Selective Bacterial Community Enrichment Between the Pitcher Plants Sarracenia Minor and Sarracenia Flava

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Microbiome Announcement

Keywords: microbial communities, metagenomics, pitcher plants, Sarracenia

Posted Date: August 11th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-55560/v1

Version of Record: A version of this preprint was published at Microbiology Spectrum on November 24th, 2021. See the published version at https://doi.org/10.1128/Spectrum.00696-21.

Abstract

Background

The interconnected and overlapping habitats present in natural ecosystems remain a challenge in determining the forces driving microbial community composition. The cup-like leaf structures of some carnivorous plants, including the family *Sarraceniaceae*, are self-contained ecological habitats that represent systems for exploring such microbial ecology questions. We investigated whether *Sarracenia minor* and *Sarracenia flava*, when sampled at the same geographic location and time, cultivate unique microbiota; an indication of biotic selection of microbes due to eliminating many of the environmental variable present in other studies comparing samples harvested over several time points.

Results

DNA was extracted from the decomposing detritus trapped in the base of each *Sarracenia* leaf pitcher. We profiled a portion of the 16S rRNA gene across the bacterial community members present in this detritus using Illumina MiSeq technology. We identified a surprising amount of diversity within each pitcher, but also discovered that the two *Sarracenia* species each contained distinct, enriched microbial community members. This suggests a non-random establishment of microbial communities within these two *Sarracenia* species.

Conclusions

Overall, our results indicate that microbial selection is occurring within the pitchers of these two closely related plant species, which is not due to factors such as geographic location, weather, or prey availability. This suggests that specific features of *S. minor* and *S. flava* may play a role in fostering specific insect-decomposing microbiomes. These naturally occurring microbial ecosystems can be developed to answer important questions about microbial community succession, disruption, and member contributions to the community. This study will help further establish carnivorous pitcher plants as a model system for studying confined, naturally occurring bacterial communities.

Background

A central question in the field of microbial ecology is how specific environments deterministically shape their microbial communities [1]. One challenge has been that most natural habitats are spatially continuous, often leading to dynamic mixing of adjacent communities. Systems comprised of naturally established yet spatially defined microbial communities are therefore valuable for addressing the question of how natural habitats influence microbial community composition. Phytotelmata (compartments of terrestrial plants that collect and retain rain water) provide ideal systems for interrogating such self-contained microbial communities [2]. One group of phytotelmata that have a functional purpose are those formed by the pitfall traps of carnivorous plants of the *Sarraceniaceae*

family [3,4]. These plants are therefore an ideal natural system in which to study the potential effects that plants and/or founder microbial communities may have on the establishment of a microbial community.

Previous *Sarracenia* pitcher plant microbiome studies have focused on a range of temporal [5-8], and geographic [7,9-11] questions. One outstanding question that has only previously begun to be investigated [12] is whether *Sarracenia* microbiomes are more impacted by plant-specific features or geographic location (which impacts both environmental factors and prey pools). This pilot study seeks to add additional information to this line of questioning by comparing the microbiomes of two *Sarracenia* species to identify different or distinct microbial communities or community members that are selected for by related plant species in the same geographic location that were collected at the same time; meaning no weather changes could influence the microbiomes between samplings. This is a critical question because the source of the microbes within these pitcher plant communities has not been clearly established. From bacterial culturing studies it is believed that *Sarracenia* pitchers are sterile prior to opening [13,14]. If true, the resident microbiota of the prey themselves may then act as the source of the pitcher plant's microbiota; thus, the geographically defined prey pools accessible to *Sarracenia* plants could strongly influence their resulting microbiota. In contrast, there are also phenotypic and chemical differences between different *Sarracenia* plant species [3,15,16] that could also influence microbial survival within the pitchers. To begin to address this question we examined the microbiota of two different *Sarracenia* species grown in the same geographic location and sampled at the same time.

We compared the bacterial communities contained in the pitchers *Sarracenia minor* and *Sarracenia flava*. Although phylogenetically related, these two plant species are structurally distinct: *S. minor* (the hooded pitcher plant) has an operculum that folds forward over the front of the pitcher opening and closes off much of the pitcher to the surrounding environment, while *S. flava* (the yellow pitcher plant) has an operculum that is raised above the pitcher opening, leaving it more open to the environment (see Figure 1A). Using 16S ribosomal RNA (rRNA) gene profiling, we analyzed the microbial communities of *S. minor* and *S. flava* plants growing in a natural setting in the same location. This sampling strategy removed the confounding temporal and geographic variables, thus mitigating differences in abiotic environmental factors or potential prey availability on the microbial community composition of these two *Sarracenia* species. This sampling design allowed us to attribute any observed differences to plant-species-specific factors. As described below, our results indicate that these two plant species do, either directly or indirectly, enrich for different bacterial community members within their pitchers, and these results provide a foundation to begin questioning the influences that may be altering the microbial communities between these two closely related plant species.

