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# Endophytic Mycobiota of Jingbai Pear Trees in North China

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**Abstract:** Endophytic fungi exist in all known plants and play an important role for plant growth and health. As an important forest tree the Jingbai pear (the best quality cultivar of *Pyrus ussuriensi* Maxim. ex Rupr.) has great ecological as well as economic value in north China. However, the mycobiota of the pear tree is still unknown. In this study, the fungal communities in different organs of the tree and in rhizosphere soils were investigated by Illumina Miseq sequencing of ITS rDNA. For organs, the roots had the highest fungal richness and diversity, while the flowers had the lowest richness and diversity. The results demonstrated that each of the organs investigated harbored a distinctive fungal assemblage. Overall, Ascomycota was the most abundant phyla, followed by Basidiomycota and Zygomycota. Fungal communities from the different soils also differed from each other. The redundancy analysis (RDA) showed that fungal community structure correlated significantly with soil temperature, soil pH, soil nitrogen and soil carbon contents. The results indicate that plant organs, site conditions and soil properties may have important influences on the endophytic fungal community structure associated with Jingbai pear trees.

**Keywords:** endophytic fungi; community diversity and structure; Jingbai pear trees

## 1. Introduction

The plant can be regarded as a holobiont comprising of the host plant and its microbiota with functions, adaptation and interactions between the host and microbiota [1]. Endophytic fungi are an important component of plant microbiota [2–4]. They are ubiquitous and inhabit within the plant organs without causing visible harm to the host [5,6]. Endophytic fungi have received extraordinary attention [4,7,8] due to the ability to produce bioactive products [9–11], promote host growth, resist environmental stress [12,13], and even litter decomposition [14]. Many studies have focused on fungal endophytes from agricultural crops, such as wheat, soybeans and tomatoes [15,16], medicinal plants [4,17] and forest trees [18,19]. Although, endophytic fungi of apple and kiwifruit [20,21], wild bananas [22], sour cherry [23] and plums [24] have been reported, information on endophytes from fruit trees is still very limited [23,25]. Culture-dependent and culture-independent approaches may provide a different picture of microbial communities and both methods should be used in fungal research [3,6,26]. Currently, high-throughput sequencing has the great advantage of generating huge data on species as well as detecting unculturable species at a much lower price, therefore making it easier to extend knowledge of microbial diversity [6,26–28].

The Jingbai pear is one of the best quality pears of the Chinese National Products of Geographic Identification brand in Beijing. As an important forest tree, the Jingbai pear has great ecological and economic value in north China [29]. Presently, some pear diseases seriously threaten the yield of the Jingbai pear tree. Chemicals are mainly used to deal with these pathogens, which could cause pollution and harm to people and the environment [29]. Studies on endophytes of Jingbai pear trees might provide novel pathogen biocontrol and growth-promotion agents. However, information on endophytic fungi of Jingbai pear trees is still very little.

A number of driving factors including environmental conditions, the host and microbial genetic background and interactions within plant microbes can influence the endophytic community structure [4,30]. Phytosanitary conditions of host plants (health status) also represent a pivotal driving factor of fungal endophytic community structures [18]. The composition of endophytic mycobiota of fruit trees can be diverse depending on the tree species [23]. Liu et al. [31] suggested a genotype-specific influence of different rootstock/scion combinations on apple endophytic microbiota. Another study revealed limited geographic differentiation of fungal communities in apples trees, while a high diversity of fungal community structures was found in blackcurrant from different regions [32]. More research is needed to investigate the endophytic community.

The main aim of this study is to investigate the differences on the endophytic mycobiota composition of different plant organs (flower, leaf, fruit, stem and root). Correlation between the fungal community structure, the sampling sites and the soil properties are also speculated and elucidated. In addition, the study will provide a comprehensive view of the endophytic fungal community of pear trees and useful information for further exploitation and utilization of these communities.

## 2. Materials and Methods

### 2.1. Sample Collection

Sampling sites were located in three main Jingbai pear producing provinces in North China. The first site (site 1) was located in Mentougou District, Beijing (40°0′8.11″ N, 116°05′40.10″ E). The second site (site 2) was located in Gu'an, Hebei (39°26′11.52″ N, 116°18′33.07″ E). The third site (site 3) was located in Jianchang, Huludao, Liaoning (40°52′36.44″ N, 119°50′31.73″ E). Samples were collected from flowers, leaves, stems, roots and soils (rhizosphere soils, 15–25 cm deep) in mid-April and from fruits in September 2017, according to Ren et al. [33]. In brief, nine Jingbai pear trees, separated at least 200 m apart, were selected randomly in each site. Organs and soils were evenly mixed and three biological replicates were chosen for each sample. Samples were collected in sterile plastic bags and processed within 24 h. After being washed in tap water, organs were surface-disinfected by washing in sequence with 75% ethanol for 1 min, 2% sodium hypochlorite for 3 min, 75% ethanol for 1 min, and then rinsed in sterile distilled water for 0.5 min and dried [33]. Soil pH was determined with a glass electrode by stirring the soil suspensions in demineralized water with a ratio of 5 g soil and 25 mL water, while moisture content was determined by oven drying (105 °C, 24 h). The Walkley–Black method [34] and Kjeldahl method [35] were used to test soil carbon (C) and total nitrogen (N) concentration, respectively.

