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Evaluation of Chinese *Saccharomyces cerevisiae* wine strains from different geographical origins

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Abstract: Interdelta sequence typing was used to investigate the genetic diversity of 54 Chinese indigenous wine strains of *S. cerevisiae* selected on the basis of preliminary sequence analysis from 349 strains that were isolated previously from 15 spontaneous fermentations in Shanshan, Xinjiang and Qing Tongxia, Ningxia, China. Of the 54 strains tested 78% (42/54) were confirmed as genetically distinct. Dendrograms based on strain similarity revealed differences in the genetic relationships of Xinjiang yeast populations between table and wine grape varieties in addition to differences between red and white grape varieties in Ningxia (Dice coefficients of 0.448 and 0.674, respectively). When data from *Saccharomyces* strains collected from California, France, Italy, Northern Europe, and Spain were included in the analysis, the dendrogram revealed 5 groups containing 51, 4, 48, 3, and 1 strain respectively. Ningxia and Xinjiang provinces displayed local specific *S. cerevisiae* biotas that show a clear separation from other strains. Cluster XJ19 isolated from Xinjiang displayed a high level of similarity with UCD587, UCD2515, and UCD2516 from California. Clusters XJ2, XJ7, XJ20, and XJ3 also isolated from Xinjiang had a lower degree of similarity with other Chinese indigenous genotypes and strains from other regions. This study compares, for the first time, the genetic diversity and relationships between indigenous *S. cerevisiae* wine strains collected from Xinjiang and Ningxia provinces in China with wine strains from different geographic regions.

Introduction

Saccharomyces cerevisiae is an important experimental model organism in addition to its commercial significance as the predominant yeast species during wine fermentation. Modern strains of *S. cerevisiae* are thought to have arisen in Asia given the diversity of strains and reproductive isolation observed in a study of *S. cerevisiae* isolates from human-associated and non-human-associated environments in China (Wang et al. 2012, Liti 2015). Distinct linages were observed for isolates from primeval and secondary forests (Wang et al. 2015). However this study considered few isolates from wine environments and found fewer isolates of *S. cerevisiae* from fruit sources and more from tree bark, rotting wood and soil samples than from fruit samples. Of the fruits evaluated the fewest isolates were obtained from grapes (Wang et al. 2012). The authors concluded that grape and orchard isolates were similar to those of the wine European lineage. Our goal was to evaluate in greater depth the diversity of natural vineyard isolates from two wine regions in China.

Several studies have reported on the genetic diversity of *S. cerevisiae* strains in different wine-producing regions. These studies revealed that geographic region (Versavaud et al. 1995, Goddard et al. 2010), climatic conditions (Valero et al. 2007), vintage (Sabate et al. 1998, Mercado et al. 2007), grape varieties and must characteristics (Blanco et al. 2012), inoculation of starter yeasts (Santamaria et al. 2005), and SO_2 addition (Wang and Liu 2013) affected the diversity of *S. cerevisiae* observed. In many cases genetically distinct strains of *S. cerevisiae* were isolated from the same fermentation during wine fermentation (Mercado et al. 2007, Wang and Liu

2013). The diversity of *S. cerevisiae* strains present in fermentations has been shown to play an important role in the characteristics of the final product (Egli et al. 1998, Pérez-Cooello et al 1999). Numerous molecular methods have been developed to study the ecology and population dynamics of *S. cerevisiae* strains (reviewed in Querol and Ramon 1996, Fernández Espinar et al.2006). Interdelta sequencing typing uses the variation of the number and position of the delta element, a repeated sequence that flanks the Ty1/Ty2 retrotransposon (Ness et al. 1993), that allows interpreting strain similarities and evolutionary or adaptive distance (Legras and Kerst 2003, Liu et al. 2014).

A succession of different *S. cerevisiae* strains are established during native as well as inoculated fermentations that could have positive or negative effects on the course of fermentation and wine quality (Mercado et al 2007,Wang and Liu 2013). Vezinhet et al. (1992) analyzed the evolution of *S. cerevisiae* strains isolated from spontaneous fermentations during six consecutive years. These authors concluded that the wide distribution of some strains in the studied areas and their presence over years, constitute evidence for the occurrence of specific indigenous strains representative of an enological region.

