

Diversity and Identification of Yeasts Isolated from Tumultuous Stage of Spontaneous Table Grape Fermentations in Central China

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Table grapes are of increasing interest for wine production in China. In this study, 480 yeast isolates were isolated from the tumultuous stage during the spontaneous fermentation of six table grape varieties, which were cultivated in an ecological environment that was not industry-influenced, in Central China. The 26S rDNA D1/D2 domain sequence analysis was more efficient for yeast species identification than the 5.8S-ITS region RFLP analysis in the present study. All the tested strains belonged to nine species from six genera: *Hanseniaspora guilliermondii*, *H. opuntiae*, *H. uvarum*, *Pichia terricola*, *Kazachstania hellenica*, *K. zonata*, *P. occidentalis*, *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*. The yeast species and populations differed notably among the grape varieties. *S. cerevisiae* was found in the samples of four grape varieties (*Vitis amurensis*, Iona, Moldova and *V. davidii*), but not detected in Cuihong and Alimandeng Rose. Interdelta sequence fingerprinting analysis was used to discriminate between 128 *S. cerevisiae* isolates. Eight *S. cerevisiae* genotypes (G1 to G8) were distinguished. Genotypes G1, G2, and G3 were the most dominant strains, accounting for 32.03%, 24.22% and 28.13% of the isolates respectively. This study shows the diversity of yeast species associated with spontaneous fermentations of different table grape varieties grown in an ecological environment without any wine industry effect or footprint.

INTRODUCTION

Table grapes are ranked one of the most economically important fruit in China, and made up 83% of total grape production in 2015 (OIV, 2017). As the world's largest table grape producer, China contributes over one-third of global productivity (FAO & OIV, 2016). However, there are some problems behind the rapid increase in table grape production; one is that there was an obvious production-consumption surplus in some regions (Feng *et al.*, 2014). To further increase the value, the interest in the production of wine using table grapes has been growing, especially since there is an oversupply of table grapes in the market. Yeasts are mainly responsible for the alcoholic fermentation of grape juice, which can be a sequential development of various genera, species and strains (Valero *et al.*, 2007; Lopandic *et al.*, 2008; Sun *et al.*, 2014). Generally, non-*Saccharomyces* yeasts such as *Hanseniaspora/Kloeckera*, *Metschnikowia*, *Candida*, *Pichia* and *Kluveromyces* are present in the early stages of grape must fermentation (Fleet, 1998). As fermentation progresses, *Saccharomyces cerevisiae* dominates the middle until the end stage of wine fermentation, with an increase

in ethanol concentration (Bisson & Kunkee, 1993; Goddard & Greig, 2015). Non-*Saccharomyces* yeasts also participate in the fermentation process (Torija *et al.*, 2001; Di Maro *et al.*, 2007; Zhang *et al.*, 2010). The diversity of yeast communities present on grapes and in must is affected by climatic conditions, age of the vineyards, grape variety, viticulture and oenological practices (Renouf *et al.*, 2007; Mercado *et al.*, 2010; Drumonde-Neves *et al.*, 2017).

While several studies have focused on monitoring indigenous yeasts during spontaneous wine grape fermentations in several viticulture regions in China (Sun *et al.*, 2009; Li *et al.*, 2011; Wang & Liu 2013; Sun & Liu 2014; Sun *et al.*, 2014), such studies have not been published about table grapes. The red grapes *Vitis amurensis*, Cuihong, Alimandeng Rose, Moldova and *Vitis davidii*, and the white grape Iona are table grape cultivars grown at the Zhengzhou Fruit Research Institute (ZFRI) in the central region of China. ZFRI is an important grapevine preservation institute in China and contains abundant germplasm resources of grapes (Jiang *et al.*, 2015). It experiences a monsoon-

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influenced, four-season humid subtropical climate, with cool, dry winters and hot, humid summers. Table grapes are intended for consumption and those used in winemaking are different from wine grapes. Although they are mainly used as table grapes for consumption, many local growers also use them for wine fermentation, obtaining better value from wines, especially when table grapes are in excess supply and the price drops substantially. Although some work has been done on the table grape microbiome, most researchers have focused on the surface of the berry during the mature stage, and concentrated on the control of postharvest diseases (Kántor & Kačániová, 2015; Carmichael *et al.*, 2017). Little has been done to study yeast species involved in table grape fermentations. No winery has been established in this region, indicating that the chance of commercial yeast occurring is scarce. In other words, it is good to investigate the yeast biodiversity in those vineyards without a wine industry influence.

