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RESEARCH ARTICLE

GENETIC DIVERSITY AMONG NATURE POPULATION OF SACCHAROMYCES BAYANUS HARBORED IN SHANGRI-LA, YUNNAN, CHINA

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ABSTRACT

Background Strains of yeast have been analyzed from vineyards, wineries and forest in Europe, North America, South America, South Africa, Asia and Australia. However, the genetical diversity of natural population of *Saccharomyces bayanus* in Southwest of China has not been studied yet. [Methods] The sequences of *ITS*, *HIS3*, *SSU1* and *MET2* genes, microsatellites analysis and AFLP analysis were used to access the genetic diversity of the *S. Bayanus* isolated from vineyards in Shangri-la, Yunnan, China. [Results] Our sequences of *ITS*, *HIS3*, *SSU1* and *MET2* genes come from vineyard *S. bayanus* in Shangri-la, Yunnan, China, which are quite similar to a Europe strain CBS7001, and they look to be derived from Europe. There were only 3 SNPs among the isolates. Microsatellites analysis results illustrated that all isolates appeared to belong to families of closely related genotypes. Seven colonies obtained from two different vineyards from Deqin County (three from Chizhong, and four more from Liutongjiang) all had genotypes that are very similar to a winery-derived strain 7A6 from New Zealand. The polymorphism level of AFLP analysis is much more than of the microsatellite. The results of AFLP analysis indicated that 63.72% of the variation was found among the different geographic origin isolates, while 36.28% was found among the same geographic origin ones. [Conclusion] Both microsatellites and AFLP analysis results showed that genetic diversity among these isolates was quite low, and *S. bayanus* populations were geographically partitioned generally.

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INTRODUCTION

Saccharomyces bayanus is a kind of yeast of the genus *Saccharomyces*. It is closely related to *Saccharomyces cerevisiae*. Although *S. cerevisiae* is the predominant yeast in most fermentation processes, *S. bayanus* has also been described to be involved in wine fermentation, and is used in winemaking and cider fermentation Thus, *Saccharomyces bayanus* var. *bayanus* (according to Naumov, 2000) or simply *S. bayanus* (Nguyen and Gaillardin, 2005) has been isolated from beer, and *S. bayanus* var. *uvarum* (Naumov, 2000) or *Saccharomyces uvarum* (Pulvirenti *et al.*, 2000; Nguyen and Gaillardin, 2005) has been found mainly associated with winemaking (Demuyter *et al.*, 2004) and cider production (Naumov *et al.*, 2001) at low temperature in colder areas of Europe. *S. bayanus* has a fermentation profile in grape must that is different from that of *S. cerevisiae*, producing less acetic acid and ethanol but more glycerol and succinic acid, while synthesizing malic acid without posterior degradation (Bertolini *et al.*, 1996). Moreover, *S. bayanus* produces volatile fermentative compounds such as phenylethanol and its acetate or volatile thiols (Masneuf-Pomare *et al.*, 2010). These phenotypic differences between *S. bayanus* and *S. cerevisiae* are associated with pronounced proteomic differences (Blein-Nicolas *et al.*, 2013). The wines typically fermented by *S. bayanus* are Tokaj (Hungary, Slovakia), Amarone (Italy), and Txakoli (Spain); and in France, Sauternes and the whites of northern French vineyards in Burgundy, Champagne, Val de Loire and Alsace (Naumov *et al.*, 2000; Sipiczki *et al.*, 2001; Rementeria, *et al.*, 2003; Demuyter *et al.*, 2004; Naumov *et al.*, 2002). *S. bayanus* is also frequently responsible for cider fermentation, which is usually conducted at low temperatures (Naumov *et al.*, 2001; Valles *et al.*, 2007). Strains of yeast have been analyzed from vineyards, wineries and forest in Europe (Nisiotou and Nychas, 2007), North America (Pallmann *et al.*, 2001), South America (Mercado *et al.*, 2007), South Africa (Jolly *et al.*, 2003), Asia (Wang *et al.*, 2012) and

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Australia (Zhang *et al*., 2010). Here we present results of some strains of *S. bayanus* from grape juice samples from vineyards in Shangri-la, Yunnan, China. *S. bayanus* is identified using RFLP and DNA sequences analysis. In addition, microsatellite and AFLP procedures were used to assess the amount of polymorphism detected among *S. bayanus* isolates and to estimate relationships and similarities, in order to evaluate the potential of these molecular markers in breeding programs as well as in germplasm conservation.

