Divergence of Phyllosphere Microbial Communities Between Females and Males of the Dioecious *Populus cathayana*

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Females and males of dioecious plants have evolved sex-specific characteristics in terms of their morphological and physiological properties. However, the differentiation of phyllosphere microbiota in dioecious plants remains largely unexplored. Here, the diversity and composition of female and male *Populus cathayana* phyllosphere bacterial and fungal communities were investigated using 16S rRNA/ITS1 gene-based MiSeq sequencing. The divergences of bacterial and fungal community compositions occurred between females and males. Both females and males had their unique phyllosphere bacterial and fungal microbiota, such as bacterial *Gemmata* spp. (5.41%) and fungal *Pringsheimia* spp. (0.03%) in females and bacterial *Chitinophaga* spp. (0.009%) and fungal *Phaeococcomyces* spp. (0.02%) in males. Significant differences in the relative abundance of phyla *Proteobacteria* and *Planctomycetes* bacteria and phyla *Ascomycota* and *Basidiomycota* fungi ($P < 0.05$) were also found between females and males. Some bacterial species of genera *Spirosoma* and *Amnibacterium* and fungal genera *Venturia*, *Suillus*, and *Elmerina* spp. were significantly enriched in males ($P < 0.05$). In contrast, levels of fungal genera *Phoma* and *Aureobasidium* spp. were significantly higher in females than in males ($P < 0.05$). The mineral,

inorganic, and organic nutrients content contributed differently to the divergence of female and male phyllosphere microbial communities, with 87.08 and 45.17% of the variations being explained for bacterial and fungal communities, respectively. These results highlight the sexual discrimination of phyllosphere microbes on the dioecious plants and provide hints on the potential host-associated species in phyllosphere environments.

Keywords: dioecious plants, microbial community, MiSeq sequencing, phyllosphere, *Populus cathayana*

Liling Liu and Lu Lu contributed equally to this work.

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Dioecious plants consist of more than 14,620 angiosperm species (Renner and Ricklefs 1995). Among these species, *Populus cathayana* is extensively distributed in temperate zone. As a common afforestation species, *P. cathayana* are fast-growing trees with the characteristic of strong adaptability, easy reproduction, and wide application, and it is widely used in ecological management, environmental improvement, and wood processing (Xu 2008). Recently, the differences between female and male plants have become a topic of considerable interest. The sexual differences in morphology, resource allocation, and reproductive allocation, stress adaptive capacity, and nutrient availability in stressful environments have been extensively addressed (Cipollini and Whigham 1994; Leigh et al. 2006; Wu et al. 2018; Zhang et al. 2010a, 2014b). Female and male *P. cathayana* have been found to differ in leaf morphology (Zhang et al. 2011), N use efficiency (Zhao et al. 2009), leaf senescence speed (Chen et al. 2010), total leaf area per unit of stem mass (Yang et al. 2015), and net photosynthesis rate (Xu et al. 2008b). Zhao et al. (2011) also found that the chlorophyll a/b content in male *P. cathayana* was higher than that in females. Male *P. cathayana* possess a more-efficient antioxidant system, a higher anthocyanin content to alleviate UV-B radiation, and a better self-protection mechanism than females (Xu et al. 2008a and b, 2010; Zhang et al. 2010a, 2012). Moreover, whether the morphological and physicochemical differences drive the divergence of phyllosphere bacterial and fungal communities in male and female *P. cathayana*, particularly in complex natural forests, is still unclear.

The phyllosphere, comprising the aerial surfaces of terrestrial plants, is considered the largest biological interface on Earth (Bailey et al. 2006). Reports conservatively estimate that the

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global leaf surface is approximately 6.4×10^8 per square kilometer in area and harbors more than 10^{36} microorganisms (Leveau and Lindow 2001; Lindow and Brandl 2003; Vorholt 2012). Although exposed to highly variable environmental factors, including UV radiation, temperature, water availability humidity, and heterogeneous nutrient availability (Lindow and Leveau 2002; Rastogi et al. 2013), the leaf surface is still colonized by highly diverse microorganisms, such as bacteria, fungi, and archaea, which are called phyllosphere microbes (Lindow and Brandl 2003). Phyllosphere microbiota were found to be in symbiosis with hosts and influence growth and ecological function in many ways, such as increasing plants stress tolerance (Delmotte et al. 2009; Kaczmarczyk et al. 2011), production of growth-promoting nutrients and hormones of plants (Bulgarelli et al. 2013; Fümkrantz et al. 2008; Reed et al. 2010), and protection of hosts against pathogen (Balint-Kurti et al. 2010; Innerebner et al. 2011). For dioecious plants, previous studies have shown that there are significant sexual differences in the physiological responses of *P. cathayana* under stressful conditions, such as nutritional deficiencies, drought, light, and temperature (Xu et al. 2008b; Zhang et al. 2014b; Zhao et al. 2009). These studies underscore the possibility of the differentiation of phyllosphere microbiota in *P. cathayana*. Additionally, the diversity of phyllosphere microbial communities is closely related to the physicochemical properties of leaves. For example, previous studies showed that different leaf-surface features, such as stomata, hydathodes, water, and phosphorus contents, and mesophyll thickness resulted in an uneven microorganism distribution on leaf surfaces (Kembel and Mueller 2014; Mariano and McCarter 1993; Mew 1984; Yadav et al. 2005). Hunter et al. (2010) revealed that plant morphology and soluble carbohydrate content also significantly influenced the diversity of the phyllosphere bacterial community. In addition, recent studies have reported that plant genotype is a critical factor influencing the microbial composition of the phyllosphere microorganisms (Redford and Fierer 2009; Whipps et al. 2008). However, most of the current studies on phyllosphere microorganisms are on crops and monoecious plants, and systematic comparisons of the phyllosphere bacterial and fungal communities of dioecious plants are still lacking.

