Contrasting microbial communities on male and female flowers of a dioecious plant, *Mallotus japonicus* (Euphorbiaceae)

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**Abstract**
Flowers play a central role in plant reproduction by dispersing and receiving pollen grains delivered by animal vectors or air. They are rich in various nutrients, and therefore, provide an ideal habitat for many microbes. Recent studies have revealed that flower microbial communities can be highly variable among species, individuals, or even floral parts. However, sexual differences in flower microbial communities have rarely been investigated. In this study, we analyzed the flower prokaryotic communities of a dioecious plant (male and female flowers are produced on different individuals), *Mallotus japonicus*, in its natural habitat in Otsu, Japan. Using 16S rRNA amplicon sequencing, we found a differentiation in microbial communities between male and female flowers. Procaryotes on male flowers were relatively diverse, and included several dominant amplicon sequence variants (ASVs), mainly from the Gammaproteobacteria. These ASVs were also found on the body surface of flower visitor insects, suggesting that the visitors dispersed these microbes when they visited the flowers. On the other hand, female flower samples were overwhelmingly dominated by a single bacterial sequence from the Alphaproteobacteria, which showed a peak of relative abundance at the middle of the flowering season. The bacterium had already been present at anthesis, and its relative abundance on flower visitors was low. Flower visitors may have little effect on the microbial composition on female flowers. The lower diversity of microbes on female flowers than on male flowers suggests that the female flowers function as a stronger filter, possibly related to a strong defense against antagonists. These differences may be associated with different reproductive strategies of male and female flowers. This study indicates that dioecious plants provide unique opportunities to study roles of microbes in the evolution of floral traits that have mostly been overlooked in pollination ecology.

**KEYWORDS**
dioecy, flower microbial community, *Mallotus japonicus*, pollination, sexual difference
Flowers are the reproductive organs that disperse and receive pollen of plants and develop into fruits to produce seeds. During anthesis (i.e., the sexually functional period of an individual flower), flowers are directly exposed to microbes in the environment. Besides, flowers have rich resources, such as floral nectar and stigma exudate, a complex mixture of different compounds, such as proteins, saccharides, and fatty acids (Clarke et al., 1979; Cresti et al., 1986). While floral nectar is composed mainly of sugar, it often contains other nutrients, such as amino acids, lipids, and proteins (Roy et al., 2017). Therefore, flowers are colonized and inhabited by various microbes. In agriculture, it has been reported that flowers can be a gateway for some pathogens in crops of various groups, including rice (Kim et al., 2018; Sun et al., 2020), wheat (Del Ponte et al., 2007), apple (Bubán et al., 2003; Vanneste, 2000), blueberry (Chang et al., 2009; Dedej et al., 2004), kiwifruit (Kim et al., 2017), and cucumber (Sasu et al., 2010; Sasu et al., 2010). The pathogens infect the interior of the plant through a nectary (Bubán et al., 2003) or stigma (Farkas et al., 2012; Spinelli et al., 2005). One of the most well-studied examples is a gram-negative bacterial pathogen, Erwinia amylovora (Vanneste, 2000), a causal agent of fire blight disease in apple. It is disseminated among trees by flower visitors, mainly pollinator bees, wind, and rain. On susceptible cultivars, if climate and physiological conditions are favorable, the disease can migrate from one infected flower down to the rootstock, killing the tree in a season (Vanneste, 2000).

While many studies have been conducted on particular pathogens infecting the flowers of the cultivated plants, and other microbes than could control the pathogens (e.g., Llontop et al., 2020; Mikiciński et al., 2016; Pusey et al., 2009; Sun et al., 2020; Vanneste, 2000), studies focusing on the diversity of microbes on flowers have started only recently (e.g., Uschio et al., 2015; Wei & Ashman, 2018). Progress of amplicon sequencing techniques has made it possible to examine the compositions of microbial populations without the need for culturing (Gohl et al., 2016; Goodrich et al., 2014). Pioneering studies revealed that flowers harbor diverse microbes, but are often dominated by limited lineages that are less phylogenetically diverse compared with plant-associated microbes in the soil (reviewed in Müller et al., 2016 and Vannette, 2020). More recent studies have focused on the heterogeneity of microbial communities among plant species (e.g., Herrera et al., 2009), among individuals of the same species, and among flowers of a single individual (Vannette & Fukami, 2018).

Microbial community differences might partly arise from colonization processes on flowers (Vannette, 2020 and references therein). Some microbes may already be present as endophytes even before flower-bud formation and reach the flower through plant tissue migration. Shade et al. (2013) showed that unopened apple flowers in an orchard already harbor a detectable microbial community. Other microbes might colonize flowers before or after anthesis from the nearby environment via insects, wind, or rain drops. Pollinators and herbivores carry microbes on their body surface, in their gut system, or on the surface of the pollen attached to their body (Herrera et al., 2009; Manirajan et al., 2018; Sasu et al., 2010; Uschio et al., 2015), though their effects may vary among visitor groups and individuals. A recent study (Wei et al., 2020) showed that different pollinator guilds had distinct effects on both the alpha and beta diversity of floral microbes on cultivated strawberries. Bees, which are extensive foragers, increased alpha diversity by enhancing colonization, while true bugs and flies contributed to microbial community differentiation and increased beta diversity. Effects of visitors also depend on their behaviors. Russell et al. (2019) compared acquisition and deposition of microbes during different foraging behaviors of the same pollinator species. They found that scrambling for pollen resulted in acquisition of many more microbes than nectaring or buzzing for pollen.

