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Diversity of epiphytic fungi on the surface of Kyoho grape berries during ripening process in summer and winter at Nanning region, Guangxi, China

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ABSTRACT

The two-harvest-per-year farming system allow table grape to be harvested a year both in summer and winter in southern China. Herein, we used high-throughput sequencing to investigate the diversity of fungi on grape fruits surface during the ripening process in summer and winter at subtropical Nanning region, Guangxi, China. The results showed that 23 fungal species existed in all samples. Among them, the five most dominant species were *Cladosporium ramotenellum*, *Pseudozyma aphidis*, *Gyrothrix* spp., *Gibberella intricans* and *Acremonium alternatum*, with abundance from 61.62 % to 91.26 %. Analysis using the student's t-test for Shannon index indicated that components of fungal community varied significantly between the two ripening seasons. The dominant genera of core fungal community were *Cladosporium*, *Gyrothrix*, *Paramycosphaerella*, *Acremonium*, *Penicillium and Tilletiopsis* in the summer and *Cladosporium*, *Pseudozyma*, *Gibberella*, *Colletotrichum*, *Sporobolomyces*, *Rhodosporidium*, *Alternaria* and *Aspergillus* in the winter. Overall, fungi diversity on grape fruits surface at Nanning showed significantly differences between different ripening seasons. Our results ennrich the understanding of epiphytic communities of grape fruits in subtropics.

1. Introduction

Some fungi are important epiphytes, which are vital for plant health as well as fruits quality and yield. Previous researches have mainly focused on the pathogenic fungi causing grape diseases, including *Erysiphe necator*, *Colletotrichum acutatum* and *Plamospara viticola*, which are the causal agents of grapevine powdery mildew, bitter rot and downy mildew, respectively. Moreover, researchers have also found that saprophytic molds, like *Aspergillus* spp., *Cladosporium* spp. and *Penicillium* spp., could produce mycotoxin, which were responsible directly for grape rots and indirectly for food spoilage (Martins et al., 2014). Epiphytic microorganisms colonizing grapes especially from traditional wine regions with latitude between 30 and 50° in both the Northern and Southern hemispheres have also been widely studied due to their impacts on wine quality (Kecskemeti et al., 2016; Lorenzini et al., 2016; Martins et al., 2014; Portillo et al., 2016; Pretorius, 2010). Some authors have isolated epiphytic yeasts and bacteria for biocontrol of pathogens on table grape (Diguta et al., 2016; Nally et al., 2012, 2013). Nevertheless, few researches have concentrated on epiphytic fungal diversity of table grape.

Previous studies on associated microbiota have been limited by methodological biases of culture-based techniques (Nisiotou and Nychas, 2007; Vincent et al., 2010) and low resolution of early molecular techniques (Martins et al., 2012; Portillo et al., 2016), which might miss up to approximately 95 % of the community in some samples (Setati et al., 2015; Taylor et al., 2014). In recent years, more efficient high-throughput sequencing technology has been used to investigate microbial diversity. However, so far it has not been applied to investigate epiphytic fungal diversity of table grape cultivation in sub-tropical regions.

Guangxi province in southwest China is too hot and humid for growing grape. However, with the development of new technologies such as using hydrogen cyanamide to break the dormancy of

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grape buds (George et al., 1988) and rain-shelter cultivation (Meng et al., 2012), table grape has been extensively cultivated in Guangxi. Benefited from novel cultivation methods, since 2006, Guangxi has become an advantageous region for the table grape growth in China, where table grape could be harvested with high yield twice per year both in summer and winter. However, due to the high humidity, this region also suffers from high incidence of diseases mostly related to fungal infections, such as ripe rot, downy mildew, powdery mildew, etc.

Our goal was to investigate the diversity of epiphytic fungal communities of grape berries at two different harvest seasons in Guangxi under two-harvest-per-year farming system. In detail, the current research characterized the fungal communities of Kyoho grape that was cultivated by rain-shelter method and harvested twice per year in summer and winter in the same vineyard at Nanning region (a major producing area located in south Guangxi) using ITS amplicon sequencing.