Materials And Methods

Pitcher plant collection and detritus extraction

In collaboration with the North Carolina Botanical Gardens, we were given access to a semi-curated, employees-only bog garden where *S. flava* and *S. minor* were both growing in the same area. In this area,

individual *Sarracenia* species' rhizomes had been separated into pots and placed in a sunken bog and were periodically maintained/propagated by the NC Botanical Gardens. On June 17, 2016 we identified mature pitcher leaves that had opened that season and were actively capturing prey; they did not have any damage to the length of the pitcher and did not have their cup opening obstructed. The pitchers of the same species were not all from the same rhizome, but some could potentially be clonal pitchers where the rhizomes had been split in previous years. Based on these restrictions, we collected seven *S. minor* pitchers with average heights of 16.36 ± 1.51 and ten *S. flava* pitchers with average heights of 19 ± 4.14 inches. Each pitcher was severed from the plant at ground level using a sterilized clipper. The pitchers were collected in sterile bags and placed on ice in a cooler for transport. Pitchers were stored on ice for no more than 2 hours before processing. For each pitcher, the outside of each pitcher was sterilized and cleaned with 70% ethanol. One side of each pitcher was sliced vertically using a new, sterile scalpel and the detritus at the bottom was collected and weighed. Up to 0.1 g of detritus was taken for DNA isolation to be used for 16S rRNA gene sequencing.

Bacterial community profiling of pitchers

Detritus was weighed and immediately processed using the PowerSoil DNA isolation kit (Qiagen). The resulting DNA was sent to the UNC – Chapel Hill High-Throughput Sequencing Facility for paired-end 16S rRNA sequencing on an Illumina MiSeq using a previously described molecular-tagging protocol [17] with the 515F and 806R 16S rRNA gene primers covering the V4 region. The sequence data have been submitted to the EMBL databases under accession number PRJEB22641. MTToolbox [18] was used to remove the molecular tags from the sequencing reads as well as remove low quality reads based on the program's default settings.

Identifying Operational Taxonomic Units (OTUs)

Using a 97% identity cutoff and filtering of chimeric sequences, high-quality reads were clustered into 98,584 OTUs using open reference picking with version 7.0.1090 of the Usearch algorithm [19] as implemented in the metagenomics plugin of MTToolbox [18]. Chloroplast and mitochondrial sequences were removed using BLAST. OTUs with fewer than 25 reads in at least two samples were removed; this was a conservative cutoff to minimize false positives in our analyses and resulted in 644 measurable OTUs. This set of OTUs contains 89% of the total high-quality sequence reads obtained (6,071,825 out of 6,849,737) and was used for all further analysis. Taxonomic assignments were made for each OTU using the assign_taxonomy.py script implemented in QIIME [20] in conjunction with the May 2013 version of the GreenGenes [21] database.

Custom analysis scripts

Analyses were not performed on rarefied data (Additional file 1) unless otherwise noted. All custom scripts are accessible via github at https://github.com/islandhopper81/pitcher_plant_utils. Names of specific scripts used in our analyses are noted in parentheses below.

Identification of enriched OTUs using DESeq2

The DESeq2 library [22] was used to call OTUs enriched in either plant species. DESeq2 models OTU read counts using a negative binomial distribution and is a tool commonly used to identify condition-specific OTUs. DESeq2 accounts for differences in sequencing depth between samples [22]. Our model included the plant species as the only factor. Custom scripts were used to streamline this process (model_main.R, make_tax_table.pl, make_otu_boxplots.R).

Beta diversity

A custom script (cap_main.R), presenting a canonical analysis of principal (CAP) coordinates [23, 24], utilizing the vegan package [25] capscale function was used to calculate beta diversity between samples. This allowed us to identify the variance in microbiome community composition that could be accounted for by the *Sarracenia* sp. the sample was collected from.

Alpha diversity

The alpha diversity for each sample was calculated using the PD Whole Tree, Chao1, Shannon, and Simpson metrics as implemented in the QIIME script, alpha_diversity.py [20]. A Student's t-test was used to test if PD Whole Tree numbers differed between the two plant species (custom script make_alpha_div_fig.R).

