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DNA sequence and community structure diversity of multi-year soil fungi in Grape of Xinjiang

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Abstract:

This study is designed to understand the community structure and diversity of fungi in the rhizosphere soil of grape. As the sample for this study, the rhizosphere soil of Crimson seedless grape with different planting years was collected from Shihezi in Xinjiang to carry out high-throughput sequencing, by which the complete sequence of soil fungi DNA was identified, and accordingly, the richness and diversity index of fungi were determined. The results showed that the dominant phyla of fungi in the grape rhizosphere soil with different planting years were *Ascomycota* and *Basidiomycota*, and the dominant classes of fungi were *Sordariomycetes* and *Dothideomycetes*. Soil organic matter, total potassium, total nitrogen and available phosphorus were the main soil fertility factors affecting the abundance and diversity of soil fungal communities, among which soil organic matter had the most significant influence. In addition, the fungal diversity and richness were highest in the middle layer (20-35 cm) of the grape rhizosphere soil with 12 planting years and lowest in the lower layer (35-50 cm) of the grape rhizosphere soil with 5 planting years. Linear discriminant analysis suggested that there were more biomarkers in the vineyard rhizosphere soil with 10 planting years, which meant there were more fungal communities with significant difference in the soil, especially in the middle layer (20-35). The results of this study can provide data reference and theoretical basis for improving vineyard soil quality, evaluating soil microecological effects and improving ecological environment of vineyard soil.

Key words: soil fungi, complete DNA sequence, community structure and diversity, vineyard, Xinjiang

1 Introduction

Grape is deeply loved by many people because of its fresh taste, rich nutrition, skin-care and anti-aging effects. Xinjiang has a long history of grape cultivation, and it is the largest province and origin of grape cultivation in China. Because of its unique ecological and climatic conditions, Xinjiang is rich in high-quality grapes, which are excellent for eating directly, making raisin or making wine. As the key factor determining the value of grape products, grape quality is closely related to planting techniques and many ecological factors, which can directly affect the diversity of soil microbial community. Soil microorganisms are not only an important part of soil, but also a major contributor to soil nutrient cycling. To some extent, the change of microbial communities in soil reflects the change trend of soil quality^[1-2]. As the main members of soil microorganisms, soil fungi have important ecological status, since they can provide nutrients for crops, decompose soil organic matter, and play an important role in material circulation and energy transfer^[3-4]. Fungi in soil can also inhibit the growth of pathogenic bacteria by secreting antibacterial substances, thus reducing the incidence of crop diseases^[5]. Soil fungi can participate in the decomposition process of agricultural chemicals and enhance the bioavailability of soil elements. In the process of crop growth and development, different fungi may have different effects, some can harm the growth of crops, while some other can control crop diseases, so fungi play a vital role in agricultural ecosystems^[6]. Nowadays, most of the researches on microorganisms in grape rhizosphere soil focus on bacteria, but few reports focus on fungi, and there are a lot of unknown fungi knowledge to be discovered.

Characterized by high throughput, high sensitivity, high accuracy and low operating cost, high-throughput sequencing technology provides a convenient and efficient way for fungal ecology research. In this study, the technology was used to study the diversity and community structure of fungi in grape rhizosphere soil with different planting years and different soil depths and to explore the effects of soil physicochemical properties on the fungal

community structure and diversity, so as to provide theoretical basis for improving the ecological environment of vineyard soil, improving soil quality and preventing grape diseases.

2 Materials and methods

2.1 Location

Soil samples were taken on August 15, 2018 in the grape planting experimental base in Shihezi Grape Research Institute, Xinjiang. The shortest planting age of grape was 5 years and the longest was 15 years. The geographical coordinates of sample taking places were 44°17' - 44°35' north latitude, 86°11' - 86°20' east longitude, and the altitude was 480m. The annual average temperature is 6.5 - 7.2°C, the annual precipitation is 125.0 - 207.7mm, and the annual sunshine hours are 2721 - 2818 h. The vineyard soil type is sandy loam, and the main cultivated variety is Crimson seedless grapes for fresh eating.

2.2 Sample collection

Grapes are perennial plants, and their absorption roots are mostly distributed within 50 cm horizontal distance from the trunk, so the sampling points were selected within 30-40 cm from the trunk. Grape root depth is usually distributed within 60 cm vertical distance below the surface, so the sampling depths were selected within 50 cm below the surface. From the same variety of grape plants with different ages (5, 8, 10, 12 and 15 years, denoted as ZN, XZ, HY, WH and GM, respectively), three plants were randomly selected for each age group. Around 30-40 cm from each grape plant, three points were selected for taking soil samples. After removing debris about 5cm from the surface, the upper layer (5-20cm), middle layer (20-35 cm) and lower layer (35-50cm) of soil were taken with earth boring augers at each point. With disposable sterilized rubber gloves, the soil was manually mixed, subpackaged and marked to make samples (about 500 g each) through quartering method. Each sample was divided into two parts, which were immediately put into two sterilized self-sealing bags with labels denoting the sampling time, place, soil depth and serial number. The samples were coded with a two-digit system, where the first digit indicated the replicate number and the second indicated the depth (1, 2 and 3 denoted the depth of 20, 35 and 50 cm, respectively). There were 5 age groups, each group included 3 plants, and for each plant the soil was taken at three depths; so, in total there were 45 soil samples (5 x 3 x 3). The fresh soil was immediately screened by 20-mesh sieve and divided into two parts, in which one was stored with liquid nitrogen and sent to Beijing Compass Biotechnology Co., Ltd for high-throughput sequencing, and the other was air-dried and stored for determining the physicochemical properties of soil. (See table 1 for sample coding information)

Table 1 Samples Coding Information

Sample code			Soil depth	Planting age/year	North Latitude	East Longitude	Elevation	Remarks
GM1.1	GM1.2	GM1.3	5-20cm					
GM2.1	GM2.2	GM2.3	20-35cm	15				
GM3.1	GM3.2	GM3.3	35-50cm					
HY1.1	HY1.2	HY1.3	5-20cm					
HY2.1	HY2.2	HY2.3	20-35cm	10				
HY3.1	HY3.2	HY3.3	35-50cm					
XZ1.1	XZ1.2	XZ1.3	5-20cm		44°17'	86°11'		
XZ2.1	XZ2.2	XZ2.3	20-35cm	8	-	-	480m	
XZ3.1	XZ3.2	XZ3.3	35-50cm		44°35'	86°20'		
ZN1.1	ZN1.2	ZN1.3	5-20cm					
ZN2.1	ZN2.2	ZN2.3	20-35cm	5				
ZN3.1	ZN3.2	ZN3.3	35-50cm					
WH1.1	WH1.2	WH1.3	5-20cm					
WH2.1	WH2.2	WH2.3	20-35cm	12				

2.3 Determination of soil physicochemical properties

The air-dried soil samples were tested for the following physicochemical factors: organic matter (SOM), total nitrogen (TN), total phosphorus (TP), total potassium (TK), nitrate nitrogen (XN), ammonium nitrogen (AN), available phosphorus (SP), available potassium (SK), pH value (1:5) and electrical conductivity (EC). The soil properties were determined in accordance with the methods in the textbook *Soil Agrochemical Analysis* compiled by Bao Shidan ^[7]. Specifically, SOM was determined by potassium dichromate titration with external heating; TN was determined with FOSS 1035 fully automatic nitrogen determinator by perchloric acid-sulfuric acid digestion method; TP was determined with Agilent Cary 60 UV-Vis spectrophotometer by perchloric acid-sulfuric acid digestion - molybdenum antimony D-iso ascorbic acid colorimetry (MADAC); TK was determined with Thermo Fisher Scientific S series atomic absorption spectrometer by perchloric acid-sulfuric acid digestion - atomic absorption method; XN and AN were determined with BRAN+LUEBBE continuous flow analyzer after extracting by 0.01 M calcium chloride; SP was determined with MADAC after extracting by sodium bicarbonate; SK was determined by atomic absorption method after extracting by ammonium acetate; pH was determined by Mettler Toledo FiveEasyTM Plus pH meter; and EC was determined by HANNA H1 2315 conductivity meter.

The 3 samples taken from the same layer of soil around each grape plant were mixed into one sample to determine their physicochemical properties. There were 5 age groups, and for each group the soil involved 3 depths, so there were 15 soil samples being determined for physicochemical properties. The soil physicochemical data were analyzed using SPSS Statistics 22.0.

2.4 DNA extraction and PCR amplification

Genomic DNA of the samples was extracted by CTAB method. Then, the purity and concentration of the DNA were detected with 1% agarose gel electrophoresis. A proper amount of sample DNA was taken into a centrifuge tube, where the sample was diluted to 1 ng/μl with sterile water. Using the diluted genomic DNA as template, specific primers with Barcode were selected according to the sequencing region to be used; Phusion[®] high-fidelity PCR master mixing with GC buffer (New England Biolabs) was used together with NEB high-efficiency and high-fidelity enzymes to ensure the amplification efficiency and accuracy for carrying out PCR. The internal transcription spacer (ITS) 36 technique was used to analyze the nucleic acid sequence, and the fungal primers were ITS1 primers (ITS5-1737F and ITS2-2043R). PCR products were detected by 2% agarose gel electrophoresis; according to the determined concentration, the samples were mixed up for isoconcentration, and then the PCR products were purified by electrophoresis with 1×TAE buffer and 2% agarose gel. Finally, the target bands were recovered by tapping.

GeneJET gel recovery kit (Thermo Scientific) was used for product purification. Ion plus Fragment Library Kit 48rxns (Thermo Fisher) was used to construct the library, which was then quantified and qualified by Qubit, and sequencing was carried out with Ion S5TMXL (Thermofisher).