METHODS

Yeast isolation and identification: The selective protocol used for *Saccharomyces* isolations was based on the selective enrichment in a raffinose–ethanol liquid medium as previously described (Sampaio & Gonçalves, 2008; Libkind, *et al*., 2011). All samples were plated directly, with dilution as appropriate, on YPD plates containing chloramphenicol (100 g/mL). Putative *Saccharomyces* isolates were confirmed by the observation of *Saccharomyces*-type ascospore production. The ITS1–5.8S-ITS2 (internal transcribed spacer, ITS) regions of these colonies were initially analyzed by restriction fragment length polymorphism (RFLP) (with the restriction endonucleases HaeIII and a more detailed analysis was undertaken for Saccharomyces sensu stricto colonies using MspI) and then by sequencing in order to identify the isolates to the species level.

PCR

DNA was isolated according to Ling *et al*. (1995). Oligonucleotide primers for PCR amplification of gene regions are listed in Table 1. PCR was performed in a mixture containing 1 × Taq polymerase buffer, 100 μM deoxynucleotides, 1 μM of each primer and 2 U of Taq polymerase. A volume of 4 μL of DNA, diluted to 1–50 ng μL, was transferred to a PCR tube before adding the reaction mixture, to a final volume of 100 μL. PCR amplifications were carried out in a 2400 Perkin-Elmer Thermocycler. PCR amplification was performed as follows: initial denaturing at 95°C for 5 min, then 40 PCR cycles of three steps (denaturing at 94°C for 1 min, annealing at 54-65°C for 2 min and extension at 72°C for 2 min), followed by final extension at 72°C for 10 min. PCR products were separated on a 1.4% agarose gel in 0.5 × TBE (4.5 mM Tris-borate, 1 mM EDTA, pH 8) buffer. After electrophoresis, gels were stained with ethidium bromide (0.5 μg mL) and visualized under UV light. A 100-bp DNA ladder marker served as the size standard.

Microsatellite analysis: The microsatellite analysis was performed according to the method of Zhang *et al*. (2014). The PCR products were assayed for their yield on agarose gels and analyzed on an Applied Biosystems DNA Sequencing 3130XL machine using Genescan software.

AFLP analysis: The AFLP analysis was performed following the manual of the AFLP kit (Life Technologies). The pre and the selective amplifications were performed in a 2400 Perkin-Elmer Thermocycler. An equal volume of loading dye (95% v/v formamide and 0.08% w/v bromophenol blue in 20 mM EDTA) was added to each sample, which was then denatured at 95°C for 3 min and placed on ice for 2 min before loading. Amplification products were analyzed by electrophoresis in a 6.5% Polyacrylamide Gel. The electrophoresis parameters were set to 1500 V, 40.0 mA, 40.0 W, 50°C and the run time was set to 2.0 h. In this study, we used nine pairs of primers (T/CT, A/CG, A/CC, T/CA, T/CG, C/CA, C/CC, G/CT, and C/CG) for AFLP analysis. Separated AFLP products were visualized by silver staining as described in the Promega Silver Staining kit and gel images were saved as TIF files for further analysis.

Data analysis: For microsatellite and AFLP data, each isolate was scored 1for presence or 0 for the absence of each polymorphic band. Bands present in all isolates were not scored. Of cause, the genetic loci which were invariant are also important for their potential to detect polymorphism in other flue-cured tobacco genotypes. Bands within genotypes were scored as missing data if poorly resolved on the gel or if template DNA did not amplify well. Only bright, clearly distinguishable bands were used in the genetic analysis. All statistical analyses were performed by NTSYS-pc, Version 1.8. Pair similarity coefficients range from 0 (all bands between isolates were different) to 1 (all bands between isolates were identical). Dendrograms were generated with the unweighted pair-group method, arithmetic average (UPGMA) algorithm as described by Sneath and Sokal (1973). AMOVA and Fst calculations were performed with the software package Arlequin (Schneider *et al*., 2000).

Purification of PCR Products and sequencing*:* PCR products for sequencing were purified using the High Pure PCR Product Purification Kit (Roche) according to the manufacturer's instructions and analyzed on an Applied Biosystems DNA Sequencing 3130XL machine. All isolates number sequences were sequenced completely on both strands.

Sequence analysis: Sequences were edited using Vector NTI, transformed into FASTA format, and compared to the NCBI database using BLAST.