China is an important wine-producing country and while some studies have investigated indigenous yeast species and population dynamics during wine fermentation within local viticulture regions (Sun et al. 2009, Li et al. 2010, Li et al. 2011, Wang and Liu 2013, Sun et al. 2014); few studies (Wang and Liu 2013, Sun et al. 2014) have focused on the breadth of the diversity of *S. cerevisiae* wine genetic

resources of China. A study of human- and non-human-associated strains of China found novel distinct lineages only distantly related to the wine strain linages (Wang et al. 2012). The genetic diversity and relatedness of indigenous wine *S. cerevisiae* resources have not been extensively compared with that of wine strains isolated from other geographic regions. Ningxia and Xinjiang provinces, where the strains in this study were isolated, are two of the oldest wine producing regions in China. Shanshan, Xinjiang in northwestern China belongs to a temperate continental climate, with an average temperature of 12˚C. It is situated 92°22′E, 42°87′N with an average altitude of 3986 m. Qing Tongxia, Ningxia in north central China also belongs to a temperate zone (average temperature is 9˚C) with an arid and semi-arid climate. It is situated 105°21′ to 105°21′ E, 37°36′ to 38°15′N with an average altitude of 1118 m. A comparison of the genetic diversity of *S. cerevisiae* resources in different viticulture regions of China with isolates from other diverse geographical regions is of importance to the study of global *S. cerevisiae* ecology.

In the present study, interdelta sequence typing with improved primers (Legras and Karst 2003) was used as genetic marker for the distinction of *S. cerevisiae* strains. Dendrograms were constructed based on similarity among different patterns of bands and the genetic relationships of all strains were evaluated. The strains used in the study were either isolated from fermentations of different grape varieties in the Ningxia and Xinjiang Provinces in China or obtained from the Department of Viticulture and Enology Culture Collection at the University of California, Davis. The aims of the present work were to evaluate the genetic diversity and relatedness among

Saccharomyces strains of different geographic origin, to establish a strain collection to preserve the *S. cerevisiae* genetic resources of China, and to identify strains useful for further development for commercial wine production in China.

Materials and Methods

Yeast strains. Fifty-four isolates collected from fifteen spontaneous fermentations of grapes grown in China and one commonly used commercial yeast, Lavin RC212, were used in this study and obtained from the collections of the College of Enology, Northwest A&F University, Yangling, Shaanxi, China. This set of strains was selected on the basis of interdelta sequence profiles from a total of 349 isolates collected from fifteen spontaneous fermentations of grapes grown in Shanshan, Xinjiang and Qing Tongxia, Ningxia. Fifty-nine yeast colonies were isolated from six spontaneous fermentations of different commonly used grape varieties: Red Globe, Small-berry Thompson Seedless, Big-berry Thompson Seedless, Merlot, Mixed red (Cabernet Gernischt, Cabernet Franc, and Cabernet Sauvignon) and Mixed white (Small-berry Thompson Seedless, and Big-berry Thompson Seedless) in Xinjiang. Two hundred ninety isolates were obtained from nine spontaneous fermentations of the grape varieties Cabernet Gernischt, Cabernet Sauvignon, Cinsault, Merlot, Pinot Noir, Riesling, Sauvignon Blanc, Semillon, and Yan73 in Ningxia (Pei 2009). The grape must fermentations were allowed to proceed spontaneously at 25~28˚C for 7~11 days until dry. Fermentations were sampled at early, mid and the final stage of fermentation, and serial ten-fold dilutions were inoculated onto WLN (Pallmann et al. 2001) and incubated for five days at 28˚C.

These yeasts were differentiated and classified according to colony morphology and color. *S. cerevisiae* isolates were purified and then maintained in 20% (v/v) glycerol at -80 °C until further analysis, resulting in the selected set of 349 isolates for the interdelta sequence analysis. The composition of the different grape musts is reported in Supplemental Table 1 for Ningxia and in Supplemental Table 2 for the fermentations from Xinjiang.