2.5 Biological information analysis

Cutadapt(V1.9.1)^[8] was used to cut off the low-quality part of the reads; then, based on the reads obtained by Barcode, the data for each sample were separated. After cutting off Barcode and the primer sequences, raw reads were obtained. The raw reads were then detected with UCHIME Algorithm ^[9] and compared with gene annotation database to remove chimeric sequences^[10], so as to obtain the final clean reads.

Sequence clustering was carried out to all clean reads of the samples by using Uparse v7.0.1001^[11] at the default identity of 97% to obtain OTUs (Operational Taxonomic Units). The sequences with the highest frequency of occurrence were selected as the representative sequences according to the principle of algorithm. Specie annotation was

done to the representative sequences by the Blast method of Qiime version 1.9.1^[12], and specie annotation analysis were conducted as per the Unit database^[13] to calculate the community composition of each sample at each classification level. Using MUSCLE [14] (Version 3.8.31), the phylogenetic relationships of all OTUs' representative sequences were obtained. Finally, the data of each sample were homogenized.

Qiime (Version 1.9.1) was used to calculate OTU abundance, Alpha diversity and UniFrac distance. R software (Version 2.15.3) was used to plot PCA, PcoA and NMDS diagrams. LEfSe software was used for LDA Effect Size analysis at the default LDA score of 4.

3 Results and analysis

3.1 Soil physicochemical properties

The test results of physicochemical factors of the soil are shown in Table 2. In the soil with 15-year vines, the average contents of TK and SK were highest and the contents of SOM and TN were lowest. In the soil with 5-year vines, the contents of XN and SK were relatively higher, and the soil pH was between 7.86 and 7.98, thus it is alkaline soil.

The analysis of variance showed that grape planting year had significant effect on TK and SP ($P < 0.05$), and soil depth had significant effect on SN and SK ($P < 0.05$). Among the physicochemical indices, organic matter had an extremely significant effect on TN and TK ($P < 0.01$), while TN had a significant effect on TK and XN ($P < 0.05$). AN was closely related to XN ($P < 0.05$), and XN had a significant effect on pH value ($P < 0.05$).

Table 2 Determined results of soil physicochemical properties

Sample Name		XZ	HY	ZN	GM	WH
planting years		8	10	5	15	12
		Mean \pm standard deviation				
SOM	g/kg	49.9 \pm 4.66a	50.5 \pm 4.25a	52.4 \pm 3.72a	46.46 \pm 3.33a	53.37 \pm 2.13a
TN	g/kg	3.42 \pm 0.07a	3.36 \pm 0.22a	3.81 \pm 0.34a	3.29 \pm 0.32a	3.81 \pm 0.35a
TP	g/kg	1.16 \pm 0.06a	1.24 \pm 0.05a	1.2 \pm 0.01a	1.18 \pm 0.00a	1.19 \pm 0.06a
TK	g/kg	15.36 \pm 0.76ab	15.45 \pm 0.64ab	15.42 \pm 0.39ab	16.56 \pm 0.34a	15.28 \pm 0.28b
XN	mg/kg	36.8 \pm 22.93a	48.89 \pm 4.29a	57.81 \pm 31.86a	49.65 \pm 31.52a	52.57 \pm 21.63a
AN	mg/kg	11.39 \pm 2.65a	10.73 \pm 1.79a	13.53 \pm 0.44a	14.7 \pm 6.71a	16.51 \pm 1.50a
SP	mg/kg	44.59 \pm 10.04b	47.95 \pm 11.62b	65.11 \pm 4.72b	59.05 \pm 5.60b	89.71 \pm 10.68a
SK	mg/kg	137.78 \pm 24.8a	123.85 \pm 18.84a	139.69 \pm 3.39a	141.9 \pm 23.58a	124.18 \pm 0.64a
pH(1:5)		7.86 \pm 0.15a	7.98 \pm 0.03a	7.91 \pm 0.10a	7.93 \pm 0.04a	7.96 \pm 0.04a

Note: SOM: soil organic matter content; TN: total nitrogen content in soil; TP: total phosphorus content in soil; TK: total potassium content in soil; XN: nitrate nitrogen content in soil; AN: ammonium nitrogen content in soil; SP: available phosphorus content in soil; SK: available potassium content in soil. The data in the same row with different lower case letters denote that there is significant difference between the groups ($P < 0.05$).

3.2 Basic analysis of sequencing data

From the 45 soil samples, we obtained 3625859 original sequences, in which 3068591 were valid ones; so, from each sample, 68199 valid sequences were obtained on average, with the minimum number 59999 and the maximum number 76469. Based on the abundance of OTUs at 97% level, we used QIIME to process the OTUs for the valid sequences, and obtained a total of 31533 OTUs, among which the highest number of OTUs from single sample were 871. Then, the data of all samples were statistically analyzed in the terms of soil depth, and the result showed that the highest number of OTUs was 11144, occurring in the middle layer (25-35 cm) and the lowest number of OTUs was 9745, occurring in the lower layer (35-50 cm); and the data were also analyzed in the terms of grape planting year, and the result showed that the 12-year vine group (WH) had the highest number of OTUs, which was 7251, while the 5-year vine group (ZN) had the lowest number of OTUs, which was 5382. See Table 3 for the sequence number and OTU number of each soil sample, and see Table 4 for the sequence number and OTU number of soil samples in the terms of soil depth and grape planting year.

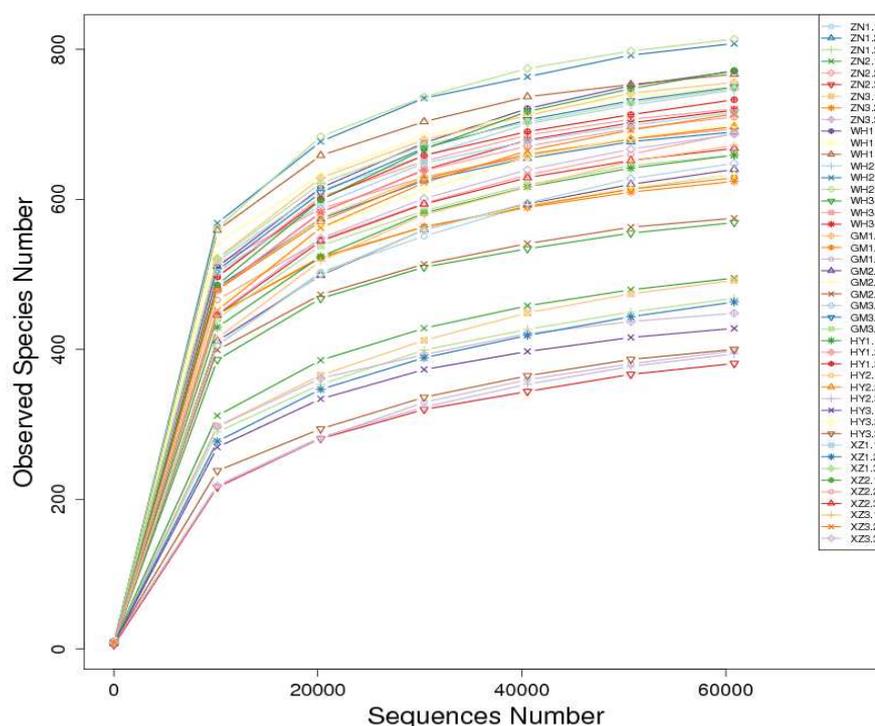
1 QIIME (Version 1.9.1) was used to generate rarefaction curves for the 45 samples (Fig. 1). Rarefaction curves can
 2 reveal the representativeness of samples, and they can be used to evaluate whether the current sequencing depth is
 3 enough to reflect the microbial diversity contained in the community samples. It can be seen from Fig. 1 that when the
 4 sequencing amount exceeded 60000 reads, there were still new OTUs appearing, but the curve had become flat,
 5 indicating that the sampling was basically reasonable, which meant the confidence of the bacterial community structure
 6 in the real environment was relatively high, in other words, the current sequencing depth was enough to reflect the
 7 diversity of fungal communities contained in the community sample.

8 Table 3 Sequence number and OUT number of soil samples

Sample_name	Total_tag	Taxon_tag	OUT_number
ZN1	236744	201439	1969
ZN2	241434	203245	1694
ZN3	244536	205176	1719
WH1	249005	207468	2496
WH2	240490	199669	2547
WH3	240451	200653	2208
GM1	248544	211312	1963
GM2	240600	200315	2052
GM3	240511	208883	2232
HY1	240458	202479	2309
HY2	246140	207273	1970
HY3	240621	214273	1697
XZ1	243371	205961	2128
XZ2	232276	196156	2270
XZ3	240678	204649	2279
Total	3625859	3068951	31533

9 Table 4 Sequence number and OTU number of soil samples in the terms of soil depth and grape planting year

Sample_name	Total_tag	Taxon_tag	OTU_num
ZN (5-year vines)	722714	609860	5382
XZ (8-year vines)	716325	606766	6677
HY (10-year vines)	727219	624025	5976
WH (12-year vines)	729946	607790	7251
GM (15-year vines)	729655	620510	6247
Upper layer (5-20 cm)	1208003	1018659	10644
Middle layer (25-35 cm)	1205536	1028143	11144
Lower layer (35-50 cm)	1212320	1022149	9745