Differences in the microbial community compositions among flowers may also arise from plant defense systems and other floral traits that impose various filters on microbes by limiting or promoting growth of some microbial groups (Vannette, 2020). For example, different sugar concentrations of nectar between male and female flowers change the abundance of yeasts inhabiting the nectar (Tsujii & Fukami, 2018). In Arabidopsis thaliana, sesquiterpene in the floral volatile is considered to serve as a defense against pathogens that invade floral tissues, since a mutant that lacks sesquiterpene is vulnerable to bacterial pathogens (Huang et al., 2012). Suppression of a particular microbe on flowers may also be assured by the presence or dominance of other microbes through resource competition or production of antimicrobial compounds (Lindow & Suslow, 2003; Pujol et al., 2005).

As a result of the colonization and proliferation of microbes and filtering processes on flowers, the microbial communities on a flower can be highly dynamic over time, though it has rarely been investigated. A limited study (Shade et al., 2013) examined microbial dynamics on apple flowers and identified microbial groups that characterize different successional stages of the microbial communities during a short period of 1 week. If such a dynamic nature is a rule, the age and longevity of the flower may also be important factors in considering the flower microbial community.

Furthermore, in a dioecious plant, male and female flowers may differ in many traits that potentially affect microbial compositions, such as flower longevity (Kaltz & Shykoff, 2001; Yamasaki & Sakai, 2013), defense against florivores (Tsujii & Sota, 2010), the number of flowers produced by a plant (Espírito-Santo et al., 2003), presence and chemistry of nectar (Tsujii & Fukami, 2018; Yamasaki & Sakai, 2013), visit frequency of pollinators and other flower visitors (Yamasaki & Sakai, 2013), and flower volatiles (Ashman, 2009). This is partly because of the different fates of male and female flowers after flowering: male flowers are often abscised after pollen dispersal, and colonization of them by antagonistic microbes may cause fewer problems. On the other hand, female flowers are retained for a longer time to produce seeds. Thus, they may be more strongly protected by chemical defenses (Boachon et al., 2019; Tsujii & Sota, 2010) or other mechanisms (Valdivia et al., 2006) against antagonistic invertebrates or microbes. Other reasons may be because male flowers tend to attract more pollinators compared with female flowers by secreting more nectar or by larger displays (Bell, 1985;
Vaughton & Ramsey, 1998; Yamasaki & Sakai, 2013). However, the differences of microbial communities between male and female flowers have rarely been examined. This is partly because most of the studies on floral microbes have been performed in hermaphrodites or only examined microbes in floral nectar of a dioecious plant (Golonka & Vilgalys, 2013; Tsuji & Fukami, 2018). A limited study of whole flower microbial community reported significant difference in the microbial compositions and diversity between the sexes in dioecious strawberries, but the study did not examine detailed taxonomic differences below the phylum and their underlying mechanisms (Wei et al., 2020).

In this study, we examined between- and within-sex variation in microbial communities on flowers of a dioecious tree, Mallotus japonicus (Euphorbiaceae), and explored factors related to the variation. This plant flowers during the rainy season in Japan (Yamasaki & Sakai, 2013), when it is wet and hot and microbes easily proliferate. The objectives of the study were (1) to evaluate variation between the sexes; a strong sexual difference in microbial composition is expected to be observed, because male and female flowers of this plant differ in many characteristics, such as longevity and presence or absence of nectar; (2) to examine variation among trees within the same sex, and the role of dispersal limitation in the variation by testing whether the dissimilarity of the microbial communities is correlated with the geographical distance between the trees. This allowed us to evaluate the importance of tree-specific filters rather than neutral processes, such as a drift and dispersal limitation, which may cause spatial correlations (Rebolleda-Gómez & Ashman, 2019); (3) to examine if the flower visitors acquire bacteria from male and female flowers by identifying microbial communities on the body surface of the flower visitors. We expected that the microbial communities of pollinators that visit male flowers are more similar to the microbial communities of male flowers relative to female flowers, and vice versa; and since we found that the microbes that dominated on female flowers were not frequently carried by flower visitors, to (4) examine if these microbes were already present before flowering based on bagging experiments.

2 | METHODS

2.1 | Study site and study species

The study was conducted from May to July 2018, in Seta Park, Otsu, Shiga Prefecture, Japan (34°50′N, 135°50′E). The mean temperatures in May, June, and July are 22°C, 26°C, and 27°C, respectively. Annual total rainfall is ~1530 mm (Japan Meteorological Agency, https://www.data.jma.go.jp/). The park is mostly covered by a young secondary forest. Mallotus japonicus is one of the dominant tree species in the park, especially around frequently cut managed vegetation.

The height of a fully grown M. japonicus can reach 15 m, while trees start reproduction from when they are approximately 1-m high. M. japonicus flowers bloom from mid-June until mid-July, and are pollinated by wind and insects. Both male and female have apetalous flowers forming non- or several-branched panicles 8–20 cm long. On a male tree, flowers open almost synchronously within a tree, and remain receptive for approximately 2 weeks. Male flowers open asynchronously, and fall off 1 or 2 days after anthesis. Flowering of a male tree lasts around 3 weeks. The female flower has a 3-lobed dry stigma (~0.8 cm each), which is covered by sepals before anthesis. It is infrequently visited by insects, and does not secrete nectar. In contrast to female flowers, they secrete nectar and are frequently visited by hymenopterans and dipterans. Some visitors to male flowers, such as native honey bees, rarely visit females, and thus are nectar and pollen robbers rather than pollinators. The habits and pollination ecology of the species described above are based on Yamasaki and Sakai (2013), in which more details are available.

