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Variation in the bacteriome of the tropical liverwort, *Marchantia inflexa*, between the sexes and across habitats

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Abstract Plant-microbe interactions impact ecosystem function through nutrient cycling, community interactions, and individual costs/benefits to the organisms involved. However, information on the establishment, diversity, and function of plant-microbe interactions remains limited, especially for non-vascular plants. We hypothesized that variation in the environment and sex of a host plant, impact the composition and diversity of associated microbial communities. To test this hypothesis, we characterized the bacteriome of the non-vascular plant, *Marchantia inflexa*, in both males and females across multiple habitats by targeted sequencing of the bacterial 16S rRNA gene. We describe the bacteriome for *M. inflexa*, and find that it is abundant and diverse, showing some similarities with other non-vascular plant lineages. Using these data, we detected a habitat specific component of the bacteriome, and sex differences in the bacteriome under common garden conditions. On the basis of known microbial functions, our analyses suggest that the specific taxonomic assemblages of bacteria detected in particular habitat types may serve functional roles; allowing plants to better acclimate to their local environment, and that sex differences in the bacteriome may correspond to subtle differences in the physiology and morphology of the sexes. Our initial characterization of variation in bacteriome composition of this tropical liverwort lineage provides valuable information for better understanding the patterns of plant-microbe interactions across land plants.

Keywords 16S sequencing · *Marchantia inflexa* · Microbiome · Sex differences

1 Introduction

Associations between prokaryotes and larger life forms are both ubiquitous and important (van der Heijden et al. 2016). The microbiome in animals is known to influence nutrient uptake, digestion (Hooper et al. 2002), metabolism (Claus et al. 2008), and even behavior (Ezenwa et al. 2012). Likewise, the plant microbiome can dramatically impact plant performance, physiology, and plays a significant role in nutrient acquisition, plant-water relations, and stress responses (Turner et al. 2013; Panke-Buisse et al. 2015; Haney et al. 2015; Vandenkoornhuyse et al. 2015; Agler et al. 2016). Because the plant microbiome can dramatically impact plant health and performance, understanding the factors that modulate microbial community establishment and composition has implications for food security, agricultural productivity (Sessitsch & Mitter 2015) and ecological stability in natural systems (Vandenkoornhuyse et al. 2015).

That being said, we are only beginning to appreciate the complex dynamics of plant-microbe interactions. To date, most research efforts have focused on subsurface

Data Accessibility
Sequence data have been deposited in the NCBI SRA database (accession numbers: SRR5429634-56). Environmental data is available on Figshare (DOI:https://doi.org/10.6084/m9.figshare.4823530).

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(rhizosphere) interactions with a particular concentration on agricultural species. Consequently, we have developed a sophisticated understanding of associations between plant roots and soil microbes (Berendsen et al. 2012; Vandenkoornhuyse et al. 2015). However, the microbiome in aboveground tissues (phyllosphere) is less well characterized, especially in non-agricultural systems (Turner et al. 2013). Nevertheless, recent technological advances have made this area of investigation more accessible, and a number of contemporary studies have addressed related questions (Turner et al. 2013; Delaux et al. 2013; Bragina et al. 2014; Knack et al. 2015; Agler et al. 2016; van der Heijden et al. 2016). Some of these studies provide evidence to suggest that plant microbiomes are determined, at least in part, by associations that span the deep evolutionary history of plant lineages (Knack et al. 2015). In parallel, others have shown that random colonization (Lebeis 2014), nutrient availability (Turner et al. 2013), local ecology (Lundberg et al. 2012; Schlaeppi et al. 2014; Koua et al. 2014; Bragina et al. 2014), host genotype (Manter et al. 2010; Ofek et al. 2014; Agler et al. 2016), and host sex (Vega-Frutis & Guevara 2009; Ali Balkan 2016) can influence the community structure of plant microbiomes.