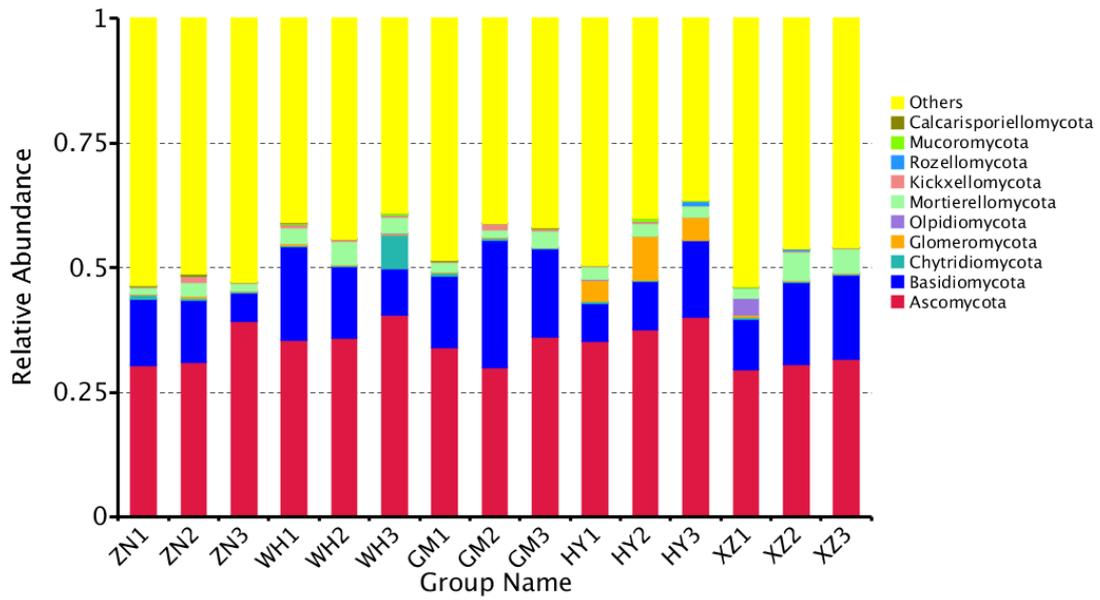
1 Fig. 1 Rarefaction curves at the OTUs similarity of 97%

2 In Fig. 1, the OTUs similarity of the samples is 97%. The abbreviations ZN, XZ, HY, WH, and GM represent the
 3 planting years of 5, 8, 10, 12 and 15, respectively. The first digit of sample name indicated the replicate number and the
 4 second indicated the soil depth (1, 2 and 3 denoted the soil depth of 5-20, 20-35 and 35-50 cm, respectively).

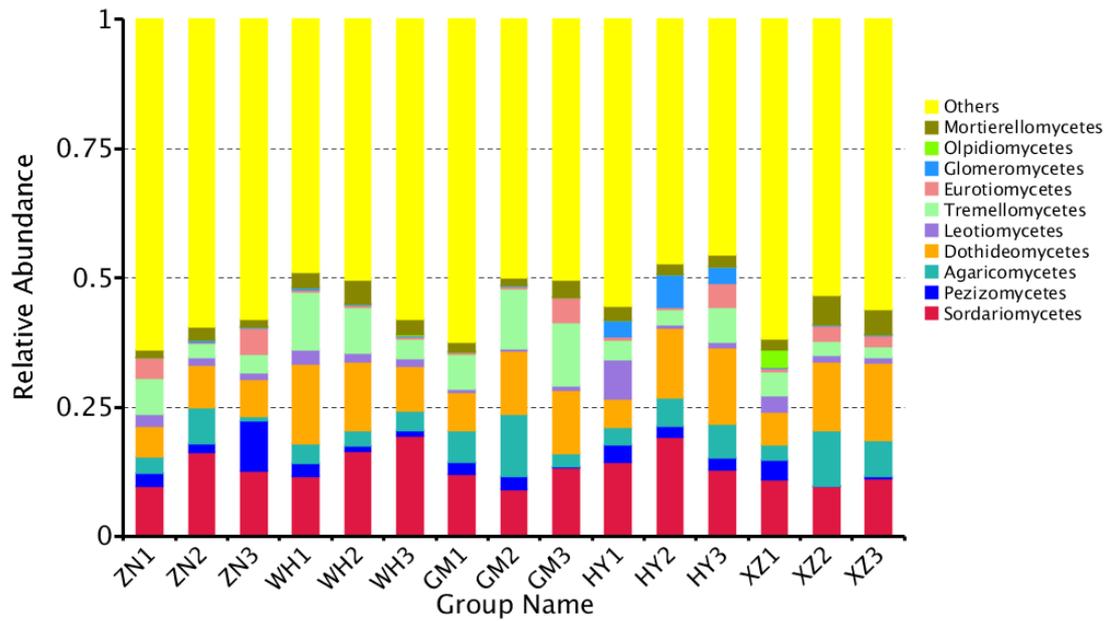
5 3.3 Analysis of composition and abundance of soil fungi

6 Species composition analysis reflects the community structure of samples at different taxonomic levels. At the
 7 phylum level, as shown in Fig. 2, the main dominant fungi in soil were *Ascomycota* with an average relative abundance
 8 of 34.49%, *Basidiomycota* with an average relative abundance of 13.85%, *Mortierellomycota* with an average relative
 9 abundance of 2.89%, *Glomeromycota* with an average relative abundance of 1.39%, and *Chytridiomycota* with an
 10 average relative abundance of 0.75%. The other dominant fungi included *Kickxellomycota*, *Olpidiomycota*,
 11 *Mucoromycota*, and *Rozellomycota*. The undetermined taxa of fungi in soil samples accounted for 45.67%. Analysis of
 12 variance (ANOVA) showed that the grape planting year had a significant effect on the abundance of *Glomeromycota*
 13 and *Mucoromycota* ($P < 0.05$) and the soil depth had a significant effect on the abundance of *Ascomycota* ($P < 0.05$).

14 At the class level, as shown in Fig. 3, the main dominant taxa were *Sordariomycetes*, *Dothideomycetes*,
 15 *Tremellomycetes*, *Agaricomycetes*, and *Tremellomycetes*, and their average relative abundance were 13.23%, 10.60%,
 16 6.07%, 5.22%, and 2.43%, respectively. The undetermined taxa at class level accounted for 54.69%. ANOVA analysis
 17 showed that grape planting year had a significant effect on the abundance of *Tremellomycetes* ($P < 0.05$), and soil depth
 18 had a significant effect on the abundance of *Agaricomycetes* ($P < 0.05$).



1 Fig. 2 Relative abundance of top 10 fungal phyla



2 Fig. 3 Relative abundance of top 10 fungal classes

3 Fig. 2 shows the relative abundance of top 10 fungal phyla in grape rhizosphere soil, and Fig. 3 shows the relative
 4 abundance of top ten fungal classes in grape rhizosphere soil. The abbreviations ZN, XZ, HY, WH, GM represent the
 5 grape planting years of 5, 8, 10, 12 and 15, respectively; and the numbers 1, 2 and 3 represent the rhizosphere soil
 6 depths of 5-20, 20-35 and 35-50 cm, respectively.

7
 8

3.4 The effect of soil physicochemical properties on fungal community

Redundancy analysis (RDA) at phylum level was carried out to reveal the relationship between soil physicochemical factors and fungal communities (Fig. 4). The first two axes of RDA analysis explained the 70.44% variations of fungal communities, in which the first axis explained 50.7% and the second axis explained 19.74%. SOM had the most significant effect on fungal communities, followed by TK, and the effects of soil physicochemical properties on fungal communities were in the order of SOM > TK > TN > TP > SK > pH > XN > SP > AN.

In this study, Spearman analysis was used to evaluate the correlation between the abundance of top 13 dominant fungal taxa at the phylum level and the soil physicochemical properties, as shown in Fig. 5. The results showed that the abundance of *Basidiomycota* was negatively significantly correlated with TP ($P < 0.05$) and extremely negatively correlated with SOM ($P < 0.01$); the abundance of *Glomeromycota* was negatively significantly correlated with AN ($P < 0.05$) and extremely positively correlated with XN ($P < 0.01$); the abundance of *Kickxellomycota* was positively significantly correlated to TK, SP and pH ($P < 0.05$); the abundance of *Zoopagomycota* was positively significantly correlated with SP ($P < 0.05$) and extremely positively correlated to AN ($P < 0.01$); the abundance of *Chytridiomycota* was positively significantly correlated to XN ($P < 0.05$); the abundance of *Mortierellomycota* was positively significantly correlated to pH ($P < 0.05$); and the abundance of *Blastocladiomycota* was negatively significantly correlated with TN ($P < 0.05$). For other dominant fungal phyla, the correlation between their abundance and soil physicochemical factors were not significant.

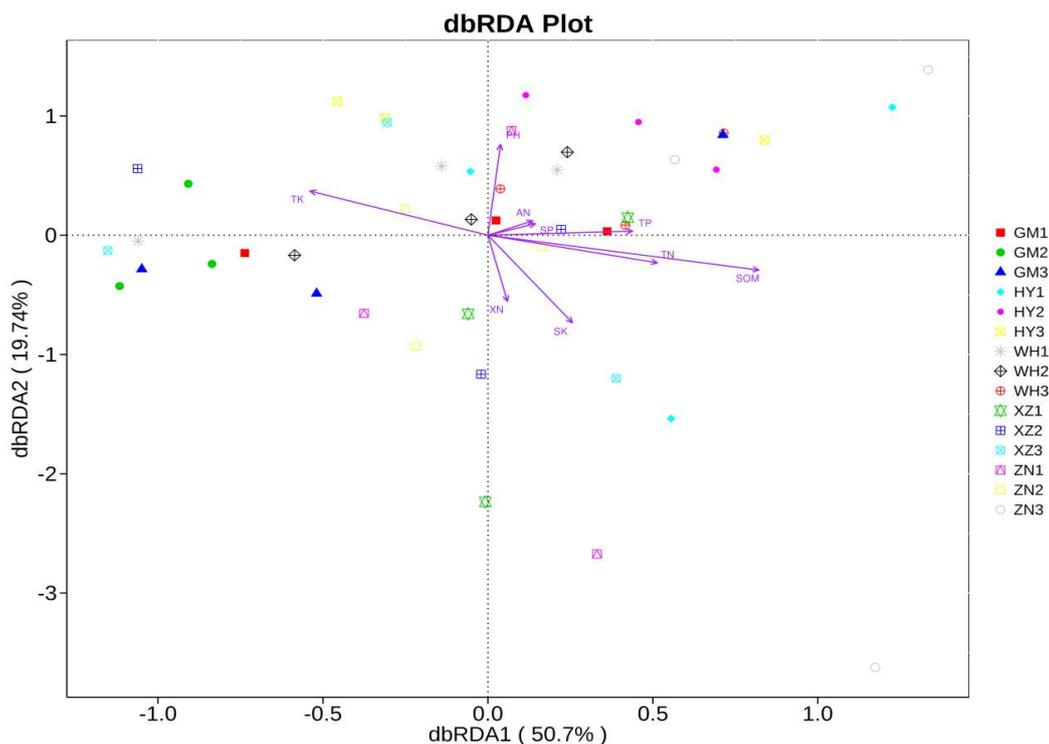


Fig. 4 Redundancy analysis of soil physicochemical indices and fungal phyla

The abbreviations ZN, XZ, HY, WH, GM represent the grape planting years of 5, 8, 10, 12 and 15, respectively; and the numbers 1, 2 and 3 represent the rhizosphere soil depths of 5-20, 20-35 and 35-50 cm, respectively.