The objective of this study was to investigate the yeast populations of spontaneous table grape fermentations and their possible correlation with grape varieties grown in an ecological environment where there is no wine industry effect. In this study, autochthonous yeasts were isolated from the fermenting must of six different table grape varieties grown in ZFRI. Information generated from this study is of value for the development of table grapes for wine production.

MATERIALS AND METHODS

Yeast isolation

Vitis amurensis (Rupr.), Iona, Cuihong, Alimandeng Rose, Moldova, and *V. davidii* were grown in the same ecological environment in the vineyard of ZFRI (113°42' E, 34°43'N). The grape samples were collected from each variety in duplicate in 2012. No commercial dry yeast for wine fermentation has ever been used in this region. Approximately 2 kg of grapes with stems were harvested in aseptic conditions from each sampling point and placed directly into sterile bags, which were transported to the laboratory in portable refrigerators with ice bricks and processed within 1 h. The initial sugar concentrations ranged between 120 and

210 g/L, and the physicochemical parameters were listed in Table 1. Each grape sample was hand squeezed directly into sterile bags using a different pair of sterile gloves for each sample, and then the grape must was aseptically transferred into sterile flasks (1.5 L). The bottles were covered with sterile two-layer gauze to avoid contamination (Clemente-Jimenez *et al.*, 2004; Sun & Liu, 2014), and the grape must fermentation was allowed to proceed spontaneously at 24°C to 26°C for each grape variety in duplicate. Fermentations were monitored for weight loss (as gram of CO₂) per 12 hours (Fig. 1). Wines were sampled during the tumultuous stage (sugar consumption was about 2/3, as shown in Fig. 1) of the fermentation. These samples were serially diluted before plating out on YPD agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar, natural pH), which was supplemented with 100 mg/L chloramphenicol to inhibit bacterial growth. Triplicate samples were plated out. The plates were inoculated at 28°C for two to three days, and 10 to 15 colonies from each plate were randomly selected (80 colonies/variety) and preserved at -20°C in 20% (v/v) glycerol. Thus, the 480 pure cultures selected were differentiated and classified according to colony morphology and colour on Wallerstein nutrient (WLN) agar (Pallmann *et al.*, 2001) and grown for five days at 28°C.

Identification of yeast culture

Yeast DNA was extracted according to the method of Sun and Liu (2014). Five to eight representative colonies of each phenotype on WLN agar were identified by sequence analysis of the 26S rDNA D1/D2 domain and 5.8S-ITS-RFLP. The 26S rDNA D1/D2 domain was amplified using primers NL1 and NL4 according to Kurtzman and Robnett (1998). The PCR procedure and the subsequent visualisation steps were done as described as Wang and Liu (2013). The products that gave positive results were sent to Beijing Sunbiotech Co. Ltd. for purification and sequence determination. The sequences were analysed using the Blast method of NCBI (<http://www.ncbi.nlm.nih.gov/blast>). The 5.8S-ITS rDNA was amplified by PCR using primers ITS1 and ITS4, as described by White *et al.* (1990). The PCR procedure and subsequent visualisation steps were carried out as described by Sun and

TABLE 1
Physicochemical parameters of the must and wine in different fermentations.

