Analysis of the Diversity of Yeast Communities in Xinjiang Honey

Yumei Wei

Shihezi University

Yonghui Lei

Shihezi University

Ailing Liu

Shihezi University

Shanshan Zhu

Shihezi University

Yang Li

Shihezi University

Yanli Cai

Shihezi University

Yanfei Sun (✉ 81711308@qq.com)

Shihezi University <https://orcid.org/0000-0003-1947-9245>

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Abstract

Honey is widely produced in China as a natural health food with high nutritional value. In this study, high-throughput sequencing (HTS) and culture-dependent methods were used to assess the yeast diversity of wild honey from three different regions of Xinjiang. The analysis of HTS data of 26S rDNA NL1F and NL2R region genes revealed the highest number of operational taxonomic units (OTUs) of the yeast genus *Zygosaccharomyces* in honey, followed by the genera *Aureobasidium* and *Clavispora*. Multifloral honey is not only different in yeast structure and composition from monofloral honey, but also more diverse than monofloral honey. Thirty-five yeast strains were obtained by traditional isolation and culture methods, and morphological, physiological and biochemical, and 26S rDNA D1/D2 domain genes were sequenced to identify 11 yeast species, including *Zygosaccharomyces siamensis*, *Aureobasidium pullulans*, *Naganishia albida*, *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Rhodotorula glutinis*, *Pichia kluyveri*, *Barnettozyma californica*, *Rhodotorula kratochvilovae*, *Candia tropicalis*, *Pichia terricola*, and these results are not entirely consistent with those obtained using high-throughput sequencing methods. Different species of honey may have contributed to the diversity of yeast. The results of this study provide basic information on the yeast diversity of native honey in Xinjiang, which will help to develop germ plasm resources of yeast in honey.

Introduction

Honey is a natural sweet substance collected by bees from plant nectar, secretions or the honeydew, mixed with their own secretions (Roshan et al. 2017; Danila et al. 2018). Honey is rich in nutrients, including sugars, proteins, amino acids, vitamins, organic acids, trace elements and some biological enzymes (Meo et al. 2017; Shapla et al. 2018). Depending on the nectar plant, honey can be divided into monofloral honey, which is honey made from the nectar or secretions of one or more nectar plants, and multifloral honey, which is honey made from the nectar or secretions of two or more nectar plants. In addition, the composition of monofloral honey and multifloral honey may differ, such as 680.2 mg/g of fructose and glucose in Oleaster honey (Zhu 2021), 812 mg/g of fructose and glucose in sunflower honey (Zhao et al. 2015), and 765.6 mg/g of fructose and glucose in multifloral honey (Zhao et al. 2015). 4-Terpineol is the most significant characteristic volatile substance of sunflower honey (Su et al. 2010), Oleaster honey contains the most phenolic substances, while multifloral honey contains the most aldehydes (Di et al. 2016; Wei et al. 2020).

Generally speaking, most honey contains high sugar content and low water content, which is extremely unsuitable for microorganisms. Thus, the maximum level allowed by this legislation (commercial honey ruled by the Mercado Común del Sur (MERCOSUR) legislation) for molds and yeasts with trading purposes is 100 CFU/g of honey (A et al. 2017). But so far, whether the yeast in honey is detrimental to health is still unknown (Devi et al. 2021). Some people have reported that yeast that produces erythritol from honey has been found (Deng et al. 2013) ethanol-producing yeast (Joseph et al. 2018), lipase-producing yeast (Palilu et al. 2019), but yeast can also affect the quality of honey (Hajimohammadi et al. 2017). Therefore, we are quite interested in the biodiversity of yeast in honey.