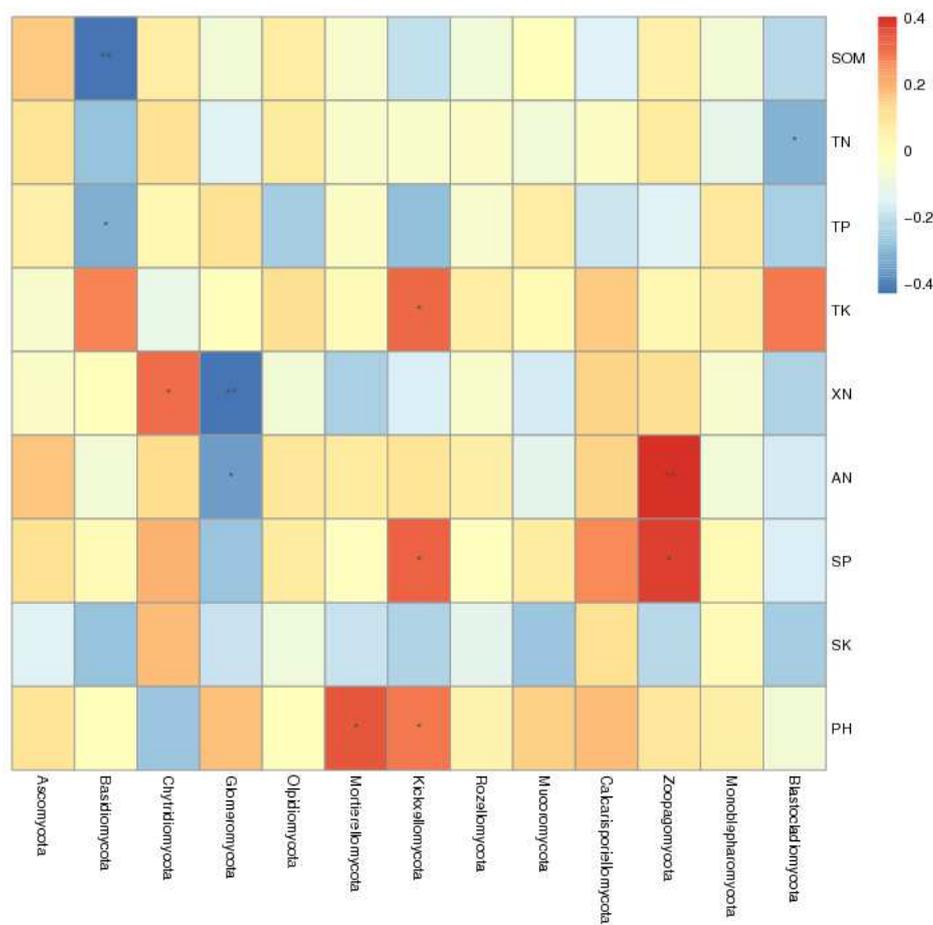


Fig. 5 Spearman analysis between soil physicochemical factors and fungal phyla

SOM: soil organic matter content; TN: total nitrogen content in soil; TP: total phosphorus content in soil; TK: total potassium content in soil; XN: nitrate nitrogen content in soil; AN: ammonium nitrogen content in soil; SP: available phosphorus content in soil; SK: available potassium content in soil; PH: soil pH value.

3.5 Analysis of soil fungi diversity

Alpha diversity represents the diversity of species in a specific ecosystem, and it is mainly related to two factors, one is the number of species, i.e., richness, and the other is diversity, i.e., the uniformity of individual distribution in the community. The community richness indices mainly include Chao index and Ace index. The larger Chao and Ace, the more OTUs in the community, and the higher the community richness. Community diversity indices mainly include Shannon index and Simpson index. The greater Shannon index, the higher community diversity; on the contrary, the higher Simpson index, the lower community diversity [13, 14].

The community richness indices Chao1 and ACE and diversity indices Shannon and Simpson of each sample are shown in Tables 5-1 and 5-2.

Table 5-1 Alpha diversity indices of fungal communities in the soil with different years of grape planting

Sample name	Observed species	Shannon	Simpson	Chao1	ACE	Good's coverage
ZN	547 ± 114c	5.99 ± 1.15a	0.94 ± 0.07a	597 ± 112b	605 ± 108c	0.99 ± 0.000a
WH	740 ± 72a	6.71 ± 0.32a	0.97 ± 0.01a	787 ± 79a	791 ± 77a	0.99 ± 0.000a
GM	632 ± 105abc	6.19 ± 0.51a	0.96 ± 0.02a	683 ± 103ab	690 ± 100abc	0.99 ± 0.000a
HY	602 ± 147bc	6.15 ± 0.65a	0.96 ± 0.04a	653 ± 144b	659 ± 138c	0.99 ± 0.001a
XZ	680 ± 88ab	6.16 ± 0.48a	0.96 ± 0.04a	785 ± 141a	761 ± 92ab	0.99 ± 0.000a

Table 5-2 Alpha diversity indices of fungal communities in different soil depths

Sample name	Observed species	Shannon	Simpson	Chao1	ACE	Good's coverage
5-20cm	646±4107ab	6.21±0.58a	0.96±0.036a	704±115ab	710±112ab	0.99±0.005a
20-35cm	684±78a	6.55±0.37a	0.97±0.008a	759±115a	746±77a	0.99±0.004a
35-50cm	590±159b	5.96±0.93a	0.95±0.055a	640±114b	648±149b	0.99±0.005a

Note: The abbreviations ZN, XZ, HY, WH, GM represent the grape planting years of 5, 8, 10, 12 and 15, respectively. The data in the same column with different lower case letters denote that there is significant difference between the groups ($P < 0.05$).

According to the analysis in the terms of grape planting years, as per the value of Chao1 and ACE indices, the sample groups were in the order of WH (12-year vines) > XZ (8-year vines) > GM (15-year vines) > HY (10-year vines) > ZN (5-year vines), indicating the richness of fungi in the soil with 12-year vines was the highest and that of the 5-year vines was the lowest. As per the value of Shannon index, the sample groups were in the order of WH (12-year vines) > GM (15-year vines) > XZ (8-year vines) > HY (10-year vines) > ZN (5-year vines), indicating the diversity of fungi in the soil with 12-year vines was the highest and that of the 5-year vines was the lowest. On the whole, the diversity and richness of fungi in the soil with 12-year vines was the highest, while that of 5-year vines was the lowest.

According to the analysis in the terms of soil depth, as per the values of Chao1, ACE and Shannon indices, the sample groups were in the order of middle layer group (20-35 cm) > upper layer group (5-20 cm) > lower layer group (35-50 cm), indicating that the fungal diversity and richness were highest in the middle layer and lowest in the lower layer of soil.

Through correlation analysis of soil physicochemical indicators on the Alpha diversity indices of fungal communities, the results showed that SP had a positively significant correlation with Shannon index, and SP had an extremely positive correlation with fungal community diversity, while other physicochemical indicators had no significant correlation with the Alpha diversity indices.

Nonmetric Multidimensional Scaling (NMDS) is often used to compare the differences between sample groups. In this study, NMDS analysis was applied to reflect the species information contained in samples into multi-dimensional space in the form of points. The degree of differences between different samples is reflected by the distance between points, which can reflect both the differences between and within sample groups. From Fig. 6, it can be seen that samples ZN1 (the upper layer of soil with 5-year vines), ZN3 (the lower layer of soil with 5-year vines) and HY3 (the lower layer of soil with 10-year vines) are far away from other samples, indicating that the fungal community structure of those samples was quite different from that of other samples. The close distance between other samples indicates that their similarity of fungal community structure was higher.