	must			wine			
	Total sugar (g/l) ^a	Total acidity (g/l) ^b	pH	Residual sugar (g/l) ^a	Total acidity (g/l) ^b	pH	Ethanol (%vol)
<i>V. amurensis</i>	129.76 ± 8.21	8.19 ± 0.55	3.13 ± 0.19	2.96 ± 0.11	7.58 ± 0.39	3.25 ± 0.12	6.9 ± 0.3
Iona	173.65 ± 2.08	4.90 ± 0.06	3.38 ± 0.04	3.24 ± 0.10	4.36 ± 0.16	3.36 ± 0.13	9.6 ± 0.4
Cuihong	207.56 ± 4.16	3.21 ± 0.13	3.66 ± 0.13	3.80 ± 0.13	3.30 ± 0.13	3.58 ± 0.18	10.5 ± 0.4
Alimandeng Rose	190.69 ± 5.06	4.72 ± 0.17	3.46 ± 0.23	3.53 ± 0.26	4.59 ± 0.18	3.48 ± 0.15	10.1 ± 0.5
Moldova	152.50 ± 3.52	5.13 ± 0.16	3.31 ± 0.09	2.68 ± 0.11	5.20 ± 0.13	3.33 ± 0.08	8.5 ± 0.2
<i>V. davidii</i>	113.82 ± 2.53	6.41 ± 0.27	3.26 ± 0.07	2.72 ± 0.15	6.52 ± 0.07	3.24 ± 0.09	5.9 ± 0.2

^a Concentration represented by glucose

^b Concentration represented by tartaric acid

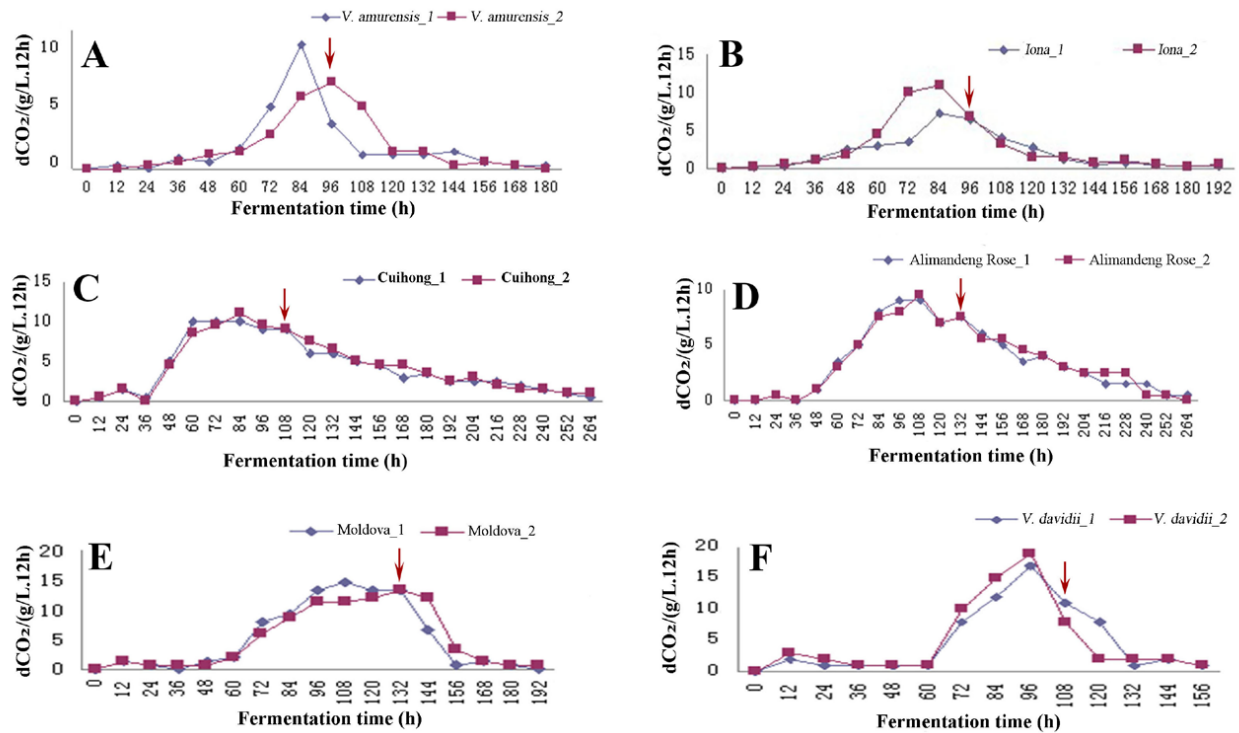


FIGURE 1

The spontaneous fermentation curves of six table grape varieties. The red arrow indicates the sampling point