### 2.2. DNA Extraction, Amplification of ITS rDNA Region and Illumina Sequencing

Genomic DNA of the organs was extracted with a standard cetyl–trimethyl ammonium bromide (CTAB) method with modifications as described in Chang et al. [36]. FastDNA® Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) was used to extract DNA from homogenized soil samples. Concentrations of the DNA were measured with a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Fungal primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2R (GCTGCGTTCTTCATC GATGC) were used to amplify ITS1 region of rDNA [37]. The PCR products were purified and sequenced with Illumina MiSeq. platform at Shanghai Majorbio Science and Technology Ltd (Shanghai,

China). All sequences were deposited at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under project accession number SRP154990.

### 2.3. Pre-Processing and Analysis of ITS rDNA Sequences

Raw read quality was pre-processed with FLASH [38] and Trimmomatic [39]. Mothur standard operation pipeline (SOP, v.1.37.6) [40] was used to analyze the data and classify sequences into OTUs (Operational Taxonomic Units) at 97% similarity against UNITE Database v. 7.2 [41]. Sequence reads were subsampled for each sample with the minimum number of reads among all samples before comparative analysis. The species richness (Sobs), diversity (Invsimpson) and evenness (Simpsoneven) [42] were calculated in Mothur. Data for rarefaction curves were also generated in Mothur. One-way ANOVA (analysis of variance) was used to identify differences in community richness, diversity and evenness, and fungal abundance among organs, the ANOVA assumptions were verified before use and post hoc tests were also carried out with Scheffe and Welch uncorrected (0.95) separately. R language platform [43] was used for analysis and visualization of data sets of the microbial diversity and abundances in different samples (Rarefaction curves, Venn, bar chart, PCoA, RDA, PERMANOVA).

## 3. Results

### 3.1. Information on MiSeq Sequencing Data

In total, 2,075,780 high quality sequences were generated across all samples after sequence denoising and quality filtering. The number of sequences in each sample ranged from 30,345 to 44,599 with an average of 38, 440 ± 3996 (mean ± SD) sequences. The average sequence length was 263 bp.

### 3.2. Fungal Richness, Diversity and Evenness

Quality-filtered fungal sequences were clustered into 1856 OTUs (excluding singletons). The roots had the highest fungal richness (159.33) and diversity (8.95), and the flowers had the lowest richness (64.22) and diversity (2.83) (Table 1). The highest evenness of fungal communities was found in leaves (0.07), while the lowest evenness was observed in flowers (0.047) (Table 1). Fungal richness, diversity and evenness of tissues and soils showing statistically significant differences are shown in Supplementary Figure S1 labeled with an asterisk (*). Rarefaction curve showed that the OTUs abundance were saturated in all samples (Supplementary Figure S2).

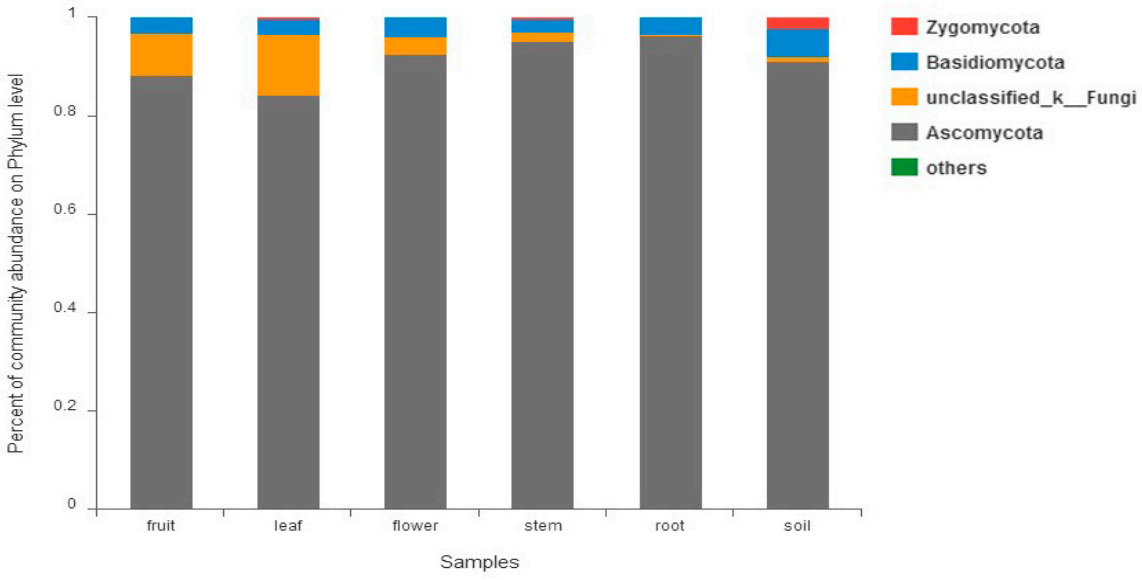
**Table 1.** Richness, diversity and evenness indexes of fungal communities (mean ± SD).