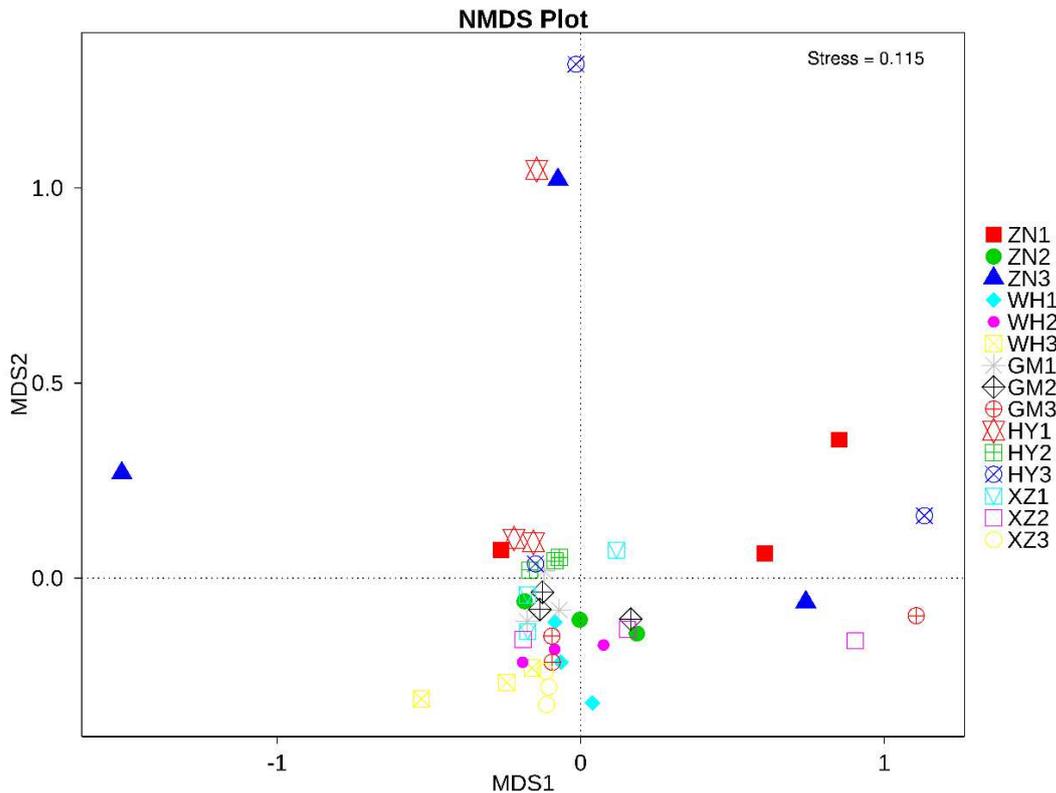


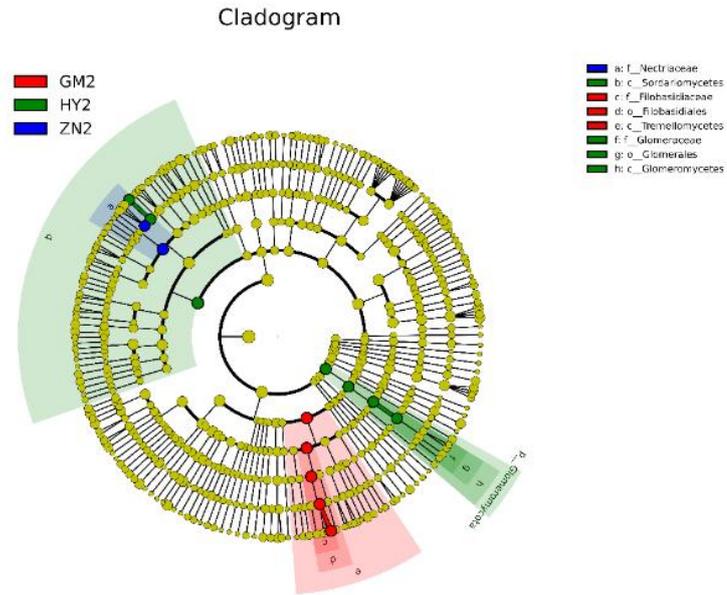
Fig. 6 NMDS analysis of soil fungi

Note: Each point in the figure represents a sample, the distance between points indicates the degree of difference, and the samples in the same group are represented by the same color. When *Stress* is less than 0.2, NMDS can accurately reflect the difference between samples. The abbreviations ZN, XZ, HY, WH, GM represent the soil sample groups with the grape planting years of 5, 8, 10, 12 and 15, respectively, and the numbers 1, 2 and 3 represent the soil depths of 5-20 cm, 20-35 cm and 35-50 cm, respectively.

3.6 LEfSe (LDA Effect Size) analysis

LEfSe was used to identify fungal species with significant differences in the terms of grape planting years and soil depths. Here, only the cladogram is showed. Firstly, the LEfSe analysis was carried out for different samples from the same soil depth, and the LDA score of 4 was used to identify statistically significant difference between fungal taxa. In the middle layer (20-35 cm), there were 14 taxa with LDA scores higher than 4, including 5 taxa of GM2 (15-year vine), 7 taxa of HY2 (10-year vine), and 2 taxa of ZN2 (5-year vine) (**Fig. 7a**). In the upper layer (5-20cm) and the lower layer (35-50 layer), no taxa with significant difference were observed.

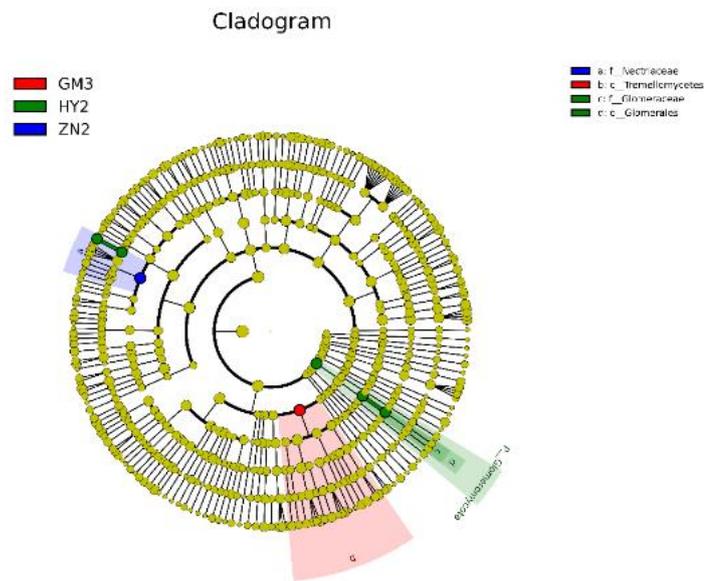
Then, the LEfSe analysis was carried out for samples from different soil depths, the results found there were 7 fungal taxa whose LDA scores were higher than 4, indicating they had statistically significant difference. The 7 fungal taxa included 1 taxa in the lower layer (35-50 cm) of GM3 (15-year vine), 5 taxa in the middle layer (15-25 cm) of HY2 (10-year vine), and 1 taxon in the middle layer (20-35 cm) of ZN2 (10-year vine), as shown in **Fig. 7b**. Generally, HY (15-year vine) sample group had more biomarkers than the groups of other planting years, especially in the middle layer (20-35 cm) there were more fungal communities with significant difference.



1

2

Fig. 7a Cladogram of LEfSe analysis



3

Fig. 7b Cladogram of LEfSe analysis

4 In the cladograms, circles radiated from inside to outside represent the taxonomic ranks from phylum to genus (or
 5 species). Each circle at different taxonomic ranks represents a classification at that level, and the diameter of the circle
 6 is proportional to the relative abundance. The coloring principle is that the species with no significant difference are
 7 uniformly colored in yellow, and the Biomarkers of different species follow the group for coloring. The red nodes
 8 indicate the fungal taxa that play an important role in the red group, while the green nodes indicate the fungal taxa that
 9 play an important role in the green group. If a certain group in the picture is missing, it means that there is no species
 10 with significant difference in that group. The names of species represented by English letters in the figure are displayed
 11 in the legend on the right. The abbreviations ZN, XZ, HY, WH, GM represent the grape planting years of 5, 8, 10, 12

1 and 15, respectively; and the numbers 1, 2 and 3 represent the rhizosphere soil depths of 5-20, 20-35 and 35-50 cm,
2 respectively. Statistically significant difference is defined by $LDA > 4$.

3 **4 Discussion**

4 In this study, 45 soil samples from vineyards were tested for ITS rRNA gene of fungi by high-throughput
5 sequencing, obtaining 31533 OTUs at 97% similarity. In the terms of soil depth, the number of OTUs was highest for
6 the samples from the middle layer and lowest for the samples from the lower layer. In the terms of grape planting year,
7 the number of OTUs was highest for the WH group (12-year vines) and lowest for the ZN group (5-year group). The
8 results indicated that the distribution of fungal communities were different in different depth of soil with different
9 planting years of grape. From the vertical direction of soil, the 10-30 cm layer was the cultivated layer as well as root
10 distribution zone, and we found there were most microbial species living in that layer, and the finding is similar to the
11 research of Luan Liying et al ^[15]; the reason may be that the cultivated layer has stable temperature and moisture,
12 abundant organic matter, sufficient water and fertilizer, which are all beneficial for the reproduction of microorganisms
13 ^[16].

14 In this study, the dominant fungal phyla in the soil samples were *Ascomycota*, *Basidiomycota*, *Mortierellomycota*,
15 *Glomeromycota*, and *Chytridiomycota*, in which *Ascomycota* was the absolute dominant fungal phyla, followed by
16 *Basidiomycota*. The result is consistent with the findings of Wang Yanyun, Li Yuekun and Fu Yajuan, et al^[17-19],
17 indicating that *Ascomycota* and *Basidiomycota* have wide adaptability and are distributed under different soil conditions.
18 The two fungal phyla are the key floras for decomposing cellulose in soil organic matter^[20], and most *Ascomycota* are
19 saprophytic fungi, which play an important role in degrading soil organic matter and are liable to decompose degradable
20 components in organic materials ^[21]. Yelle D J et al. ^[22] reported that *Basidiomycota* can degrade lignocellulose greatly
21 to form mycorrhiza with plants, thus promoting the absorption of water and mineral elements by roots and improving
22 the physical properties of soil. In addition, *Basidiomycota* can secrete antibacterial substances to inhibit the growth of
23 soil pathogenic bacteria to protect host plants ^[23].

24 At the class level, the dominant taxa were *Sordariomycetes*, *Dothideomycetes*, *Tremellomycetes*, and
25 *Agaricomycetes*, in which *Sordariomycetes* and *Dothideomycetes* were the most dominant fungal classes, having their
26 relative abundance higher than 10%. *Ascomycetes* play a key role in decomposition of SOM as well as a dominant role
27 in fungal communities through participating in the secretion, assimilation and SOM degradation of roots.