Liu (2014). The PCR products were digested separately with two different restriction endonucleases, *Hae*III and *Hinf*I. The digestions were performed according to the instructions of the supplier, TaKaRa Biotechnology (Dalian) Co. Ltd. The size of the restriction fragments was obtained by separation on 3% (w/v) agarose gels and comparing them to the 2000 DNA Marker (TaKaRa).

Interdelta sequence typing

Interdelta sequence typing of all the *S. cerevisiae* isolates was carried out as described by Sun *et al.* (2014), using delta12 and delta21 primers according to Legras and Karst (2003).

RESULTS AND DISCUSSION

Identification of yeast isolates

Yeast were isolated and identified from the spontaneous fermentations of six table grape cultivars, namely *V. amurensis*, Iona, Cuihong, Alimandeng Rose, Moldova and *V. davidii*. As shown in Table 2, nine species (groups), viz. *H. guilliermondii*, *H. opuntiae*, *H. uvarum*, *P. terricola*, *Kazachstania hellenica*, *K. zonata*, *Pichia occidentalis*, *S. cerevisiae* and *Zygosaccharomyces bailii* were identified by sequencing of the 26S rDNA D1/D2 region. In addition, the PCR products of 5.8S-ITS rDNA were digested with *Hae*III and *Hinf*I, whereby eight different profiles were obtained. Seven of the nine species were identified after comparing the molecular weight of the restriction products digested by restriction endonuclease *Hae*III and *Hinf*I with those previously described by Esteve-Zarzoso *et al.* (1999) and the Yeast-id database (<http://yeast-id.com/>). *H. guilliermondii* (Group I), *H. uvarum* (Groups II and III), *P. terricola*

(Group IV), *T. delbrueckii* (Group V), *S. cerevisiae* (Group VIII), and *Z. bailii* (Group IX). Group VII was identified as *C. sorbosa*/*P. terricola*/*P. galeiformis*, while Group VI showed no match to any species in the database.

For *H. guilliermondii* (Group I), *P. terricola* (Group IV), *S. cerevisiae* (Group VIII) and *Z. bailii* (Group IX), the two methods coincided with regard to yeast species assignment. Similar to the findings of Wang and Liu (2013), *H. opuntiae* (Group II) and *H. uvarum* (Group III) shared similar 5.8S-ITS rDNA restriction patterns. However, this study showed that the 26S rDNA D1/D2 domain sequence of these two species differed. Isolates from group VI could not be identified with 5.8S-ITS rDNA-RFLP analysis, but were identified as *K. zonata* with the 26S rDNA D1/D2 sequence analysis. For isolates from groups V and VII, the 26S rDNA D1/D2 sequences were used for species identification, because no information about 5.8S-ITS rDNA restriction patterns for *K. hellenica* and *P. occidentalis* was available. Therefore, we recommend that the 26S rDNA D1/D2 sequence analysis should be the first choice for yeast species identification. It is not necessary to perform 5.8S-ITS rDNA-RFLP analysis, as the estimation of the sizes of fragments may vary using electrophoresis in different laboratories, and the 5.8S-ITS rDNA RFLP profile database may not cover all known yeast species.

Population diversity of yeasts from different table grape varieties

Generally, three to five different yeast species were isolated from wines produced from each of the various grape varieties (Fig. 2). The yeast species and the corresponding populations

TABLE 2
Identification of yeast species isolated from spontaneous table grape fermentations by 5.8S-ITS-RFLP analysis and 26S rDNA D1/D2 sequence.