2.2 | Experimental treatments and sampling

We selected five female (Sample ID = F1 to F5) and four male (Sample ID = M1, M3, M5, M6) trees for this study (Figure 1). Three of the male trees (M1, M3, and M5) were the nearest males to the female trees with the same numbers (F1, and F3 and F4), while M6 did not have a corresponding female sample. For each female tree, 15 inflorescences were tagged prior to flowering. Among them, five were assigned as “Control” and left open. Another five were covered with a mesh bag (mesh size of about 1 mm) to exclude insect visitors as “Net” treatment. The remaining five inflorescences were covered with a waterproof paper bag (Grape Bag; Daiich Vinyl Co., Ltd.) to prevent both wind and insect visitors from reaching the flowers as “Bag” treatment.

To assess temporal changes of microbial communities, we sampled flowers from the Control inflorescences at four different stages of flowering: S1 is bud stage, when the flower is still closed; S2 is early flowering stage on the very first day after flower opening; S3 is mid, and S4 is late flowering stage. Sampling of stages S2 and S3 was separated by 2–3 days, and that of S3 and S4 stages was separated by 5–6 days. At each sampling, five flowers were collected from each of the five Control inflorescences. Five flowers each from the Net and Bag inflorescences were sampled only at the last flowering stage (S4), because we were interested in the effect of these treatments when carried out for the entire flowering period. Therefore, we had 20 samples (4 stages × 5 inflorescences) of the Control treatment in addition to five samples of the Bag and Net treatments for one female tree. For comparison with female flower microbial communities, we also collected male flowers at three different times separated by approximately 4 days during flowering from each of the four male trees (S2, S3 and S4). Five flowers each from five inflorescences were taken to make a total of 25 male flowers per sample. These male and female flower samples were put into a plastic tube using forceps sterilized with 70% EtOH. Lastly, we caught flower visitors with an insect net sterilized with 70% EtOH to assess the microbial community on their body surface. Insects were opportunistically caught on both male and female flowers of the target trees and non-target trees near a target tree between June 14th and June 30th, when
at least one target tree was flowering. These flower and insect samples were brought back to the laboratory on ice and kept in a freezer (–20°C) until further processing. Because the abundance of microbes on small insects tends to be low (Ushio et al., 2015), only insects larger than 2 mm in length (6 and 39 visitors to female and male flowers, respectively) were analyzed. After DNA extraction, the insects were pinned, dried, and labeled. They were identified to at least the order, and to species when it was possible to identify species under a binocular microscope (not shown), and are kept at Center for Ecological Research, Kyoto University.

### 2.3 Sample filtering and DNA extraction

Each flower sample was transferred to a 5-ml microtube, and 3 ml of Phosphate-Buffered Saline solution (PBS buffer, pH 7.4, Nippon Gene Co., Ltd.) was added to the tube. Microbes were detached from the flower surface using ultrasonic dispersion for 30 s at 50% power of a Handy Sonic UR-21P (Tomy Seiko Co., Ltd.). Once sonicated and homogenized, samples were filtered using a Sterivex Millipore filter (Sterivex-HV 0.22, Merck). DNA was extracted using a Qiagen DNeasy Blood and Tissue Kit (QIAGEN) following the manufacturer's instructions and then stored at –21°C until further processing. Insect samples were processed with the same procedure, while the amount of PBS buffer for the extraction was changed depending on the body size of the insect.

### 2.4 DNA purification, amplification, and sequencing

Amplification and sequencing were performed for the V4 region of the 16S rRNA using the 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R primer pair (5′-GGACTACVSGGGTATCTAAT-3′) (Caporaso et al., 2011). A first round PCR was conducted in triplicate for each sample in a 12-µl reaction containing 3.6 µl of Milli-Q water, 6 µl of KAPA Hifi HotStartReadyMix (Kapa Biosystems), 0.7 µl of each forward and reverse primer, and 1 µl of DNA template. The PCR thermal cycle was as follows: an initial denaturation step at 95°C for 3 min followed by 35 cycles of denaturation at 98°C for 20 s, annealing at 60°C for 15 s, and elongation at 72°C for 30 s, followed by final elongation at 72°C for 5 min. After checking amplification success on a 2% agarose gel, triplicates of a sample were combined and purified using AMPure XP (Beckman Coulter) (Greenwald et al., 2019). We performed the AMPure purification step using the ratio of PCR product: AMPure = 1:0.8. This ratio removed only primers and primer-dimers (< ca. 200 bp). Larger fragments including the target-size amplicon were retained (there was no significant variation in the amplified fragments). Second round PCR was performed in a 24-µl reaction containing 7.2 µl of Milli-Q water, 12 µl of KAPA HiFi HotStart ReadyMix, 1.4 µl of each forward and reverse primer with Nextera adapter and sample-specific 8-mer indices, and finally 2 µl of purified first round PCR product obtained as described above. The PCR thermal cycle

![Figure 1](image-url)
was as follows: an initial denaturation step at 95°C for 3 min followed by 12 cycles of denaturation at 98°C for 20 s, annealing at 60°C for 15 s, and elongation at 72°C for 15 s, followed by final elongation at 72°C for 5 min. Different samples were then combined and size-selected to 350–450 bp using the E-Gel SizeSelect System II Agarose Gel 2% (Life Technologies), representing the size of the V4 region of the 16S rRNA, including Illumina sequencing adaptor. The concentration of size-selected purified PCR was estimated with a Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific Inc.). The final library concentration was adjusted to 1 nM. Sequencing was performed on the Illumina MiSeq® Platform (Illumina Inc.) using a 500 cycles V2 reagent kit.