2. Material and methods

2.1. Sample collection

This study was performed in the Modem Agriculture Demonstration Base of Guangxi Academy of Agricultural Sciences (N 22.6079, E 108.2345, 113 m altitude) in the summer and winter of 2016. This base is characterized by hot summers with the highest temperature of 41 °C, warm winters with lowest temperature of 2 °C and annual rainfall of more than 1300 mm. Samples harvested in the summer and winter were assigned into the summer harvest group (Sum) and the winter harvest group (Win), respectively. To evaluate changes in the microbial ecosystem during grape maturation, samples were collected with three replications in the vineyard using the method reported previously (Martins et al., 2014) with modifications at four different growth stages of beginning of berry ripening (BRB), berry veraison (BV), berries not quite ripe (BQR), and harvest ripe (HR), corresponding to stages 34, 35, 37 and 38, respectively, in the modified E-L system proposed by Coombe (1995) for identifying major and intermediate grapevine growth stages. At each sampling date and location, approximately 10 kg of undamaged grapes with their pedicels attached were aseptically removed from several bunches, put in sterile bags, transported to the laboratory in refrigerated boxes, pooled into a composite sample and processed within 12 h of post-collection.

2.2. Microbial biomass recovery

Epiphytic microbial biomass was recovered as previously reported (Martins et al., 2014) with slight modifications. Each sample was composed of 250 undamaged berries randomly and aseptically removed from bunches, placed in a sterile flask with 1500 ml 0.9 % saline solution containing 0.1 % peptone and 0.01 % Tween 80. Elution was performed by orbital shaking at 150 rpm for 1 h. The cell suspensions were separated from the berries by filtering through cellulose acetate filters with pore size of 0.22 μm and diameter of 47 mm (Tianjin Jinteng Experimental Equipment Co. LTD, China) for downstream analysis.

2.3. DNA extraction, PCR and sequencing

Genomic DNA was extracted from each sample with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The ITS regions were amplified using bar-coded primer pairs of ITS1F (5′-CTT GGT CAT TTA GAG GAA GTAA-3′) and ITS2 (5′-GCT GCG TTC TTC ATC GAT GC-3′) synthetized by Major-bio. To achieve the sample multiplexing during pyrosequencing, barcodes were incorporated in the 5′ end of primers ITS1F and ITS2. PCR reactions were performed in a 20 μL mixture containing 0.8 μL of each primer at 5 μmol L^{-1}, 1.5 μL of template DNA (10 ng), 4 μL 5×FastPfu Buffer and 2 μL dNTPs at 2.5 μmol L^{-1} at the following thermal program: 95 °C for 3 min, 33 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, and an extension step at 72 °C for 10 min. Each sample was amplified in triplicate, and the PCR products were purified using an Agarose Gel DNA purification kit (TaKaRa, Dalian, China). An equal amount of the PCR product from each sample was combined in a single tube and run on an Illumina Miseq PE250 sequencing platform at Major-bio Bio-Pharm Technology Co., Ltd., Shanghai, China.

2.4. Data analysis

The raw sequences were trimmed to remove the sequences with poor quality. After trimming, qualified data were clustered to obtain the operational taxonomic units (OTUs) as described by Li et al. (2015) and analyzed using the script of Mothur v.1.30.1 (http://www.mothur.org) to obtain rarefaction curve, abundance base coverage estimator (ACE), richness (Chao), Shannon diversity and Simpson diversity. Good's coverage analysis was used to assign the sequences, and operational taxonomic units (OTUs, 97 % similarity) were further analyzed using the furthest neighbor approach. For taxonomy-based analysis, the Unite database project (http://unite.ut.ee/index.php) was used as a repository for aligned ITS sequences.

3. Results

3.1. Overall analysis of sequencing

A total of 681 646 sequences with good quality were obtained. Each sample had (20 306–37 232 sequences with mean of 28 402). Among them, 99.2 % were classified at the phylum level, indicating that the design of Illumina sequencing method was reasonable and sufficient, and could be used for analyzing the fungal diversity on the surface of Kyoho grape berries.

3.2. Fungal α-Diversity

The ecological diversity of epiphytic fungal communities on the surface of Kyoho grape berries was estimated by various indexes (Table 1). All qualified sequences were grouped into OTUs at a distance level of 3 % to estimate the phylogenetic diversities of fungal communities. Student's t-test for Shannon index indicated that the components of fungal community varied significantly during both two ripening seasons in a year (Fig. 1). Fig. 2 shows the rarefaction curves of the 8 groups (three duplicates per group) generated at cutoff of 0.03. It is clear from Fig. 2 that all curves approached plateaus, indicating that the sequencing depths were enough to cover the whole microbial diversity of each sample.

3.3. Taxonomic composition

All 396 OTUs from the 8 groups (three duplicates per group) were classified into 2 phyla, 76 families and 162 genera, including unclassified. Table 2 shows the taxonomical classification at the phylum level. It is clear that the Ascomycota were the dominant type, comprising approximately from 55.04 % to 99.50 % reads across all samples with mean OTUs abundance across all groups from 60.88 % to 99.20 %. Basidiomycota were the second most abundant phylum in all samples with abundance from 0.49 % to 44.81 % across all samples and mean abundance from 0.77 % to 38.56 % across all groups. The average reads of unclassified group were insignificant, ranging from 0.01 % to 0.69 % across all samples.