| Samples | Sobs (Richness) | Invsimpson (Diversity) | Simpsoneven (Evenness) |
|---|---|---|---|
| leaf | 109.78 ± 7.37 | 5.92 ± 0.27 | 0.074 ± 0.006 |
| flower | 64.22 ± 1.55 | 2.83 ± 0.08 | 0.047 ± 0.002 |
| fruit | 115.56 ± 6.03 | 5.98 ± 0.35 | 0.057 ± 0.003 |
| stem | 120.00 ± 6.84 | 4.58 ± 0.19 | 0.048 ± 0.004 |
| root | 159.33 ± 6.27 | 8.95 ± 0.54 | 0.057 ± 0.003 |
| soil | 487.44 ± 15.82 | 9.95 ± 1.24 | 0.018 ± 0.002 |

### 3.3. Fungal Community Composition among Different Organs and Soils

The sequences assigned to fungi kingdom were classified into 6 fungal phyla (excluding unclassified fungi). Ascomycota was the most abundant group (91.2%) followed by Basidiomycota (3.6%) and Zygomycota (0.5%). Chytridiomycota, Rozellomycota and Glomeromycota were much less (<0.1%). The abundances of phyla exceeding 1% in each organ are shown in Figure 1a. Abundance of Zygomycota and Chytridiomycota showed significant differences among all samples, while the abundances of Ascomycota and Basidiomycota did not (Supplementary Figure S3A). Thirteen classes had a relative abundance of more than 1% (Figure 1b), which include Dothideomycetes,

Sordariomycetes, Eurotiomycetes, Leotiomycetes, Saccharomycetes, Agaricomycetes, etc. Class Dothideomycetes were predominant in all five organs. However, Sordariomycetes was the most abundant in tree soils. The abundance of Leotiomycetes was much higher in roots. Abundance of 11 classes (including classes mentioned above) showed significant differences among all samples (Supplementary Figure S3B).