In this study, the common native dioecious species *P. cathayana* Rehd, in the Xiaowutai Mountain Nature Reserve, Hebei Province, China, was chosen to characterize and compare the diversity and composition of the female and male phyllosphere bacterial and fungal communities using 16S rRNA/ITS1 gene-based MiSeq sequencing. Previous studies revealed that the definition of endophytic and epiphytic microorganisms was ambiguous and some epiphytic fungi can penetrate into the epidermis or stomata and colonize internal leaf tissues (Cordier et al. 2012a; Viret and Petrini 1994). The sequenced phyllosphere microbial communities comprise both

the surface and the interior of leaves (Delmotte et al. 2009; Jumpponen and Jones 2009). This work addressed two key questions. i) Are there sex-specific variation in the phyllosphere bacterial and fungal communities between females and males, and ii) do physicochemical properties of leaves contribute to the divergence of bacterial and fungal communities between females and males. Our results could provide insights into the genetic resources that are host-associated in dioecious plants.

RESULTS

Sexual differences in leaf physicochemical properties.

The 12 physicochemical characteristics of female and male leaf samples were shown in Table 1. The total carbon (TC) content in male leaves was significantly higher than that in female leaves ($P < 0.001$), with concentrations of 454.98 and 428.23 mg/g, respectively. There was no significant difference in pH, moisture content, total nitrogen (TN), total phosphorus (TP), starch, soluble sugar contents, and mineral content (K, Ca, Mg, Cu, and Fe) between female and male leaves ($P > 0.05$).

Sexual differences in richness and diversity of phyllosphere microbial communities.

In total, 894,616 and 779,162 high-quality bacterial and fungal sequences were obtained from eight female leaf samples and eight male leaf samples (Supplementary Table S1). The rarefaction analyses indicated that the diversity of all samples was well-represented by the number of sequences analyzed (Supplementary Fig. S1). The bacterial sequences in the female and male phyllosphere microbial communities were clustered into 510 and 584 operational taxonomic units (OTUs) at a 97% sequence similarity, respectively. The fungal sequences were clustered into 470 and 550 OTUs in females and males, respectively (Supplementary Table S1). Both females and males had their shared and unique bacteria and fungi OTUs (Supplementary Fig. S2). As to the alpha-diversity indexes, no significant differences between female and male phyllosphere bacterial and fungal communities were detected in the Chao1, abundance-based coverage estimator (ACE), Shannon, and Simpson indexes ($P > 0.05$) (Fig. 1A). Similarities of the phyllosphere bacterial and fungal communities between sexes were compared by non-metric multidimensional scaling (NMDS) and the adonis test based on Bray-Curtis. Stress values of <0.2 and <0.1 indicated good to potential useful interpretation and appropriate representativeness, respectively. NMDS analysis showed that phyllosphere bacterial and fungal communities of females and males were distributed in different quadrants (Fig. 1B). To verify the dominant role of sex selection on microbial communities, the variations between the individual samples and females vs. males were determined using principal component analysis and adonis test (P values) (Table 2; Supplementary Figs. S3 and S4). As shown in Table 2, the degree of variation inferred by the mean square values was greater between females and males (mean squares of 0.332 and 0.167 for bacterial and fungal communities, respectively) than among all individual samples (mean squares of 0.002 and 0.059 for bacterial and fungal communities, respectively). These results indicated that the interplant (females vs. males) variation was greater than that of intraplant (among individuals) in microbial communities.

Sexual differences in the composition of the phyllosphere bacterial community.

