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Investigating of yeast species in wine fermentation using terminal restriction fragment length polymorphism method

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ABSTRACT

The objective of this study was to examine the potential of terminal restriction fragment length polymorphism (T-RFLP) in monitoring yeast communities during wine fermentation and to reveal new information on yeast community of Chinese enology. Firstly, terminal restriction fragment (TRF) lengths database was constructed using 32 pure yeast species. Ten of these species were firstly documented. The species except for *Candida vini*, *Issatchenkia orientalis*/*Candida krusei*, *Saccharomyces bayanus*, *Saccharomyces pastorianus*, *Saccharomyces cerevisiae*, *Saccharomyces kudriarzevii* and *Zygosaccharomyces bisporus* could be distinguished by the T-RFLP targeting 5.8S-ITS rDNA. Moreover, the yeast communities in spontaneous fermentation of Chardonnay and Riesling were identified by T-RFLP and traditional methods, including colony morphology on Wallerstein Nutrient (WLN) medium and 5.8S-ITS–RFLP analysis. The result showed that T-RFLP profiles of the yeast community correlated well with that of the results identified by the traditional methods. The TRFs with the highest intensity and present in all the samples corresponded to *Saccharomyces* sp. Other species detected by both approaches were *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Pichia minuta* var. *minuta*, *Saccharomycodes ludwigii*/*Torulaspora delbrueckii* and *Candida zemplinina*. This study revealed that T-RFLP technique is a rapid and useful tool for monitoring the composition of yeast species during wine fermentation.

1. Introduction

Alcoholic fermentation of the grape juice is a complex ecological and biological process which mainly depends on the sequential development of various yeast species and strains. The yeast communities during wine fermentation have been investigated in different winemaking regions of the world (Chavan et al., 2009; Combina et al., 2005; Di Maro et al., 2007; Li et al., 2011; Lopandic et al., 2008; Wang and Liu, 2013; Zhang et al., 2010). The traditional method of monitoring the yeast community is very laborious and time-consuming and includes isolation, cultivation and characterization of each species. The method exists inconsistent results and limits culturable yeasts only in wine samples. However, the presence of viable but non-culturable (VBNC) yeasts in wine samples has been previously described (Cocolin and Mills, 2003; Divol and Lonvaud-Funel, 2005). Although the non-culturable VBNC cells remain viable and metabolically active, they are not able to be detected by the traditional method. The recovery of the yeasts from the VBNC state may lead to additional fermentation during wine maturation and bottle-aging (Divol and Lonvaud-Funel, 2005; Divol et al., 2005), resulting in wine spoilage and economic losses. Therefore, identification and controlling of VBNC yeasts becomes one of the challenges in the contemporary yeast ecology. It has been accepted that a more complete picture of yeast community can be obtained by culture-independent, PCR-based methods (Cocolin and Mills, 2003; Urso et al., 2008). In recent years, various culture-independent methods have also been successfully used to characterize yeast communities, such as single-strand conformational polymorphism (SSCP; Callon et al., 2006), denaturing gradient gel electrophoresis (DGGE; Mills et al., 2002; Renouf et al., 2007a), real-time quantitative PCR (QPCR; Hierro et al., 2004, 2007) and terminal restriction fragment length polymorphism (T-RFLP; Bokulich et al., 2012; Hamby et al., 2012). However, most of these molecular methods require complicated procedures and are not suitable for handling large quantity of samples. In addition, the DGGE needs a sequencing step to complete the identification. The QPCR, on the other hand, needs the species-specific primer pairs for different yeast species (Zott et al., 2010).

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Compared with those methods, T-RFLP is a rapid and effective molecular method that have been recently applied to characterize fungal communities and particular fungal species such as ascomycete community, arbuscular mycorrhizal fungi and basidiomycete communities (Buchan et al., 2002; Mummey et al., 2009; Robinson et al., 2009). This technique has many advantages over other culture-independent methods on analyzing microbial community structure owing to its higher efficiency and sensitivity (Marsh, 1999; Nocker et al., 2007). However, using T-RFLP to monitor communities of yeast flora, especially the yeast communities in wine fermentation, has not been well studied. Moreover, the yeast flora of Chinese enology has not been investigated by T-RFLP yet.

In this study, a T-RFLP technique was developed and applied to rapidly and accurately detect the ecological yeast shifts that occurred in wine fermentations. A TRF database of 32 yeast species from strains of three culture collections was firstly constructed. The TRF profiles from the mixed yeast communities during spontaneous fermentation of grape musts were obtained and compared these profiles with the TRF database to measure its capability of identification. New information of the yeast community in Chinese wine was generated as well. In general, the potential of T-RFLP in monitoring mixed yeast communities during wine spontaneous fermentation was examined and compared with the traditional methods.

2. Materials and methods

2.1. Yeast sample collection

Pure yeast cultures were obtained from the Phaff Yeast Collection (University of California, Davis), the Viticulture and Enology Culture Collection (University of California, Davis), and our laboratory (Table 1). The yeast strains were cultured in 3 mL of YEPD (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose, natural pH) for 40 h at 28 °C with shaking speed at 160 rpm.