### 2.5 Sequence data processing

We processed the sequence data following Ushio (2019). The raw MiSeq data were converted into FASTQ files using the bcl2fastq program provided by Illumina (bcl2fastq v2.18). The FASTQ files were then demultiplexed using the command implemented in Claident (http://www.claident.org; Tanabe & Toju, 2013). Demultiplexed FASTQ files were analyzed using the ASV (amplicon sequence variants) method implemented in dada2 v1.10.1 (Callahan et al., 2016) package of R (R Core Team, 2020).

At the quality filtering process, forward and reverse sequences were trimmed at the length of 215 and 160, respectively, using DADA2::filterAndTrim() function.

Taxonomic identification was performed for ASVs (Callahan et al., 2017) inferred using dada2 based on the query-centric auto-k-nearest-neighbor (QCauto) method (Tanabe & Toju, 2013) and subsequent taxonomic assignment with the lowest common ancestor algorithm (Huson et al., 2007) using “semial” database and clidentseq and classigtax commands implemented in Claident v0.2.2018.05.29. The reads identified as prokaryote (bacteria and archaea) were used for further analysis. Additionally, the most abundant ASVs for which the above procedure did not provide the identification of the order or lower taxonomic levels were searched against the Nucleotide database of the National Center for Biotechnology Information (NCBI) with BLAST (Altschul et al., 1990). Top matches of the sequences were referred in order to assign taxonomic identities (Order, Family, Genus) of the ASVs.

We obtained a total of 10,812,990 reads of the partial 16S rRNA sequences from flower samples. Among the reads, 459,644 or 4.5% were identified as archaea or bacteria sequences. The rest of the reads were identified as plant DNA, and then were removed from the analysis. Archaea and bacteria sequences were clustered into 1546 ASVs. Samples with fewer than 30 reads were removed from further analysis. This reduced the number of the samples to 143 and 11 for female and male flowers, respectively. The median number of reads in the samples was 365.

From 49 samples of insects, we obtained a total of 416,139 reads of the target 16S rRNA region. Among the reads, 202,342 or 48.6% were identified as prokaryote sequences, which were clustered into 1262 ASVs. Samples with less than 30 reads were removed from further analysis. This reduced the number of visitor samples to 6 and 39 for female and male flowers, respectively. The visitors belonged to Hymenoptera (22 insects), Diptera (12), Coleoptera (5), and Lepidoptera (2). The median number of reads in the samples was 773.

Before investigating community composition, we evaluated how much ASV richness was captured by the sequence depth by visualizing rarefaction curves. According to the rarefaction curves (Figure S1), the sequencing captured most prokaryotic diversity, and thus we focused on the proportional data in order to evaluate the composition of the prokaryotic community on flowers and insects.

### 2.6 Statistical analysis

#### 2.6.1 Diversity within samples (alpha diversity)

Diversity of prokaryote reads of each flower sample was evaluated by Shannon diversity index calculated using the phyloseq:estimate_richness() function. The index did not show significant association with the total numbers of the reads (Pearson’s correlation test, $p = 0.152$). Significant differences in the index among treatments and among female trees and between the Control female and male flowers were tested using GLMMs. Prior to the analysis, we transformed the Shannon index by Box–Cox transformation ($\lambda = 1.3$) (Box & Cox, 1964) using the bcPower() function of the R package car (Fox & Weisberg, 2019), since the distributions of both indices deviated significantly from the normal distribution (Shapiro–Wilk test, $W = 0.979$, $p = 0.019$). This improved the deviation from a normal distribution ($W = 0.98663$, $p = 0.146$).

For the comparison among the female flower samples under the different treatments, we constructed a GLMM with the treatment as a fixed term and Tree ID as a random factor. Significance of the fixed term was tested by comparing the full model and the model without the fixed term using the anova() function. Only the S4 samples were used for the test. For the comparison among the stages, we constructed a GLMM with the stage as a fixed factor and tree and inflorescence ID as random factors. Significance of the fixed term was tested by comparing the full model and the model without the fixed term using anova() function.

To compare the male and female samples, we used only samples collected at the flowering peak (S2 and S3), since female flowers showed significant changes along the flowering stages (see Results). We averaged the Shannon index across the female samples collected from the same tree on the same day to adjust uneven sample sizes of male (one sample per stage per tree) and female (five samples per stage per tree) flowers prior to the Box–Cox transformation. We constructed a GLMM with the tree sex as a fixed term and Tree ID as a random factor. Significance of the fixed term was tested by comparing the full model and the model without the fixed term using anova() function.
2.6.2 | Variation among samples (beta diversity)

To compare prokaryotic communities among samples, we calculated the Bray–Curtis dissimilarity index using the package vegan (Version 2.5.6) (Oksanen et al., 2010) of R, and ordinations were plotted with non-metric multidimensional scaling (NMDS) using vegan’s metaMDS procedure. To statistically examine the contribution of factors to the variation, we conducted a permutational multivariate analysis of variance (PERMANOVA) using the vegan::adonis function. For this analysis, only ASVs that were present in five or more flower samples of the Control female or male flowers were included (115 ASVs). Since the filtering of the ASVs had reduced the total reads of the samples, we removed samples with total reads <30. The frequency of each ASV was divided by the total frequencies of each sample to convert it to a proportion.

Using this procedure, we first examined variation among the female flower samples. We tested if the community structure was significantly different among the stages within a tree using the samples of the Control treatment. We then tested the significance of the differences among the treatments within the same tree using the samples of S4.