28 In order to understand the effect of soil physicochemical factors on soil fungal communities, redundancy analysis
29 (RDA) was carried out to soil fungal communities at the class level. The result showed that SOM had the most
30 significant effect on fungal communities, followed by soil TP and TN. Spearman analysis showed that the dominant
31 *Basidiomycota* was negatively significantly correlated with TP and extremely negatively correlated with SOM. The
32 *Kickxellomycota* were positively significantly correlated with TK, SP and pH. The correlation analysis between soil
33 physicochemical properties and Alpha diversity of fungal communities showed that SP was positively significantly
34 correlated with Shannon index. The above results indicated that SOM, TK, TN and SP were the main soil fertility
35 factors affecting the abundance and diversity of soil fungal communities, suggesting that such soil factors played an
36 important role in the genetic diversity of soil fungal communities in this study. The result is similar to the findings of
37 Yang Junbo ^[24] and Wu Lisha et al^[25].

38 Alpha diversity analysis showed that the diversity and richness of fungi was highest in the soil with 12-year vines
39 and lowest in the soil with 5-year vines. The diversity and richness of fungi were highest in the middle layer and lowest
40 in the lower layer (25-35cm). Currently, most studies believe that, after continuous cropping, the number of bacteria and
41 *Actinomycetes* in soil will decrease and the number of fungi will increase, which means continuous cropping can
42 transform the soil from "bacterial type" with high fertilizer to "fungal type" with low fertilizer ^[26]. With the increase of

1 grape planting years, the accumulation of toxic substances secreted by roots was beneficial to the reproduction of fungi
2 in soil and increased the diversity and abundance of fungi. Xiao Hong et al. found that the number of bacteria in the soil
3 of new orchard was higher than that in the soil of continuously cropped orchard, but the number of fungi in the former
4 was lower than that in the latter, and the overall soil condition of new orchard was better than that of the continuously
5 cropped orchard [27]; meanwhile, the structure of microbial communities in the orchard soil also showed certain
6 regularity with the changes of time and space. Wang Shutong et al. found that with the extension of apple tree life, the
7 number of rhizosphere microorganisms first increased and then decreased [28]; rhizosphere bacteria and other
8 microorganisms in orchard mainly concentrate in the 0-40 cm layer of soil, showing a decreasing trend from top to
9 bottom [29].

10 LEfSe (LDA Effect Size) analysis showed that there were more Biomarks in the soil with 10-year vines than in the
11 soil with other planting years, which means there were significant difference fungal taxa in that group, especially in the
12 middle layer. With the increase of grape planting years, the number of biomarkers increased, indicating that continuous
13 cropping of grapes changed the community structure of soil fungi. Some studies have pointed out that the microbial
14 communities in plant rhizosphere are closely related to plant growth time and root exudates [30-31]. Root exudates not
15 only provide the necessary energy for rhizosphere microorganisms, but also directly affect the quantity and community
16 structure of rhizosphere microorganisms, and their effects sometimes are positive and sometimes negative. With the
17 increase of vine planting years, rhizosphere exudates gradually increase, which is beneficial to the growth and
18 reproduction of some fungi.

19 5 Conclusion

20 According to high-throughput sequencing, we found that in grape rhizosphere soil there was a large number of
21 fungi that adapted to environmental changes, and the structure of fungal communities showed difference both in the soil
22 with different planting years and in the different soil depths. Soil physicochemical properties, grape planting year and
23 soil depth all have certain effects on fungal diversity and community structure. The fungal taxa showed more significant
24 difference in the middle layer of soil with longer planting years, and the formation of those specific fungal groups may
25 be closely related to the root exudates of grape plants.

26 In this study, the community structure and diversity of rhizosphere soil fungi in vineyards were analyzed by high-
27 throughput sequencing , and more in-depth study needs to be done on the evaluation on microecological functions in
28 soil caused by the change of fungal abundance and the interaction between rhizosphere exudates and fungi.

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22 Author Contributions:

23 ProfessorTongLiu and Dr Feng Xue designed the experiment. Feng Xue analyzed the data and wrote the article.

24 Competing Interests:

25 The authors declare no competing interests

26

Figures

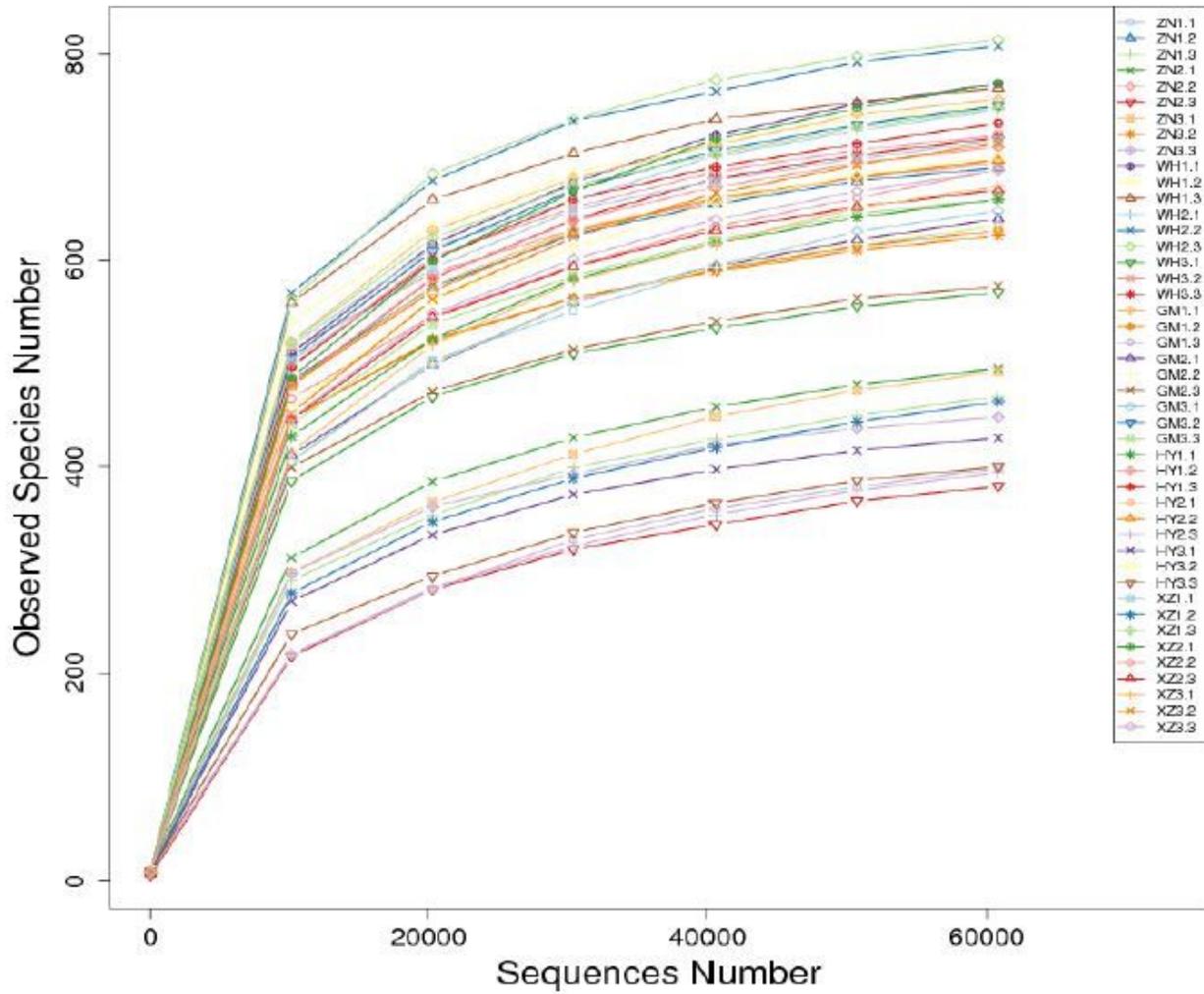


Figure 1

Rarefaction curves at the OTUs similarity of 97%

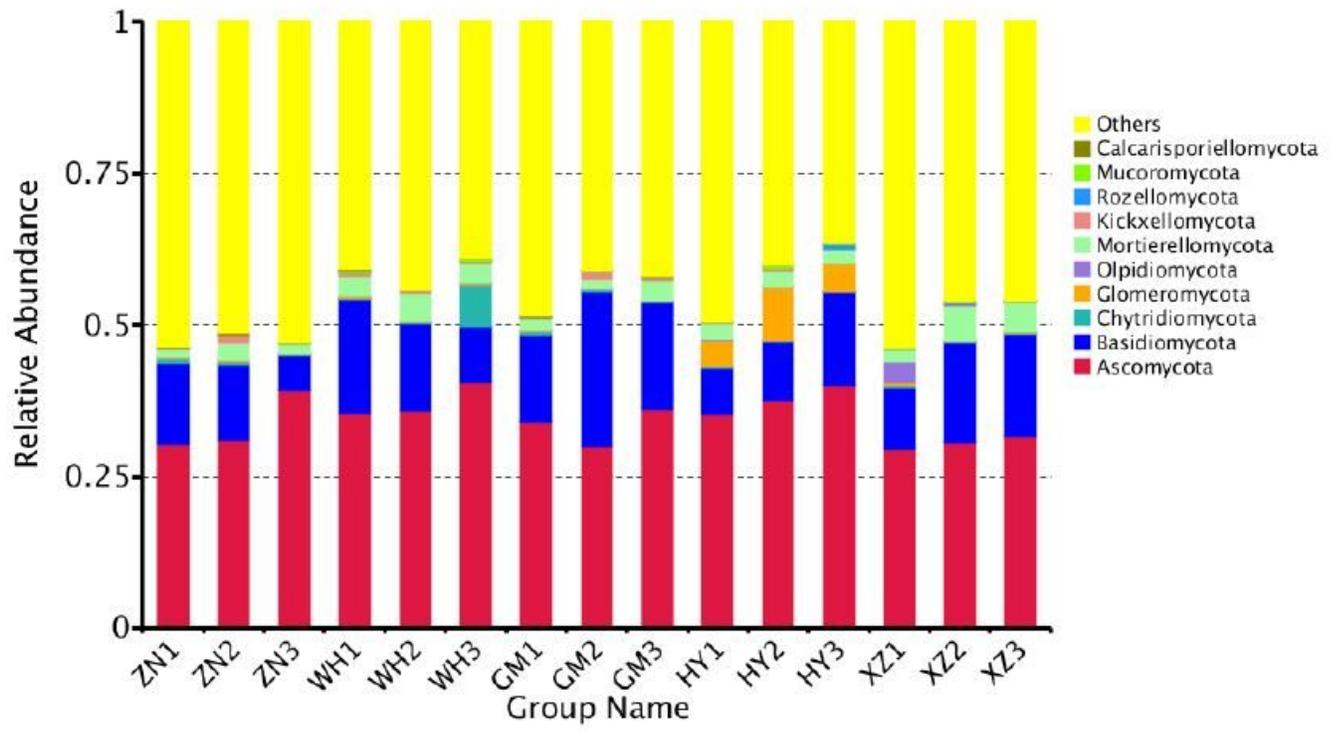


Figure 2

Relative abundance of top 10 fungal phyla

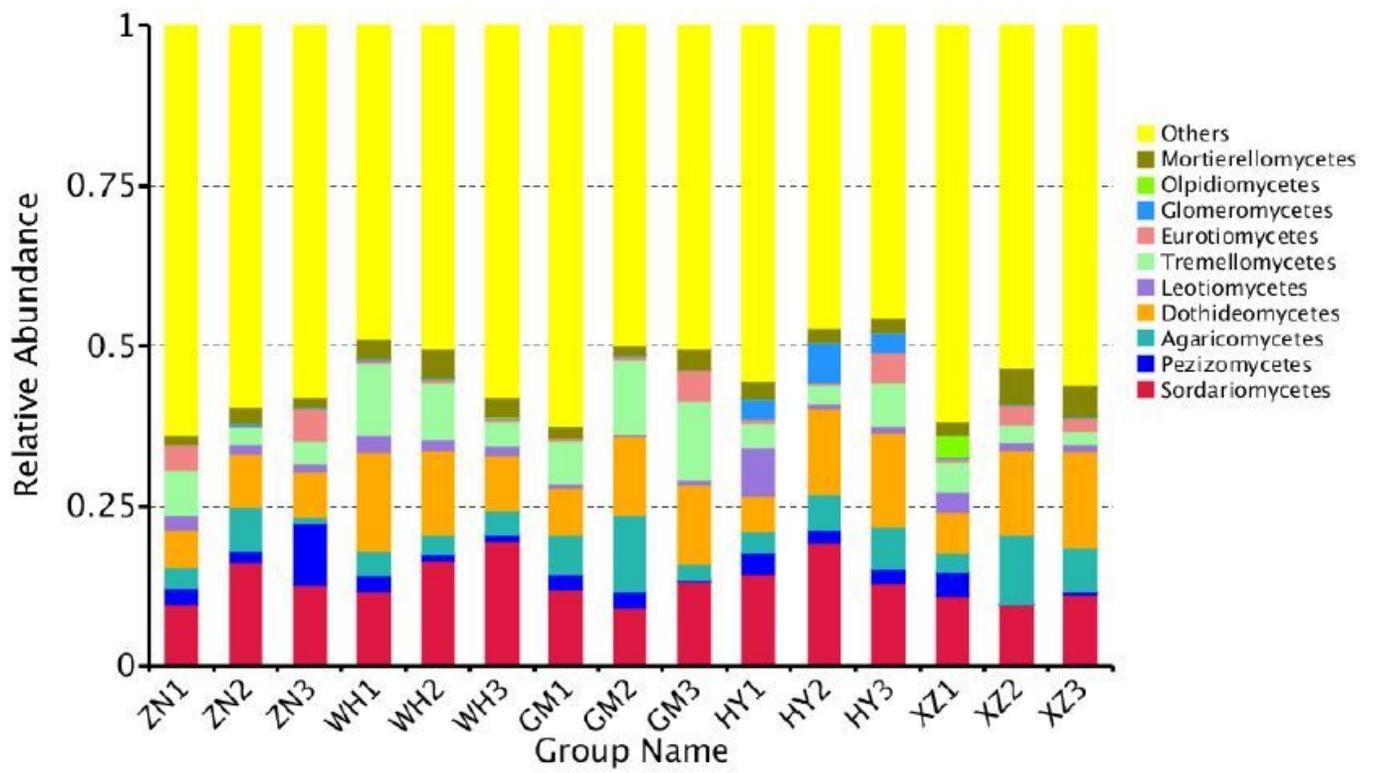


Figure 3