RESULTS

Sequences analysis: The 5.8S-ITS region of those isolates only exhibited an *S. bayanus* restriction pattern. And these 5.8S-ITS sequences displayed identical with the strain CBS7001. Further analysis showed that the nucleotide sequence of *HIS3*, *SSU1* and *MET2* genes of all isolates were very similar to that of CBS7001. There were only 3 bp differences among these 3 genes, one appeared in *HIS3* (*in* LTJ 19), the other two were in *SSU1* (*in* CZ 14 and BZL5 respectively), and *MET2* of all isolates was identical to the CBS7001. It indicated that these *S. bayanus* strains might original from Europe or might have the same ancient with the European isolate CBS7001. The sequencing results also indicated that the relationships among these strains were very close, and genetic diversity among these isolates was quite low.

Microsatellite analysis: Microsatellite amplified very well, and using this system, we could distinguish 43 genotypes in total. One (XJ5) matched the sequenced strain of *S. bayanus* (ACY338), only 2 bp different at two loci. A total of 125 bands were scored from the comparison of amplification with 13 primers of DNAs from 43 isolates, with an average of 9.6 bands scored per primer.

Three to fifteen bands generated by a single primer of variable lengths were detected. Portions of gels showing typical amplification products are shown in Figure 1. The polymorphic bands were 78 (62.4%), and one primer detected a mean of 6 polymorphic bands per reaction. Microsatellite-based pair similarities ranged from 0.84 (BZL5 and CZ10, BZL7 and CZ10) to 0.98 (CZ11 and CZ14, LTJ26 and LTJ 29). At the 0.88 phenon level, three distinct clusters were apparent in the dendrogram (Figure 1). Ⅰincluded 60.47% of the isolates encompassing pair similarity values ranging from 0.90 to 0.98. Ⅱ included 23.26% of the isolates and encompassing pair similarity ranging from 0.90 to 0.97. Ⅲ included 16.27% of the isolates and encompassed encompassing pair similarity values ranging from 0.95 to 0.98. Interestingly, XJ11, a Xinjian isolate, did not cluster with two other isolates from Xinjian in group III, but with Benzhilan isolates in group II (Figure 1).

Figure 1 showed the relationships among these isolates. All isolates appeared to belong to families of closely related genotypes. The Buchun vineyard from Deqin County also had its own cluster of ten very closely related strains. Separate fruits from the same vineyard in Xingjian, Shangri-la County diverted in two different benches: one came from the Buchun vineyard sampling, while the other came from different juices at the Benzilan vineyard. The most surprising finding was that seven colonies obtained from two different vinyards from Deqin County (three from Chizhong, and four more from Liutongjiang) all had genotypes that are very similar to a winery-derived strain 7A6 from New Zealand Zhang *et al*. (2014).

AFLP analysis: Using nine primer pairs, 563 different fragments were obtained，with an average of 5.47 polymorphic loci per primer pair. The average polymorphism rate was 16.84%. A range of different strains was present in Shangri-la. Two (E-ACT/M-CTG) to eleven polymorphic bands (E-AGC/M-CTC) of variable lengths were detected. And the polymorphic rate ranged from 1.42% (E-AAC+M-CTA) to 10.12% (E-ACG+M-CAA). AFLP-based pair similarities ranged from 0.84 to 0.97. The dendrogram based on AFLP data was quite similar to the dendrogram based on microsatellite data, although there were some little differences (data not shown). The 3D plot of the 43 *S. bayanus* isolates was shown in Figure 2. Analysis results illustrated that *S. bayanus* populations were geographically partitioned generally (Figure 2), and all the accessions could be divided into 4 groups.

When the genetic variation of the isolates was partitioned by AMOVA, 63.72% of the variation was found among the isolates that had different geographic origins while 36.28% was found among the ones that had the same geographic origin, suggesting a significant genetic differentiation among populations. The overall average Fst of the 43 isolates was 0.117. In 9 Chizhong isolates, the mean Fst of each isolate against the rest ranged from 0.082 to 0.118 and the mean was 0.104. Those in Liutongjiang isolates were 0.114 (mean), 0.072 (min.), and 0.123 (max.). In Benzhilan isolates, they were 0.122 (mean), 0.071 (min.) and 0.131 (max.). The means were largest in Xingjian isolates and smallest in Buchun isolates. A low value of mean Fst revealed the narrow genetic diversity of the populations.