Table 1
Richness and diversity indexes relative to each sample (OTU cutoff of 0.03).

ID	Growth stages	Reads	OTUs	Diversity and richness index			
				Shannon	Chao	ACE	Coverage
S1A	BRB	30 072	124	1.2907	147.71	194.85	0.9988
S1B	BRB	36 302	162	1.1338	218.08	305.86	0.9986
S1C	BRB	33 342	183	1.6091	177.20	186.23	0.9988
S2A	BV	25 021	142	1.6922	224.50	246.11	0.9980
S2B	BV	28 513	180	1.7377	172.00	169.75	0.9987
S2C	BV	26 757	202	1.8400	197.05	201.71	0.9983
S3A	BQR	24 157	151	1.8416	255.65	311.66	0.9975
S3B	BQR	27 006	192	1.9376	252.12	352.83	0.9978
S3C	BQR	35 620	218	2.0090	195.94	210.66	0.9987
S4A	HR	28 330	165	1.9282	207.13	217.30	0.9982
S4B	HR	23 740	205	2.0932	221.27	261.31	0.9978
S4C	HR	27 731	227	2.1534	236.62	256.11	0.9980
W1A	BRB	31 559	178	2.3053	214.16	223.41	0.9988
W1B	BRB	29 732	211	2.1852	214.36	230.01	0.9986
W1C	BRB	20 306	228	2.1164	202.14	205.68	0.9979
W2A	BV	31 981	154	2.4668	209.36	221.45	0.9986
W2B	BV	21 949	192	2.4531	180.17	193.18	0.9981
W2C	BV	23 714	211	2.4892	184.22	190.63	0.9984
W3A	BQR	29 707	139	2.1543	172.30	175.40	0.9988
W3B	BQR	31 023	166	2.3098	195.79	224.61	0.9986
W3C	BQR	27 987	184	2.1654	163.71	154.66	0.9988
W4A	HR	37 232	121	2.0873	203.75	222.66	0.9989
W4B	HR	22 778	148	1.9498	159.50	188.90	0.9984
W4C	HR	27 087	163	2.0596	156.62	174.59	0.9988

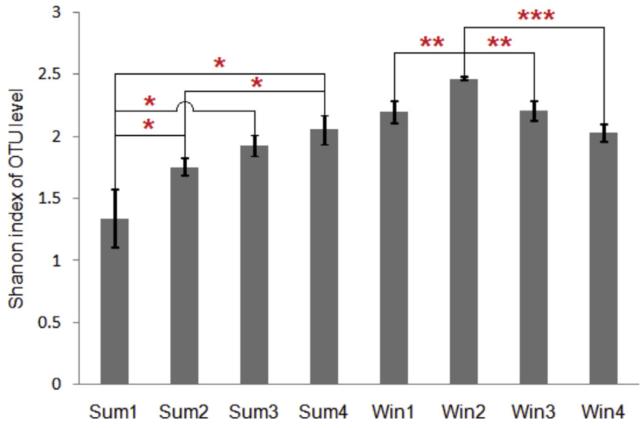

Fig. 1. Changes in diversity of epiphytic fungi communities during grape berries ripening process in summer and winter. Data are shown as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ by Student's *t*-test.

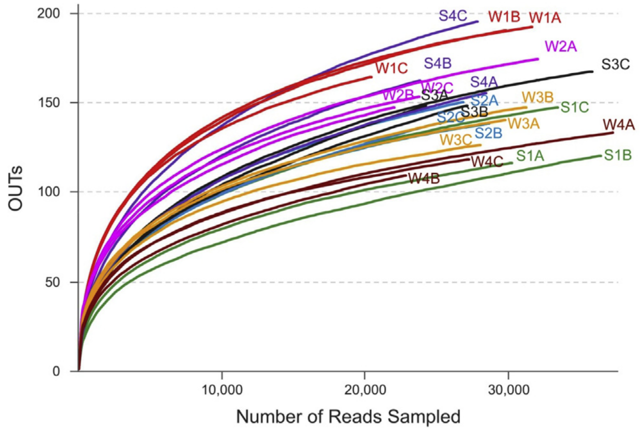

Fig. 2. Rarefaction curves of OTUs (Operational Taxonomic Units) clustered at 97 % phylotype similarity level in different samples.

Table 2
Fungal composition of the 8 groups of samples at phylum level.

