 0.08^{ad}	3.15 ± 0.01^{b}	3.14 ± 0.19^{b}	3.08 ± 0.18^{ce}	–
	Chardonnay	188.81 ± 1.27^{ci}	7.20 ± 0.40^{b}	3.56 ± 0.01^{c}	4.26 ± 0.17^{c}	3.96 ± 0.07^{d}	–
Changli	Cabernet Sauvignon	183.05 ± 0.88^{b}	8.25 ± 0.05^{c}	3.20 ± 0.01^{d}	4.12 ± 0.03^{c}	4.10 ± 0.05^{dg}	–
	Merlot	184.93 ± 1.41^{bc}	8.42 ± 0.03^{c}	3.25 ± 0.01^{e}	4.16 ± 0.07^{c}	4.18 ± 0.09^{dg}	–
	Chardonnay	142.86 ± 0.53^{d}	6.53 ± 0.05^{d}	3.26 ± 0.01^{f}	3.41 ± 0.10^{b}	3.39 ± 0.17^{e}	–
Wuwei	Cabernet Sauvignon	227.85 ± 1.36^{e}	8.82 ± 0.12^{ae}	3.00 ± 0.01^{g}	2.42 ± 0.01^{d}	2.36 ± 0.32^{f}	1.21 ± 0.17^{a}
	Merlot	248.86 ± 1.62^{f}	8.81 ± 0.27^{ae}	3.04 ± 0.01^{h}	3.37 ± 0.10^{b}	2.76 ± 0.28^{cf}	1.16 ± 0.15^{a}
	Chardonnay	242.16 ± 1.53^{g}	8.50 ± 0.02^{e}	3.21 ± 0.01^{d}	3.10 ± 0.09^{b}	2.90 ± 0.16^{c}	1.52 ± 0.46^{a}
Penglai	Cabernet Sauvignon	195.65 ± 1.00^{h}	4.55 ± 0.07^{f}	3.21 ± 0.00^{d}	5.05 ± 0.06^{a}	4.99 ± 0.07^{a}	–
	Merlot	185.57 ± 0.91^{ci}	3.76 ± 0.15^{g}	3.28 ± 0.02^{e}	4.85 ± 0.05^{a}	4.46 ± 0.15^{bg}	–
	Chardonnay	189.48 ± 0.94^{i}	3.52 ± 0.20^{g}	3.29 ± 0.01^{i}	4.27 ± 0.03^{c}	3.96 ± 0.10^{d}	–

* Data are means ± standard deviation. Means within a column with different superscript letters are significantly different by Student–Newman–Keuls test ($P < 0.05$).
\# TY, total yeast counts by the spread plate method on Wallerstein Laboratory nutrient agar; NS, non-*Saccharomyces* counts by the spread plate method on lysine medium; S, *Saccharomyces* counts by the spread plate method on ethanol sulfite agar.

Table 2
Nucleotide fragments length of the new 5.8S-ITS profile described in the study.

Yeast species	5.8 S-ITS region amplified product size (bp)	Size of the restriction fragment (bp)[a]		
		*Hin*6I	*Bsu*RI	*Hin*fI
Sporidiobolus pararoseus	550	270 + 280	550	250
Cryptococcus carnescens	550	200 + 280	90 + 350	250 + 250
Cryptococcus flavescens	460	200 + 260	460	170 + 220
Candida inconpicua	600	100 + 100 + 100 + 190	500 + 100	100 + 220 + 270
Candida quercitrusa	596	200 + 300	90 + 100 + 400	120 + 200 + 270
Cryptococcus magnus	600	250 + 350	80 + 460	140 + 230 + 240
Hanseniaspora guilliermondii	690	300 + 320	690	120 + 190 + 320
Zygosaccharomyces fermentati	650	280 + 320	80 + 210 + 300	310 + 350

[a] Restriction fragments smaller than 80 bp could not be visualized.

Table 3. The yeast communities present on the samples are complex and rich in different species. A total of 17 different yeast species belonging to eight different genera were found by PCR-RFLP analysis combined with sequence analysis of the 5.8S-ITS rDNA region.