While some specific plant-microbe relationships have been well studied, the bacteriome (the collection of bacteria that inhabit a given environment) in bryophytes (mosses, hornworts and liverworts) is not well characterized. Only a few studies have described bacteriomes in liverworts (Koua et al. 2014; Knack et al. 2015) and mosses (Opelt & Berg 2004; Opelt et al. 2007a, b, Bragina et al. 2012, 2014, 2015; Knack et al. 2015), despite the potential insight provided by such studies. It is worth noting that bryophyte microbiomes may differ substantially from the microbiomes of other plants in relevant ways. For example bryophytes are often early colonizers in ecological succession, and their propensity for drastic changes in water content may necessitate specific adaptations in the accompanying microorganisms (Opelt & Berg 2004; Proctor et al. 2007). Additionally, many bryophytes are found in harsh and nutrient limited environments (Goffinet 2008), which likely impacts any microbes associated with these plants. A comprehensive understanding of the bryophyte microbiome may therefore offer insight into relationships that aid in stress tolerance and nutrient acquisition, both of which have practical applications in environmental and crop management.

In this study, we hypothesized that because the plant bacteriome can affect plant fitness, changes in the composition of a plant's bacteriome would be associated with the environment, possibly increasing local fitness. We further hypothesized that host plant sex would be associated with changes in bacteriome composition, due to differences in the specific functions of each sex. To test these hypotheses, we examined the bacteriome of the tropical liverwort, *Marchantia inflexa*, in male and female plants from multiple habitats (native stream sides, recently colonized roadsides, and a greenhouse common garden) by targeted sequencing of the bacterial 16S rRNA gene. We predicted that particular bacterial taxa would be enriched in the bacteriome of plants from different habitats and sexes. Others have demonstrated that the plant bacteriome is influenced by environmental variation, and we expected our study system to exhibit a similar pattern. Identification of habitat specific associations would point toward functional relationships that may aid in plant performance under specific environmental conditions, such as drought or nutrient limitation. Due to differences in the function, physiology and morphology of the sexes we expected to detect sex-specific differences in the bacteriome of *M. inflexa*. Although sex specific fungal interactions have been noted in angiosperms (Varga et al. 2013) and mosses (Ali Balkan 2016), a sex difference in the bacteriome of liverworts has not been documented.

2 Materials and methods

2.1 Study organism

Marchantia inflexa Nees & Mont is a New World liverwort with unisexual individuals found from northern Venezuela to the southern United States (Bischler 1984). *Marchantia inflexa* grows as a dichotomously branching thallus with dorsiventral organization (Fig. 1), and can reproduce asexually by fragmentation and the production of gemmae (asexual propagules), or sexually by spores. The dominant life phase of *M. inflexa* is the haploid gametophyte, which produces gametes, and sex is chromosomally determined (Bischler1984). Male and female gametophytes have unique morphology during reproductive life stages (Fig. 1) and can easily be sexed. *Marchantia inflexa* is typically found in low light, high humidity environments along streams, but has recently been found colonizing more exposed and disturbed sites, such as roadsides (Groen et al. 2010; Brzyski et al. 2014).

2.2 Sampling and growth conditions

Specimens of *M. inflexa* were collected from the island of Trinidad, Republic of Trinidad and Tobago in 2009 and 2015 (Table 1). In 2009, vegetative thallus tissue from individual male and female plants was collected from populations along one stream (East Turure) and one road (Cumaca) (Table 1). Samples were physically separated from one another to ensure that individuals were genetically distinct, and the uniqueness of each isolate was confirmed by microsatellite analysis (Brzyski et al. 2014). Specimens were vouchered at the Missouri Botanical Garden (St Louis, MO, USA, specimen numbers M092113 and M092115) and at the National Herbarium of the Republic of Trinidad and Tobago (St Augustine, Trinidad, specimen number TRIN34616, D. N.