For the comparison between male and female flowers, the female flower samples of the same stage of the same tree were combined and averaged to balance the number of samples of female and male flowers. We examined if samples from the same sex had similar microbial communities, and if microbial communities from the same tree individual were more similar than those from different tree individuals using PERMANOVAs.

We also assessed if closely located trees had more similar microbial communities than distantly located trees did by conducting the Partial Mantel Test. The proportions of 115 ASVs of different stages were averaged for each tree. The dissimilarity matrix of the Bray–Curtis dissimilarity index and spatial distances conditioned on the sexual difference (0, same sex; 1, different sexes) were assessed based on Spearman’s correlation. The analysis was conducted using partial.mantel() implemented in vegan.

Finally, we analyzed the prokaryotic amplicon sequence dataset from the insect samples collected from male and female flowers together with the averaged dataset for the male and female comparison above. PERMANOVAs were conducted for the insect–flower group pairs with same or different plant sexes: “insects from female flowers” versus “female flowers”; “insects from male flowers” versus “female flowers”; “insects from male flowers” versus “female flowers”; and “insects from male flowers” versus “male flowers”.

2.6.3 | Frequencies of dominant ASVs

We compared frequencies of the 10 most abundant ASVs in the NMDS analysis between male and female flowers. These ASVs accounted for 2.1–16.7% (Table 1) of the total reads of the 115 ASVs used in the NMDS analysis of the male–female comparison. To statistically evaluate differences in the proportions of the ASVs among samples, we employed negative binomial and zero-inflated mixed models (NBZIMMs) implemented in the R package NBZIMM (Zhang et al., 2018; Zhang & Yi, 2020). Microbiome sequencing data have the characteristics of varied total sequence reads across samples, over-dispersion, and zero-inflation. The NBZIMMs were developed for the analysis of longitudinal microbiome data while accounting for these characteristics. We examined if the frequencies of the 10 ASVs were different between female and male flowers. For the comparison, 92 female flower samples from the Control treatments of all stages and 11 male flower samples were included. Frequencies of each ASV read were modeled with the flower sexes (female or male flowers) as a fixed factor, the tree identities as a random factor, and the log-transformed number of the total prokaryote reads as the offset term. If the estimated parameter for the fixed effect significantly deviated from zero, we considered the difference between the sexes to be significant.

We also examined if Net or Bag treatments decreased the frequencies of ASVs compared with that of the Control. We modeled ASV frequencies in 71 S4 stage female samples of the Control, Net and Bag treatments with the treatments as a fixed factor, the tree identities as a random factor, and the log-transformed number of the total prokaryote reads for the offset. A bag could decrease the focal ASVs by reducing colonization, or enhance their growth and/or survival by protecting them from UV exposure, temperature fluctuations, desiccation, or being washed away by rain. The analysis was conducted for six ASVs among the 10 above. This was because the other four ASVs were rarely found on female flowers, while they were abundant on male flowers (see Results).

2.6.4 | Temporal variation of ASV01S1 over flowering period

We examined if the frequencies of the most dominant ASV (ASV01S1) changed from S1 to the following stages (S2, S3, S4) using NBZIMM. The ASV accounts for 28.9% of the prokaryotic reads of the Control treatment in the S1 stage female flowers (see Results). ASV01S1 frequencies were modeled with the stages as a fixed factor, the tree identities as a random factor, and the log-transformed total prokaryote reads as the offset term.

3 | RESULTS

3.1 | Diversity within samples (alpha diversity)

Shannon diversity index showed a large variation among samples, ranging from 0.24 to 3.92 (Figure 2). We did not detect significant differences among the treatments (GLMM analysis, \( \chi^2 = 1.16, p = 0.5588 \)). Shannon diversity index of female samples of the Control treatment was significantly different across the flowering stages \( (\chi^2 = 26.836, p < 0.0001); \) it was lowest at the flowering peak (S2 and S3). The index at these stages in the female trees was significantly lower than that of the male trees \( (\chi^2 = 4.934, p = 0.0263) \).
3.2 | Variation among samples (beta diversity)

Variation among the female samples based on the Bray–Curtis dissimilarity index was visualized in an NMDS plot (Figure S2). PERMANOVA showed that the control flowers harbored different prokaryotic communities among the trees and among the stages within a tree ($R^2 = 0.2049$, $p = 0.0001$ for the trees, and $R^2 = 0.25249$, $p = 0.0001$ for the stages). It also showed that the prokaryotic communities on S4 flowers differed significantly among the trees and among the treatments within a tree ($R^2 = 0.2282$, $p = 0.0001$ for the trees, and $R^2 = 0.1690$, $p = 0.0106$ for treatment).

The NMDS plot of the male and female flower samples showed that male and female flowers of Mallotus japonicus present different prokaryote compositions (Figure 3a). The corresponding plot of ASVs suggested that female flowers tend to have more Alphaproteobacteria and Betaproteobacteria, which are plotted in the left side of the plot, while males have more Gammaproteobacteria, which are plotted in the right side (Figure 3b). Besides, samples collected from the same...
individual were closely located on the plot although they were collected on different days. PERMANOVA supported the conclusion that the prokaryote compositions were different between the sexes \((R^2 = 0.1567, p < 0.0001)\) and among the individual trees within the sex \((R^2 = 0.3675, p < 0.0001)\). The contribution of the tree identity to the variation was larger than that of the sex. The prokaryote communities on the female trees F3 and F5 were quite similar and the physical distance between these two trees was only 25 m. In contrast, tree F1 had similar prokaryote composition to those of female trees F2 and F3, while it was the most isolated tree (430 m from the closest focal female tree). Partial Mantel test did not detect significant correlations between the prokaryotic composition dissimilarity and the physical distance (Mantel Statistic \(r = 0.0920, p > 0.05\)).