The dominant bacterial phyla were *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Firmicutes*, and *Planctomycetes* (Fig. 2A). The phylum *Proteobacteria* accounted for 91.46 and

Table 1. The physicochemical characteristics of leaves^z

Leaf physicochemical properties	Female	Male
pH	5.97 ± 0.15a	5.83 ± 0.13a
Moisture content (%)	66.11 ± 1.03a	64.62 ± 2.60a
TC (mg/g)	428.23 ± 4.68b	454.98 ± 6.27a
TN (mg/g)	15.06 ± 3.56a	18.33 ± 6.37a
TP (mg/g)	1.04 ± 0.26a	1.13 ± 0.41a
Starch (mg/g)	87.03 ± 17.70a	120.46 ± 33.20a
Soluble sugar (mg/g)	79.37 ± 20.77a	69.61 ± 14.24a
K (mg/g)	11.83 ± 4.52a	8.70 ± 2.14a
Ca (mg/g)	25.72 ± 2.68a	25.21 ± 2.51a
Mg (mg/g)	2.60 ± 0.41a	2.37 ± 0.45a
Fe (mg/g)	0.58 ± 0.14a	0.49 ± 0.17a
Cu (mg/kg)	7.97 ± 0.80a	6.98 ± 0.73a

^z Different letters following values within the same lines indicate significant differences between females and males ($P < 0.05$).

95.48% of the total sequences in the female and male phyllosphere communities, respectively, and exhibited a significant difference in abundance between females and males based on the Metastats analysis ($P < 0.05$). The relative abundance of planctomycetes in females (4.81%) was significantly higher than that in males (1.77%) ($P < 0.05$). The dominant bacterial classes were *Alphaproteobacteria*, *Actinobacteria*, *Betaproteobacteria*, and *Cytophagia*, representing 98.4 and 98.7% of the total sequences in females and males, respectively (Fig. 2C). *Alphaproteobacteria* was the most abundant class,

accounting for 88.86 and 92.68% of the sequences both in females and males, respectively, showing no significant difference in abundance between them. At the genus level, nine bacterial genera were unique in the female phyllosphere, such as genus *Gemmata* (5.41%), and some rare (relative abundance $< 0.1\%$), such as genera *Rhodococcus*, *Brevundimonas*, *Devosia*, *Modestobacter*, *Xenophilus*, and *Vibrionimonas* (Supplementary Fig. S5A). Two rare bacterial genera, *Candidatus_Hodgkinia* (0.03%) and *Chitinophaga* (0.009%), were unique in male phyllosphere (Fig. 3A). Moreover,

Fig. 1. A, Alpha diversity indexes of bacterial and fungal communities in the phyllosphere of female and male *Populus cathayana*. F and M represent female and male, respectively. Each group was conducted in eight repetitions. Error bars represent standard errors of the mean of the eight repetitions. **B,** Non-metric multidimensional scaling analysis, based on Bray-Curtis distance, showing microbial community composition differences between females and males.

Table 2. Permutational multivariate (adonis) analysis of variance of the phyllosphere bacterial and fungal composition between the individual samples and females versus males^z

Source	df	Sums of squares	Mean squares	F	R ²	P
Bacteria						
Females vs. males (inter-variation)						
Between sexes	1	0.332	0.332	1.628	0.320	0.120
Among individuals (intra-variation)						
Within females	7	0.194	0.194	1.115	0.027	0.715
Within males	7	0.144	0.144	1.031	0.031	0.532
All samples	15	0.002	0.002	0.354	0.039	0.498
Fungi						
Females vs. males (inter-variation)						
Between sexes	1	0.167	0.167	1.290	0.234	0.205
Among individuals (intra-variation)						
Within females	7	0.091	0.091	1.381	0.185	0.515
Within males	7	0.020	0.020	0.563	0.022	0.836
All samples	15	0.059	0.059	1.123	0.028	0.671

^z The compositional dissimilarity between assemblages was assessed with the Bray-Curtis dissimilarity index.

significantly higher relative abundances of the genera *Spiriosoma* (male 1.38%, female 0.32%) and *Amnibacterium* (male 2.58%, female 1.09%) were observed in males than in females ($P < 0.05$) (Fig. 4A).

Sexual differences in the composition of the phyllosphere fungal community.