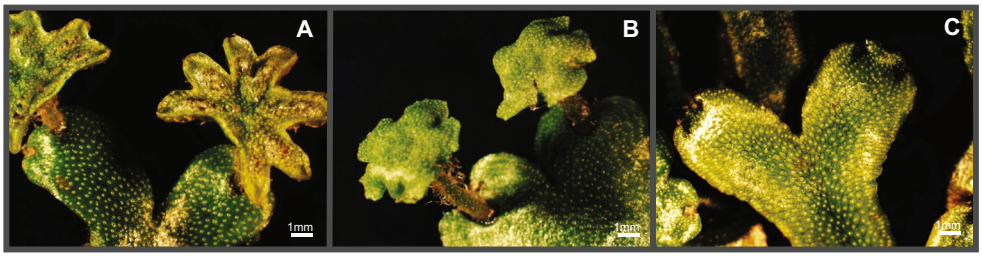

Fig. 1 Anatomy and morphology of *Marchantia inflexa* plants. (**a**) two fully developed male sex structures (antheridiophores) emerging from the thallus. The antheridia are embedded in the antheridiophore and sperm is released onto the top of the structure (as can be seen on the surface of the right most antheridiophore above). (**b**) two fully developed, but unfertilized female sex structures (archegoniophores) and supporting thallus tissue. The female archegonia are located on the underside of the archegoniophore Fertilization occurs when water dispersed sperm reaches the female archegonia. (**c**) vegetative thallus tissue. Greenhouse collections consisted of vegetative tissues only, whereas field samples included male or female sex structures (used to determine the sex of each sample)

McLetchie, collector). Specimens were transported to Kentucky, USA, planted on steam-sterilized soil in a climate-controlled greenhouse and maintained via vegetative propagation. All individual genotypes were maintained in lidded pots to prevent contamination or cross fertilization among specimens. Pots were placed on capillary mat, kept wet by daily watering with dH_2O, and under shade cloth to mimic field light conditions. Plants were rotated periodically, and new clones of each genetic line were propagated by vegetative fragmentation on a semi-annual schedule. When sampled for the current study, greenhouse specimens had been in culture in Kentucky for over five years. For the current study, three vegetative thallus tips (~5x8mm) from each genetic line were collected for DNA extractions. Only tips growing aerially with no soil contact and no evidence of reproductive structures were sampled, and tissue was collected directly into 100% EtOH. Total tissue sampled amounted to ~30 mg per genotype. Although female plants of this species have higher growth rates than males (McLetchie & Puterbaugh 2000), the individual thalli sampled were similar in size across sexes and genotypes.

Additional specimens were collected directly from field sites in Trinidad, Republic of Trinidad and Tobago in March 2015 (Table 1). Plants were collected at three sites (Quare stream, Cumaca road and Guanapo road). Thus, there is potential genetic overlap among greenhouse and field samples from Cumaca road. Study sites in separate drainages were targeted to increase variation between populations. The closest and farthest sites were ~1 and ~11 km apart, respectively. Vegetative tissues (growing aerially with no soil contact) and attached reproductive structures (used to determine the sex of the specimens) were collected at each field site. In order to minimize the potential of collecting clones, all samples were at least 1 m apart. At each site a minimum of one and maximum of three plants of each sex were collected directly into 75% ethanol and transported to the University of Kentucky for subsequent DNA extraction. Field collections included reproductive structures, but greenhouse specimens were comprised of vegetative tissue only. Environmental soil samples were not collected due to the restrictions on the transport of soil across national borders.

In order to demonstrate that the field habitats were significantly distinct from one another in relative humidity and temperature, we collected environmental data in June 2016. We monitored the relative humidity and temperature at each field site at five minute intervals for 4–6 days (overlapping dates when possible) using sensors integrated in the WatchDog™ model 450 data logger (Spectrum® Technologies, Inc. Plainfield, IL, USA). The resulting data were analyzed with a mixed effect linear model in JMP®, Version 10 (SAS Institute Inc., Cary, NC) to test the effect of habitat type and site (nested within habitat type) on relative humidity and temperature (date and time of day were included as random effects). Relative to the road sites, the stream sites were significantly more humid (mean: 99.8 ± 0.02% vs. 95.2 ± 0.17%, $F_{1,11} = 920.8$, $P = 0.0019$) and cooler (mean: 24.6 ± 0.04 °C.