The NMDS plot obtained with the insect samples added suggested that insects collected from female flowers had microbial communities on their body surface that were more similar to the communities on the female flowers, and the microbial communities on insects collected from male flowers were more similar to the microbial communities on male flowers (Figure 4a). PERMANOVA showed that the insect-flower comparisons between the different sexes were significantly different (insects from males vs. female flowers, \(R^2 = 0.1493, p = 0.0001\); insects from females vs. male flowers, \(R^2 = 0.1094, p = 0.0435\)), while the comparisons between the same-sex pairs were not significantly different (female flower pair, \(R^2 = 0.0895, p = 0.069\); male flower pair, \(R^2 = 0.0227, p = 0.4202\)) (Figure 4b).

### 3.3 Frequency of dominant ASVs

The ten most abundant ASVs mainly belonged to eight families of three classes in the phylum Proteobacteria (Table 1). Among the 10, three ASVs were significantly more associated with male flower samples (ASV04E1, \(t = 2.726, p = 0.0295\); ASV09E2, \(t = 4.281\), ...)
p = 0.0037; and ASV10E3, t = 3.338, p = 0.0124) (Figure 3). ASV04E1 and ASV9E2 belong to Erwinia and Enterobacteriaceae, respectively, both belonging to the class Gammaproteobacteria (Table 1). Although Cladent did not assign the family or genus of ASV10E3, BLAST search identified the sequences of *Klebsiella* of the family Enterobacteriaceae, or *Pantoea*, Erwiniaaeceae, as the groups with the highest sequence matches (Table 1). Although Cladent did not assign the family or genus of ASV10E3, BLAST search identified the sequences of *Klebsiella* of the family Enterobacteriaceae, or *Pantoea*, Erwiniaeaeae, as the groups with the highest sequence matches (Table 1). On the other hand, female samples had significantly higher frequencies of ASV01S1 (t = −3.278, p = 0.0135) and ASV07C1 (t = −3.357, p = 0.0121) (Figure 5). The former was identified as *Sphingomonas*, Sphingomonadaceae by BLAST search, while the latter was identified as *Delftia* of the family Comamonadaceae by Cladent (Table 1). The other four ASVs did not show a significant difference between the sexes (ASV02P1, t = −0.476, p = 0.6485; ASV03M1, t = 2.1199, p = 0.0717; ASV06O1, t = 0.0264, p = 0.9796; and ASV08O2, t = 0.4781, p = 0.6471) (Figure 5).

The ten most abundant ASVs were also found in the samples of the flower visitor body surface, while their proportions among the prokaryote reads in the flower visitor samples varied among the ASVs (Figure 4b). In particular, ASV02P1, ASV03 M, ASV04E1, and ASV09E2 were found from more than 35% of the insects, and accounted for up to 44%, 78%, 66%, and 72% of the prokaryotic reads.
Among the six ASVs that were present in 20 or more S4 samples, a significant decrease in the Bag treatment was observed for ASV02P1 ($t = -2.195$, $t = 0.0317$), ASV06O1 ($t = -1.999$, $p = 0.0498$), and ASV08O2 ($t = -2.104$, $p = 0.0393$) (Figure S3). ASV06O1 and ASV08O2 in most samples from the Bag treatment were zero, while many samples under the Bag treatment had ASV02P1 reads with lower frequencies (Figure S3). A significant increase in Bag treatment compared to Control flowers was detected for three ASVs: ASV01S1 ($t = 2781$, $p = 0.0071$), ASV04E1 ($t = 5087$, $p < 0.0001$), and ASV07C1 ($t = 2.958$, $p = 0.0043$). ASV01S1 and ASV07C1 were also present on female flower buds (S1 samples) of multiple trees. On the other hand, ASV04E1, which was absent in flower samples under the Bagged treatment from four of the five trees, was not found in the flower bud samples.
3.4 | Temporal variation of ASV01S1 over flowering period

ASV01S1 was the most abundant ASV on female flowers (Figure 3b). The relative proportion of this single ASV in the female samples of the Control had mean and maximum values of 24.2% and 95.9%, respectively. The proportion of ASV01S1 showed a similar pattern of temporal variation across the five female trees (Figure 6). A relatively low proportion of this ASV at the beginning of the flowering was followed by a rapid increase in proportion in S2 or S3 or both (Figure 6). This increase was followed by a drastic decrease which led it to reach close to 0% in S4 (Figure 6). The frequency of ASV01S1 at stage S2 was significantly higher than that at S1 (t = 4.327, p < 0.0001). No significant difference was detected between S1 and S3, but the frequency at S4 stage was significantly lower than that at S1 (t = -5.623, p < 0.0001).

4 | DISCUSSION

4.1 | Differences in microbial compositions between the sexes

In the dioecious plant Mallotus japonicus, we found distinct microbial communities in male and female flowers. The difference in the microbial compositions was mainly driven by a small number of ASVs which belong to different families. Male flowers were dominated by three closely related ASVs belonging to Erwinia (ASV04E1), Enterobacteriaceae (ASV09E2), and Erwinia or Enterobacteriaceae (ASV10E3). Bacteria belonging to these two families are commonly found on plant tissues and flowers (Junker & Keller, 2015; Junker et al., 2011; Wei & Ashman, 2018) and flower visitors (Zemenick et al., 2018). Erwinia includes many members that are able to colonize plant tissues asymptotically or symptomatically (Vannette, 2020). Well-known examples include pathogens, such as Erwinia amylovora, the causal agent of bacterial fire blight disease in Rosaceae (Pujol et al., 2005; Vanneste, 2000), and its biocontrol agents, such as Pantoea agglomerans (Llontop et al., 2020; Mikiciński et al., 2016; Pusey et al., 2009). Knowledge about the distribution and habits of these bacteria in natural systems may contribute to better control of pathogens in agriculture systems.