Group	Profiles of 5.8S-ITS-RFLP analysis (bp)			Species identified by 5.8S-ITS-RFLP	Species identified by 26S rDNA D1/D2 sequence	Accession no. ^a
	Size of the PCR products	Hae III	Hinf I			
I	775	775	360 + 200 + 160	<i>H. guilliermondii</i>	<i>H. guilliermondii</i>	KR069091
II	775	775	360 + 200 + 180	<i>H. uvarum</i>	<i>H. opuntiae</i>	KP966890
III	775	775	360 + 200 + 180	<i>H. uvarum</i>	<i>H. uvarum</i>	KP966891
IV	450	290 + 125	240 + 105	<i>P. terricola</i>	<i>P. terricola</i>	KP966885
V	800	800	410 + 380	<i>T. delbrueckii</i>	<i>K. hellenica</i>	KP966887
VI	750	750	360 + 330 + 220	—	<i>K. zonata</i>	KP966897
VII	450	290 + 80	240 + 120 + 100	<i>C. sorbose/P. terricola/P. galeiformis</i>	<i>P. occidentalis</i> ^b	KP966878
VIII	850	320 + 230 + 180 + 150	365 + 155	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>	KP966895
IX	790	690 + 90	340 + 225 + 160	<i>Z. bailii</i>	<i>Z. bailii</i>	KP966888

^a GenBank

^b *I. occidentalis* was renamed *P. occidentalis*

exhibited differences among the grape varieties. *K. hellenica* (51.25%) was dominant in *V. amurensis* fermentations, and its high numbers could be a result of the humid summers in Zhengzhou. A study by Nisiotou and Nychas (2008) showed the linkage of *Kazachstania* sp. with Botrytis-affected fermenting grape juice. *S. cerevisiae* was predominant in the Iona and Moldova fermentations (53.75% and 96.25% respectively), while *H. uvarum* was predominant in the Cuihong, Alimandeng Rose and *V. davidii* fermentations (62.50%, 58.75% and 66.25% respectively). These findings are in agreement with other studies (Di Maro *et al.*, 2007; Zhang *et al.*, 2010; Li *et al.*, 2011; Sun *et al.*, 2014). It is widely reported that *H. uvarum* is one of the most frequently encountered species in wine fermentations, and it shows positive oenological properties for wine production (Li *et al.*, 2011). Other minor non-*Saccharomyces* species, such as *H. opuntiae*, were isolated in the fermentations of three grape varieties (Cuihong, Alimandeng Rose and Moldova). *P. terricola* and *P. occidentalis* were isolated from four grape varieties (*Vitis amurensis*, Iona, Cuihong and *V. davidii*). Species of *H. guilliermondii*, *K. zonata*, and *Z. bailii* occurred at extremely low populations in the fermentation of Iona, Cuihong and Alimandeng Rose, and only one colony among the 80 random yeast isolates was obtained from the corresponding samples. This association could be attributed to differences in the physicochemical properties of the different grape varieties, which affect the adaptation of wild yeasts (Rojas *et al.*, 2003).

Although the biodiversity of wine yeasts has been analysed in different viticultural regions in China (Li *et al.*, 2011; Wang & Liu, 2013; Sun *et al.*, 2014), this is the first study conducted on autochthonous yeast biodiversity in table grape vineyards that have never been exposed to commercial wine yeasts. This result contributes to the understanding of yeast biodiversity within and between vineyards located in the same ecological environment, as described by Mercado *et al.* (2010).

S. cerevisiae diversity determined by interdelta sequence typing

S. cerevisiae strain diversity with regard to the four grape varieties is listed in Table 3. The banding patterns that were generated for the 128 isolates showed eight different genotypes, named as G1 to G8 (Fig. 3). Among these *S. cerevisiae* isolates, predominant genotypes were G1, G2 and G3, with proportions of 32.03%, 24.22% and 28.13% respectively (Table 3). G4 accounted for around 10% of the total isolates, and the other three genotypes occurred rarely (below 2%). The degree of variability, measured as the percentage of different strains found amongst the colonies analysed (Torija *et al.*, 2001), was 6.25%. Moreover, the distribution of *S. cerevisiae* genotypes differed among the grape varieties. *V. amurensis* and Iona shared the same predominant genotype (G3), with a proportion of 81.82% and 62.79% respectively. *S. cerevisiae* isolates with the G1 genotype dominated as a proportion of 73.08% in *V. davidii* fermentations, while in Moldova fermentations, 47.92% of *S. cerevisiae* isolates had the G2 genotype.