Yeast is an important industrial microorganism and has important applications in the fields of food, feed, medicine and environmental management(Huang et al. 2020). Screening excellent yeast strains in honey is of great significance for products with regional characteristics and unique flavors in Xinjiang. Rering Caitlin C et al. found that pollen and yeast may work together to alter the chemical composition of nectar, thereby affecting honey quality(Rering et al. 2021). Echeverrigaray Sergio et al. isolated yeast from 17 stingless bee honey samples in southern Brazil and found that some yeasts were only identified in honey samples from specific bee species, indicating that these yeasts are closely related to insects(Echeverrigaray et al. 2021), and the isolated yeast species exhibited high osmotic pressure (Palilu et al. 2019)and low sugar assimilation (Park et al. 1996)capacity. Osmophilic yeasts such as Yong Kun Park isolated from honey and pollen not only convert sucrose to polymers but also to fructooligosaccharides in good yields. It has been reported that osmophilic yeast produce lipase due to their low water content in their natural habitat. Palilu Prayolga Toban (Palilu et al. 2019)Lipase-producing yeast isolated from wild forest honey in Sulawesi. Xinjiang is a traditional beekeeping province with an extensive mass beekeeping base, and it has a typical continental climate with large diurnal temperature differences(ZHANG et al. 2020) providing unique conditions for the formation and evolution of species(Zhu et al. 2021). By studying the community structure of yeast in honey, the germplasm resources of yeast in honey were developed and utilized.

The ultimate goal of this study was to determine the basic structure of the honey yeast community suitable for the special environmental characteristics of Xinjiang. High-throughput sequencing techniques and culture-dependent methods were combined for accurate identification. The reported data will be useful for the study of yeast diversity in honey and as a guide for the development and utilization of yeast germ plasm resources.

Materials And Methods

Sample Collection

Honey was collected from Shawan, Shihezi and Yili Kazakh Autonomous Prefecture in Xinjiang, and all samples were obtained directly from beekeepers during the flowering season. The samples are kept fresh in the sampling box and shipped to the laboratory. Honey samples for cultural isolation were stored at 4°C, and genome-extracted honey samples for high-throughput sequencing were stored at -80°C. There were 3 samples of Oleaster honey (3 from Shawan area), 3 samples of sunflower honey (3 from Shihezi area), and 3 samples of multifloral honey (3 from Ili area).

High-throughput sequencing

Three replicates of each sample were taken and mixed together in equal amounts(Zhang et al. 2021). Using the MoBio Power Fecal® DNA Isolation Kit (MOBIO, USA), total DNA was extracted directly from each sample according to the manufacturer's protocol. The yeast 26S rDNA was amplified with a pair of specific primers with barcode NL1F (forward primer) (5'-GCAT ATCAATAAGCGGAGGAAAAG-3') and NL2R (reverse primer) (5'-CTTGTTTCGCTATCGGTCTC-3') (Liu et al. 2018).The PCR reaction system (20 µL)

contained 5× FastPfu Buffer (4 µL), 2.5 mM dNTPs (2 µL), primer (5 µM; 0.8 µL each), FastPfu Polymerase (0.4 µL), BSA (0.2 µL), and template DNA (10 ng). (Zhu et al. 2021). The PCR reaction was performed using a thermocouple PCR system as follows: 5 min at 95°C (denaturation), 30 cycles at 95 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min, and finally, at 72 °C for 10 min (elongation). The PCR products were analyzed using 2% agarose gel electrophoresis, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA). The DNA fragments were quantified using QuantiFluor™-ST (Promega, USA) (Zhang et al. 2018). Equimolar amounts of purified DNA fragments were pooled after individual samples were tagged with indexes through an index PCR, and the Illumina MiSeq PE2500 platform (Illumina, USA) was used to perform paired-end sequencing (2×250) following the protocol by Personalbio Biomedical Technology (Shanghai, China).

Raw reads are prime clipped and paired reads are combined into a single sequence. The data for each sample are identified and distinguished by barcodes. They then quality filter using QIIME (version 1.8.0). All sequences were clustered based on the distance between sequences. Sequences were assigned to operational classification units (OTUs) based on 97% similarity (Zhao et al. 2011). The RDP Classifier method was adopted to annotate the species of OTU representative sequence (threshold is 0.8–1), and the yeast diversity of samples was counted at the classification level of domain, phylum, class, order, family, and genus (Zhang et al. 2021). Excel 2007 (Microsoft Corp., United States) was used for yeast species abundance (mean values) (Li et al. 2019). Origin Pro 2018 (OriginLab., United States) was used to make a heatmap of yeast species in honey. Raw fungal ITS sequence data were deposited at the NCBI database under accession number SUB10550652.

