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Survey of Annual and Seasonal Fungal Communities in Japanese *Prunus mume* Orchard Soil by Next-Generation Sequencing

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Abstract

Fungi play a vital role in the management of soil environment. Although various fungal communities are found in soil, it is difficult to determine the fungal community structure in soil. In this study, we conducted a comprehensive survey of fungal communities in Japanese *Prunus mume* orchard soil from 2010 to 2012 growing seasons using next-generation sequencing technology. Fungal DNA was directly extracted from the soil samples and the internal transcribed spacer 1 region was amplified by PCR and sequenced. We identified 34,826 fungal clone sequences from the soil samples. The fungal clones were sorted into 2132 operational taxonomic units and a majority of the discriminated clone sequences were classified as *Ascomycota* and *Basidiomycota*. The number of fungal species belonging to *Ascomycota* showed increases in June in the three growing seasons. That belonging to *Glomeromycota* showed increases in August in the three growing seasons. As *Ascomycota* fungi are wood decomposers and saprotrophs, the results suggested that the number of plant pathogenic fungi increased in Japanese *P. mume* orchard soil in June. These findings show for the first time the annual and seasonal fungal community structures in Japanese *P. mume* orchard soil, and are expected to provide valuable clues for improvement when planting new *P. mume* trees in Japanese orchards.

Keywords

Fungal Community Structure, Japanese *Prunus mume* Orchard, Next-Generation Sequencing,

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Operational Taxonomic Unit, Soil Microorganism

1. Introduction

In Japan, fungal disease is prevalent in plants because of considerable temperature fluctuations throughout the year, coupled with frequent rains and high humidity. Particularly, there is much blight due to the soil fungus with the fruit tree. Once disrupted, the balance of soil microbial communities for fruit tree cultivation cannot be easily restored. The disruption of soil microbial balance increases the susceptibility of plants to fungal disease. Therefore, it is necessary to perform soil diagnosis regularly. However, studies of orchard soil have not been pursued due to the complexity of soil microbial communities. The elucidation of microbial communities in orchard soil for perennial plant cultivation remains a difficult and complex task. Soil microbial communities are analyzed conventionally by culture-based methods. However, culture-based methods only allow for the isolation of fungal hyphae and/or spores and provide no clues to help us understand exhaustively the fungal community structures in soil. As an alternative, molecular biology techniques, including polymerase chain reaction (PCR) and real-time PCR, were developed to analyze DNA or RNA of microorganisms in soil samples [1] [2]. To comprehend fungal community structures and their diversities, PCR products are analyzed by denaturing or temperature gradient gel electrophoresis, terminal restriction fragment length polymorphism, and automated ribosomal intergenic spacer analysis [3] [4]. Using molecular biology techniques, microbial communities in soils collected from grasslands, forests, alpine areas, and orchards have been identified, and the effects of chemical composition [5], soil particle size [6], cultivated plant [7], seasonal condition [8], and agricultural management system on the diversity of soil microorganisms have been evaluated.

Genetic analysis of soil microbial communities is carried out comprehensively in Japan [9]. However, annual fungal community structures have not been exhaustively evaluated in Japanese orchard soil. Negishi *et al.* [10] isolated pathogenic fungi causing root rot decline from Japanese *Prunus mume* orchard, including *Cylindrocarpon*, *Cladosporium*, and *Fusarium*. In this study, we surveyed annual and seasonal fungal communities in Japanese *P. mume* orchard soil by next-generation sequencing (NGS) in an attempt to understand the relationship between soil microbial community and soil cation content in Japanese seasonal orchard soil. Sixty soil samples were collected from *P. mume* orchard in Atami City, Shizuoka prefecture over a three-year period and subjected to NGS, to understand fungal community structures and their diversities in the soil. In addition, changes in soil cation content for each season were investigated to clarify the relationship between soil cation content and seasonal fungal community. Atami City is well known for viewing *P. mume* blossoms in Japan. The *P. mume* orchard which we used then is used for the purpose of a study among other things. Because of this, fenitrothion alone is applied to this orchard once a year in April, and no other agricultural chemical or manure is applied until April of the next year. Therefore, it can be said that this *P. mume* orchard has “pure” microbial community and cation content.

We expect that this *P. mume* orchard would become the standard of Japanese *P. mume* orchards in terms of both microbial community and cation content. The results obtained enabled us to understand the seasonal variation of root rot decline caused by pathogenic fungi in soil, and suggested the appropriate season and place for planting new *P. mume* trees in Japanese orchards.