	Ascomycota (%)	Basidiomycota (%)	Unclassified (%)
Sum1	99.20 ± 0.34	0.77 ± 0.32	0.03 ± 0.02
Sum2	97.75 ± 0.78	2.22 ± 0.78	0.03 ± 0.00
Sum3	92.68 ± 0.37	7.29 ± 0.37	0.03 ± 0.01
Sum4	93.35 ± 1.29	6.56 ± 1.31	0.09 ± 0.02
Win1	74.11 ± 4.13	25.74 ± 4.14	0.15 ± 0.02
Win2	62.23 ± 1.58	37.25 ± 1.53	0.51 ± 0.07
Win3	60.88 ± 8.20	38.56 ± 8.10	0.56 ± 0.14
Win4	61.70 ± 7.53	38.20 ± 7.50	0.10 ± 0.04

At family level, there were 6 shared families (abundance > 1 %) among the total 75 families existing in all samples (Fig. 3), namely, *Davidiellaceae*, *Ustilaginaceae*, *Mycosphaerellaceae*, *Nectriaceae*, *Trichocomaceae* and *Pleosporaceae*. *Davidiellaceae* was the most abundant family with abundance ranging from 66.14 % to 33.71 % across all groups. *Mycosphaerellaceae* was the second most abundant family in Sum 1, Sum 2, Sum 3 and Sum 4 with relative abundances of 0.65 %, 5.04 %, 12.19 % and 18.77 %, respectively. Unlike the Sum groups, the second most abundant family in Win groups was *Ustilaginaceae*, with abundances of 22.22 %, 24.95 %, 27.18 % and 34.54 % in Win 1, Win 2, Win 3 and Win 4, respectively. From Fig. 3 it was obvious that there were significant differences in fungal community between Sum and Win groups at the family level.

At genus level, *Cladosporium* was the dominant genus in Sum and Win groups, with abundance from 71.41 % to 32.06 % and 49.08 %–29.28 %, respectively (Fig. 4). Beyond that, epiphytic fungi communities varied significantly during grape berries ripening process in summer and winter. The second dominant genus in Sum groups was *Gyrothrix* with mean abundance from 4.84 % to 16.60 %. The second dominant genus in Win groups was *Pseudozyma* with mean abundance from 20.76 % to 33.45 %. Moreover, other genera with relatively high abundance were *Acremonium*, *Paramycosphaerella*, *Penicillium* and *Tilletiopsis* in Sum groups and *Gibberella*, *Colletotrichum*, *Acremonium*, *Sporobolomyces*, *Rhodosporidium*, *Alternaria* and *Aspergillus* in Win groups. Except for *Cladosporium*, *Acremonium* was the only common dominant genus between both harvest periods, with abundance from 11.13 % to 3.68 % in the summer and 3.76 %–0.66 % in the winter.

At species level, 23 common fungal species were found in all samples. The five dominant fungal species were *Cladosporium ramotenellum*, followed in turn by *Pseudozyma aphidis*, *Gyrothrix* spp., *Gibberella intricans* and *Acremonium alternatum* with abundance from 61.62 % to 91.26 % in our samples.

3.4. Fungal community variation

To compare the similarity and dissimilarity among all samples, we generated a heat map with hierarchical cluster based on Bray–Curtis distance (Fig. 5). Hierarchical cluster analysis showed that different patterns of community structure were observed between Sum groups and Win groups, while samples collected at the same or similar periods within the same ripening processes had similar community structure. The Bray–Curtis indices showed a higher similarity of fungal communities in the Sum groups than in the Win groups.

Concomitantly, principal component analysis (PCA) clearly showed variations between these two ripening processes (Fig. 6A). The first two principal components can explain 91.04 % of the total fungal community variations among individual samples. Fungal community compositions shift greatly between the Sum and Win