Relative abundance of top 10 fungal classes

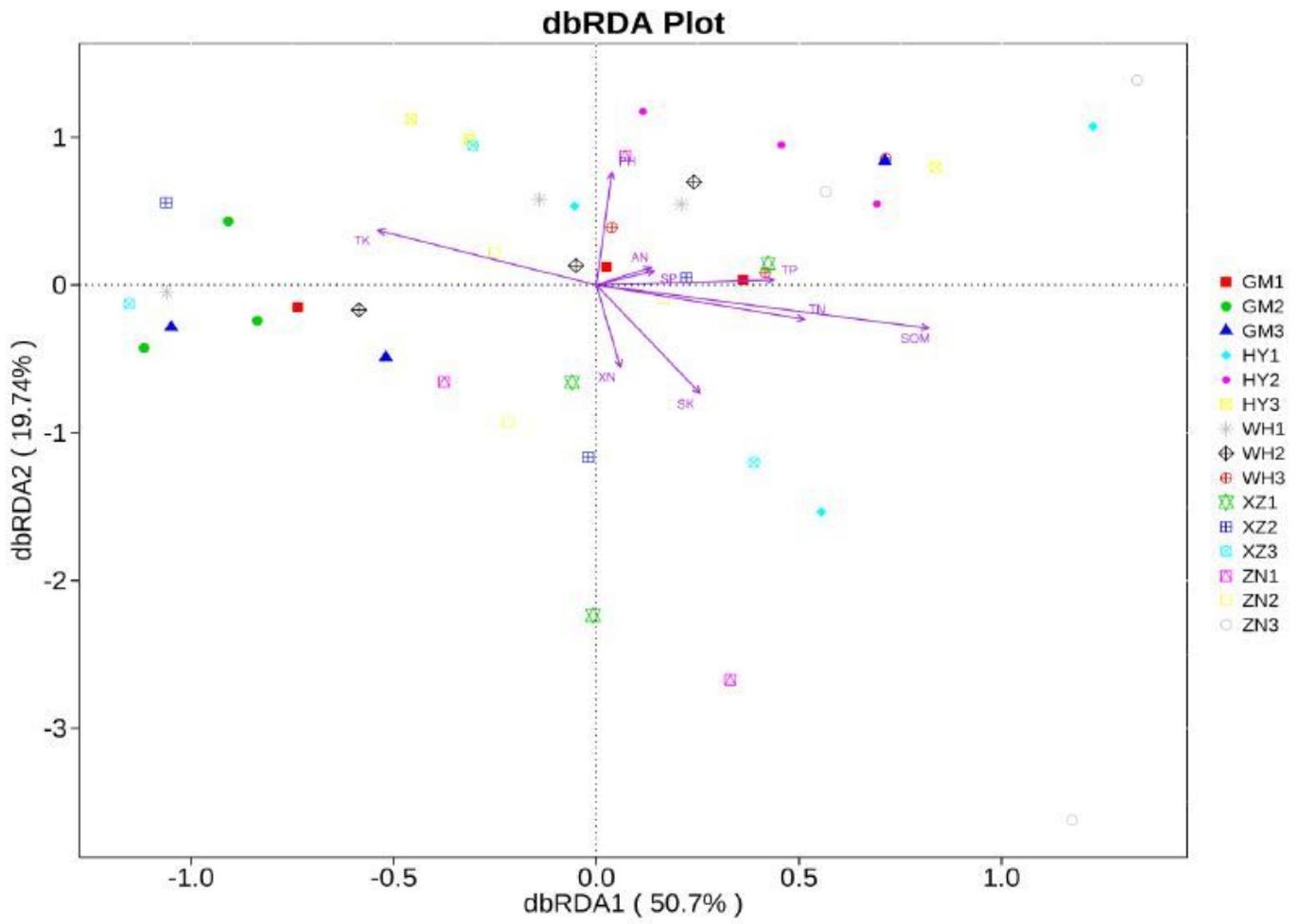


Figure 4

Redundancy analysis of soil physicochemical indices and fungal phyla

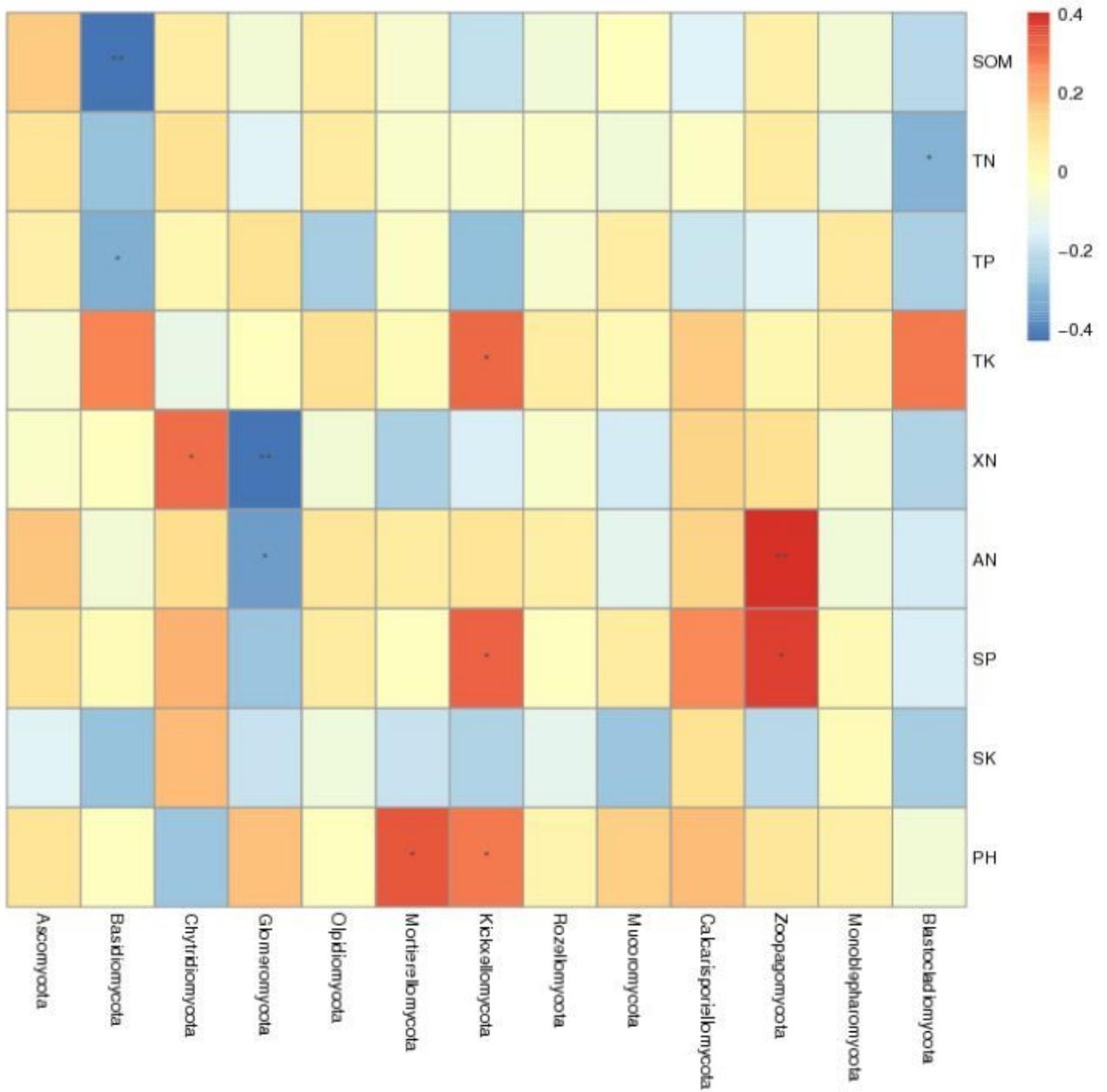


Figure 5

Spearman analysis between soil physicochemical factors and fungal phyla

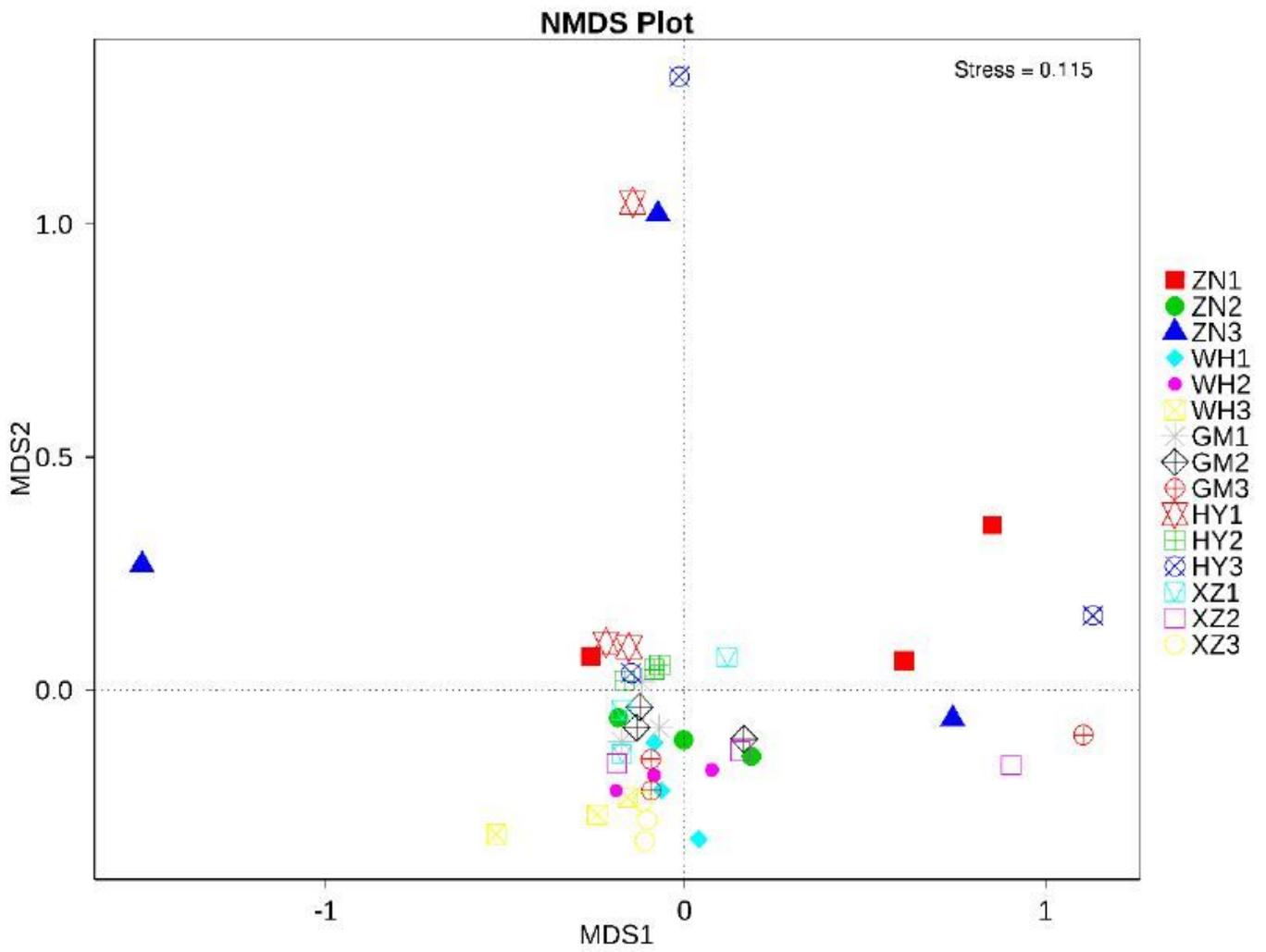


Figure 6

NMDS analysis of soil fungi

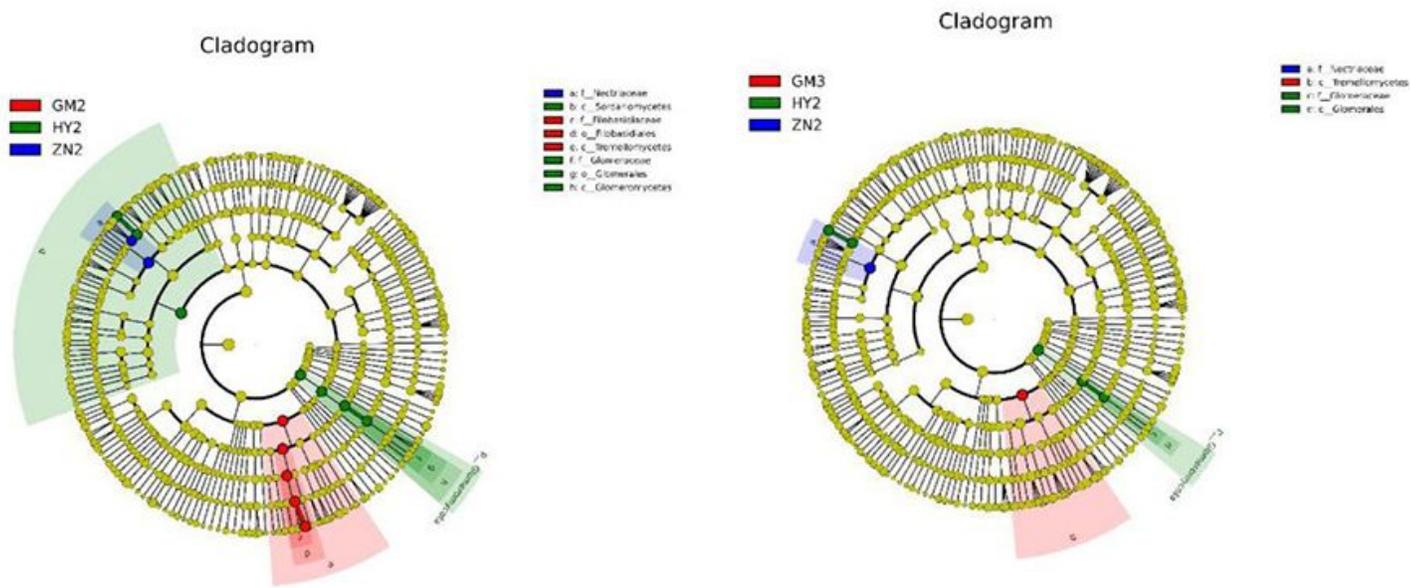


Figure 7

7a Cladogram of LfSe analysis 7b Cladogram of LfSe analysis