**a**

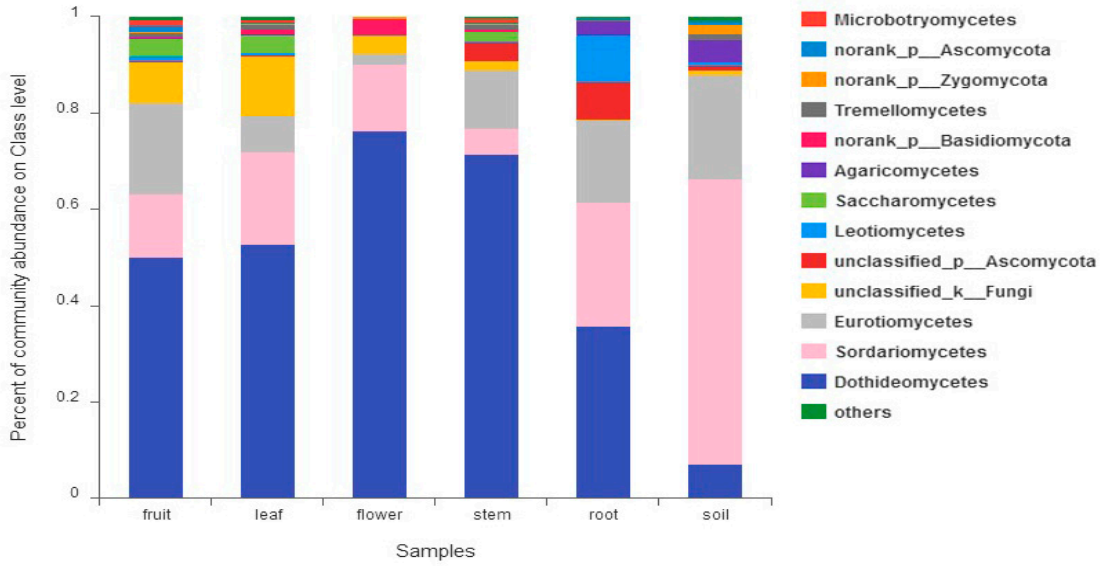



**Figure 1.** Fungal abundance in different organs and soil: (**a**) at phylum level; (**b**) at class level.

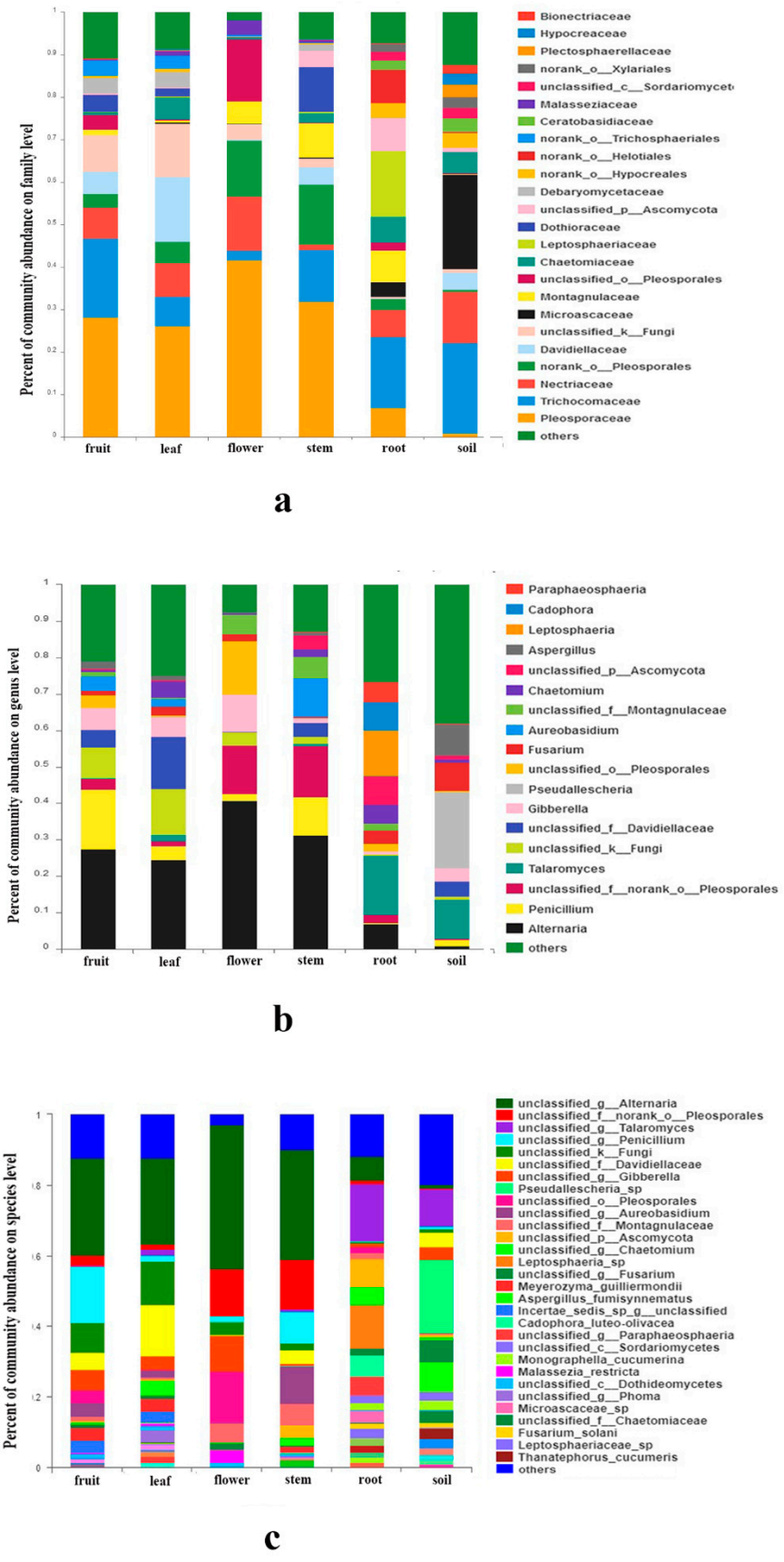



**Figure 2.** Fungal abundance in different organs and soil: (**a**) at family level; (**b**) at genus level; (**c**) at species level.