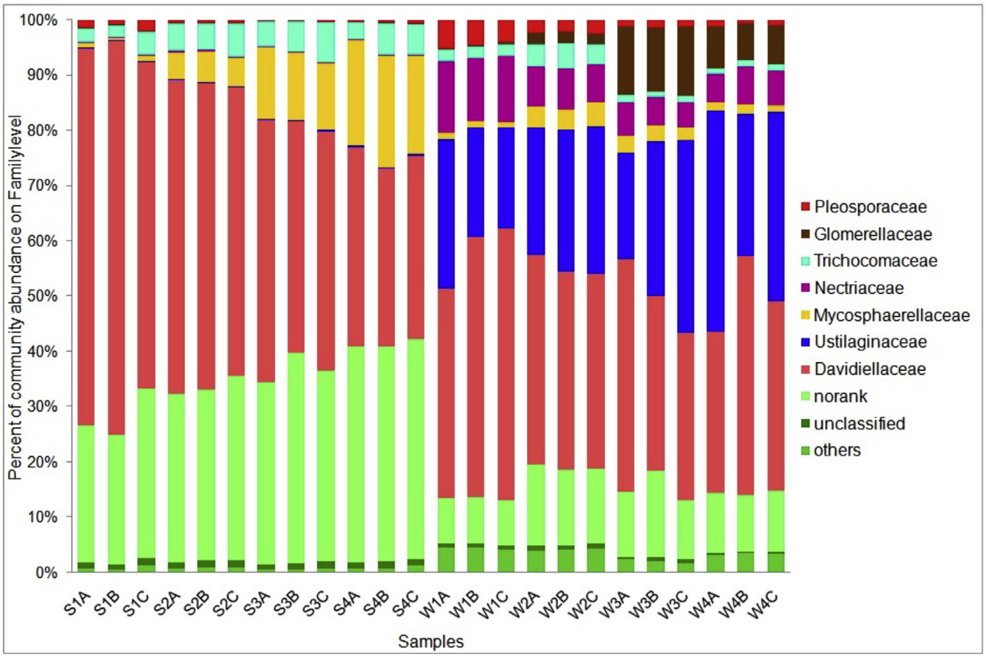

Fig. 3. Composition of different fungal communities at the family level. Shown are the relative read abundances of different fungal families within different communities. Sequences that could not be classified into any known group were assigned as 'unclassified'. _p_, phylum; _c_, class; _o_, order.

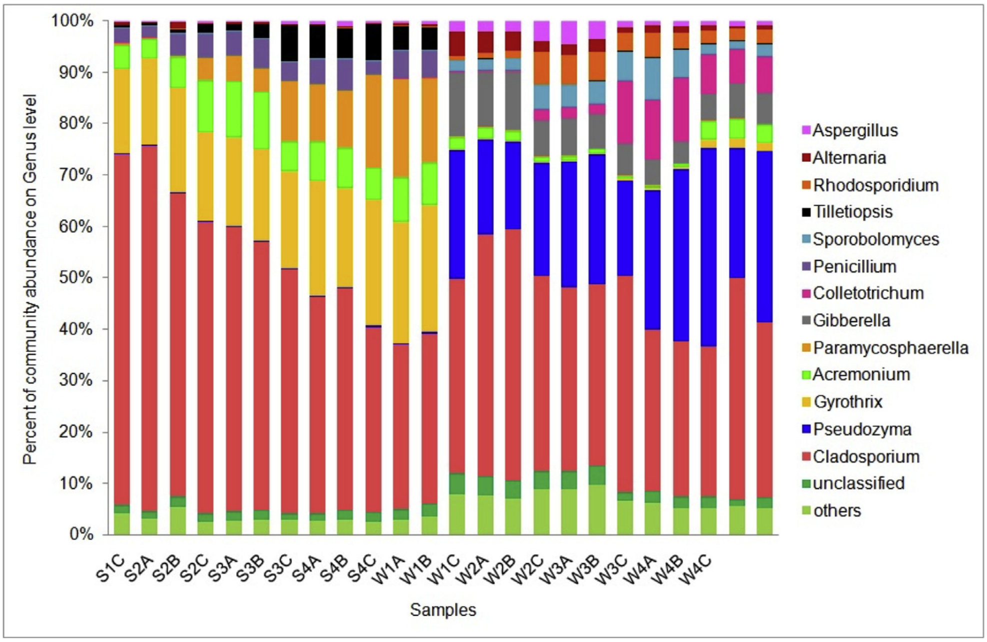

Fig. 4. Composition of different fungal communities at the genus level. Shown are the relative read abundances of different fungal genera within the different communities. Sequences that could not be classified into any known group were assigned as 'unclassified'. _p_, phylum; _c_, class; _o_, order; _f_, family.

ripening processes along the second principal component axis and this finding was confirmed by NMDS analysis (Fig. 6B).

3.5. Core fungal community of grape berries during ripening process in summer and winter

Two four-way Venn diagrams were created to visualize the core fungal communities in the Sum groups and Win groups. As can be seen from Fig. 7 that 64 genera were shared by all samples in the Sum groups (Fig. 7A), and 77 genera were shared by all samples in the Win groups (Fig. 7B). The structure of core fungal communities was significantly different between the ripening process in the summer and the winter (Fig. 7C, D). The dominant genera of core fungal community in the summer ripening process were *Cladosporium*, *Gyrothrix*, *Paramycosphaerella*, *Acremonium*, *Penicillium and Tilletiopsis*, while those in winter ripening process were *Cladosporium*, *Pseudozyma*, *Gibberella*, *Colletotrichum*, *Sporobolomyces*, *Rhodosporidium*, *Alternaria* and *Aspergillus*.