The fifty-four yeast strains selected from this larger population of isolates represented the major strain clusters of interdelta sequence profiles identified in the earlier preliminary study. The origins of the 54 isolates used in this study are shown in Table 1. Identification of *S. cerevisiae* was confirmed by PCR-RFLP of the 5.8S-ITS rDNA using restriction enzymes *Hae*III, *Hpa*II, and *Scr*FI as described by Li et al. (2012). Strains were maintained in frozen stocks (glycerol, 20% v/v) at -80˚C before use. Note that a similar strain numbering system was independently used by Wang et al. (2012) in their study but the strains are unrelated. We retained our numbering system since that is the designation given to the strains in the Northwest A&F University strain collection.

Other strains were obtained from the Wine Yeast and Bacteria Collection of the Department of Viticulture and Enology at the University of California, Davis. The data from all fifty-two *Saccharomyces* isolates listed in Table 1 of Liu et al. (2014) were included in this study as the method of interdelta sequence analysis was identical. These yeast strains were collected from California, France, Italy, Northern Europe, and Spain (Liu et al. 2014).

DNA extraction. DNA from *S. cerevisiae* in the collection at the Northwest A&F

University was extracted as described by Zhou et al. (2004).

Interdelta sequence typing. PCR amplification of the interdelta sequence was carried out using primers δ12 (5'-TCAACAATGGAATCCCAAC-3') and δ21 (5'-CATCTTAACACCGTATATGA-3') (Legras and Karst 2003). Amplification reactions and the DNA fragment separations were performed according to Liu et al. (2014). In brief, PCR amplifications were performed in a 25 µL reaction volume containing 2.5 µL of 10× PCR buffer, 2.5 µL 25 mM $MgCL_2$, 2.0 µL 2.5 mM dNTPs, 1.25 µL of 10 µmole/L of each primer, 0.4 µL of 5 U Taq DNA polymerase, 30-100 µL of template DNA (amount varied dependent upon DNA quantification value of the sample) and double distilled H_2O to make the volume up to 25 µL. The same volume of DNA sample (1µL) was added to each lane. PCR and interdelta typing analysis was performed multiple times (3 to 6 replicates) for each isolate in order to obtain a replicated stable interdelta pattern prior to analysis. Band positions were determined by reference to a standard DNA Ladder (100 bp Plus DNA Ladder (Fermentas)) to enable comparisons across gels. PCR reactions were repeated in cases where a low DNA content was evident on the gel.

Cluster analysis of the strains. The interdelta sequence patterns obtained after gel electrophoresis were used for the construction of a presence/absence matrix, taking into account the total number of different bands observed. The interdelta sequence patterns were obtained following electrophoresis. All visible bands were assigned a number based upon relative position to the DNA ladder. Each position was then assigned a "0" or a"1" to indicate absence or presence of the band, respectively.

The 0/1 matrix was then used to generate the dendrograms. Similarities based on the Dice coefficient were calculated and UPGMA clustering was obtained using NTSYS software (Mercado et al. 2010).

The comparative cluster analysis of different strains integrates banding pattern data from two separate experiments. The data were combined in a single matrix. Although banding patterns differed there were some band positions in common in some strains across the two studies. The reagents used in the two studies were identical; however the PCR thermal cycler was different, a GeneAmp PCR System 2700 was used in this study.

Results

Interdelta sequencing typing of *S. cerevisiae* isolates *in* Xinjiang and Ningxia, China. In a previous study 349 strains isolated from different native fermentations in China were screened by interdelta sequence typing. The number of strains tested in this analysis did not allow definitive comparisons of highly similar strains as each strain was only run once. However based on this preliminary analysis 54 different banding patterns were identified. These 54 strains were studied in more detail under conditions enabling conclusive comparisons of DNA band profile. Samples of the strains were run on gels multiple times and band positions calculated in comparison to the DNA ladder on each gel. Strains were run on different gels and yielded identical patterns on the replicate gels. This method generated stable replicate banding patterns for each isolate. The replicated interdelta sequence typing methodology enabled clear differentiation of forty-two distinct *S. cerevisiae* genotypes among the 54 *S.*

cerevisiae isolates of the first study. Twenty-one strains of *S. cerevisiae* isolated from Xinjiang revealed eighteen different patterns (Figure 1). The analysis of thirty-three *S. cerevisiae* isolates from Ningxia revealed the existence of twenty-four distinct profiles (Figure 2) in other words, 24 differentiated strains. The results also showed that the interdelta profile of genotype NX10 was identical to that of commercial yeast Lalvin RC212, which has been used in this region as a commercial starter strain. In general, the Xinjiang and Ningxia regions evaluated in this study exhibited different *S. cerevisiae* populations. There were no identical strains between the regions. In addition, comparison with the forty-four interdelta genetic patterns found among the fifty-two *Saccharomyces* strains from Liu et al. (2014) revealed that no identical strains were present between the studies.