The dominant fungal phyla were *Ascomycota*, *Basidiomycota*, and *Neocallimastigomycota*, accounting for more than 99.83 and 99.91% of the fungal sequences in females and males, respectively (Fig. 2B). The relative abundance of *Ascomycota* was significantly higher in females than in males, whereas *Basidiomycota* showed the opposite trend ($P < 0.05$). *Dothideomycetes* and *Leotiomyces*, belonging to the phylum *Ascomycota*, were the main classes in both females and males (Fig. 2D). The relative abundance of class *Agaricomycetes* in males was significantly higher than that in females ($P < 0.05$). With respect to the genus level, six fungal genera were unique in females (Fig. 3B), including genera *Pringsheimia*, *Psathyrella*, *Farysizyma*, and *Sarocladium*, with a relative abundance level of $<0.1\%$. Ten fungal genera were only present in male phyllosphere. Among them, genus *Phaeococcomyces* (0.02%)

was the most dominant, and the relative abundances of other 9 genera were less than 0.01%, such as genera *Phaeococcomyces*, *Stachybotrys*, and *Tremella* (Supplementary Fig. S5B). By comparing the relative abundance of shared genera, significantly higher relative abundances of genera *Aureobasidium* (1.75%, $P < 0.05$) and *Phoma* (19.21%, $P < 0.001$) were detected in females than in males. In contrast, significant higher relative abundances of genera *Elmerina*, *Suillus*, and *Venturia* were observed in males than in females ($P < 0.05$) (Fig. 4B).

Multivariate correlation analysis between bacterial and fungal communities and leaf physicochemical properties.

Variation partitioning analysis (VPA) based on partial redundancy analysis (RDA) illustrating the effects of environmental factors on the female and male phyllosphere microbial communities (Fig. 5; Supplementary Fig. S6; Supplementary Table S2). The VPA result showed that nonmetal parameters (including TC, TN, TP, soluble sugar, starch, and moisture content) were the most important contributors to the variance of bacterial communities between females and males, with 80.97% of the observed variation being explained. Mineral factors contributed 6.10% alone (Fig. 5A). As to the fungal

Fig. 2. A, Phylum-level taxonomic composition of the bacterial and B, fungal communities. C, Class-level taxonomic composition of the bacterial and D, fungal communities. F and M represent female and male, respectively.

communities, only 45.17% variation in fungal profiles of females and males was explained, and nonmetal parameters (29.25%) were identified as the main driver for the differences in fungal communities of females and males. Metal contents contributed 5.92% of the total variance in fungal communities of females and males (Fig. 5B). Among these metals, Mg and Fe content contributed mostly to the variance with 3.45% of the total metal contribution (Supplementary Fig. S7). RDA results also showed that Mg and Fe content of leaves was the significant constraining variable shaping fungal community composition of the overall samples ($P < 0.05$) (Supplementary Fig. S6B).

DISCUSSION

In this study, no significant differences between the alpha diversity indexes of the female and male *P. cathayana* phyllosphere microbial communities was observed, whereas there were, indeed, some taxa discriminatingly inhabiting on females and males. The leaf physicochemical properties contributed differently to the differentiation of the microbial community compositions in females and males. The study could provide typical data for microbial sexual discrimination in dioecious plants and, thus, contribute to the increasing knowledge of microbial ecology in phyllosphere environment.

Fig. 3. **A**, The shared and unique bacterial and **B**, fungal genera between female and male *Populus cathayana*. F and M represent female and male *P. cathayana*, respectively.

Fig. 4. **A**, Taxon relative abundance of bacterial and **B**, fungal genera that was significantly different between the phyllosphere of female and male *Populus cathayana* ($P < 0.05$).

The TC content of the male leaves was higher than that in female leaves. The TC content of leaves is an indicator of plant carbon sequestration ability during photosynthesis (Boerner 1984), and photosynthesis is directly related to the availability of nutrients for microorganisms on the leaf surface and in the leaf tissues (Vacher et al. 2016). Compared with females, males exhibited a higher photosynthetic rate and carbon fixation rate (Xu et al. 2008a), which could explain the higher TC content in male leaves observed here. The other leaf properties measured here did not differ between the females and males, which was consistent with the results of previous studies on *P. cathayana* (Chen et al. 2014; Zhang et al. 2010a).

There were no significant differences in the alpha diversity indexes between females and males, which was consistent with the findings of a previous study showing that the phyllosphere microbial community structure of the same species showed little variability (Yang et al. 2001). Previous study had also shown that there was far more variability in phyllosphere microbial community structure across tree species than within species in the same habitat (Redford et al. 2010). The samples in this study were collected from the same hillside with similar elevation, temperature, rainfall, or solar, which might explain the undifferentiated diversity indexes between females and males. However, the differences in leaf-surface structure, element contents, stomatal distribution, gas exchange, and net photosynthetic rates (Liu 2003; Wang et al. 2010; Xu et al. 2008a) between females and males might lead to the divergence of the phyllosphere microbial communities (Yadav et al. 2005). The microbial OTU composition variation between female and male trees was much higher than that among individual trees, supporting the role of sex-selection on phyllosphere microbial communities (Table 2; Supplementary Figs. S3 and S4). As to the OTU result, the data showed that fungal species of most samples outstrip bacteria (Supplementary Table S1). This result is consistent with many previous studies (Kembel and Mueller 2014; Kembel et al. 2014). The OTU of each sample was 218 to 1,040, and 207 to 869 OTUs for bacterial and fungal communities were detected in all the samples at a 97% sequence similarity, which was much lower than the OTU number in soil or water samples (Chen et al. 2020; Wang et al. 2018). Many previous studies also found that only hundreds of OTUs were detected in phyllosphere microbial communities (Cordier et al. 2012a; Delmotte et al. 2009; Gomes et al. 2018; Kim et al. 2012; Ren et al. 2014).