Yeast isolation and cultivation

Yeasts were isolated from honey samples by published method under aseptic conditions (Boundy-Mills 2006). Honey was diluted by 10 being with sterile water and vortexed for 1 minute (P et al. 2003). One hundred microliters of successive decimal dilutions were spread on yeast extract-Malt extract (YM) agar (Qingdao Hope Bio-Technology Co., Ltd) supplemented with 100mg l-1 chloramphenicol (Saksinchai et al. 2012). A representative purified yeast colony was selected from each sample, and each colony was streaked on Wallerstein Laboratory (WL nutrient agar) Nutrient Agar (ShanDong Tuopu Biol-engineering Co., Ltd) to better identify their morphological type (Pallmann et al. 2001). Then store them in YM slope or 30% (v/v) glycerin.

Physiological and biochemical identification

The composition of all media used in this study and the methods of fermentation and assimilation tests were referred to Kurtzman (Kurtzman and Cletus 2011; Kurtzman et al. 2011; Xu et al. 2019). Both sugar fermentation and assimilation tests of the yeast were performed at 25°C and recorded after 1, 2, 3 weeks (S et al. 2013).

(1) Sugar fermentation test: The sugar to be fermented (e.g. glucose, sucrose, maltose, lactose) was added to a 0.6% solution of yeast extract to bring the sugar content to 2%, and then the prepared solution

was added separately to test tubes containing Duchenne tubes, and the activated test strain was accessed and incubated in a constant temperature incubator at 28°C for 3 weeks. During incubation, if bubbles appear at the top of the Duchenne tube, the test strain can use the sugar, which can be recorded as "+" (positive reaction), if there is no change, it is recorded as "-" (negative reaction).

(2) Carbon source assimilation test: The test strain in the growth phase was inserted into the test tube containing carbon source liquid medium (Xu et al. 2019) and incubated in a constant temperature incubator at 28°C for 3 weeks to observe the growth and the change of the solution, observe the growth and the change of the solution, and control with the blank test to observe whether the solution is turbid, whether the formation of atolls and bacteria Pu.

(3) Nitrogen source assimilation test: the test strains in the growth period into the nitrogen source liquid medium, incubated at 28°C for 3 weeks in a constant temperature incubator, observe the growth and solution changes as in the carbon source assimilation test.

Molecular biology identification

In the experiment, acetyl trimethyl ammonium bromide (CTAB) method was utilized to extract yeast genomic DNA (M et al. 2010). PCR amplification of yeast 26S rRNA D1/D2 sequences using fungal universal primers s NL1 (GCATATCAATAAGCGGAGGAAAAG) and NL4 (50 GGTCCGTGTTTCAAGACGG30) (Úbeda et al. 2014; H Tansel Yalçın 1 2021). PCR reaction was performed as described by Wang and Liu (2013) with a fiducial volume of 50 µL mixture containing 0.2 mmol/L of each dNTP, 1× buffer without Mg²⁺, 1.5 mmol/L MgCl₂, 0.2 µmol/L of each primer, 1.5 U Taq polymerase (TaKaRa, Dalian, China), and 1 µL DNA template (Wang et al. 2019). The amplification procedure: pre-denaturation at 94 °C for 5 min; denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, extension at 72 °C for 1 min, 30 cycles; extension at 72 °C for another 10 min, store at 4 °C. The quality of the PCR products was verified by GeneGreen-stained 1% agarose gel electrophoresis at 120 V for 30 min. The PCR products were directed to Shanghai Biotech for purification and sequencing. The sequences analyzed in this study were blasted in the NCBI database (<http://www.ncbi.nlm.nih.gov/BLAST>), and the registration numbers of the yeast strains are shown in Table 6 and compared with those in the NCBI's GenBank database.