Table 1 The name, location, and habitat type of collection locations in Trinidad, Republic of Trinidad and Tobago are listed along with the collection year. Cultivation indicates whether the plants were cultured in a common garden at the University of Kentucky or processed directly from field sites

Habitat type	Collection location	Coordinates	Collection Year	Cultivation
Stream	East Turure	10°41′04″N 61°09′39″W	2009	Yes
Stream	Quare	10°40′37″N 61°11′40″W	2015	No
Road	Guanapo	10°41′08″N 61°15′49″W	2015	No
Road	Cumaca	10°41′11″N 61°09′45″W	2009 and 2015	Yes 2009 No 2015

vs. 25.3 ± 0.08 °C., $F_{1,13} = 5.7$, $P = 0.032$). While these data clearly do not capture all differences between these two habitats, they demonstrate that the habitats differ significantly in temperature and humidity. Environmental data is available at Figshare (DOI:https://doi.org/10.6084/m9.figshare.4823530). Historical weather data for June 2009, June 2015, and June 2016 indicate that mean monthly temperatures varied by only 0.5 °C across sampling years (© Copyright 2017 The Weather Company, LLC).

2.3 *Characterization of the* M. Inflexa *bacteriome*

Given evidence for a tightly associated and functionally diverse bacteriome in *M. inflexa* (unpublished data), we sought to characterize the taxonomic diversity of the *M. inflexa* bacteriome and assess variation across habitats and among the sexes. The composition and diversity of the *M. inflexa* bacteriome (including both surface dwelling and endophytic bacteria) was characterized in mature male and female plants from three habitat types: 1) streams in Trinidad (native habitat), 2) roadsides in Trinidad (recently colonized habitat), and 3) the greenhouse at the University of Kentucky (common garden environment). The reproductive structures of field samples were included, but greenhouse samples consisted of only vegetative tissue.

DNA was extracted from 20 plant samples via a modified CTAB extraction method (Doyle & Doyle 1987). Prior to DNA extraction samples were washed three times in distilled water to reduce surface contamination and remove any residual soil on samples. This washing was not intended to remove all surface bacteria, but rather to enrich the proportion of tightly associated and endophytic bacteria in our samples. In addition, we performed three DNA extractions from 100% EtOH without plant tissue to serve as negative controls. 16S rRNA gene sequencing libraries were generated for each sample by PCR amplification of the V4 region of the 16S bacterial rRNA gene. Barcoded primers 501-507F (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG)/701-706R (5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) were used to index samples. Sample names, collection site, host sex and indices can be found in Table S1. PCR reactions were conducted using Promega GoTaq 5X PCR buffer, 0.8μM of each primer (F/R), and 150–200 ng genomic DNA. The PCR cycling conditions consisted of initial denaturation for 5 min at 94 °C followed by 34 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s and a final extension at 72 °C for 5 min. Samples that did not amplify were cleaned using AMPure magnetic beads, until adequate amplification was attained. All barcoded samples were pooled in approximately equi-molar concentration for sequencing (estimated by florescence on agarose gels). Sequencing of the 16S rRNA gene was conducted at the Advanced Genetic Technology Center, University of Kentucky on an Illumina MiSeq platform. Samples were demultiplexed by the Advanced Genetic Technology Center. Sequence data are available on the Sequence Read Archive of NCBI (BioProject accession number: PRJNA381821).

Read quality of the resulting sequence data was assessed with fastQC (Andrews 2010), and reads were merged using FLASH (Magoč & Salzberg 2011) with a minimum overlap of 20 base pairs (bp) and a maximum overlap of 250 bp. Merged reads were quality filtered with FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Bases with a quality score below 25, entire reads in which >20% of the bases had quality scores below 25, and samples with <1000 reads were removed. The total number of reads retained for each sample post quality filtering is presented in Table S1.