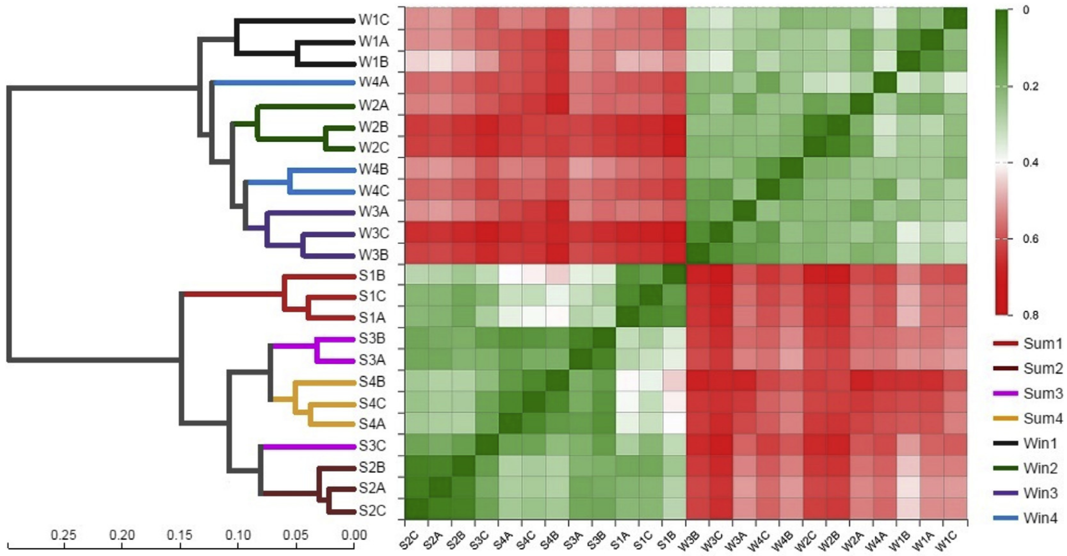

Fig. 5. Heat map of fungal communities based on Bray–Curtis distance indices. Clustering of samples based on Bray–Curtis distance indices were calculated by OTUs at a distance of 3 %. *Color from red to green indicated increased similarity.* (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