Results

Determination of yeast community structure distribution by high-throughput methods

In order to characterize the structure of the yeast community in honey from three regions of Xinjiang, the OTU sequences were clustered and annotated with species. 40 genera belonging to 2 phyla, 10 orders, 13 families and 15 families were included in the OTU of honey from the three sampling sites. The top 20 genera in relative abundance and the lower genera in relative abundance were selected and grouped into others. *Zygosaccharomyces*, *Aureobasidium*, *Clavispor*, *Pichia* and *Filobasidium* were the main yeast genera in honey. The results for the main yeast genera levels in honey (Fig.1) showed that *Zygosaccharomyces* had the highest relative abundance of 85.86% and was the most dominant

genus among the three honey, followed by *Aureobasidium* and *Clavispora*. And the top 20 genera were distributed in all three honey. The relative abundances of Oleaster honey and sunflower honey differed little at the yeast genus level, but there were large differences in the dominant yeast and their relative abundances with multifloral honey. The highest relative abundance of *Zygosaccharomyces* was found in sunflower honey at the Shihezi sampling site (96.64%), followed by *Clavispora* (1.24%) and *Filobasidium* (1.06%), while the relative abundance of *Zygosaccharomyces* reached (44.71%) in multifloral honey at the Ili sampling site, and followed by *Aureobasidium* (14.07%) and *Pichia* (9.80%).

Heat map analysis showed the relative abundance of the last 20 yeast genera in the three honey groups (Figure 2). The relative abundance of these yeast genera was generally low in all three samples. Among them, the genus *Barnettozyma* was present in FM1 and FM3 yeast, but no tested were available in FM2. *Eremothecium* was present in both FM2 and FM3, but not in FM1.

Morphological identification of yeast

Through isolation and purification of YM medium, 15, 13 and 7 yeast strains were isolated and purified from Shawan oleaster honey, Shihezi sunflower honey and Yili multifloral honey, and a total of 35 yeast strains were obtained (Table 1). According to the phenotypic characteristics of yeast on WL medium, it can be classified into 11 different phenotypes (Figure 3), and then, the cell morphology of each phenotypic representative strain was observed, a total of three microscopic forms were obtained. Single-ended sprouting of pike (phenotype FM1-1), round single end sprouting (phenotype FM1-2), Oval single-ended sprouts (phenotype FM1-3, FM1-4, FM1-8, FM1-10, FM1-12, FM2-10, FM3-1, FM3-3, FM3-7).

Physiological and biochemical experiments

Based on morphological characterization, further physiological and biochemical experiments were conducted on yeast, including sugar fermentation experiments, carbon source assimilation experiments, nitrogen source assimilation experiments, and maximum growth temperature experiments. In the sugar fermentation experiment, strains FM1-1, FM1-2, FM1-4 and FM1-8 were unable to ferment glucose, galactose, sucrose, maltose, lactose, raffinose and trehalose. Except glucose, FM1-3, FM3-3 and FM3-7 cannot ferment other sugars. FM1-10, FM1-12, FM2-12 and FM3-2 have different sugar fermentation conditions (Table 2). In addition to various organic acid salts as the sole carbon source, including lactate, succinate and citrate, they also assimilated a wide range of carbon sources (Table 3). They assimilated differently on various nitrogen sources, with FM1-1, FM1-2, FM1-3, FM1-4, FM3-2 being able to utilize sodium nitrate and sodium nitrite, while the rest of the strains were unable to assimilate (Table 4). In addition, in the maximum temperature growth experiment, it was found that all strains except FM1-4 and FM1-8 could grow at 35°C (Table 5). According to the morphological, physiological and biochemical characteristics of the above isolate combined with the "Characteristics and Identification Manual of Yeast" (Kurtzman and Cletus 2011; Kurtzman et al. 2011), it can be preliminarily judged that the FM1-1 strain is *Aureobasidium*, the FM1-2 strain is *Naganishia*, and the FM1-3 strain is *Barnettozyma*, Strains FM1-4 and FM1-8 belong to the genus *Rhodotorula*, strain FM1-10 belongs to the genus *Zygosaccharomyces*, strain FM1-12 belongs to the genus *Saccharomyces*, strain FM2-10 belongs to the

genus *Candida*, strain FM3-1 belongs to the genus *Wickerhamomyces*, FM3-3 and The FM3-7 strain belongs to the genus *Pichia*.