2. Materials and Methods

2.1. Soil Samples

We sampled soil from a *P. mume* orchard located in Atami City, Shizuoka Prefecture, Japan for three years (2010 to 2012 growing season). Five samples each were collected in June, August, November, and February of each growing season. Soil pits located 50 cm from a *P. mume* trunk were dug to a depth of 30 cm. Approximately 50 g of soil was sampled from each pit.

2.2. Soil pH, Electrical Conductivity, and Cation Content

Approximately 20 g of soil was vigorously shaken in 100 mL of distilled water for 1 minute, and the mixture

was left to stand for 10 minutes. Soil pH and electrical conductivity were measured with a pH and electrical conductivity tester (Combo l; HANNA Instruments, Padova, Italy). Soil cation content was analyzed with a Soil and Plant Analyzer Development-Spectro-Flame Photometer (SFP-3; Fujihira Industrial Co., Tokyo, Japan).

2.3. DNA Extraction from Soil

Large rocks and root tips were removed from the soil samples. Total DNA was directly extracted from 0.4 - 0.3 g of soil samples using a Power Max Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's instructions.

2.4. Fungal Amplicon Libraries

The PCR reaction mix consisted of 1 μL of 10 × PCR buffer, 0.4 μL of 2.5 mM each dNTP, 0.25 μL of 20 mM each ITS1 (internal transcribed spacer 1) primer with tag and/or adaptor sequences (**Table 1**), 0.1 μL of *Ex* Taq Hot Start Version (Takara, Shiga, Japan), 7 μL of ddH_2O, and 1 μL of DNA solution. The primers (**Table 1**) were reused in each of the three growing seasons. PCR conditions were as follows: 94˚C for five minutes (one cycle); 94˚C for 30 seconds, 55˚C for 30 seconds, and 72˚C for 40 seconds (25 - 39 cycles depending on amplification efficiency); and 72˚C for seven minutes (one cycle). The PCR products were separated on 2.0% agarose gel. The 250 - 600 bp fractions corresponding to the predicted fungal ITS1 amplicons were recovered from the agarose gel with a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The fractions were combined into a microtube as the fungal amplicon libraries for each growing season.

2.5. Sequence Analysis

The fungal amplicon libraries were checked by electrophoresis and densitometry. The libraries were amplified by emulsion-PCR with adaptor sequences and analyzed by a pyrosequencing technique using a Roche 454 GS Junior Bench Top Sequencing Platform (Roche Diagnostics, Tokyo, Japan). DNA sequences outputted by NGS were classified by tag arrangement. Then, adaptor, tag, and primer sequences were removed from the DNA sequences. The absolute DNA sequences were subjected to a homology search using BLAST search program of National Center for Biotechnology Information (100% - 81% identity). Based on BLAST search results, irrelevant sequences, such as plant sequences, were rejected from the data. Fungal ITS1 clones were sorted by the Bioinformatics Toolkit BLASTclust (http://toolkit.tuebingen.mpg.de/blastclust) into operational taxonomic units (OTUs). Fungal ITS1 clones in OTUs had 250 - 600 bp lengths and >95% sequence lengths to be covered, and >98% sequence similarities.

The diversity test was performed on the sequencing data to see how our system compares to others and if the diversity is influenced by season. We performed the diversity test by using PAST software based on the number of each data the species of fungal and a population (ITS-clone), and calculated eight kinds of diversity indexes. We calculated the average diversity indexes for the three growing seasons and ranked them thereafter.

3. Results and Discussion

From the orchard soil samples, we collected 7906 reads from 2010 growing season, 20,549 reads from 2011

Table 1. PCR primer pairs used in the present study.