To characterize the bacteriome composition and address questions of sex and habitat specificity, bacteriome composition and diversity were analyzed using QIIME (Caporaso et al. 2010). FASTA files were labeled, combined into a single file and de novo operational taxonomic units (OTUs) were picked using a 97% similarity threshold. All OTUs found in negative controls and all chloroplast sequences were removed from downstream analyses.

To test for differences in the magnitude of bacterial diversity among habitat types, alpha diversity was assessed. In order to account for differences in the number of reads recovered for each sample, rarefaction analysis was conducted. The data set was normalized to 7174 counts/sample (the median number of counts/sample) before calculating the Chao1 metric, and the difference in the magnitude of diversity between habitats was tested for significance using a dissimilarity matrix.

To test for differences in bacteriome composition between habitats, beta diversity was analyzed using the Unweighted UniFrac metric (Caporaso et al. 2010; Lozupone et al. 2011), and the number of counts/sample was normalized to the 1139 (the minimum number of counts/sample in our dataset). Principal coordinates analysis (PCoA) plots were generated to characterize the differences between all samples. The diversity between habitats was compared to the diversity within habitats using a 999 Monte Carlo permutations based on the Unweighted UniFrac metric, and *P*-values were adjusted using the Bonferroni correction to correct for multiple comparisons.

Differences in the relative abundance of each OTU among habitats were tested for significance using the Kruskal Wallis test. To reduce the statistical problems associated with multiple comparisons the OTU table was filtered to retain only OTUs observed in 25% or more of the samples prior to running this test (Caporaso et al. 2010). P-values were corrected for false positives resulting from multiple comparisons with the False Discovery Rate (FDR) adjustment.

We tested for a sex difference in the bacteriome of the entire data set, but also in a reduced OTU table containing only

greenhouse samples. Analyses of sex differences in alpha diversity, beta diversity, and differential abundance of taxa (analogous to those described above) were conducted using the complete OTU table and an OTU table containing only the greenhouse samples. Again, all *p*-values were adjusted using the Bonferroni correction in order to account for multiple comparisons.

To characterize similarities in the bacteriome, we described the shared bacteriome of *M. inflexa.* We defined a shared bacteriome that includes all OTUs found in at least 75% of samples. This definition is analogous to the definition of the core bacteriome (Bragina et al. 2015), but because our study included only a limited number of *M. inflexa* populations we prefer to be conservative and refrain from defining a core bacteriome. Differences in taxon abundance among habitats within the shared bacteriome were identified using a Kruskal Wallis test, and the FDR correction was employed to correct for false positives.

3 Results

3.1 *Characterization of the* M. Inflexa *bacteriome*

Targeted sequencing of the 16S rRNA gene in *M. inflexa* males and females from three habitats revealed high diversity in the bacteriome of *M. inflexa*. Overall, we identified 10,337 unique OTUs, representing 618 bacterial genera. The most abundant phylum was Proteobacteria, and within that phylum, the order Rhizobiales was prevalent. Other abundant phyla included Bacteroidetes, Verrucomicrobia, Cyanobacteria, Acidobacteria and Actinobacteria (Fig. 2). Although hundreds of different bacterial genera were present at all collection sites, there was no difference in the magnitude of alpha diversity among habitats (Chao1 values for each habitat: stream = 2050; road = 1827; and greenhouse = 1.383).

Comparisons among samples derived from stream, roadside and greenhouse habitats revealed substantial differences in community composition. There was significantly higher beta diversity among habitats compared to within habitat diversity ($P < 0.0001$), and samples from the same habitat clustered with one another and were distinct from other habitats in PCoA plots of bacteriome composition (Fig. 3). The variation detected may be driven by standing differences in the bacterial communities present at these collection sites, or differences in plant-microbe interactions that are impacted by habitat differences, but we are unable to distinguish among these alternate explanations. Greenhouse plants were originally collected from different habitats (roads and streams) ~five years prior to the study, but habitat of origin did not significantly impact bacteriome composition of common garden plants.