Molecular biology identification and phylogenetic tree construction

Sequence analysis of 26S rDNA D1/D2 region was performed on yeast isolates in honey. Amplification of the NL1 and NL4 regions produced 400 to 600 bp fragments. According to the sequencing results, submit to the GenBank database of the National Center for Biotechnology Information (NCBI), and use the basic local alignment search tool (BLAST) for homology search, similarity> 99% was applied to the species-level identification of isolates (Fang et al. 2012). Identified strains represented 11 species belonging to 9 genera, namely *Aureobasidium*, *Naganishia*, *Barnettozyma*, *Rhodotorula*, *Zygosaccharomyces*, *Saccharomyces*, *Wickerhamomyces*, *Candida* and *Pichia*. (Table 1). The main species are designated as *Zygosaccharomyces ssiamensis*, *Aureobasidium pullulans* and *Naganishia albida*. Among the 35 identified yeast strains, 24 belonged to Basidiomycetes (approximately 68.6%), while the remaining 11 strains were designated as Ascomycetes (approximately 31.4%). The phylogenetic tree constructed based on the 26S rDNA D1/D2 region further confirmed its taxonomic status (Figure 4).

Yeast community structure composition

Yeast communities from different sources show different species diversity. The majority of strains isolated from oleaster honey are *Z.siamensis* and *N. albida*, a colony frequency of 33.33% and 26.67%; the strains isolated from sunflower honey are mainly *Z.siamensis*, with a colony frequency of 76.92%. The main strain isolated from honey of various flowers are also *Z.siamensis*, with a colony frequency of 42.86%. Although *Z.siamensis* is the dominant strain in the three honey, it is more common in sunflower honey. It is worth mentioning that there are rich types of yeasts in oleaster honey, including 7 species, including *A.pullulans*, *C.albidus*, *B.californica*, *R.glutinis*, *Z.siamensis*, *R.kratochvilovae*, *R.dioboratum*; There are only 3 species in Shihezi sunflower honey, while there are 5 species in Yili honey of various flowers (Table 6).

Discussion

Analysis of yeast diversity in wild honey from Xinjiang by the Illumina MiSeq cloud platform with on-line sequencing revealed the presence of abundant yeast resources in honey. Among three honey containing 40 genera and 148 species, the phylum Stenotrophomonas was the dominant fungal phylum, of which *Zygosaccharomyces* was the dominant genus followed by *Aureobasidium*, *Pichia*, which was consistent with the culturable results. The genus *Zygosaccharomyces* is commonly found in honey (Sujinan et al. 2011; Fan et al. 2014; Liu et al. 2016) and is resistant to hyperosmolarity. Nils Arneborg found that *Zygosaccharomyces bailii* was resistant to short-term pH changes caused by acetic acid (Arneborg et al. 2000). Sugumaran has produced commercially important biodegradable polymers, pullulans, from agricultural waste through *Aureobasidium pullulans* (Sugumaran et al. 2013). *Pichia pastoris* can be used for the production of Ganoderma immunomodulatory proteins, industrial enzymes and chemicals (Mao et al. 2019; Zhu et al. 2019). Savitree Limtong isolated a new *Pichia* sp, from soil and found it to be

thermotolerant and methylotrophic(Savitree et al. 2005). In the heat map, yeasts of the genera *Barnettozyma* and *Eremothecium* are relatively less abundant in honey, and both genera have been reported in the literature to have some functional potential as well. Saha found that *Barnettozyma populi* Y-12728 selectively produces xylitol from mixed sugar and corn stover hemicellulose hydrolysate(Saha and Kennedy 2020). Nouri can improve ethanol production through improved barnettozyma californiac(Nouri et al. 2018). A new essential oil obtained from the fermentation broth of Eremothecium ashbyii H4565 by Feng He showed some antibacterial activity(He et al. 2018). All these results indicate that yeast germplasm resources are of great significance to humans.