Primer name	Adaptor sequence	Tag sequence[a]	ITS1 sequence
ITS1-J Fw (June)[b]	5'-CGTATCGCCTCCCTCGCGCCATCAG	ACGTA	CTTGGTCATTTAGAGGAAGTAA-3'
ITS1-A Fw (August)[b]	5'-CGTATCGCCTCCCTCGCGCCATCAG	ATGCT	CTTGGTCATTTAGAGGAAGTAA-3'
ITS1-N Fw (November)[b]	5'-CGTATCGCCTCCCTCGCGCCATCAG	CACGT	CTTGGTCATTTAGAGGAAGTAA-3'
ITS1-F Fw (February)[b]	5'-CGTATCGCCTCCCTCGCGCCATCAG	CGATA	CTTGGTCATTTAGAGGAAGTAA-3'
ITS1 Rv[c]	5'-CTATGCGCCTTGCCAGGCCCGCTCAG		GCTGCGTTCTTCATCGATGC-3'

[a]The tag sequences were used to classify the DNA sequences outputted by NGS for each growing season. [b]Forward primers. These were reused in the three growing seasons. [c]Reverse primer. This was reused in the three growing seasons.

growing season, and 6854 reads from 2012 growing season. However, the designed primer pairs also amplified the plant ITS region. Therefore, reads of plant ITS region sequences were omitted from the data. Because, 51 reads (0.6% of total) of plant ITS region sequences were collected from 2010 growing season, 343 reads (1.7%) from 2011 growing season, and 89 (1.3%) reads from 2012 growing season. Finally, we recovered 2132 OTUs from 34,826 fungal clone sequences Japanese *P. mume* orchard fungal amplicon libraries in the course of three years: 7855 reads from 2010 growing season, 20,206 reads from 2011 growing season, and 6765 reads from 2012 growing season. For those three years, the seasonal amplicon libraries ranged from 139 to 217 OTUs. Those fungal clone sequences were examined for similarity to sequences of known fungi in the database. Clone sequences with BLAST scores exceeding 200 (100% - 81% identity) accounted for 95.3% in 2010 growing season, 97.8% in 2011 growing season, and 93.3% in 2012 growing season, suggesting that several percent of the fungal population existing in the orchard soil irrespective of year or season were unidentified fungi. As the rarefaction curves of the 34,826 fungal clones did not plateau (data not shown), we could estimate only a small portion of fungi existing in the soil. More fungal clones are required to estimate the precise seasonal fungal species. Nevertheless, our results suggested that on average, at least 710 fungal species exist in the soil and most of them belong to *Ascomycota* and *Basidiomycota* (**Figure 1**). The effects of permanent swards and bare soil on soil microbial count in two Australian orchards were examined using culture methods [6]. However, fungal composition in the orchard soil was not determined in that study and as far as we know, there are hardly any studies on the comprehensive identification of fungal clone sequences in seasonal orchard soil. Our results suggested that NGS was able to identify exhaustively fungal species in soil.

The fungal amplicon libraries indicated that the soil fungal community had annual and seasonal structures that consisted of unique and variously sized clones (**Figure 1**). For instance, except for unclassified fungi, *Ascomycota* had the most abundant amplicon libraries in the three growing seasons, whereas *Chytridiomycota* had only one very small amplicon libraries in the three growing seasons (**Figure 1**, November in 2011 growing season). However, when viewed in terms of predominance by season, *Ascomycota* was replaced by *Basidiomycota* in November and February in the three growing seasons (**Figure 1**). The total number of OTUs was larger in June and August than in November and February in the three growing seasons; *Glomeromycota* showed the greatest increase in number in August in the three growing seasons (**Figure 1**, *Glomeromycota* and **Figure 3**). *Glome-*