Chardonnay (C) and Riesling (R) musts were from the vintage of Winery Imperial Horse, located in Ningxia, China, in 2010. For each variety, 6 kg of ripe and physically undamaged grape clusters were randomly and aseptically collected into sterile plastic bags. The grapes were de-stemmed and crushed manually by hand in a sterile bio-hood (BCN 1360B, Hadonglian Company, Harbin, China). Two liters of the grape must along with the peel and seeds were transferred to 5 L stainless glass fermenters in duplicate after SO_2 was adjusted to 40 mg/L level. The reducing sugars content and pH of the Chardonnay and Riesling grape musts were 216 g/L and pH 3.40 and 218 g/L and pH 3.44, respectively. The spontaneous fermentations of the two grape musts were carried out in the sterile bio-hood at 16 °C–18 °C and monitored by measuring their specific gravities. Five samples were taken from each glass fermenter at different specific gravity during the wine fermentation: 1.077–1.079 (beginning of the fermentation, stage 1), 1.048–1.050 (stage 2), 1.030–1.032 (stage 3), 1.000–1.002 (stage 4), and 0.997 (end of the fermentation, stage 5). Based on the stages and variety, these samples were labeled as C1, C2, C3, C4, and C5 for the Chardonnay samples and R1, R2, R3, R4, R5 for the Riesling samples, respectively. Yeast growth was analyzed by the classical plate count method to measure viable cells enumeration. At each stage, aliquots of several dilutions (from 10^{-1} to 10^{-6}) were spread onto plates of YEPD Agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar, natural pH) in triplicate, supplemented with 100 mg/L chloramphenicol to inhibit bacterial growth. Moreover, the total biomass grown on plates YEPD without dilution was prepared in duplicate for biomass DNA extraction. All the plates were incubated to develop colonies at 28 °C for 2 days. Forty colonies from each of appropriately diluted samples were randomly selected. All isolates were streaked on WLN medium (Pallmann et al., 2001). After incubation at 28 °C for five days, the colony morphology and color on WLN agar were recorded and sorted into different phenotypes. For reliable identification of each isolate, 1 to 5 representative colonies of each WLN biotype were selected for the PCR-RFLP analysis of 5.8S-ITS rDNA.

2.2. DNA isolation

DNA isolation of the pure cultures corresponding to 32 species of 77 yeast strains was performed using the MasterPure Yeast Purification kit (Epicenter) according to the manufacturers instructions. The total biomass grown on YEPD agar plates without dilution was harvested to use as a source of DNA for T-RFLP analysis. The DNA extraction protocol for the yeasts was adapted from Renouf et al. (2007b). DNA was qualified using DN-1000 Spectrophotometer (NanoDrop, USA). All DNA samples were stored at −20 °C before use.

2.3. T-RFLP analysis

The DNA amplifications were performed in GeneAmp PCR System2700 (Singapore). They were carried out in a 50 μL reaction volume containing 5.0 μL 10 × PCR buffer (*Taq* buffer with KCl), 2 units *Taq* DNA polymerase (Fermentas), 6.0 μL of 25 mmol/L $MgCl_2$, 1.0 μL of 10 mmol/L dNTP, 2.5 μL of 10 μmol/L of each primer, 1.0 μL of template DNA and 35 μL of ultrapure water. Primers ITS1-HEX (5′-[5HEX] TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify 5.8S-ITS rDNA gene as described by White et al. (1990). The PCR amplifications were performed using the following protocol: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, hybridization at 52 °C for 2 min, extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. A 5 μL sample of the PCR product was migrated on 1% agarose gel. After the PCR amplification, the PCR products were digested separately with two different restriction endonuclease, *Hae*III and *Hinf*I, to generate two TRF patterns. The digestions were performed according to the instructions of the supplier TaKaRa Biotechnology (Dalian) Co. Ltd. The restriction fragments were separated on 3% agarose gels and evaluated by comparison with the 100 bp marker to obtain their size (Fermentas).

The fluorescent fragments were separated using the capillary electrophoresis method provided by Beijing Sunbiotech Co. Ltd, (China) to generate the database. They were measured using an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Forster City, CA, USA) with internal size standard ROX1000 and ROX500 for the detection of PCR products and TRFs, respectively. The TRF profiles and lengths of PCR products were analyzed by the ABI GeneMapper4.0 software.

3. Results

3.1. Development of T-RFLP database

To validate the T-RFLP approach, a total of 77 yeast strains (32 species 15 genera) were used as references for the operational taxonomic units (OUT) identification. Each strain was analyzed using two restriction endonucleases independently. The database of T-RFLP patterns was showed in Table 1. For per T-RFLP restriction endonuclease, most strains had only one peak, some showed two peaks and the italics type indicated the major TRF length of the same strain (Table 1). The lengths of PCR products were also listed. A high length variation in this region for different species was about 375.72 bp for the strains of *Metschnikowia pulcherrima* to