Genetic relationships among the strains from Xinjiang and Ningxia. The PCR amplification profiles obtained by interdelta sequence typing were used to obtain independent presence-absence matrices for *S. cerevisiae* isolates from Xinjiang and Ningxia, China. The dendrograms deduced by interdelta sequence typing are presented in Figures 3 and 4, respectively. The dendrograms demonstrated that native wine fermentations were conducted by a mixture of *S. cerevisiae* strains. In general a minimum of two interdelta sequence profiles could be detected during each of the spontaneous fermentations with the exception of the fermentation of Big-berry Thompson Seedless. Some strains showed highly similar but not identical banding patterns and are thought to represent genetically related strains. Six clusters of two isolates each, cluster XJ4 and XJ5, XJ12 and XJ15, XJ2 and XJ7 (Figure 3); NX2 and

NX3, NX29 and NX33, and NX9 and NX32 (Figure 4) showed highly conserved interdelta sequence patterns indicating that they are likely genetically distinct derivatives of the same strain. Two clusters of four isolates, cluster NX1, NX4, NX5, and NX8; cluster NX17, NX30, NX31, and NX26 also displayed conserved interdelta sequencing patterns (Figure 4) again suggesting a genetic relationship among the sets of strains. The *S. cerevisiae* isolates from Xinjiang that clustered together were from the same grape variety, but, in contrast, the isolates from Ningxia that clustered together were from more than two different grape varieties. NX10 from a native Riesling fermentation was indistinguishable from the commercial strain RC212.

A difference was observed in the genetic relationships of *S. cerevisiae* among strains isolated from table and wine grape varieties planted in Xinjiang (Figure 3). Both table and wine grapes are used for wine production in Xinjiang. Four groups containing nine, eight, three, and one isolate, respectively, were distinguished with a Dice coefficient of 0.448. All nine isolates in group I and all the 3 isolates in group III were obtained from the table grape varieties Red Globe, Small-berry Thompson Seedless, Big-berry Thompson Seedless, and Mixed white (Small-berry Thompson Seedless and Big-berry Thompson Seedless). Group II had seven isolates from wine grape varieties of Merlot and Mixed red (Cabernet Gernischt, Cabernet Franc, and Cabernet Sauvignon), and one isolate from the table grape variety Red Globe. Group IV only included one genotype, XJ3, also from Red Globe (Figure 3). Thus the majority of isolates from the four different table grape fermentations were genetically similar in banding pattern and clustered together. In addition, a difference was

observed in the genetic relationships of *S. cerevisiae* among strains isolated from red and white wine grape varieties planted in Ningxia (Figure 4). Five groups are indicated in Figure 4, when the Dice coefficient is 0.674. Groups I, II, and III consisted of isolates from more than three grapes varieties, while group V only included isolates from Pinot Noir. Group IV included only 1 isolate, NX20, from Cabernet Sauvignon. Most of the *S. cerevisiae* isolates included in group I were isolated from red grape varieties (8 of the 13 isolates in group I), while isolates in group III were mainly from white grape varieties (9 of the 12 isolates in group III). All the isolates in group II were from the red grape varieties of Cabernet Sauvignon, Merlot, and Cinsault (Figure 4).

Genetic relationships among the strains from different countries. Genetic relatedness was also evaluated by constructing a dendrogram compiled from all interdelta sequence patterns in this study and Liu et al. (2014) (Figure 5). Differences were observed in the genetic relationships between the Chinese strains and the strains from the UC Davis collection. The similarity of strains was correlated with their geographical regions of origin: Ningxia strains were closer to the Xinjiang strains, while a clear separation between the indigenous Chinese and the UC Davis strains was observed. The dendrogram produced by interdelta sequence typing revealed five groups containing fifty-one, four, forty-eight, three, and one isolate each when the Dice coefficient is 0.418 (Figure 5). The largest group, group I, contained fifty isolates from China and one from California (UCD2211, *S. servazzii*). There is a difference observed in the genetic relatedness between Xinjiang and Ningxia