On the other hand, the most abundant ASV on female Mallotus japonicus flowers was ASV01S1, which was suggested to belong to Sphingomonas, Sphingomonadaceae by BLAST search. Sphingomonas is a diverse genus, within which 138 species with valid names have been documented at the time of writing (http://www.bacterio.net/sphingomonas.html, accessed on August 28, 2021). Members of this genus have been isolated from diverse sources in natural habitats, such as water, air, and sediments (Cha et al., 2019). Sphingomonas is also reported to be abundant on both flowers and other plant tissues in some plant taxa, including species of the family Euphorbiaceae (Eke et al., 2019; Junker & Keller, 2015; Wei & Ashman, 2018). On M. japonicus female flowers, ASV01S1 was overwhelmingly abundant at all of the different flowering stages analyzed except S4. To our knowledge, such a high dominance of a single ASV, with the highest value of 95.88%, has never been reported before in any flower microbial study. Interestingly, the relative abundance of ASV01S1 in female flower samples showed a unimodal temporal change, with rapid increase at the beginning of the flowering followed by a drastic decrease. This pattern was consistent across all of the female individuals. We did not find any comparable changes in the other five ASVs dominant on female flowers (ASV02P1, ASV04E1, ASV06O1, ASV07C1, and ASV08O2) (results not shown). Succession of or temporal changes in floral bacterial communities has rarely been studied (but see Shade et al., 2013). Underlying mechanisms of such temporal patterns will be an important research subject to understand what factors shape microbial communities of flowers, habitats that are rich in resources but ephemeral.

One of the limitations of this study is that our sampling was conducted in only one year at one study site. Since most studies to date on flower microbes on wild plants have been 1-year studies at a site, it is largely an open question to what extent the microbial communities are consistent across years. As discussed below, if female flowers have more intimate relationships with microbes than male flowers do, female flowers may maintain more constant microbial communities across years.

4.2 | Differences among individuals

Although some common and abundant microbes were shared among individuals of the same sex, PERMANOVA suggest that each tree harbored a unique microbial community of different diversity over time. Besides, dissimilarity of microbial communities was not significantly correlated with the spatial distance between the trees, suggesting that dispersal limitation played a limited role in structuring the variation among the trees. Some of the bacteria found on flowers may stay on the plant for a much longer time than flowers as endophytes or epiphytes of vegetative organs (Bacon & White, 2016; Hardoim et al., 2015; Rosenblueth & Martínez-Romero, 2006). Differences among trees might have already existed prior to flowering. It is also possible that shared microenvironments of the flowers on each individual, such as height, sun exposure, and soil characteristics, could play a role in the colonization and filtering of microbes (Igwe & Vannette, 2019; Tobor-Kaplon et al., 2006). Although the results of our experiments indicated that some microbes colonize flowers assisted by insects (see below), this colonization might not be sufficient to homogenize flower bacteria among neighboring trees.

4.3 | Colonization by the dominant bacteria

It has been reported that insect visitors play important roles in vectoring flower microbes in M. japonicus and many other plant species (Russell et al., 2019; Ushio et al., 2015; Wei et al., 2020), while
the importance of dispersal by flower visitors may differ between male and female flowers or among microbes. Insect visitors may be important vectors of some bacteria, such as ASV02P1, ASV03M1, ASV04E1, and ASV09E2 abundant on male flowers. These four ASVs were found from more than a third of the flower visitors, while we detected all of the 10 dominant ASVs from the body surface of some insect visitors. On the other hand, the contribution of insect visitors on the microbial community on female flowers may be less extensive compared with that on males. ASV01S1 and ASV07C1, the ASVs more frequently found on females than on males, were rarely found on the body surface of the flower visitors. On the female flowers, insect flower visitors do not collect pollen or feed on nectar, substances which are only present on male flowers. Such differences in the behaviors of the flower visitors may also explain the rarity of the female-flower-specific ASVs on flower visitors’ bodies (Russell et al., 2019). In *Mallotus japonicus*, female flowers are not as frequently visited by flower visitors as male flowers. Besides, flower visitors on females are relatively small (Yamasaki & Sakai, 2013), and thus may carry fewer microbes compared to large visitors, such as honey bees, bumble bees, and wasps that visit male flowers (Ushio et al., 2015).

The bagging treatments on the female flowers had different effects on the flower microbial communities and the frequencies of the six dominant ASVs on female flowers. The abundances of three ASVs, ASV02P1, ASV06O1, and ASV08O2, were significantly higher in the Control than in the Bag treatments. The frequencies of these ASVs were not significantly different between male and female flowers. Therefore, their abundances may mostly depend on the frequencies of colonization from other *Mallotus* trees or other environments. On the other hand, we found significantly higher frequencies of ASV01S1 and ASV07C1 in the Bag treatment than in the Control. Since the Bag treatment did not allow either wind or insects to disperse microbes to inside the flower, the presence of the bacteria indicates that they were already present on the female flowers before the flowering started (prior to the bud stage when flowers were bagged). These bacteria remained on the flowers until we sampled them at the end of flowering approximately 2 weeks later. The net and paper bags may have sheltered the flowers from UV light and kept the humidity inside high. This may explain the high abundances of ASV01S1 and ASV07C1 in the Bag treatment compared with those in the Control.