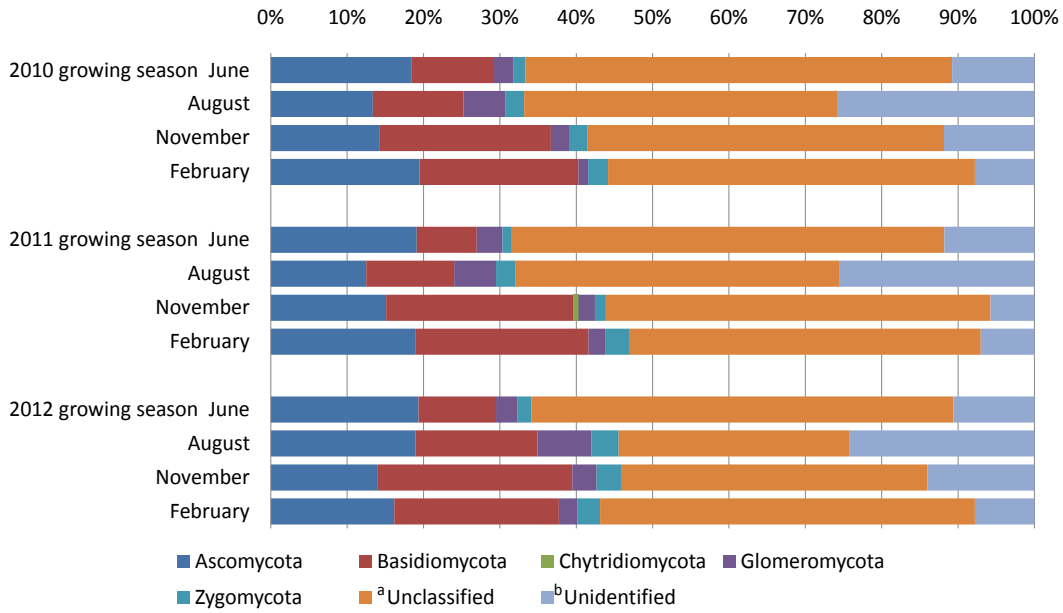

Figure 1. Frequencies of fungal ITS1 sequences belonging to each fungal phylum. 2132 OTUs from 34,826 fungal clone sequences of Japanese *P. mume* orchard fungal amplicon libraries were collected over a three-year period (7855 reads from 2010 growing season, 20,206 reads from 2011 growing season, and 6765 reads from 2012 growing season). Fungal phyla were classified based on the results of BLAST search. Bar size is proportional to the percentage of fungal OTUs for the indicated year. [a]Unclassified, fungi not classified in the current fungal phyla. [b]Unidentified, fungi showing no matches with the NCBI database.

romycota species are arbuscular mycorrhizal fungi and therefore, microorganisms that are effective against plant pathogenic fungi may be present in Japanese *P. mume* orchard soil in August [11]. Finally, we constructed and uploaded the Web database "Soil fungal database" (http://www.yamanashi-univ.com/) based on the results of the present study. The database is expected to help us develop new ideas and strategies for soil management to improve the quality of crops, cereals, and fruits.

Both the OTU patterns in the fungal amplicon libraries and the number of clones in each OTU were diverse among the fungal amplicon libraries in each growing season. This result suggested the complex distribution of the fungal community in Japanese *P. mume* orchard soil. *Basidiomycota*, the major phylum detected by pyrosequencing analyses of forest soil, consisted of mushroom-forming fungi, wood decomposers, including *Ectomycorrhizae*, and fungi feeding on decaying plant residue [12]. Regrettably, we were not able to identify the dominant fungus at the species level in the soil due to the limited sample size. However, we found a point common to the fungal amplicon libraries of the three growing seasons: only *Basidiomycota* OTUs showed an increase in November and February. It is plausible that the increase was due to the rise in humidity in the summer in Japan. Fungal species belonging to *Basidiomycota*, such as *Entoloma*, thrive in November in all the three growing seasons ("Soil fungal database", http://www.yamanashi-univ.com/). As *Entoloma* species are arbuscular mycorrhizal fungi, they are expected to be effective against pathogenic fungi of *P. mume* [11] [13]. In addition, the increase in the number of *Basidiomycota* OTUs may be correlated with K_2O, NH_4-N, and air temperature. In the Japanese *P. mume* orchard soil, K_2O concentration increased in August and November in all the three growing seasons, whereas NH_4-N concentration decreased in 2012 growing season, and both conditions were favorable for *Basidiomycota* increase (**Figure 2**). The decrease in air temperature enhanced *Basidiomycota* increase as well (**Figure 3**). Of the soil cation contents examined, only K_2O and NH_4-N showed significant differences