indigenous *S. cerevisiae* strains. Group I could be further divided into five subgroups, I-1 to I-5, when the Dice coefficient is 0.506. Most of the *S. cerevisiae* isolates included in subgroups I-1 (5 of 6) and I-5 (6 of 7) as well as all three in I-4 were isolated from Xinjiang, while isolates in I-3 (29 of 31) and I-2 (3 of 3) were mainly from Ningxia (Figure 3). Group II displayed a high similarity with XJ19 (Xinjiang), UCD587 (a winery isolate), UCD2515 and UCD2516 (both are lab strains known to be related to each other). Forty-eight out of fifty-two *Saccharomyces* isolates from the UC Davis collection were clustered in group III. XJ2, XJ7, and XJ20 were clustered in group IV. Group V consisted only of XJ3. Clusters IV and V containing XJ2, XJ7, XJ20, and XJ3 had a low degree of similarity with other Chinese indigenous genotypes and UC Davis collection strains (Figure 5). In addition, this dendrogram showed that identical isolates were found only in the same geographic regions with the exception of UCD522 (commercial yeast) and UCD514 (origin in Spain).

Discussion

Understanding the genetic diversity of *S. cerevisiae* strains from different geographical origins can make an important contribution towards delineating the genetic distance of these strains as well as providing genetic material for further strain development. The genetic diversity of indigenous *S. cerevisiae* was investigated during the spontaneous fermentations of grape must in Xinjiang and Ningxia, China. Eighteen distinct interdelta profiles were found in Xinjiang, and twenty-four in Ningxia. Forty-two different *S. cerevisiae* strains were distinguished out of a total of three hundred and forty nine isolates analyzed. All forty-two of these isolates were

unique when compared to a set of strains from other major international wine producing regions (Dendrogram 3 groups I, IV, V).

Lavin RC212, showing the same interdelta sequence patterns as genotype NX10 isolated from Ningxia was detected during the spontaneous fermentations in this study. Similar to other studies, commercial yeasts were detected in fermentations without inoculation (Santamaria et al. 2005, Mercado et al. 2007, Sun et al. 2014). In this study, the detection of Lavin RC212 colonizing a spontaneous fermentation could be explained by the winery practice of dumping grape skins on the road for drying. Valero et al. (2005) analyzed the dissemination and survival of commercial wine yeast in the vineyards near wineries and they suggested that the dispersion of commercial strains is mainly mediated by water run-off and derived from macerated grape skin at dumping sites. Drying the grape skins on the roads for further processing is a normal practice at the Imperial Horse Winery, Qing Tongxia, Ningxia, China, where the spontaneous fermentations were conducted. It is understandable that this practice could have contributed to the dissemination and survival of Lavin RC212 in the vineyards and wineries, and its occurrence in spontaneous fermentations.

In this study, comparison between eighteen and twenty-four different *S. cerevisiae* patterns by interdelta sequence typing revealed that yeast strains from Xinjiang and Ningxia did not share the same interdelta profiles. The same observations made in the Western Cape, South Africa (Khan et al. 2000) showed that different *S. cerevisiae* strains were present at different regions in the different climate zones. In addition, the differences could be attributed to the fact that the grape

varieties studied were different in these two regions: table and wine grape varieties in Xinjiang vs. wine grape varieties in Ningxia. This result agrees with a previous study that demonstrated the impact of grape variety on yeast diversity (Mercado et al. 2011). Further, this study suggests that these two winemaking regions are biologically isolated from each other.

Geographic location and ecological niches are both thought to play a significant role in *Saccharomyces* strain diversity (Bisson 2012). In comparison with strains isolated from other winemaking regions, the Ningxia and Xinjiang strains showed a high degree of similarity. This suggests that the indigenous Chinese strains are distinct from European and new world lineages. Nearly identical strains were only found in the wine samples collected in the same viticulture region with the exception of UCD522 (commercial yeast) and UCD514 (origin in Spain). These results are in agreement with previous studies on geographically close regions (Versavaud et al. 1995) and widely distant geographic regions (Goddard et al. 2010). According to Ezeronye and Legras (2009), who studied the genetics of *S. cerevisiae* strains isolated from palm wine in eastern Nigeria, geographic and/or ecological isolation results in a specific population of *S. cerevisiae*. These analyses have led to the conclusion that geographic location plays a significant role in genetic divergence. Strain XJ19 displayed a high similarity with UCD587 (a California must isolate), UCD2515 (YPH500) and UCD2516 (BY4743) (two genetically related lab strains with California origins (Mortimer and Johnston 1996). The relatedness of these four strains may imply a common origin or a commonality of evolutionary forces in the wild.