Two hundred and ninety-one bacterial genera exhibited significant differences in abundance among habitats. Of these,

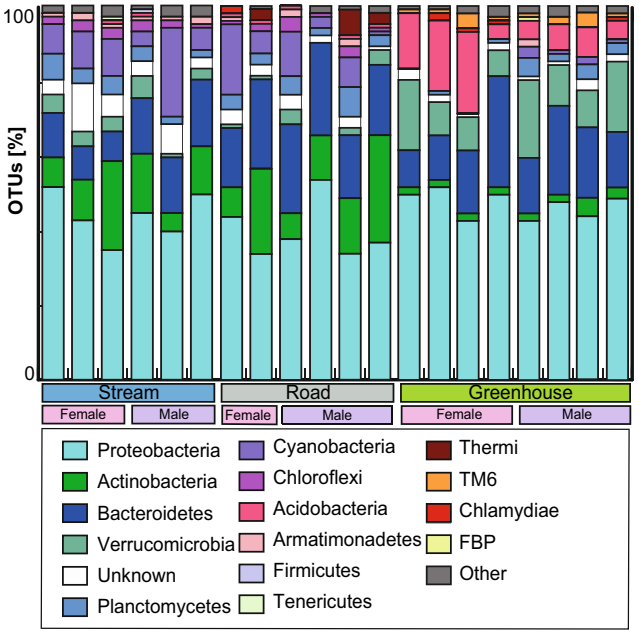

Fig. 2 The 16 dominant bacterial phyla (major groups) in the bacteriome of *Marchantia inflexa* are shown as percentage of total OTUs. Each stacked column depicts the bacteriome of a single sample and colors represent different phyla. All phyla that comprise <1% of the total OTUs for a given sample are combined and labeled as "other". Samples are grouped by habitat and then by sex

140 belonged to the phylum Proteobacteria, including the subgroups Rhizobiales (known to be involved in nitrogen fixation, stress protection, auxin and vitamin production (Erlacher et al. 2015)) and Gammaproteobacteria, which function in carbon processing and sulfur oxidation (Gifford et al. 2014). Forty-three genera belonged to the phylum Actinobacteria. Many subgroups of this phylum are involved in nitrogen fixation, antimicrobial compound production, and antioxidant production (Newton et al. 2008). These genera were most abundant in the field sites. Cyanobacteria (10 genera) some of which are known to fix nitrogen in bryophyte tissues (DeLuca et al. 2002), Amaimonadetes (5 genera) (Lee et al. 2014) and the filamentous Chloroflexi (5 genera) (Björnsson et al. 2002) were most abundant in the stream site. In the greenhouse, the carbon processing Bacteroidetes (27 genera) and Verrucomicrobia (14 genera) (Thomas et al. 2011) were abundant. Planctomycetes (14 genera), some of which can metabolize ammonia (Fuerst & Sagulenko 2011), Acidobacteria (12 genera) which are important in nutrient cycling (Naether et al. 2012), and Tenericutes (2 genera) were also more abundant in the greenhouse. The remainder of differentially abundant genera are unclassified taxa.

No significant sex differences were detected in the *M. inflexa* bacteriome when the entire data set was analyzed. Both alpha and beta diversity were assessed between the sexes and found to be non-significant. Additionally, no specific taxa were found to differ significantly in abundance between the sexes. However, analysis of only greenhouse samples