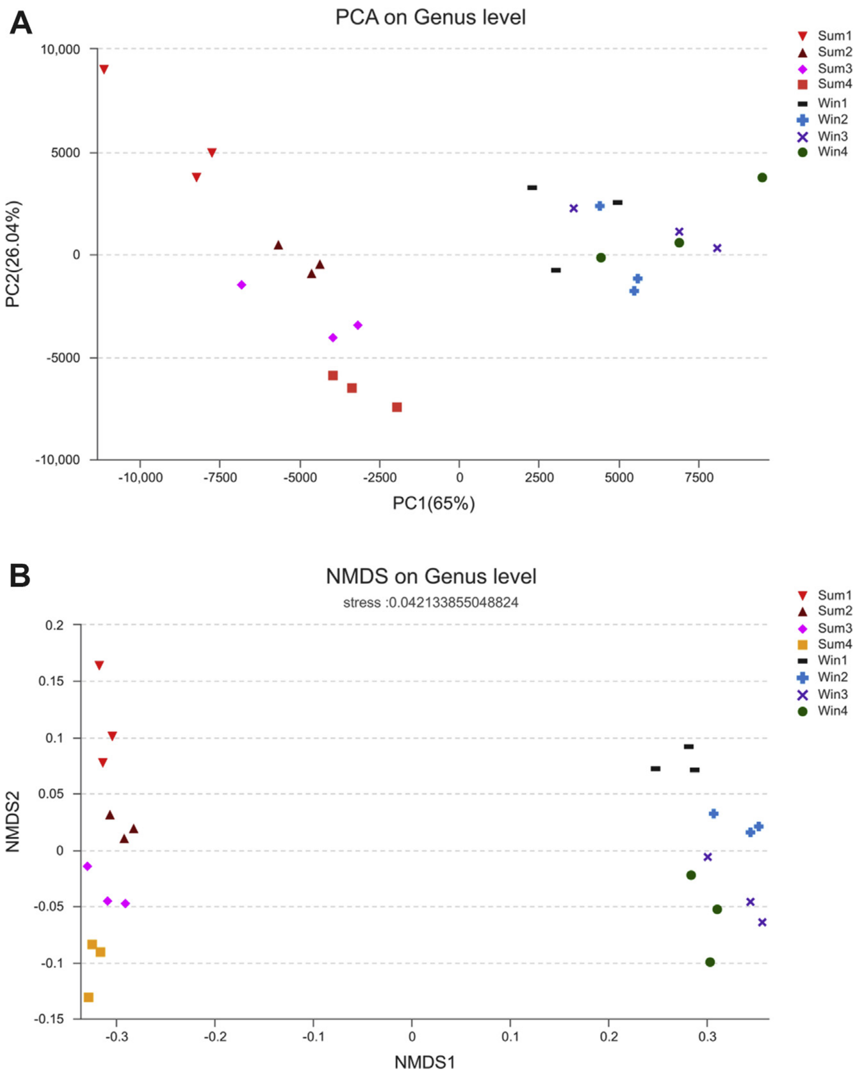

Fig. 6. Principal component analysis (PCA, Fig. 6(A)) and non-metric multidimensional scaling analysis (NMDS, Fig. 6(B)) of fungal communities based on OTUs at a distance of 3 % for individual samples from summer and winter ripening processes.

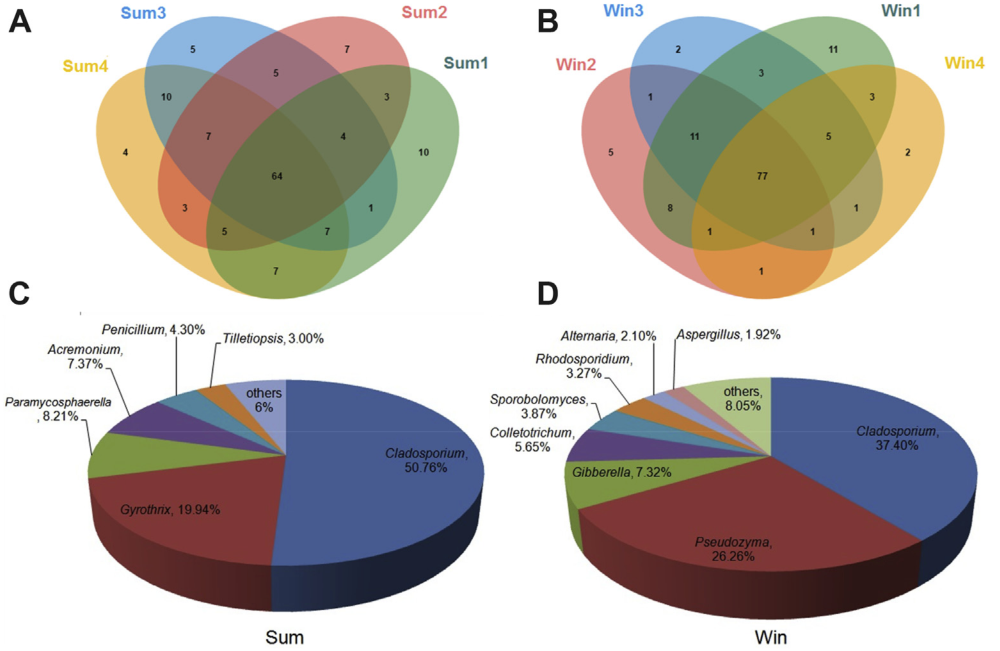

Fig. 7. Four-way Venn diagram of epiphytic fungal genera detected in grape berries during different harvest periods and the composition of corresponding core fungal communities. **(A)** Venn diagram of the summer harvest period; **(B)** Venn diagram of the winter harvest period; **(C)** Composition of core fungal community of summer harvest period; and **(D)** Composition of core fungal community of winter harvest period.

4. Discussion

The epiphytic fungal diversity in colonizing grape berries have been investigated previously (Cordero-Bueso et al., 2011; Grube et al., 2011; Martins et al., 2014; Milanović et al., 2013; Schmid et al., 2011). However, in some studies, the epiphytic microbiota were mostly analyzed using grape berries crushed with a stomacher machine. This subsequent enrichment steps inevitably induced microbial selection. In other studies, the epiphytic microbial communities were analyzed using microbial isolation and pure cultivation method. This method might miss a large number of uncultured microorganisms in epiphytic microbiome. In order to avoid these artifacts, in this study, the epiphytic community was collected directly from the grape berry surface by washing it with an isotonic solution and analyzed using culture-independent Illumina MiSeq.