DISCUSSION

Wine quality is strongly influenced by the levels of secondary compounds, which are determined in part by the yeast species or strains involved in the fermentation process (Romano *et al*. 1997). Hence, discovering new strains or new species of yeast is the focus of much research（Espinosa *et al*. 2002). Overall our results confirm that *S. bayanus* occurs in Shangri-la, where it appears to be quite common in some vineyard from Shangri-la. Genetic analysis and the sequence of the strains showed close similarity to the sequenced isolate CBS7001, which was originated in Europe. The nucleotide sequences of *ITS*, *PAD1* and *IAH1* genes of some isolates in NZ also were very similar to that of CBS7001 (Zhang *et al*., 2014). It indicated that both some Shangri-la *S. bayanus* and some NZ *S. bayanus* might be derived from Europe or might have the same ancient, and there is one globally dispersed way, properly via the grapevine, since a lot of grapevine in Shangri-la and in NZ was originated in Europe. Microsatellite and AFLP markers were employed to access the polymorphism among isolates. In this study, microsatellite and AFLP (amplified fragment length polymorphism) are genetic fingerprinting techniques suitable for the genetic evaluation of *S. bayanus*. These PCR (polymerase chain reaction) based technologies require small amounts of DNA. AFLP has been recognized as a reliable and efficient DNA marker system, compared with RFLP, RAPD, or ISSR, and has been used extensively for studying genetic diversity in different species because of their high reproducibility and multiplex ratio (De Riek *et al*. 2001). However, AFLP is quite difficult to operate, hard to master; in contrast, microsatellite is much easy, but also very reliable and efficient. Interesting enough, the strains which had SNPs among *HIS3* (*in* LTJ 19) and *SSU1* (*in* CZ 14 and BZL5 respectively) did not appear quite different with other strains among populations in the dendrograms generated by Microsatellites and AFLPs data.

Several factors can affect the number of bands per primer detected, including the species for study and the number of genotypes compared, the primer sequences, minor variations in the amplification protocol, and the scoring. Of course, the use of primer pairs selected for the reproduction of higher polymorphism in the target group of genotypes could further increase the efficiency and the applications of the AFLP approach, while the genetic loci which were invariant are also important for their potential to detect polymorphism in other *S. bayanus* genotypes. In this study, AFLPs were more efficient than microsatellite for generating polymorphisms and, therefore, also were more efficient for distinguishing among isolates. Higher levels of polymorphism per reaction detected with AFLP may have been due to the larger number of loci surveyed per reaction compared to the microsatellite. However, results from cluster analyses using microsatellite or AFLP data indicate that these two marker techniques provide similarly, but not identical phylogenetic information. The reason for this should be that microsatellites can not cover the whole genome, while AFLPs can. Hence, we concluded that both microsatellite and AFLP markers appear to provide useful genotyping systems in *S. bayanus*. However, AFLPs seem to be much more informative and efficient. In comparable, Zhang *et al*. (2015) reported that they used 10 primer pairs in analysis 65 New Zealand strains, and found that using microsatellite markers were

Table 1. Sequence of the primers and the annealing temperature used in the experiments

Fragments amplified	Primer sequence	Annealing temperature
ITS	ITS1: TCC GTA GGT GAA CCT GCG G ITS4: TCC TCC GCT TAT TGA TAT GC	55
HIS3	F1: ATG TCA GAG CAA AAG GCC CTA R1: CAT GAG AAC ACC CTT TGT GGA	54
SSU1	L1: AAA GCG ACG TCC GCT AAG TA R1: CCC CAA GCG GTT AGT AAA CA	60
MET2	L1: CGA AAA CGC TCC AAG AGC TGG R1: GAC CAC GAT ATG CAC CAG GCA G	60