At the family level, abundance of 24 families exceeded 2% (Figure 2a). Pleosporaceae (Dothideomycetes) were most abundant in fruits, leaves, flowers and stems, while Trichocomaceae (Eurotiomycetes) were richest in roots and soils. A total of 19 fungal genera of fungi had a relative abundance of more than 2% (Figure 2b), such as Alternaria, Penicillium, Talaromyces, Gibberella, Pseudallescheria, Fusarium, etc. A few species can be identified, such as *Meyerozyma guilliermondii* (Wick.) Kurtzman and M. Suzuki, *Aspergillus fumisynnematus* Y. Horie, Miyaji, Nishim., Taguchi and Udagawa, and *Fusarium solani* (Mart.) Sacc. Many sequences can be just classified to genus or higher level. The organs and soils sampled shared 59 (3.2%) of the total 1856 OTUs. The OTUs proportion unique to a certain tissue ranged from 0.9% (16 OTUs; leaves) to 8.6% (159 OTUs; stems) (Figure 3). An abundance of 14 families, 12 genera, 12 species and 14 OTUs showed significant differences among organs and soils (Supplementary Figure S3C–F).

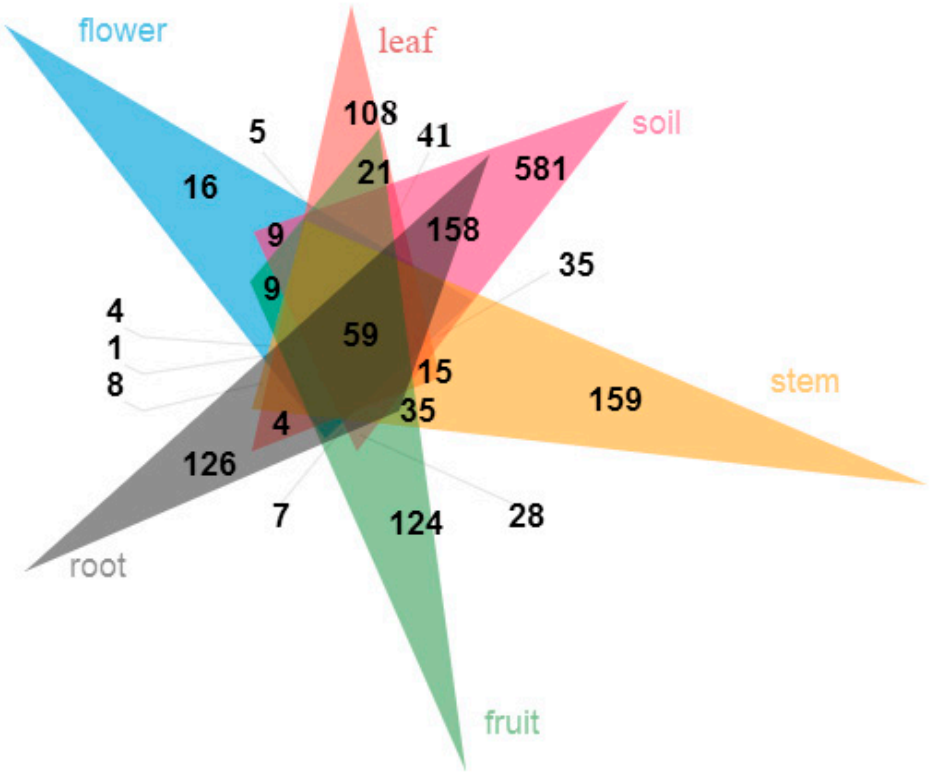



**Figure 3.** Venn diagram showing shared and unique fungal OTUs in each organ (flower, leaf, fruit, stem, root) and soil.

### *3.4. The Relationship between Fungal Community Structure and Organs, Sampling Sites and Soil Properties*

Principal Coordinate Analysis (PCoA) showed that each organ formed individual clusters as well as the soils (Figure 4a). PCoA also illustrated that the three sites formed different fungal clusters (Figure 4b). A subsequent PERMANOVA test confirmed the significant differences in community structures among the five organs, the soils ($p < 0.05$ in all possible pairs), as well as the three sites ($p < 0.05$).

The RDA tests showed that the fungal community structure was significantly correlated with soil temperature ($r^2 = 0.86$, $p = 0.001$), soil pH ($r^2 = 0.77$, $p = 0.001$), soil N ($r^2 = 0.82$, $p = 0.001$) and soil C ($r^2 = 0.75$, $p = 0.001$) (Figure 5). The soil properties of three sites can be found in Ren et al. [33]. Briefly, soil temperature values were 14.2, 13.2, 11.0 (°C); pH were 7.1, 7.2, 6.8; soil N values were 1.6, 1.5, 1.7 (mg/g); soil C values were 13.5, 12.2, 15.3 (mg/g) (mean value in Beijing, Hebei, Liaoning, respectively) [33].