It has been reported that fermentative species of *Saccharomyces* (*S. cerevisiae*) occur at extremely low

populations on intact berries, and that these species originate from the equipment surfaces in the winery during spontaneous fermentations, and even from insect vectors such as bees and wasps (Martini, 1993; Mortimer & Polsinelli, 1999). Since there is no winery in Zhengzhou, the possibility of commercial yeast colonisation in ZFRI should be very low, indicating that the *S. cerevisiae* strains isolated in this study came from the vineyard. G1, G2, G4, G6, G7 and G8 appear to be new genotypes when compared to the previous studies of Pei *et al.* (2009) and Sun *et al.* (2015). The G3 genotype is similar to genotype I found by Sun *et al.* (2015), while G5

is similar to genotype V found by Pei *et al.* (2009). Studies by Tofalo *et al.* (2013, 2014) reveal that unique *S. cerevisiae* strains are associated with particular grape varieties in specific geographical locations. Characterising the yeast community and its oenological properties in a specific wine could provide data for potential industrial applications (Li *et al.*, 2011; Tofalo *et al.*, 2014).

Eight different *S. cerevisiae* genotypes revealed considerable strain diversity among the table grapes fermentations. Moreover, differences in genotype distribution were found according to grape varieties, which is in

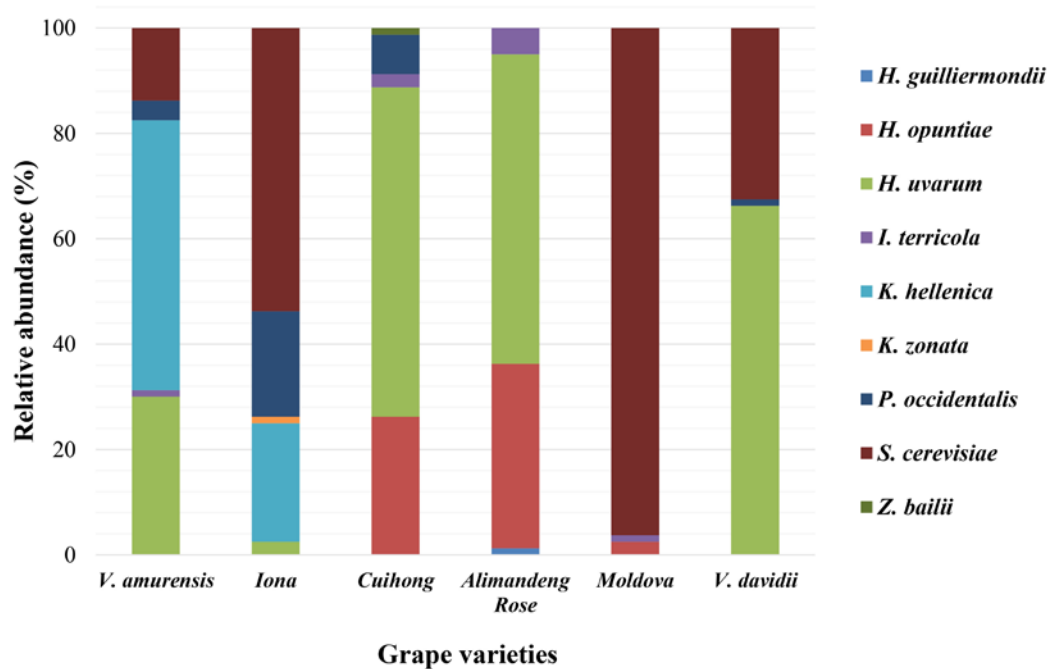


FIGURE 2

The occurrence of yeast species at the tumultuous stages in spontaneous fermentations for different table grape varieties

TABLE 3

Distribution and proportion of *S. cerevisiae* genotypes of different table grape varieties.