We found that non-*Saccharomyces* yeasts were the dominant species on the grapes in our study, which is in agreement with previous reports (Pretorius et al., 1999; Sabaté et al., 2002; Jolly et al., 2003; Combina et al., 2005; Raspor et al., 2006; Nisiotou and Nychas, 2007; González et al., 2007; Chavan et al., 2009). Recently, several studies have examined the beneficial and detrimental influence of non-*Saccharomyces* yeasts and their potential application in wine-making (Ciani and Maccarelli, 1998; Esteve-Zarzoso et al., 1998; Romano, 2002; Romano et al., 2003; Fleet, 2003; Loureiro and Malfeito-Ferreira, 2003; Clemente-Jimenez et al., 2004; Anfang et al., 2009). In order to exploit the potential benefits and to minimize potential spoilage by non-*Saccharomyces*, the yeast biodiversity on grapes needs to be known.

H. uvarum was found in all samples and was the most frequent species on grapes. The apiculate species (*K. apiculata* and its sporiferous form *H. uvarum*) have been found to be the predominant yeasts on grapes from a variety of different places (Combina et al., 2005; Raspor et al., 2006; Clemente-Jimenez et al., 2004; Nisiotou and Nychas, 2007). The apiculate yeasts are known to be high producers of esters and glycerol. Moreover they have the capability to secrete several enzymes, e.g. β-glucosidases and proteases, interacting with grape-derived precursor compounds such as glycosidically bound terpenes, thus contributing to the subsequent expression of varietal aroma thereby improving the wine-making process (Jolly et al., 2006; Zott et al., 2008). Therefore, such indigenous yeasts make a positive contribution to wine quality. However, some strains of apiculate yeasts are also known as high producers of acetic acid, making them undesirable for wine production (Ciani and Picciotti, 1995). Therefore, only selected strains of apiculate yeasts might favor aroma and flavor enhancement in wines.

C. flavescent also was a common species present on the grapes in China, and has been isolated from wheat, soil and clinical specimens (Takashima et al., 2003), but has not been found on grapes or in wines in other regions.

The proportions of other species were relatively low (<10%). Unexpectedly, one of the most dangerous wine spoilage yeast genus *Zygosaccharomyces* was found on Merlot from Penglai. Some strains of *Zygosaccharomyces* are known to produce off-flavors, and some can produce spoilage by sediment or cloudiness formation. They can reside in winery environments for their tolerance of high ethanol concentrations (>10%), which is a real threat to product quality and preservation (Fleet, 2003; Loureiro and Malfeito-Ferreira, 2003). Therefore, the origins and control measures of *Zygosaccharomyces* must be well established. *I. terricola* was detected on Cabernet Sauvignon from Changli. It is suggested *I. terricola* is to be avoided due to its low fermentative characteristics and its high ethyl acetate production, contributing to a vinegary taste (Clemente-Jimenez et al., 2004). Additionally, *I. orientalis* has produced wines with intense color and foam (Clemente-Jimenez et al., 2004). The color of a wine is an indicator of its condition, quality, age, and even style. *C. quercitrusa* was found on Cabernet Sauvignon from Shacheng and Wuwei. *C. quercitrusa* was seldom found to be associated with grape berries by

Table 3
Percentage distribution (%) of indigenous yeast species present on wine grapes of five varieties (C, Cabernet Sauvignon; M, Merlot; X, Chardonnay) from four vineyards in China.

Yeast species	Sampling location and grape variety											
	Shacheng			Changli			Wuwei			Penglai		
	C	M	X	C	M	X	C	M	X	C	M	X
Hanseniaspora uvarum	40.00	33.33	46.67	46.67	46.67	73.33	26.67	26.67	33.33	60.00	26.67	33.33
Cryptococcus flavescens	26.67	26.67	46.67	26.67	20.00	26.67	20.00	13.33	13.33	40.00	13.33	6.67
Pichia fermentans	6.67	6.67	–	6.67	13.33	–	20.00	–	–	–	–	26.67
Candida zemplinina	–	6.67	6.67	6.67	6.67	–	6.67	6.67	–	–	13.33	6.67
Cryptococcus carnescens	6.67	–	–	–	6.67	–	–	26.67	13.33	–	–	–
Candida inconpicua	–	–	–	–	–	–	–	26.67	–	–	6.67	–
Zygosaccharomyces fermentati	–	–	–	–	–	–	–	–	–	–	13.33	–
Issatchenkia terricola	–	–	–	–	6.67	–	–	–	–	–	13.33	–
Candida quercitrusa	6.67	–	–	–	–	–	13.33	–	–	–	–	–
Hanseniaspora guilliermondii	13.33	13.33	–	–	–	–	–	–	–	–	–	–
Candida bombi	–	–	–	–	–	–	–	–	–	–	–	26.67
Zygosaccharomyces bailii	–	–	–	–	–	–	–	–	–	–	13.33	–
Sporidiobolus pararoseus	–	–	–	–	–	–	–	–	13.33	–	–	–
Cryptococcus magnus	–	–	–	–	–	–	–	–	26.67	–	–	–
Metschnikowia pulcherrima	–	–	–	–	–	–	13.33	–	–	–	–	–
Issatchenkia orientalis	–	–	–	13.33	–	–	–	–	–	–	–	–
Pichia guilliermondii	–	13.33	–	–	–	–	–	–	–	–	–	–