An increasing body of studies has demonstrated the important roles of microbial communities in regulating the phyllosphere multifunctionality (Delmotte et al. 2009; Kembel et al. 2014; Stone et al. 2018). However, most of our knowledge is still based on dominant species or the diversity indexes, and less

attention has been paid to some individual species or rare microbial taxa. Here, we identified both abundant and rare taxa that were enriched in females or males, respectively. For example, a significant higher proteobacteria relative abundance was observed in males than in females. The abundance of proteobacteria was often positively correlated with nutrient availability (Fierer 2017). The higher proteobacteria abundance in males was coupled well with higher leaf nutrition (TC, TN, TP, and starch) in males (Table 1). In contrast, the higher relative abundance of planctomycetes in females might be explained by their potential capacity to resistance to chemical antimicrobial compounds (Wagner and Horn 2006). Previous studies have reported that contents of antimicrobial compounds were much higher in the phyllosphere of females than that of males (Cepeda-Cornejo and Dirzo 2010; Cornelissen and Stiling 2005). Thus, the planctomycetes could lose their competitive advantage over other bacterial groups in the phyllosphere of males (Bengtsson and Øvreås 2010), explaining their lower abundance in males. At the genus level, the higher abundance of *Spirosoma* spp. in males compared with females might be related to the higher UV-B resistance capacity of males (Xu et al. 2010). Many *Spirosoma* species revealed resistance to gamma and UV radiation, such as a *Spirosoma radiotolerans* (Lee et al. 2014) and *Spirosoma swuense* (Joo et al. 2017) (Supplementary Table S3). The ability to withstand UV radiation is thought to be an important adaptation for the survival of bacteria in the phyllosphere, and a previous study revealed that UV radiation can alter maize phyllosphere bacterial diversity (Kadivar and Stapleton 2003). Furthermore, the leaf area of males is generally larger than that of females (Cornelissen and Stiling 2005; Xu et al. 2010), so the leaves of males are exposed to more UV-B radiation compared with those of females. The higher abundance of *Spirosoma* spp. in males could be due to their high resistance to gamma and UV radiation (Lee et al. 2015). Genus *Amnibacterium* was found to be significantly enriched in males. The isolates *Amnibacterium soli* sp. and *Amnibacterium kyonggiense*, whose 16S ribosomal RNA (rRNA) gene sequences were more than 97% similar with the *Amnibacterium* in males, can produce a wide range of biologically active metabolites, possess antibacterial activity, and promote the growth of plants by fixing nitrogen (Jiang et al. 2018; Selvakumar et al. 2014). The enrichment of *Amnibacterium* in males could contribute to the more effective pathogen defense mechanism in males compared with females (Zhang et al. 2010b). Besides, the unique genus *Gemmata* in females is related with sterol biosynthesis and has the ability to uptake proteins present in the external milieu through an energy-dependent and endocytosis-like process (Lonhienne et al. 2010; Pearson et al. 2003), such as its closely related

Fig. 5. Variation partition analysis based on **A**, the phyllosphere bacterial and **B**, fungal community structure and leaves physicochemical variables.

species *Gemmata obscuriglobus* (>97% similarity) (Supplementary Table S3). Interestingly, the phytoestrogens in female plants can induce the steroid transcription to microorganisms (Maier et al. 1995), which might select the *Gemmata* spp. in females. Besides, some rare genera (<0.1%) were only present in females or males, such as genera *Rhodococcus*, *Brevundimonas*, and *Chitinophaga*, which are all found to have a potential role in organic compound metabolism (Martínková et al. 2009). Recent finding also proposed that microbial taxa with low relative abundance could have an overproportional role in biological multifunctionality and be crucial to the sustainable provision of ecosystem functions (Jousset et al. 2017). These host-specific microbes could lead to the functional divergence of female and male phyllosphere microbial communities. The individual species and rare microbial taxa have attracted well-deserved attentions (Chen et al. 2020). Therefore, the sex-biased colonization of microbes in females or males and their relationship with the division of ecological functions warrant further studies with greater details.