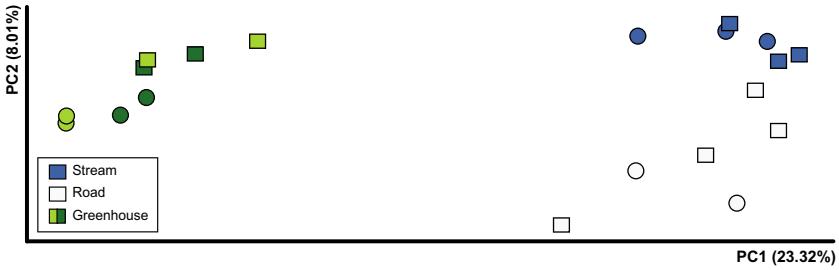

Fig. 3 Unweighted Unifrac principal coordinates analysis (PCoA) showing the diversity among samples along PC1 and PC2. Each point represents a sample, and samples are color coded by habitat. Within greenhouse plants, dark green plants were originally collected from Cumaca road, whereas light green plants were collected from East Turure stream. The sexes appear as separate shapes (squares designate males, and circles represent females). Habitat differences in the bacteriome are significant, but sex differences are evident only within greenhouse samples. No additional separation was evident when comparing PC2 and PC3, and consequently that relationship is not shown here, but can be found in Fig. S1

revealed that beta diversity between the sexes was significantly higher than the diversity within the sexes ($T_5 = 4.22$, $P = 0.03$), indicating sex specificity in the bacteriome under common conditions.

The shared bacteriome of *M. inflexa* contained 34 OTUs from 26 genera that are commonly associated with this plant. The most abundant orders of bacteria found in the shared bacteriome were Caulobacterales, Rhizobiales, Acidobacteriales, Saprospirales, Actinomycetales and Rhodospirillales (Table 2). Like the entire bacteriome, some taxa in the shared bacteriome varied in abundance among habitats (Fig. S2).

4 Discussion

Here, we demonstrate that there are significant patterns of association between bacteriome composition and habitat in *M. inflexa*. There was no comprehensive sex difference in bacteriome composition or diversity. However, we did detect a sex difference in the bacteriome of greenhouse-grown specimens, which we speculate is linked to sex function, related physiology (e.g. nutrient requirements for the production of gametes and maturation of sexual offspring) and morphology (e.g. aiding in the dispersal and capture of gametes). It is likely that this sex difference is only identifiable under controlled conditions, due to an overwhelming effect of habitat on the bacteriome. Notably, we identified multiple groups of nitrogen fixing bacteria associated with *M. inflexa*. These taxa likely serve nutrient acquisition roles, as has been shown for other non-vascular plants (Bragina et al. 2012; Knack et al. 2015), suggesting that bacterial nitrogen fixation may be prevalent among bryophyte lineages.

Table 2 The orders of the shared bacteriome and percent that each comprises are listed in decreasing abundance. The shared bacteriome was defined to include only OTUs found in 75% or more of the samples. Orders comprising less than 1% of the shared bacteriome are not shown

Bacterial Order	Percent of shared bacteriome (%)
Caulobacterales	18.59
Rhizobiales	15.73
Acidobacteriales	13.44
Saprospirales	11.50
Actinomycetales	9.03
Rhodospirillales	8.65
Xanthomonadales	7.99
Sphingobacteriales	6.10
Chthoniobacterales	2.43
BD7–3	2.02
Cytophagales	1.58

Recent studies have shown that the bacteriomes of mosses (Opelt & Berg 2004; Opelt et al. 2007a, b, Bragina et al. 2012, 2014, 2015) and liverworts (Knack et al. 2015) exhibit high diversity, and we find that the bacteriome of *M. inflexa* is similarly diverse. Variation in the taxonomic composition of the *M. inflexa* bacteriome is largely explained by habitat type. This variation may derive from standing differences in local bacterial pools across environments. Alternatively, it may arise from plant directed recruitment of symbiotic microbes, or selection within *M. inflexa*. The plant microbiome, as a reservoir of additional genes, may serve as the plant's first line of defense against changing environmental conditions (Vandenkoornhuyse et al. 2015), and therefore differences in the bacteriome correlated to habitat are expected. Interestingly, although greenhouse specimens were derived from both road and stream habitats originally, there was no persistent effect of native habitat on bacteriome composition. This suggests that current environmental conditions have a dominant effect on the bacteriome of these plants, and that a substantial fraction of the bacteriome is relatively transient.

