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Evaluation of different PCR primers for denaturing gradient gel electrophoresis (DGGE) analysis of fungal community structure in traditional fermentation starters used for Hong Qu glutinous rice wine

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ABSTRACT

Denaturing gradient gel electrophoresis (DGGE) has become a widely used tool to examine microbial community structure. However, when DGGE is applied to evaluate the fungal community of traditional fermentation starters, the choice of hypervariable ribosomal RNA gene regions is still controversial. In the current study, several previously published fungal PCR primer sets were compared and evaluated using PCR-DGGE, with the purpose of screening a suitable primer set to study the fungal community of traditional fermentation starters for Hong Qu glutinous rice wine.

Firstly, different primer sets were used to amplify different hypervariable regions from pure fungal cultures. Except NS1/FR1 + and ITS1fGC/ITS4, other primer sets (NL1 + /LS2R, NL3A/NL4GC, FF390/FR1 +, NS1/GCFung, NS3 + /YM951r and ITS1fGC/ITS2r) amplified the target DNA sequences successfully. Secondly, the selected primer sets were further evaluated based on their resolution to distinguish different fungal cultures through DGGE fingerprints. Three primer sets (NL1 + /LS2R, NS1/GCFung and ITS1fGC/ITS2r) were finally selected for investigating the fungal community structure of different traditional fermentation starters for Hong Qu glutinous rice wine. The internal transcribed spacer (ITS) region amplified by ITS1fGC/ITS2r, which is more hypervariable than the 18S rRNA gene and 26S rRNA gene, provides an excellent tool to separate amplification products of different fungal species. Results indicated that PCR-DGGE profile using ITS1fGC/ITS2r showed more abundant fungal species than that using NL1 + /LS2R and NS1/GCFung. Therefore, ITS1fGC/ITS2r is the most suitable primer set for PCR-DGGE analysis of fungal community structure in traditional fermentation starters for Hong Qu glutinous rice wine. DGGE profiles based on ITS1fGC/ITS2r revealed the presence of twenty-four fungal species in traditional fermentation starter. A significant difference of fungal community can be observed directly from DGGE fingerprints and principal component analysis. The statistical analysis results based on the band intensities of fungal DGGE profile showed that *Saccharomyces cerevisiae*, *Saccharomycopsis fibuligera*, *Rhizopus oryzae*, *Monascus purpureus* and *Aspergillus niger* were the dominant fungal species.

In conclusion, the comparison of several primer sets for fungal PCR-DGGE would be useful to enrich our knowledge of the fungal community structures associated with traditional fermentation starters, which may facilitate the development of better starter cultures for manufacturing Chinese Hong Qu glutinous rice wine.

1. Introduction

Chinese yellow rice wine (*huáng jiǔ*), one of the three most famous brewed wines (rice wine, grape wine and beer) in the world, has more than 4000 years of brewing history. It plays important roles in Chinese culture and people's daily life, and has been widely consumed in China for centuries because it is rich in amino acids and has a unique aroma, subtle flavor and low alcoholic content (Wang et al., 2014). As one of the most typical representatives of Chinese yellow rice wine, Hong Qu glutinous rice wine is produced from glutinous rice with the addition of

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two traditional wine fermentation starters—Hong Qu (hóng qǔ, also called Chinese red yeast rice) and Yao Qu (yào qǔ) (Park et al., 2016; Lv et al., 2013a). However, it is difficult to control the fermentation process of Hong Qu glutinous rice wine, because the microbial composition of traditional fermentation starters is extremely complex. The traditional making of wine fermentation starters under non-sterile and uncontrolled environmental conditions based on empirical knowledge often leads to the uncontrollability of the fermentation process and the instability of wine quality between different batches (Park et al., 2016).

The main stage of the traditional rice wine fermentation process is a typical simultaneous saccharification and fermentation process. During the fermentation process, filamentous fungi and yeasts play a vital role in the formation of organoleptic characteristics. They can secrete various enzymes (including α-amylase, β-amylase, glucoamylase and proteases) to hydrolyze starch and protein, and increase the digestibility and physiological active ingredients in rice wine, and play a prominent role in determining the fermentation speed, wine flavor and other wine qualities (Jolly et al., 2006; Zheng et al., 2011). Some fungal strains may produce mycotoxins, such as aflatoxin, citrinin and ochratoxin A (OTA), which would seriously affect the edible safety of rice wine (Haas et al., 2013). Given the biodiversity of filamentous fungi and yeasts in regard to their production levels of enzymatic activities (Lv et al., 2012a; Viana et al., 2008) and fermentation metabolites (Capece et al., 2005) of enological importance, suitable fungal strains should be selected in order to design defined mixed starter cultures capable of providing beneficial contributions to wine quality. Therefore, it is necessary to systematically valuate the fungal community structure of traditional fermentation starters for Hong Qu glutinous rice wine.