4.4 | Microbial communities and reproductive strategies of male and female flowers

We found contrasting microbial diversity and composition between male and female flowers in *M. japonicus*. The differences may stem from sexually dimorphic colonization and filtering processes related to contrasting floral characteristics and reproductive strategies between the sexes.

A few lines of evidence suggest that male flowers are more frequently colonized by microbes than female flowers in *M. japonicus*. First, male flowers, which have nectar and pollen, are far more frequently visited by many flower visitors than female flowers are (Yamasaki & Sakai, 2013). Besides, male flowers have numerous anthers, which increase the contact with flower visitors and the probability of microbial colonization. The idea is supported by the present finding that male flowers were dominated by several bacteria belonging to two closely related families, Erwiniaeaceae and Enterobacteriaceae, and that these bacteria were frequently found at high proportions on flower visitors. In contrast, the ASVs that were more frequent on female flowers than on male flowers (ASV01S1 and ASV07C1) were relatively rare in the insect samples, and the bagging experiment and flower bud samples suggested that these ASVs had already been present on the flowers prior to anthesis.

A higher rate of microbial colonization may indicate a higher risk of infection by antagonistic microbes in male plants, if colonizers include antagonists at a certain proportion. Different risks of infection by antagonistic microbes between the sexes have been suggested in a dioecious plant, *Silene latifolia*. Its male plants have larger and more abundant and rewarding flowers and receive more pollinator visits than female plants (Shykoff & Bucheli, 1995). Therefore, male plants are exposed to a higher potential for infection by an anther-smut pathogen vectored by pollinators. Kaltz and Shykoff (2001) hypothesized that male plants mitigate their higher risk of infection by abscising their flowers after anthesis. The much shorter lifespan of male flowers than that of female flowers in *M. japonicus* may also contribute to protecting the flowers from antagonist microbes. Interestingly, we confirmed the presence of *Erwinia mollitivora*, a causal agent of bacterial leaf spot (Goto, 1976) on *M. japonicus* flowers (Marre et al., in press). The effects of the floral infection by a bacterial pathogen in a dioecious plant, *Mallotus japonicus* (Euphorbiaceae). Population Ecology, in press). It is one of the isolated bacteria that matches with the sequence of ASV04E1.

On the other hand, female flowers may more strongly filter microbes than male flowers. Female flowers were dominated by a single ASV of *Sphingomonas*, and microbial diversity on female flowers was low compared with that on male flowers. Previous studies showed that *Sphingomonas* on the plant surface can have some beneficial effects on its host. Khan et al. (2017) reported that an endophyte, *Sphingomonas* sp. K11, improved the growth of tomato plants under salinity stress. Innerberner et al. (2011) showed that inoculation of a strain of *Sphingomonas* on the leaf surface suppressed leaf pathogen *Pseudomonas syringae* on *Arabidopsis thaliana*. An interesting topic for future study is whether ASV01S1 on *M. japonicus* also has a beneficial effect on the plant, and whether it contributes to filter other microbes. The flower microbiome of sexually dimorphic plants has previously been documented in dioecious or subdioecious *Fragaria* species (Wei & Ashman, 2018). That study also showed higher diversity on male-fertile (male and hermaphrodite) flowers compared with female flowers, consistent with our study. Less resources (nectar, floral volatiles) and/or smaller size have been suggested to make female flowers more selective environments (Rebolleda-Gómez
et al., 2019; Wei & Ashman, 2018). The other potential explanation is stronger defense against antagonists, as reported in plant-florivore interactions. Tsuji and Sota (2010) reported that flower size and density in Eurya japonica (Theaceae) are greater in hermaphrodite and male trees, while total concentration of phenolics in flowers, a defense against florivores, is higher in female plants, probably due to the necessity to retain flowers until fruit maturation. Such sex-specific interactions have been little explored in plant-flower microbe interactions. Different chemical compositions of floral scents between the sexes (Ashman, 2009) may be related to different strengths of flower defense against microbes (Huang et al., 2012).

5 | CONCLUSION

Our study demonstrated significant sexual differentiation in the floral microbiome in a sexually dimorphic plant species. This may be partly because male and female flowers have different organs that provide distinct habitats and resources to microbes (Golonka & Vilgalys, 2013; Tsuji & Fukami, 2018; Vannette, 2020; Wei & Ashman, 2018). Besides, it may also reflect different chances of microbial colonization and defense mechanisms: male plants are visited by many flower visitors and have higher chances of colonization, while female flowers may exert a stronger filter which results in lower prokaryote diversity (Ashman, 2009; Rebolleda-Gómez et al., 2019). This study indicates that dioecious plants provide unique opportunities to study roles of microbes in the evolution of floral traits that have mostly been overlooked in pollination ecology.

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CONFLICT OF INTEREST

None declared.

AUTHORS CONTRIBUTIONS

MM, MU, and SS designed the study; MM conducted field experiments, sampling, and amplicon sequencing; MM analyzed data assisted by MU and SS; MM wrote the first draft under the supervision of SS. All authors read and edited the manuscript.

DATA AVAILABILITY STATEMENT

Data will be available on DNA Data Bank of Japan (DDBJ, https://www.ddbj.nig.ac.jp/dra/index.html; accession numbers, DRA013108).

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