–: Not detected.

other researches. Recently, Chavan et al. (2009) reported isolating *C. quercitrusa* from grape berries of Bangalore blue and Cabernet varieties in India for the first time and that this species was associated with insects. *S. pararoseus* was detected on Chardonnay from Wuwei. This species has not been previously reported as a member of the grape ecosystem.

Unexpectedly, *Saccharomyces* were isolated from just four of the twelve groups of samples of grapes (Table 1). Possibly *Saccharomyces* were present on the other grapes but at such low populations that they were not detectable either among the *non-Sacchromyces* by plating on non-selective medium (i.e. WL medium) and even on ESA medium. In the four cases where they were found detection was achieved by plating on ESA medium. Some researchers also suggest that *S. cerevisiae* should be isolated from grapes by enrichment culture than direct agar plating (Fleet et al., 2002).

3.4. Yeast ecology present on different vine-growing locations

Geographic locations and climatic conditions had an impact on the population and diversity of yeast on grapes (Table 1, Table 3). The four areas chosen for sampling have different climatic conditions as described in Section 2. The yeast counts on wine grapes from Wuwei were significantly lower than those of samples from other locations, which may be due to the specific climatic conditions in the Wuwei region. Wuwei is the furthest inland and is located on the edge of the Tenggeli desert, which has little rainfall and cool weather. Some authors have suggested that rainfall exerts the greatest influence on the yeast population, because rainfall could have favored nutrient release, increasing their availability for yeast multiplication (Rousseau and Doneche, 2001; Combina et al., 2005). The grape must from Wuwei tended to have higher sugar content (high osmotic pressure) and lower pH values than for the other regions (Table 1), which could affect negatively the microflora populations. On the other hand, the most abundant yeast populations were found on grapes from Penglai, which may be ascribed to warmer climatic conditions (lower latitudes, higher temperatures) in Penglai.

The indigenous yeast species associated with wine grapes differed among the four locations sampled. Seven different species were found on samples from Changli, eight species from Shacheng, nine species from Penglai and ten species from Wuwei. The predominant species *H. uvarum*, *C. flavescen*, *P. fermentans* and *C. zemplinina* were found in all sampling locations. The three yeast species *S. pararoseus*, *M. pulcherrima* and *C. maqnus* were detected only on grapes collected from Wuwei, while *C. bombi, Z. fermentati* and *Z. bailii* were detected only on grapes collected from Penglai. *P. guilliermondii* and *H. guilliermondii* were detected only on grapes collected from Shacheng. The species *I. orientalis* was found only on grapes collected from Changli.

3.5. Yeast ecology present on different vine varieties

The indigenous yeast flora present on grapes differed among vine varieties as well as vine-growing locations (Table 1, Table 3). Among the 180 identified indigenous yeast isolates, the largest diversity of yeast species was found on the red variety Merlot (eleven species), followed by Cabernet Sauvignon (nine species), while eight different yeast species were isolated from the white variety Chardonnay. These results suggest that red vine varieties maybe offer an advantage for yeast diversity. Varietal factors, e.g. thickness of grape skins, can play a role (Bisson and Kunkee, 1991). The most frequent species *H. uvarum, C. flavescens, C. zemplinina*, *P. fermentans* and *C. carnescens* were found on all grape varieties. In all vine-growing regions studied, the genera *Issatchenkia* was detected only on the red varieties Cabernet Sauvignon and Merlot. The most dangerous wine spoilage yeast genus *Zygosaccharomyces* was found only on Merlot. *P. fermentans* was detected only in Cabernet Sauvignon variety together with *C. quercitrusa*.

The study of yeast biodiversity to gain more information about yeast communities present on the grape berries and their influence on the wine-making process is important. This study has provided the first information on the yeast flora associated with the grapes in China's vine-growing regions. The wine grapes in China were shown to be a principal source of different yeast species, especially non-*Saccharomyces* yeasts. The results obtained will be useful to develop good quality wine and to control wine spoilage, which have an important significance for the local wine-making industry. Moreover, further studies are necessary to obtain better knowledge about the participation of these indigenous yeast populations in spontaneous fermentation.

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