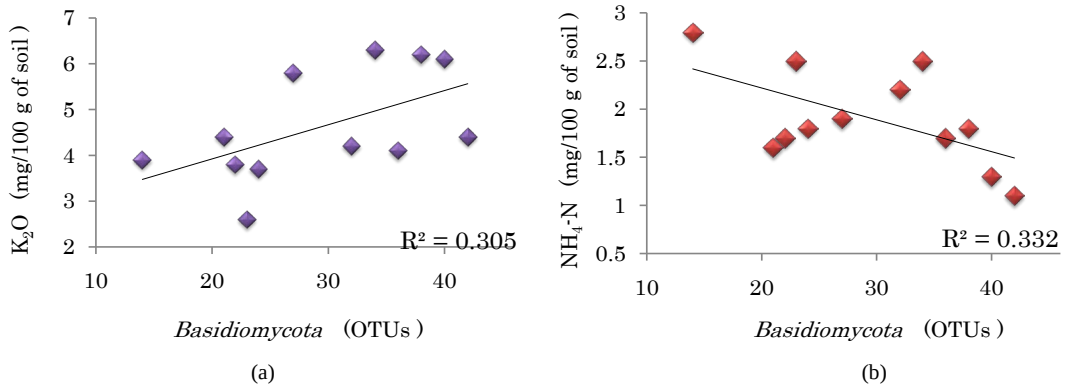

Figure 2. Correlation of soil cation contents (a) K_2O and (b) NH_4-N with *Basidiomycota* OTUs in Japanese *P. mume* orchard soil (n = 12). K_2O and NH_4-N data were extracted from **Table 2**. *Basidiomycota* OTU numbers were extracted from the Web database "Soil fungal database" (http://www.yamanashi-univ.com/).

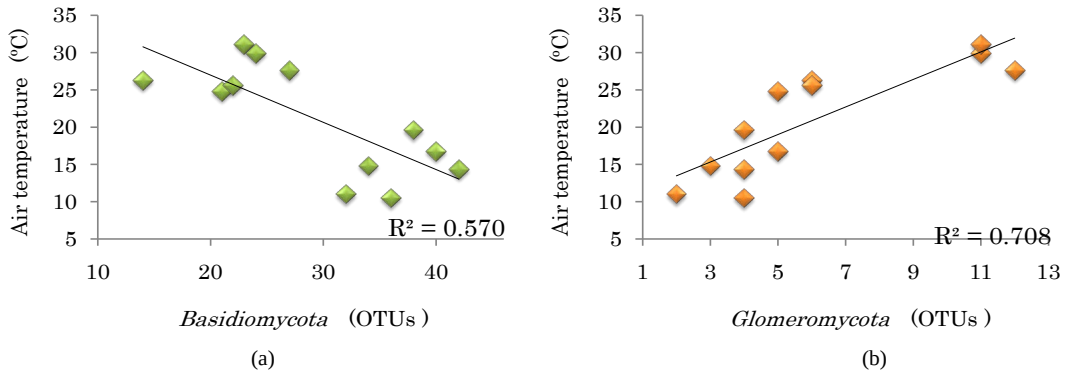

Figure 3. Correlation of (a) *Basidiomycota* OTUs and (b) *Glomeromycota* OTUs with air temperature of Japanese *P. mume* orchard (n = 12). Air temperature data were extracted from **Table 2**. *Basidiomycota* and *Glomeromycota* OTU numbers were extracted from the Web database "Soil fungal database" (http://www.yamanashi-univ.com/).

among the years or the seasons. Air temperature also varied significantly among the years or the seasons (**Table 2**). Those results offer important clues to the discovery of useful microorganisms for *P. mume*. On the other hand, fungi belonging to *Ascomycota*, such as *Fusarium* and *Cylindrocarpon*, showed population increases in June, whereas fungi belonging to *Cladosporium* showed population increases in August in all the three growing seasons ("Soil fungal database", http://www.yamanashi-univ.com/). In addition, the diversity indexes were generally higher in August than in the other months, as can be seen from **Table 3**. *Fusarium* and *Cylindrocarpon* were reported to be responsible for strawberry root rot disease [14]. As both strawberry and *P. mume* are classified under *Rosaceae*, those fungi would have deleterious effects on *P. mume* as well. *Cladosporium* was reported to be responsible for scab in *P. mume* fruit [15] [16]. *Ascomycota* species exist as *P. mume* pathogens, endophytes, wood decomposers, and saprotrophs, and consequently, plant pathogenic fungi may increase in June and August in Japanese *P. mume* orchard soil. From these results, we can elucidate the seasonal variation of plant pathogenic fungi, and this information is expected to help us decide the appropriate season and place for planting new *P. mume* trees in Japanese orchards. Further investigation is warranted to clarify their relationships with environmental characteristics and maintenance in relation to the spray calendar for Japanese *P. mume*.

Table 2. Soil cation content, pH, electrical conductivity, and air temperature of Japanese *Prunus mume* soils.