The phyllosphere fungal communities were dominated by members of phyla *Ascomycota* and *Basidiomycota* both in females and males, which were also found in other tree species (Davey et al. 2012; Jumpponen and Jones 2009; Kembel and Mueller 2014). Some fungal taxa were found to be preferentially enriched in females or males, respectively. A significant higher ascomycota relative abundance was observed in females than in males. Most genera of phylum *Ascomycota* can produce metabolites that can protect hosts from fungal pathogens (Rodriguez et al. 2009; Schulz et al. 1999). Higher concentrations of phenolic and fungal metabolites in females than in males were previously observed (Cepeda-Cornejo and Dirzo 2010; Cornelissen and Stiling 2005). The metabolites produced by fungi, such as alkaloids, and phenolic acids, in turn, have been shown to affect the growth of phyllosphere fungi (Grayer and Harborne 1994; Rauha et al. 2000), which could result in the enrichment of ascomycota in females. Besides, levels of the genera *Aureobasidium* and *Phoma* belonging to phylum *Ascomycota* were markedly higher in females than in males. Their closest isolates *Aureobasidium pullulans* and *Phoma* spp. have a key functional role in resisting pathogenic microorganisms (Vacher et al. 2016; Zhang et al. 2014a) and promoting plant growth (Wang et al. 2012) (Supplementary Table S3). In contrast, the relative abundance of basidiomycota was much higher in males than in females. Previous studies showed that the abundance of basidiomycota increased with the course of leaf senescence (Kembel and Mueller 2014; Osono 2006; Voříšková and Baldrian 2013). Moreover, our previous studies reported that male *P. cathayana* have a faster leaf senescence rate than females (Xu et al. 2008b; Zhao et al. 2009), which could explain the enrichment of basidiomycota in males. In addition, phyllosphere fungi have the ability to utilize various organic compounds and participate in leaf litter decomposition (Osono 2006). Members of the unique genus *Pringsheimia* in females possess the capacity to decompose lignin and leaf litter (Fillat et al. 2017; Voříšková and Baldrian 2013), indicating different decomposition rate of leaves might occur in female and male *P. cathayana*. This result might also be closely related with the sampling time, further studies should consider the variations among the seasons. In addition, some fungi are not commonly present in the phyllosphere environment, such as *Suillus* spp. Members of the significant enriched genus *Suillus* in males are common ectomycorrhizal fungi colonizing the roots of plants (Kurth et al. 2013). Members of genus *Suillus* are common ectomycorrhizal associates of conifers in temperate region, mostly with members of family *Pinaceae* (Sarwar 2014; Wu et al. 2000). Recent studies showed that *Suillus* spp. can be detected in many other environments by using molecular

sequencing analysis (Sarwar et al. 2015; Zhou et al. 2008). For example, the *Suillus* spp. can survive in the leaf litter of many tree types, such as genera *Abies*, *Cedrus*, *Pinus*, *Populus*, and *Quercus* (Sarwar 2014). Although *Suillus* spp. are common root ectomycorrhizal fungi, *Suillus* spp. were also found in beech leaf litter (Colpaert and Laere 1996). Some fungi, like *Phoma* spp., can rapidly emerge and sporulate on leaves during tree senescence (Weber et al. 2004). The detected *Suillus* species here could be due to spore deposition on the leaves, which provides a physical and chemical environment suitable for fungal growth and reproduction (Rodriguez et al. 2009). Although previous studies reported that *Suillus* spp. preferentially colonized in a nitrogen-limited oligotrophic environment (Lilleskov et al. 2001), how the physicochemical properties of male *Populus cathayana* phyllosphere environment select the *Suillus* species warrants further study. Furthermore, the detected *Suillus* spp. also imply that the detected microbial diversity might not only contain living and growing microorganisms but also include spores or sequenced 16S rRNA gene from extracellular DNA or dead cells (Blagodatskaya and Kuzyakov 2013). The identification of living and active microorganisms from the total sequenced communities on the female and male leaves should be taken into consideration in further studies.

The main nonmetal nutrients, such as C, N, P, or compound (sugar, starch), were the most important contributors to variance of bacterial communities between females and males with 80.97% of the variation being explained. This observation was consistent with previous studies showing that phyllosphere bacteria communities are primarily shaped by carbon-limited and secondarily nitrogen-limited conditions (Delmotte et al. 2009; Hunter et al. 2010; Kembel et al. 2014; Wilson and Lindow 1994). In contrast, these nonmetal nutrients only contributed 29.25% of the total variations in fungal communities of females and males. This result suggested that future fungal community ecology should be explored in terms of more explanatory deterministic physicochemical factors and other potential stochastic factors (Chen et al. 2019). Besides, mineral contents contributed to the variations between females and males in the microbial communities. For instance, Mg and Fe content showed important roles in shaping fungal communities (Supplementary Fig. S6B). These results were consistent with results of previous studies showing that fungal community structures significantly correlated with mineral content (such as K, Al, Fe, Zn) of leaves (Kembel and Mueller 2014; Martín-García et al. 2011). Mg and Fe are involved in numerous physiological and biochemical processes in plants, including photosynthesis, enzyme activation, synthesis of nucleic acids and proteins, or nitrogen fixation (Chen et al. 2018; Graziano et al. 2002). Mg and Fe could affect the microbial colonization by participating physiological metabolism of plants. It was found that phyllosphere fungi can acquire Mg and Fe from their host plants (Wyszkowska and Wyszkowska 2002; Johnson 2008).