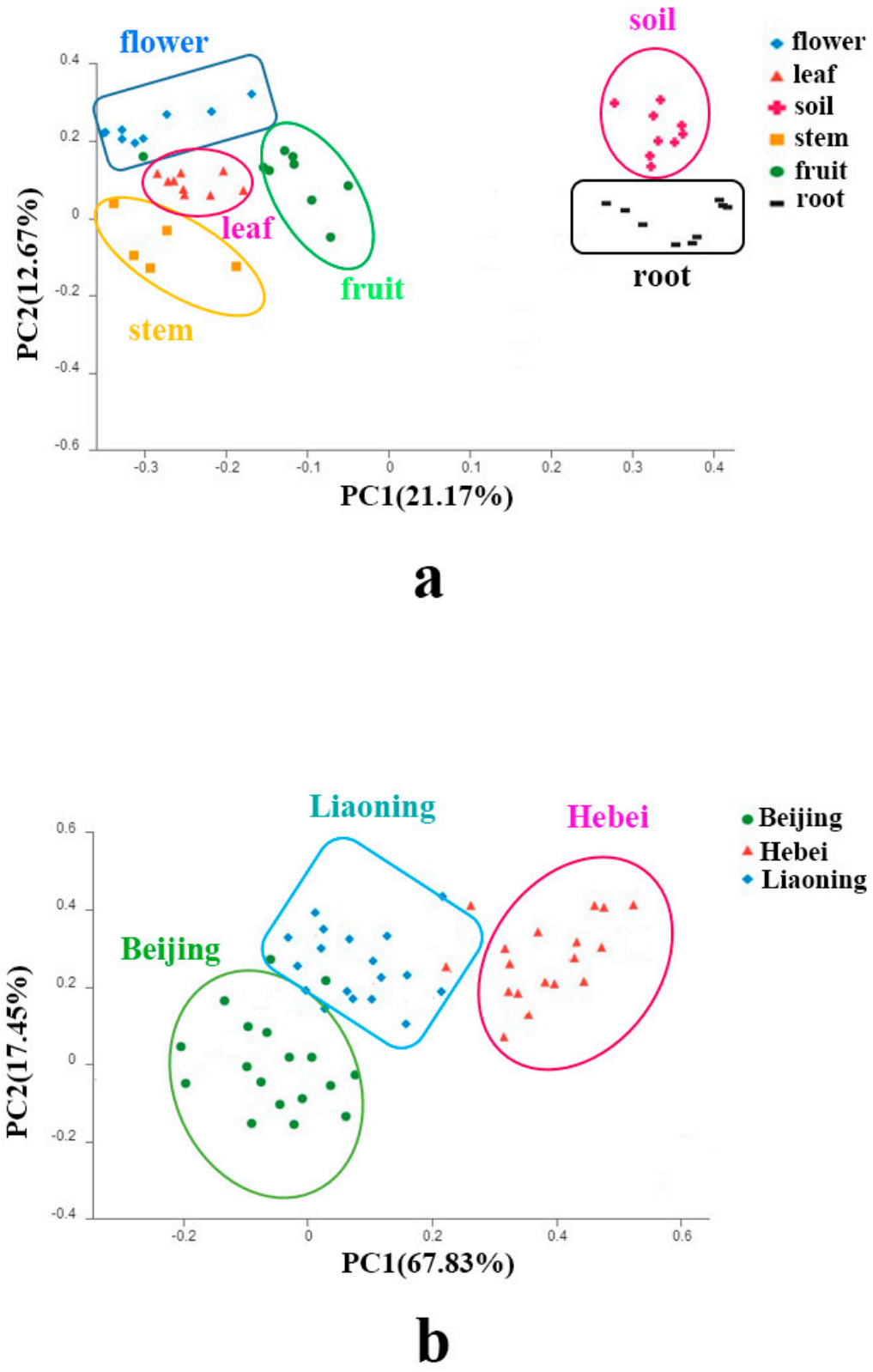



**Figure 4.** Principal coordinates analysis (PCoA) based on the relative abundance of fungal OTUs showing the fungal community structure: (**a**) in different organs and soil of Jingbai pear trees; (**b**) in different sites.