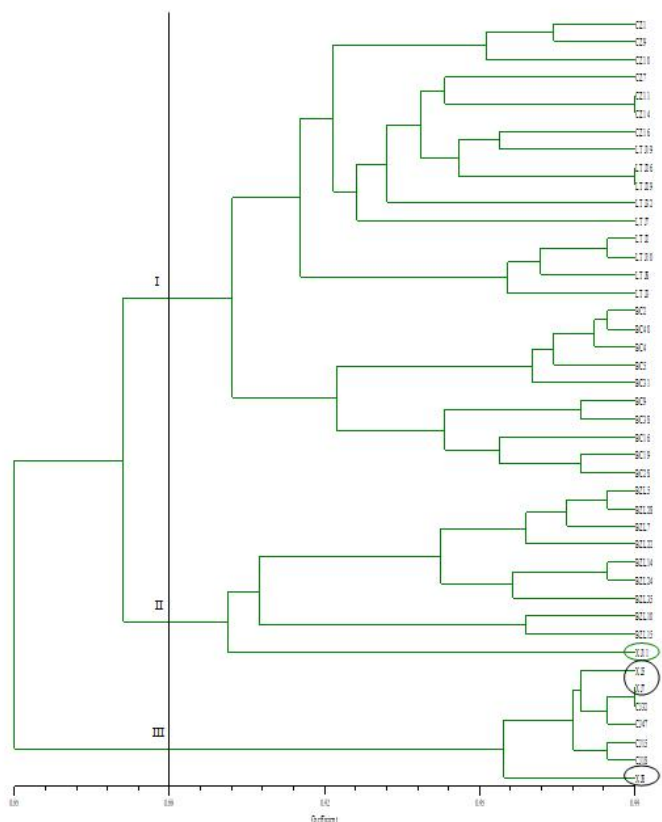

Figure1. Dendrogram of *S. bayanus* isolates based on pair-wise comparison of microsatellite data. The vertical line represents an overall similarity of alleles of 88%, using this line all the strains could be divided into three groups. All the cycle-strains were isolated from Xinjian, but the green cycle-strain XJ11 clustered with Benzilan isolates.

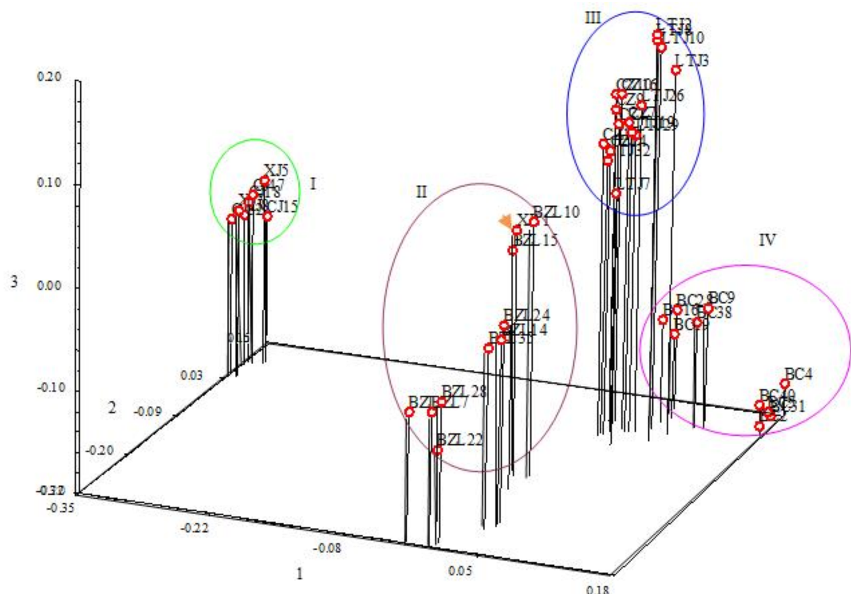

Figure 2. The 3D plot of the 43 *S. bayanus* isolates on the basis of AFLP data. All the strains could be divided into 4 groups. This time, XJ11 (the yellow arrow) did not cluster with other Xinjian isolates, but with Benzilan isolates, again.

unsuccessful because of a remarkably low level of polymorphism among the simple sequence repeats analyzed, and there was very little indication of allele sizes differing in a variable number of tandem repeats, with a total of only ~10^3 genotypes distinguishable. The genetic diversity level here is quite low in this study too. Since the genetic diversity of *S. bayanus* in both Shangri-la and New Zealand is low, and the weather in those two areas is cool, we inferred that the sexual activity of the species in those cool areas is inactive. The population genetic data suggest that there is a degree of migration of *S. bayanus* among regions within the Shangri-la area. We did not have enough time or resources to go on to test ideas as to why there are differences among regions. Climate, local adaptation, and other factors include the soil, substrate nutrient composition and availability might determine the differences (Gayevskiy and Goddard, 2012). It is likely that a combination of factors will be responsible, and it will be of interest to ascertain the relative contributions of each in the future.

CONCLUSION

Our sequences come from vineyard yeast in Shangri-la, Yunnan, China, which are quite similar to a Europe one. They may come with the grapevine which also came from Europe years ago. It is another evidence for yeast spread in the world wild aided by the human being. There is a genetically diverse natural population of *S. bayanus* isolated from vineyards in Shangri-la, Yunnan, China, where is an important place for wine production but never been reported in the area of yeast research. However, the genetic diversity level here is quite low, and those isolates may have the same origin. Both microsatellites and AFLP markers appear to provide useful genotyping systems in *S. bayanus*, although polymorphism of AFLP is much more than that of the microsatellite. And AFLPs seems to be much more informative and efficient.

Conflict of interest: The authors declare that they have no competing interests.

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