As one of the most important temperate fruit, grape is harvested once a year in temperate winemaking regions such as Europe, USA and Australia. The epiphytic microbiota colonizing wine grape berries have been investigated to understand their impacts on wine quality (Pretorius, 2010; Verginer et al., 2010). Yeasts and yeast-like fungi were the most studied fungi in epiphytic microbiota. *Crytococcus* spp., *Rhodotorula* spp., *Sporobolomyces* spp. and *Aureobasidium pullulans* were well known as the most dominant species isolated from European wine grape berries (Barata et al., 2012; Martins et al., 2014). On the other hand, epiphytic filamentous fungi are usually associated with plant diseases. Figuring out the community of epiphytic fungi may help protect potential diseases. *Botrytis fuckeliana* (*Botrytis cinerea*), *C.* spp., *Alternaria alternata* and *P.* spp. were repeatedly isolated from European wine grape as the dominant species (Kecskemeti et al., 2016; Diguta et al., 2011). Setati et al. (2015) demonstrated that *A. pullulans* and *Kabatiella microsticta* were dominant species in three South African vineyards, *B. fuckeliana*, *Neofusicoccum austral*, *Cladosporium cladosporioides*, *Davidiella tassiana*, *Lewia infectoria* and *Mucor* spp. were also abundant in their samples. They concluded that potential grapevine pathogens accounted for 50 % of the total fungi population in some samples. Bokulich et al. (2014) found that the fungal profiles of crushed Chardonnay and Cabernet Sauvignon berries from California were dominated by *Cladosporium* spp (28.2 % average relative abundance). *B. fuckeliana*, *Penicillium* spp., *D. tassiana* and *A. pullulans*. *Cladosporium* spp, *Penicillium* spp. and *Alternaria* spp. were also found in many samples from New Zealand (Taylor et al., 2014).

Our study showed that the most dominant fungal species in our samples was *C. ramotenellum* rather than *C. cladosporioides*. This phenomenon was less prevalent in previous researches about epiphytic microbiota from grape berries. The second most abundant species in the Sum groups and Win groups were *P. aphidis* and *Gyrothrix* spp., respectively. To our best knowledge, this is the first study reporting these two species as the dominant fungal species in grape epiphytic microbiota. Besides, *B. fuckeliana* accounted for less than 1 % of the total fungi population in our samples, which was significantly lower than the values of 5 %–90 % reported previously. These results indicate that the epiphytic fungal community on Kyoho grape berries surface from Nanning region was significantly different from that had been reported in other regions. Furthermore, the components of core fungal communities were significantly different between the ripening processes in summer and winter (Fig. 7C, D), indicating that even sampled from the same vineyard, the epiphytic fungal community on Kyoho grape berries surface during summer ripening process was significantly different from that during winter ripening process.

Cladosporium and *Alternaria* are most common and important fungal agents of grape bunch rot (Lorenzini and Zapparoli, 2014; Mahdian and Zafari, 2016). We have previously found that *Cladosporium* spp. and *Alternaria* spp. were the major pathogenic fungi inducing bunch rot of table grape in Guangxi province during harvest and post-harvest periods.

Colletotrichum spp. cause anthracnose in various fruits during harvest and post-harvest periods and are a particularly important problem in tropical and subtropical fruits such as avocado, guava, papaya, mango, passion fruit, banana, apple and grape (Peres et al., 2002). In previous researches, *C. gloeosporioides*, *C. capsici*, *C. fructicola* and *C. viniferum* were isolated from leaf, shoot and fruit lesions of vitaceous plants in Guangxi, Guizhou and Yunnan provinces, southwest China (Pan et al., 2016; Peng et al., 2013). In this research we found the abundance of *Colletotrichum* spp. in epiphytic microbiota on healthy berries increased significantly with grape ripening (Fig. 4). This phenomenon varies notably from the results of previous research (Kecskemeti et al., 2016) and probably causes serious ripe rot of grape berries during winter harvest periods in Guangxi region.

5. Conclusions

The most abundant epiphytic fungal taxa of Kyoho grape berries surface from Nanning region, a typical Subtropical region in southwest China, were investigated for the first time using Illumina MiSeq platforms. Our results contribute to the understanding of differences in epiphytic fungal communities of grape berries surface between subtropical region and temperate region. Regional patterns of epiphytic fungal communities also suggest that even in the same vineyard, the epiphytic fungal community on Kyoho grape berries surface during summer ripening process was significantly different from that during winter ripening process. This is of particular importance in view of the role epiphytic fungi in plant health and grape post-harvest periods. Further research is required to explore the effects of other factors such as usage of fruitbag, fungicides and plant hormones on the size and composition of the fungal communities on the grape berry surface.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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