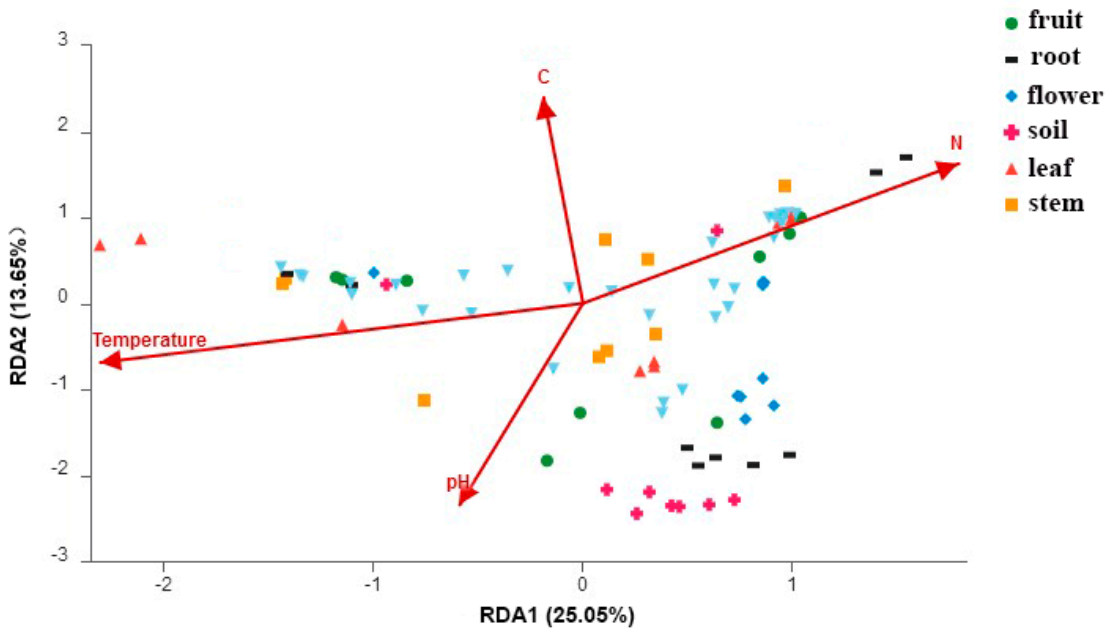



**Figure 5.** RDA plot showing the correlation between fungal community structure and soil properties.

## 4. Discussion

Although both culture-dependent and culture-independent approaches should be used for microbial studies [3,26], next–generation sequencing technology was mainly adopted in this studyas high-throughput sequencing has the great advantage of generating huge species data and detecting unculturable species at a much lower price. Isolation and isolation-related studies will be carried out in the future. As far as it is known, this is the first implementation of PCR-based Illumina Miseq technology for investigating endophytic mycobiota in Jingbai pear trees. In this study 6 phyla, 28 classes, 90 orders, 208 families, 478 genera, 832 species and 1856 OTUs were identified in the fungi kingdom, providing a comprehensive picture for the unexplored fungal diversity of Jingbai pear trees.

Previous studies have shown that mycobiota mainly consist of Ascomycota and to a lesser extent, Basidiomycota and Zygomycota [6,18,26,44]. The fungal communities in our study were also predominated by Ascomycota in all the organs and soils. Class Dothideomycetes was the most abundant in all organs, however, its abundance was much lower in roots and soils than in the other four organs. Dothideomycetes has also been reported as the largest group in *Pinus halepensis* Mill. [45], *Lycopodium annotinum* L. and *Lycopodium clavatum* L. [46], as well as blackcurrant berries in Lithuania [32]. Members of Dothideomycetes include several plant pathogens and the majority are found to be endophytes, or saprobes growing on woody debris, decaying leaves or dung [19,46,47]. The abundance of Sordariomycetes and Leotiomycetes has shown an opposite trend compared to Dothideomycetes. The two classes are sister groups [48]. Members include endophytes, saprobes, coprophilous and fungicolous, and lichenicolous taxa [49,50]. The high abundance of Sordariomycetes and Leotiomycetes in the roots of Jingbai pear trees may indicate an active role.

At family level, Pleosporaceae were the most abundant in fruits, leaves, flowers and stems, while Trichocomaceae were the most abundant in roots and soils. Pleosporaceae species were found as endophytes of plants, pathogenic or saprobic on wood, herbaceous stems and leaves [51,52]. Trichocomaceae including members of *Aspergillus* and *Penicillium* found in soils as well as plants [53,54]. *Alternaria* spp. were the most abundant in fruits, leaves, flowers and stems, which were also reported as the most abundant endophytic fungi in fruit cherry [23,55] and apple trees [25]. *Alternaria* spp. are ubiquitous in the environment and include saprobic, endophytic and pathogenic species associated with a wide variety of substrates [56]. *Alternaria* spp. can produce highly bioactive metabolites, i.e., one species derived from *Morinda officinalis* can generate thirteen compounds including