Interestingly as a group the Chinese wine strains appear to show greater overall diversity as compared to the wine isolates from the rest of the world. This is consistent with the narrowness of the Wine European lineage previously described (Wang et al. 2012) as well as the observations of overall greater diversity of natural *S. cerevisiae* isolates from China as compared to other regions (Liti 2015). The greater natural diversity of isolates of *S. cerevisiae* from China many represent an untapped genetic reservoir for strain improvement and breeding programs. Knowledge about indigenous yeast strains can also help preserve and employ the most representative strains from a wine region (Tristezza et al. 2014).

Conclusion

This study investigated the genetic diversity of *S. cerevisiae* in Ningxia and Xinjiang, China, which has not previously been examined. The results of this study showed that different *S. cerevisiae* strains were associated with different viticulture regions in China. In addition, the results demonstrated that a commercial yeast was detected in spontaneous fermentations at one winery. Ningxia and Xinjiang are two of the best known viticulture regions in China; therefore, the preservation of biodiversity and genetic resources of indigenous yeasts is very important in these regions.

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Figure Legends

Figure 1: Interdelta sequence profiles of the 21 genetically distinct *S. cerevisiae* isolates in Xinjiang, China. M: 100bp Plus DNA Ladder; 1 XJ1, 2: XJ2, 3: XJ3, 4: XJ4, 5: XJ5, 6: XJ6, 7: XJ7, 8: XJ8, 9: XJ9, 10: XJ10, 11: XJ11, 12: XJ12, 13: XJ13, 14: XJ14, 15: XJ15, 16: XJ16, 17: XJ17, 18: XJ18, 19 XJ19, 20: XJ20, 21: XJ21.

Figure 2: Interdelta sequence profiles of the 33 genetically distinct *S. cerevisiae* isolates in Ningxia, China. M: 100bp Plus DNA Ladder; 1: NX1, 2: NX2, 3: NX3, 4: NX4, 5: NX5, 6: NX6, 7: NX7, 8: NX8, 9: NX9, 10: NX10, 11: NX11, 12: NX12, 13: NX13, 14: NX14, 15: NX15, 16: NX16, 17: NX17, 18: NX18, 19: NX19, 20: NX20, 21: NX21, 22: NX22, 23: RC212, Commercial *S. cerevisiae* strain RC212, 24: NX23, i25: NX24, 26: NX25, 27: NX26, 28: NX27, 29: NX28, 30: NX29, 31: NX30, 32: NX31, 33: NX32, 34: NX33.

Figure 3: Unweighted Pair Group Method with Arithmetic mean **(**UPGMA) dendrogram showing genetic relatedness of the *S. cerevisiae* isolates obtained from Xinjiang, China. Four distinct groupings of strains were evident with a Dice coefficient of 0.448. The black line represents the Dice coefficient and groups are designated by use of Roman numerals.

Figure 4: UPGMA dendrogram showing genetic relatedness of the *S. cerevisiae* isolates obtained from Ningxia, China. Five distinct groupings of strains were evident

with a Dice coefficient of 0.674. The black line represents the Dice coefficient and groups are designated by use of Roman numerals.

Figure 5: UPGMA dendrogram generated by cluster analysis of interdelta region profiles obtained of *Saccharomyces* from our culture collection and Department of Viticulture and Enology Culture Collection in University of California, Davis. Five distinct groupings of strains were evident with a Dice coefficient of 0.418. Two of these groups were large: group I (51 isolates) and group III (48 isolates). Using a higher Dice coefficient (0.506), the two larger groups can be differentiated in to subgroups. The black line represents the Dice coefficient and groups are designated by use of Roman numerals. The subgroups are designated by use of Arabic numerals.