In summary, although the dominant bacterial and fungal taxa were of no significant difference between the female and male phyllosphere microbiota, there were some taxa discriminately inhabiting in females and males. Further verification studies regarding the differentiation of microbial community compositions and functions in dioecious plant is therefore of importance for revealing the evolutionary differences between females and males. Here, we propose four potential verification experiments (Supplementary Fig. S8). i) Metatranscriptomics analysis of microbial communities could be applied to detect all the genes expressed under the female and male phyllosphere environment and not only present in the samples (Moran et al. 2013). ii) Synthetic microbial community-based experiments by inoculating the synthetic community onto leaves could be

performed to explore whether the difference microniche or microhabitat of the female and male phyllosphere environment influence the microbial communities (Xin et al. 2016). iii) Pot experiments under given conditions (such as drought, UV light, nutrient deficiency) could be established. The experiment could further verify the role of microbial communities in stress resistance of female and male *P. cathayana* by monitoring the growth of plants and dynamic change of phyllosphere microbial communities (Methe et al. 2020). iv) Active bacteria groups responsible for specific biogeochemical cycle in female and male phyllosphere environments could be traced using the DNA-stable isotope probing technique (Neufeld et al. 2007). The present study is a good start for the following verification experiments and provides typical data for microbial sexual discrimination in dioecious plants.

Conclusion.

Taken together, the results of this study underscore the role of sex selection in driving variations in the female and male phyllosphere microbial communities of *P. cathayana*. Though there were no significant differences between the females and males in the alpha diversity indexes, the microbial composition results clearly demonstrated that some microbes discriminately inhabited on females or males. These bacterial and fungal genera appeared to have special ecological function, which could be reflected in the different physiological response to environmental changes in dioecious plants. The different leaf physiochemical properties contributed differently to the variations of microbial community structure between females and males. Our results will improve our understanding of the sex discrimination of microorganism in the dioecious *P. cathayana*.

MATERIALS AND METHODS

Site description and leaf sampling.

The sampling site is located in the Xijin Valley of the Xiaowutai Mountain Nature Reserve in Hebei, China (Supplementary Fig. S9). This area is characterized by a warm-temperate continental monsoon climate with a mean annual precipitation of 528 mm and a mean annual temperature of 3.5°C. The major soil type is alfisol. The forest vegetation is dominated by the genera *Acer*, *Cerasus*, *Betula*, *Corylus*, *Populus*, *Quercus*, *Ulmus*, or *Tilia*. The pure *P. cathayana* natural secondary forest distributes in the deciduous broad-leaf forest zone (1,400 to 1,800 m above sea level).

Leaves of *P. cathayana* were randomly collected from eight females and eight males between 1,300 and 1,400 m above sea level, in riparian and valley habitats, in September 2017. The selected trees were healthy, similar in habitat, full-grown with similar canopy sizes, more than 30 years old, and approximately 20 to 25 m tall. The trees were approximately 200 m away from each other. The sex of the trees was determined by observing the inflorescence during the beginning of the flowering season (the middle of April 2012). The sex of the trees was double identified by their leaf morphological characters. The morphologic characters of male and female leaves are different. The leaves of females are ovate with entire margin and apical acuminate, whereas the leaves of males are long elliptical with undulate margin and apical acuminate (Xu 2008) (Supplementary Fig. S10). Leaves from each tree were randomly obtained from three branches at the same height. The tree branches were chosen on the basis of their orientation (north, southeast, and southwest) to avoid the influence of lighting conditions in different directions. Healthy and similarly sized leaves were collected (50 to 100 g) with sterile gloves and were transferred to sterile plastic bags. All the

leaves were transported to the laboratory in a cooler and were then kept at -20°C for further analysis.

Sample physicochemical analysis.