isobenzofuranone A and indandione B, which have significant inhibitory activities against tumor cell lines [57]. Some species have antimicrobial activities and can be potentially used for biological control of plant diseases [58]. *Talaromyces* spp. have a wide distribution and some species are involved in leaf litter decomposition [59]. The secondary metabolites and compounds of *Talaromyces* spp. have attracted great attention, i.e., many strains from mangrove forests show bioactive effects of secondary metabolites (more than 60 compounds identified), particularly cytotoxic/antiproliferative activity against tumor cell lines, antimicrobial effects, and immunosuppressive and enzyme inhibitory aptitudes [60]. In our study, *Talaromyces* spp. were more abundant in roots and soils. The existence of *Alternaria* and *Talaromyces* members were verified. Similarly, the few species identified in the study (*Meyerozyma guilliermondii*, *Aspergillus fumisynnematus*, and *Fusarium solani*) were also cultivated from Jingbai pear organs in this experiment. *Meyerozyma guilliermondii* has been reported with antifungal, anticancer and phosphate-solubilizing activity [61,62]. Fumimycin produced by *Aspergillus fumisynnematus* can be a new target in antibacterial, antimalarial and anticancer drug discovery [63]. Bioactive dihydronaphthoquinone derivatives of *Fusarium solani* exhibit strong cytotoxic activity [64]. For the presence of *F. solani* within the samples, since ITS region is not a good molecular marker for *Fusarium* species, the molecular marker Elongation Factor (TEF 1$\alpha$) should also be analyzed [65].

The plant habitat is a dynamic environment where many factors can affect the structure of the microbial community [4,30]. Previous studies have shown that different plant organs host a different fungal community [18,27,32]. This was verified using a survey that found fungal community structures differed significantly among five organs, since each organ forms a distinctive microenvironment. It has been reported that individual microbiota of compartments consist of a selective gradient from soil, exterior root, rhizoplane, interior roots and other endosphere compartments [6]. In this study, soil and root were found to have the highest fungal richness and diversity. The fungal community in roots and soils demonstrated more similarities when compared with other organs (leaf, flower, fruit and stem), this may be due to spatially distance. Tree conditions (phytosanitary health status) can also be an important factor of fungal endophytic community structures [18]. For Scots pine trees, declining trees were found to harbor a higher number of taxa and were characterized by higher colonization frequencies than healthy–looking trees. Fungal assemblages are significantly affected by the degree of decline of trees [18] and studies on this aspect should be considered in the future.

The results demonstrated that three geographically different sites harbored a unique fungal community. Several studies have reported that site conditions can influence a fungal community [4,66]. For fruit trees, there are fewer studies concerning the influence of sites [32]. Blackcurrant from different regions had different fungal communities [32]. Regional effect was also found in grape wine microbial at different vineyards [67,68]. Endophytic fungi from medicinal plants in different sites may even affect the quality and effects of medicines [4]. Therefore, geographical locations must be a very important environmental effector.

Fungal community structures were significantly correlated with soil properties—soil temperature, pH, N and C, indicating soil may have an influence on the fungal community structure associated with Jingbai pear trees. Plants and soils have a very close mutual relationship. A plant can affect soil organic matter, soil nutrient availability and the composition of soil microbial communities. The influences on soil properties result in net positive or negative feedback effects, which influence plant and community composition [69], for example, soil type and properties may alter root development and root exudation [70]. Endophytic communities live and coevolve with trees for a long time, therefore the structure can be correlated with soil properties [6,69]. In a recent study, the endophytic bacterial communities associated with Jingbai pear trees also differed significantly among different organs and soils, and the bacterial community structure related significantly to soil properties [33]. These findings are consistent with the results of this study.

## 5. Conclusions

Each organ investigated (flower, leaf, fruit, stem and root) in this study harbored a unique fungal assemblage. Ascomycota was the most abundant phylum, followed by Basidiomycota and Zygomycota. As to the correlation between fungal community structures, the sampling sites and soil properties, the fungal communities from the different sites (soils) differed from each other. The fungal community structure remarkably correlated with soil temperature, soil pH, soil nitrogen and soil carbon contents. These results indicate that plant organs, sampling sites and soil properties may exert influence on the endophytic microbial community structure associated with Jingbai pear trees. The isolation and cultivation of core potential endophytic fungal strains with a growth-promoting effect and pathogen resistance, as well as ecological and functional roles, will require further study.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/1999-4907/10/3/260/s1. Figure S1. Statistically significant differences in the fungal species richness (A), diversity (B) and evenness (C) of the samples, * $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, *** $p \leq 0.001$. Figure S2. Rarefaction curves of all the samples. All the samples are saturated for further study. Figure S3. Significantly different taxa among samples. (A) at phylum level, (B) at class level, (C) at family level, (D) at genus level, (E) at species level, (F) at OTU level.

**Author Contributions:** F.R., H.S., D.-H.Y. conceived the study and contributed in the experimental design of the study; F.R., W.D. and D.-H.Y. collected the samples; F.R. conducted the experiment; F.R., W.D. performed the statistical analysis; F.R. wrote the first draft of the manuscript; all authors contributed to manuscript revision and approved the submitted version.

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**Ethics:** The samples in the study were collected on private land and the owners gave full permission to conduct the study on the sites. The experimental materials did not involve any humans or animals.

**Conflicts of Interest:** The authors declare no conflict of interest.

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