Molecular techniques such as terminal-restriction fragment length polymorphism (T-RFLP), 16S rRNA gene clone libraries, denaturing and temperature gradient gel electrophoresis (DGGE/TGGE) and next-generation sequencing (NGS) technology are widely used in comparative microbial ecology to assess the diversity of microbial communities and their responses to changing environments (Marzorati et al., 2008). Among these approaches, PCR-DGGE has been widely used to study the microbial diversity and dynamics of traditional fermented foods (Xu et al., 2011; Kim et al., 2009; Haruta et al., 2006) quickly and economically, which would enable development of starter cultures, and identification of major microorganisms that play a major role in flavor development and spoilage. NGS technology could certainly provide a more clarifying and precise picture of the microbial community of fermentation starters. Nevertheless, whether it is based on the DGGE analysis, or NGS technology, microbial genomic DNAs in samples should be amplified by PCR firstly, then the amplified fragments were used for subsequent DGGE analysis or high-throughput sequencing. The most critical step for accurate rDNA amplicon analysis is still the choice of appropriate primer sets, because the target variable gene regions may affect the outcomes of fungal diversity, and it may consequently result in questionable biological conclusions when using inappropriate primers. Therefore, careful selection of appropriate PCR primers is critical for the successful application of both DGGE technology and NGS technology to the analysis of fungal communities. This issue motivated us to compare the properties of the commonly used fungal primer sets. Internal transcribed spacer (ITS) region, 26S rRNA gene and 18S rRNA gene have been extensively used to study the fungal community structure in various ecosystems (Cocolin et al., 2000; Vainio and Hantula, 2000; Haruta et al., 2006; Thanh et al., 2008; Bonito et al., 2010; Liu et al., 2015). However, it is still controversial on the sensitivity and accuracy of the three variable gene regions. Until now, no systematic comparison has been made for the fungal DGGE profiles when different hypervariable regions are amplified from pure fungal isolates from rice wine starters. Little information is available about the influence of different fungal DGGE primer sets on the outcome of fungal community of traditional fermentation starters for Hong Qu glutinous rice wine brewing.

The objective of this study was to evaluate several previously published fungal primer sets for fungal community analysis of rice wine fermentation starters by PCR mediated denaturing gradient gel electrophoresis (DGGE). Evaluation criteria for the PCR primer sets involved testing the DGGE resolution of PCR products generated from pure fungal cultures. Then suitable fungal primers for better distinguishing pure fungal cultures were selected for the analysis of fungal communities in traditional fermentation starters for Hong Qu glutinous rice wine.

2. Materials and methods

2.1. Sample collection

Ten samples of traditional fermentation starters (5 Hong Qu and 5 Yao Qu) were obtained from different geographical areas of Fujian province in China (local markets or brewing companies located in northern, southern and western area of Fujian province). All of the starter samples were fermented and matured (having been stored for 6 months of maturation). Samples were stored at 4 °C immediately after collection prior to test.

2.2. Microbial strains and culture conditions

Twenty-four fungal strains were previously isolated and purified from several starters for Hong Qu glutinous rice wine brewing by spreading plate method on plates of potato dextrose agar (PDA, Difco) (as shown in Table 1). All fungal strains were cultivated in potato dextrose broth (PDB, Difco) medium in thermostatic shaker at 28 °C for 4 days. Cultures were centrifuged to collect the yeast cells or fungal mycelium (about 100 mg) used for DNA extraction.

2.3. DNA extraction from pure fungal culture and traditional fermentation starters

DNA extractions for reference fungal strains and traditional fermentation starters were carried out using a benzyl chloride method (Zhu et al., 1993). The DNAs were dissolved in 50 μL TE containing RNase. The yield and fragmentation of the DNAs were checked by agarose gel electrophoresis, followed by ethidium bromide staining and visualization under UV light. DNA extracts from each sample were also analyzed spectrophotometrically at 260 nm and 280 nm by using a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). The DNA extracts were stored at − 20 °C for future use.

2.4. PCR amplification of 18S rDNA, 26S rDNA and internal transcribed spacer (ITS) regions

The extracted DNA was then used as a template for PCR to amplify 18S rRNA genes. Almost full-length of eukaryotic SSU gene (18S rRNA gene) were amplified using the fungal universal primers NS1(F)/FR1(R) as described previously (Vainio and Hantula, 2000). The variable D1/D2 region of 26S rRNA gene was amplified using NL-1 and NL-4 primers as previously described (Kurtzman and Robnett, 1998). For amplification of the ITS1-5.8S-ITS2 region, the primers ITS1 and ITS4 (White et al., 1990) were used.

2.5. Nested PCR-DGGE analysis

Nested PCR was carried out using different primers (FF390/FR1 +, NS1/GCFung, NS3 +/YM951r, NL1 +/LS2R, NL3A/NL4GC and ITS1fGC/ITS2r) for DGGE analysis, respectively (Supplementary Table S1). The PCR products were amplified by a T3000 Thermocycler (Biometra, Germany) using the conditions as shown in Supplementary Table S2. PCR products were firstly checked by agarose gel electrophoresis with ethidium bromide staining. According to the Mass Ruler

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