Fresh leaves (0.5 g) were chopped and ground into a homogenate, and deionized water (30 ml) was added into the homogenate. After ultrasonic vibration filtration for 2 h, the pH value was determined by a pH meter (Mettler-Toledo Instruments Co., Ltd.). Leaf samples were oven-dried at 70°C for 48 h and were then weighed. The dried leaves were measured to calculate the moisture content, were ground to a fine powder, and were passed through a 100-mesh screen. The TC was measured on a TOC analyzer (Elementar-TOC & Water Analysis) by the rapid dichromate oxidation technique (Nelson et al. 1996). The concentrations of TN and TP were determined using an Auto Discrete Analyzer CleverChem 200+ (Dechem-Tech). The content of mineral elements (K, Ca, Mg, Cu, and Fe) was determined using the flame atomic absorption method, using an atomic absorption spectrophotometer (AA-7000; Shimadzu). Fine leaf powder (20 mg) in 4 ml of 80% (vol/vol) ethanol was placed in a water bath at 80°C for 30 min, followed by centrifugation at $5,000 \times g$ for 5 min. Samples were then extracted three times, and the supernatants were pooled together for sugar analysis. The residues remaining in the centrifuge tubes were stored wet at -20°C for starch analysis. After the addition of 2 ml of distilled water, the tubes were placed in a boiling water bath for 15 min and 1 ml of 9.2 M perchloric acid and 1 ml of distilled water was added after cooling and the mixture was shaken for 15 min. The supernatants were then collected after centrifugation at $5,000 \times g$ for 5 min. The residues were re-extracted and the supernatants were combined for starch analysis. Total soluble sugars and starch were both determined colorimetrically at 625 nm by the phenol-sulfuric acid method, using glucose as the standard (DuBois et al. 1956). All samples were analyzed in triplicate.

Leaf DNA extraction and real-time quantitative PCR.

Microbial cells were collected from the phyllosphere as previously described (Kembel et al. 2014). In brief, microbial cells were collected from leaf surfaces by agitating leaves (10 g) for 5 min in 100 ml of 1:50 diluted washing solution (1 M Tris-HCl, 0.5 M Na EDTA, and 1.2% cetyltrimethylammonium bromide [Kadivar and Stapleton 2003]). The washing solution was centrifuged at $4,000 \times g$ for 20 min and the supernatant was removed by pipetting. Cells were resuspended in 500 µl of the Fast DNA SPIN bead solution, and the total DNA was extracted using the Fast DNA spin kit (Qbiogene) according to manufacturer protocol. The quality and quantity of extracted DNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies). The DNA samples were stored at -20°C for later use.

Illumina MiSeq sequencing of 16S rRNA and ITS1 gene amplicons.

Illumina MiSeq sequencing was employed to investigate the diversity of phyllosphere bacterial and fungal communities in female and male *P. cathayana* leaves. The primer pairs 515/907 and ITS5F/ITS1R were used for the amplification of the bacterial 16S rRNA gene and fungal ITS1 gene, respectively. The PCR primers and conditions are listed in Supplementary Table S4. Detailed sequencing analysis for sequencing was performed using the quantitative insights into microbial ecology (QIIME) 2 software package (v1.8.0) (Caporaso et al. 2010) as described previously (Wang et al. 2018). The sequences of chloroplastic and mitochondrial origin were removed. Sequences shorter than 150 bp, containing N6 ambiguous bases or a homopolymer longer than 6 bp, having one or more mismatching bases in the

primer or barcode, or showing a quality score below 20 were eliminated from further analysis. The data were denoised using the AmpliconNoise program (Quince et al. 2011). Finally, high-quality sequences were clustered into OTUs based on 97% sequence similarity with UCLUST (Edgar 2010). Non-parametric calculations of minimum community richness, including Chao 1 and ACE (Chao 1984; Chao and Lee 1992) were performed at a genetic distance of 0.03. The Shannon and Simpson indexes were calculated to compare species diversity and evenness as described previously (Schloss et al. 2009). Besides, we defined the genus relative abundance threshold as 0.1% for rare genera and >0.1% for abundance genera.

The physicochemical properties of the leaves were compared with multiple-sample comparisons using one-way analysis of variance to check for quantitative variance between female and male samples. SPSS version 20.0 was used for statistical analysis (SPSS Inc.). Differences were considered significant at the $P < 0.05$ level. Compositional dissimilarity of phyllosphere microbiota structure between females and males were analyzed by a NMDS plots based on Bray-Curtis distance of the R vegan package (Anderson 2001). The adonis analysis test was used to compare the variations between intraplant (individual trees) and interplant (females vs. males), using a permutational multivariate analysis of variance. Analyses of variance were performed with the adonis function of the R vegan package by using 999 permutations (Anderson 2001). Correlations between leaf physicochemical properties and phyllosphere bacterial and fungal community compositions were calculated by RDA. VPA was used to quantitatively assess the individual and common interpretation of two or more groups (groups 2 to 4) of environmental factor variables to microbial community differences. The RDA and VPA were conducted using R 3.3.1 with the vegan package. Bacterial and fungal community comparisons at the phylum and genus levels were performed using Metastats (White et al. 2009). Bar charts were generated by OriginPro 81.

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