Notably, we detected an abundance of nitrogen fixing species, which were substantially enriched in putatively low nutrient environments (field sites). It is well known that some cyanobacteria associated with mosses, hornworts and liverworts aid in nitrogen fixation (During & Tooren 1990;

DeLuca et al. 2002), but it has only recently become clear that rhizobium-like bacteria may play an equally important role in nutrient acquisition for the living relatives of early diverging land plants (Knack et al. 2015). In the current study, Rhizobiales were among the most abundant and consistently detected microbial taxa. Additionally, the richness of Actinobacteria in the field, but not the greenhouse, might suggest a role for the bacteriome in stress tolerance. In addition to antimicrobial compounds, some Actinobacteria species are known to produce mycothinol, a glutathione like antioxidant, that could be important in reactive oxygen species scavenging during stress response (Newton et al. 2008). These taxa would aid plants growing in field sites where stressful drying events are more common than in the climate-controlled greenhouse.

We did not detect comprehensive evidence of an association between bacteriome composition and plant sex. However, under common conditions we identified a sex difference in the bacteriome. This general pattern is analogous to studies of plant rhizosphere interactions, showing that the strongest driver of microbial community composition is soil type (or land use in the case of air born microbial communities (Bowers et al. 2011)), yet within a single soil type plant genotype, cultivar and species can influence microbiome assemblage (Manter et al. 2010; Ofek et al. 2014). Because the plants in this study were grown under common conditions, the identified sex difference cannot be explained by exposure to different microbial communities. Thus, we speculate that the detected difference derives from sex specific plant-microbe interactions, or sex specific morphology and physiology. It seems possible that these differences impact the retention and recruitment of associated microbes. However, these effects are subtle enough to be overwhelmed by the dominant effect of habitat on the bacteriome composition. In the common garden, we detected a higher abundance of Rhizobiales in females, including Brydyrhizobium, Agrobacterium and Rhizobium. In addition, females harbored a substantially more Terriglobus and Pseudonocardia. Males, on the other hand, had a greater abundance of the nitrogen fixing cyanobacteria, Nostoc, multiple groups of the aquatic Planctomyces and Chitinophagaceae. Taken together these differences in taxon abundance point towards alternative strategies for nitrogen acquisition among the sexes. While females host more Rhizobiales, males appear to associate preferentially with Nostoc. Additionally, the detection of Pseudonocardia in females suggests that the bacteriome may aid in stress tolerance, as these taxa have been implicated in detoxification and protection roles (Jafari et al. 2014). Consequently, we speculate that sex differences in microbial communities may be correlated to a previously identified sex difference in dehydration tolerance under identical growth conditions (Marks et al. 2016), but additional studies will be needed to confirm this.

We identified 26 bacterial genera shared in the majority of our samples, indicating that specific taxa are closely associated with *M. inflexa*, despite changing habitats and sexes (Table 2), although it is possible that these taxa are simply ubiquitous. The composition of the shared *M. inflexa* bacteriome shows similarities with other bryophytes, including an abundance of Proteobacteria and a richness of Rhizobiales, Actinobacteria, and methanogenic bacteria (Juottonen et al. 2005; Knack et al. 2015). Actinobacteria are also abundant in seed plant bacteriomes indicating that this group may be a common feature of all plant bacteriomes (Schlaeppi et al. 2014). These genera are interesting candidates for future studies investigating relationships that are critical for *M. inflexa* across habitats.

In summary, we present evidence supporting the hypothesis that the *M. inflexa* bacteriome varies across habitats, and we show that under common conditions, host sex can modulate bacteriome composition. Furthermore, our data suggest that habitat dependent differences in the *M. inflexa* bacteriome may be functionally relevant because particular taxa that may aid in plant performance in specific conditions were enriched in these environments, but this speculation must be confirmed by additional studies. Other work in the field has indicated that plant bacteriome composition can be determined by lineage (Knack et al. 2015), and also that community composition depends on environmental factors (Bulgarelli et al. 2012; Schlaeppi et al. 2014). Our study suggests that both of these factors influence bacteriome diversity and assemblage in *M. inflexa*.

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