Year	Season	MgO[a]	CaO[a]	K_2O[a]	NO_3-N[a]	NH_4-N[a]	Br-P_2O_5[a]	Mn[a]	Fe_2O_3[b]	pH	Ec[c]	Air temperature[d]
2010	June	13.2	140.7	4.4	2.4	1.6	8.4	0.2	3.2	5.9	17.7	24.8
	August	8.9	143.3	3.7	1.7	1.8	8.4	0.2	3.3	5.8	17.3	29.9
	November	9.7	121	6.2	1.6	1.8	10.1	0.2	3.5	5.6	17.7	19.6
2011	February	14.4	140.3	4.2	1.8	2.2	10.1	0.1	3.6	5.7	19.3	11
2011	June	10.6	146.3	3.9	1.1	2.8	10.5	0.1	3.2	5.7	15.7	26.3
	August	11.9	139.3	2.6	1.2	2.5	11.5	0.1	3.6	5.6	17.3	31.1
	November	13.3	141.3	6.3	1.4	2.5	13.2	0.1	2.8	5.6	18.7	14.8
2012	February	13.4	123.3	4.4	0.9	1.1	11.2	0.2	3.9	5.9	17.3	14.3
2012	June	14.6	111.3	3.8	1.9	1.7	12.2	0.1	2.6	5.6	15.3	25.6
	August	16	129	5.8	1.5	1.9	7.1	0.2	3.1	5.5	18	27.6
	November	12.8	112	6.1	1.4	1.3	9.8	0.1	2.8	5.4	18	16.7
2013	February	10.1	103	4.1	0.6	1.7	8.6	0.2	2.8	5.6	16.7	10.5

[a]mg/100 g of soil. [b]Percentage. [c]μS/cm. [d]°C.

Table 3. Averages of diversity indexes calculated based on sequencing data of seasonal fungal communities in *P. mume* orchard soil for three years.

Diversity index	June		August		November		February	
	Average[c]	Rank[d]	Average[c]	Rank[d]	Average[c]	Rank[d]	Average[c]	Rank[d]
Taxa_S[b]	176.67 ± 15.14	(1)	167.01 ± 14.73	(2)	143.33 ± 15.82	(3)	133.01 ± 17.35	(4)
Shannon_H[b]	3.23 ± 0.48	(2)	3.61 ± 0.01	(1)	2.97 ± 1.01	(4)	3.14 ± 1.15	(3)
Simpson_1-D[a]	0.89 ± 0.05	(2)	0.93 ± 0.01	(1)	0.83 ± 0.19	(4)	0.86 ± 0.16	(3)
Menhinick[b]	3.52 ± 0.66	(2)	3.69 ± 0.32	(1)	3.09 ± 1.33	(3)	2.93 ± 1.25	(4)
Margalef[b]	22.41 ± 2.51	(1)	21.78 ± 1.92	(2)	18.44 ± 3.59	(3)	17.09 ± 2.33	(4)
Equitability_J[a]	0.62 ± 0.08	(3)	0.71 ± 0.01	(1)	0.61 ± 0.18	(4)	0.64 ± 0.24	(2)
Fisher_alpha[b]	43.47 ± 7.87	(1)	43.09 ± 4.98	(2)	35.18 ± 11.21	(3)	32.01 ± 8.22	(4)
Berger-Parker[a]	0.23 ± 0.07	(2)	0.21 ± 0.03	(1)	0.31 ± 0.26	(4)	0.26 ± 0.22	(3)

[a]Diversity index mostly biased toward evenness. [b]Diversity index mostly biased toward richness. [c]The avarage value of the seasonal diversity indexes for three years. [d]The number in parenthesis indicates rank in four seasons for each item.

4. Conclusion

We analyzed the seasonal variation of plant pathogenic fungi and the microorganisms effective against them in Japanese *P. mume* orchard soil. The results suggested that a large number of fungal species exist in Japanese *P. mume* orchard soil and the fungal community structure shows annual and seasonal diversity and complexity. *Ascomycota* was the main phylum in the soils, but was replaced by *Basidiomycota* in November and February in the three growing seasons. Therefore, the dominant fungal groups found in this study might be similar to the previously reported dominant fungal groups in forest and agricultural soil. Determining the seasonal variation of plant pathogenic fungi would give us information on the appropriate season and place for planting new *P. mume* trees in Japanese orchards. Further investigations of other *P. mume* orchards in Japan are expected to yield the precise biological characteristics of *P. mume* orchards.

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Abbreviations

ITS1: Internal transcribed spacer 1;
NGS: Next-generation sequencing;
OTU: Operational taxonomic unit.