Genotypes	Numbers of each genotype/proportion (%) ^a				Total/ (%) ^b
	<i>V. amurensis</i>	<i>Iona</i>	<i>Moldova</i>	<i>V. davidii</i>	
G1	—	—	22/45.83	19/73.08	41/32.03
G2	1/9.09	—	23/47.92	7/26.92	31/24.22
G3	9/81.82	27/62.79	—	—	36/28.13
G4	1/9.09	13/30.23	—	—	14/10.94
G5	—	2/4.65	—	—	2/1.56
G6	—	1/2.33	—	—	1/0.78
G7	—	—	1/2.08	—	1/0.78
G8	—	—	2/4.17	—	2/1.56

^a Percentage calculated from each variety

^b Percentage calculated from the total 128 isolates

—: undetected

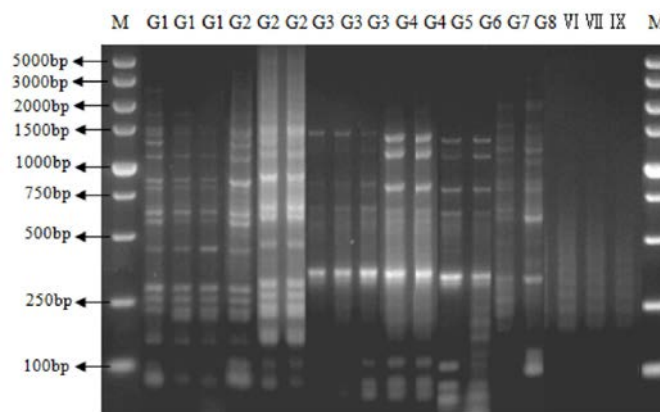


FIGURE 3

The interdelta sequence profiles of the 128 *Saccharomyces cerevisiae* isolates studied. M: 5 000 bp DNA Ladder; G1 to G8 represent different genotypes. Roman numbers VI, VII, IX indicate the non-*Saccharomyces* species *K. zonata*, *P. occidentalis* and *Z. bailii* respectively.

agreement with previous studies showing that the presence or absence of *S. cerevisiae* differed according to each plant and grape cluster (Pretorius *et al.*, 1999). It is well known that, in most cases, *S. cerevisiae* is the main species occurring in the tumultuous stages of fermentation, and that it prevails until the fermentation has finished. However, in this study, two-thirds of the samples of table grapes were not predominantly fermented by *S. cerevisiae*. *S. cerevisiae* was not found at the tumultuous stages in the fermentations of Cuihong and Alimandeng Rose. Drumonde-Neves *et al.* (2017) reported similar results, namely that 25% of fermentations were finished by non-*Saccharomyces* species on five islands of the Azores Archipelago, Spain. The reasons why *S. cerevisiae* was not found were not clear, but one possible explanation is that its presence and diversity during wine fermentations are complex and unpredictable (Sun *et al.*, 2015). Many factors, such as climatic conditions, age of the vineyards, grape varieties and viticulture, can affect the diversity of yeasts during wine fermentation (Mortimer & Polsinelli, 1999; Pretorius *et al.*, 1999; Clemente-Jimenez *et al.*, 2004; Drumonde-Neves *et al.*, 2017). Consequently, to obtain more reliable results, other information, such as the nitrogen concentration of the grapes as well as the composition of the microbial community in the phyllosphere, should be known in order to do further analysis (Oliveira *et al.*, 2017).

CONCLUSIONS

Table grapes are of increasing interest for wine production in China. However, little has been done to study the yeast species involved in table grape fermentations. The present study has shown the yeast biodiversity involved in the spontaneous fermentations of different table grape varieties. Differences between cultivars were apparent, as two-thirds of the samples were predominantly fermented by non-*Saccharomyces* species in the tumultuous stages – a distinct difference compared to wine grape fermentation. This would provide a starting point for future table grape wine ecology/alcoholic beverage research. Further research

on the oenological properties of these yeast species could be valuable for the evaluation of wine quality during native fermentations.

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