From the yeast community structure, it can be seen that among different honeys, the difference between yeast diversity in Oleaster honey and sunflower honey is not much, which may be related to the geographical environment, because Shihezi is adjacent to Shawan and has similar environmental conditions. However, the diversity and abundance of yeast in multifloral honey was significantly higher than that in Oleaster honey and sunflower honey, which may be due to the fact that monofloral honey and multifloral honey differ in composition and structure, and the microorganisms on the nectar source are more abundant compared to monofloral honey. Mohammed (Mohammed et al. 2013) reported that the chemical composition of honey produced by different species of bees is different, which is of reference for the above study.

Xinjiang honey, as a traditional natural health product, contains a large number of microbial resources (Herrera et al. 2010; Lenaerts 2017). In the experiment. Three honey products were selected as samples of research materials, a total of 15 strains and 7 species of yeast were isolated from date honey, 13 strains and 3 species of yeast from sunflower honey, and 7 strains and 5 species of yeast from peperomia honey. This may be due to geographical conditions, climatic environment and production technology, and traditional culture can be cultivated. The limitations of the method and other factors have caused differences in the microbial composition of wild honey in different regions. Among 35 yeast strains isolated *Zygosaccharomyces* was found as the dominant yeast genus, followed by *Aureobasidium* genus, and all 11 yeast species identified were found in high throughput. Physiological characteristics of *Zygosaccharomyces* yeast including their ability to ferment sugar, osmotic pressure, resistance to preservatives, and heat tolerance of ascospores are very significant (James and Stratford 2003).

In this study, Illumina MiSeq sequencing technology was applied to discover the complex and diverse structure of the wild honey yeast population in Xinjiang, and several yeast genera such as *Clavispora*, *Holtermanniella*, and *Filobasidium* were detected that were not isolated by traditional methods. This further confirmed that Illumina MiSeq sequencing technology not only broadens the depth of microbial diversity in environmental samples, but also provides a comprehensive picture of the flora structure in the samples and presents the true level of microbial diversity (Haifeng et al. 2017; Wu et al. 2020). In the conventional culture, the selectivity of the medium and the restrictive visual observation of the colonies led to errors in the selection of characteristic colonies, and more species of yeast were not isolated. Although the conventional pure culture method has major limitations, it has the advantage that pure cultures can be isolated, such as the genera *Barnettozyma*, *Saccharomyces* and *Wickerhamomyces*,

which are relatively low in abundance in high-throughput sequencing, were also isolated in the culturable method. Traditional pure culture methods can not only enrich the strain resources, but also provide resources for the application of microorganisms. Therefore, the effective combination of traditional isolation and culture methods with high-throughput sequencing technology can, on the one hand, systematically reveal the diversity of brewer's yeast in wild honey in Xinjiang, and on the other hand, lay the foundation for the enrichment and exploitation of the yeast strain resource base.

Declarations

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Tables

Table 1 to 6 xlsx are available in the Supplemental Files section.

Figures

Figure 1

Relative abundance of species at the level of honey yeast genera.

The top 20 dominant genera were selected from the three honey samples, and the remaining genera were grouped into others, and the relative abundance of species at the yeast genus level was plotted.

Figure 2

Heat map of the last 20 OTUs based on relative abundance in the three honey samples. The color intensity in each panel indicates the relative abundance of a genus in each sample, and the abundance was defined as the number of sequences assigned to that taxon divided by the total number of sequences per sample.

Figure 3

Colonial morphology (left) and cell morphology (right) of different types of representative strains on WL nutrient agar medium.

Figure 4

Neighbor-joining phylogenetic tree of the isolates and related reference strains. GenBank accession numbers for the 26S rDNA sequences used in the tree reconstruction are given in the parentheses next to each taxon.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.xlsx](#)
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- [Table3.xlsx](#)
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