Results

While a relatively small number of *Sarracenia* samples were collected for this pilot study, this was the limit of samples that could be collected and be processed on the same day, allowing us to remove abiotic influences, such as weather changes between samplings, from our results. The detritus from nine pitchers of *S. flava* and seven pitchers of *S. minor* were processed for bacterial community analysis using 16S rRNA gene sequencing. While 10 *S. flava* pitchers were sampled, one of the library preparations failed and had to be removed from the analysis. We obtained varying number of sequencing reads depending on the pitcher sample (Fig. 1B). When assigning these reads to OTUs, we chose a relatively conservative cutoff, only considering those OTUs that were represented by at least 25 sequence reads in at least two separate samples; this eliminated many singleton reads (Additional File 2) and resulted in 644 OTUs (representing 89% of the total 6,068,766 reads obtained). The overall phylogenetic composition of the bacterial communities within the pitchers' detritus consisted of eight main bacterial phyla (representing 98.6% of OTUs), with Bacteroidetes, Firmicutes, and Proteobacteria dominating (Fig. 1C).

We then examined the diversity of the two plant species' microbiomes. Calculations of phylogenetic diversity indicated that there were no significant differences in alpha diversity between the microbial communities of these plant species (Additional File 3). Beta diversity was calculated using the weighted Bray-Curtis dissimilarity metric as part of a canonical analysis of principal coordinates (CAP) analysis that was constrained by plant species. We elected to use this approach since all samples were harvested on the same day from the same location, and we were specifically interested in determining whether there were bacterial community differences dictated by the plants themselves. This CAP analysis indicated that 10.24% of the variance between samples was attributable to the plant species (Additional File 4).

We next identified specific OTUs that were differentially abundant between the two pitcher plant species. To do so, we built a negative binomial generalized linear model using DESeq2, which takes into account differences in sequencing depth between samples. This model identified 35 OTUs enriched in *S. flava* and 74 OTUs enriched in *S. minor* (Fig. 2A). For both species, the enriched OTUs most frequently fell within the Gammaproteobacteria, Alphaproteobacteria, and Clostridia classes (Fig. 2B). Thus, the same bacterial classes that were most abundant in the overall pitcher plant communities (Fig. 1C) also contained the OTUs that were enriched in a specific plant species. Due to the variability observed in microbial community composition across samples – even those from the same plant species – we next examined how individual enriched OTUs were distributed across each pitcher sample. Figure 2C shows the distributions of representative enriched OTUs and their normalized counts within each sampled pitcher. These data demonstrate that enriched OTUs are indeed more abundant across the majority of samples from a single plant species (Fig. 2C). While different bacterial clades were enriched in the two plant species, no single phylogenetic class showed enrichment in only one of the pitcher plant species (Fig. 2B).

Conclusions

Our results indicate that *Sarracenia* carnivorous pitcher plants can harbor distinct microbial community members unrelated to geographic location, weather, or prey availability. Utilizing this data can lead to larger questions about factors influencing microbial succession and diversity between these species, using carnivorous pitcher plants as unique, defined-area, model systems for studying microbial ecology, or how the uniquely abundant clades of bacteria may influence the bacterial communities they inhabit. These microbiomes presented in this article will help add to the unique niche of observed naturally occurring microbial communities that carnivorous plants harbor; as their microbiomes hover somewhere between plant microbiome and human gut microbiome.

With the clear indication that there are enriched bacterial members between the two *Sarracenia* species' environments not effected by time or geographical location, a second temporal study has been planned to study plant and insect influences that could explain the microbial succession that develops within the pitchers. Various physical changes to the pitchers have been planned which can indicate the influence of the operculum, the insect species being lured, and the natural rainfall or passive influx of debris into the plant.

Declarations

Ethics approval and consent to participate –

Not Applicable

Consent for publication –

Not Applicable

Availability of data and material –

The data generated and analyzed during this study have been submitted to the EMBL databases under accession number PRJEB22641.

Competing interests –

The authors declare that they have no competing interests.

Funding –

This work was supported by the National Institute of General Medical Sciences, National Institutes of Health [grant number GM112981 to E.A.S.] and the NSF INSPIRE Track II award IOS-1343020 to Jeffery L. Dangl. N.M.S. was supported by the Training Workforce Development & Diversity division of the National Institute of General Medical Sciences, National Institutes of Health [grant number K12GM000678]. S.Y. was supported by NSF INSPIRE Track II award IOS-1343020 to Jeffery L. Dangl.

Authors' contributions –

NS conceived and designed the study along with acquiring and processing samples, contributing to data interpretation, and drafting the manuscript. SY was involved with analysis and interpretation of data and contributing to the final manuscript. IW and EA were involved in data acquisition, processing, and analysis. ES contributed to the design of the project as well as data interpretation and manuscript preparation. All authors ready and approved the manuscript

Acknowledgements –

We thank Christine Liloia at the North Carolina Botanical Gardens for coordinating the collection of pitcher plants from their bogs and Corbin D. Jones for his comments on the manuscript.

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Figures

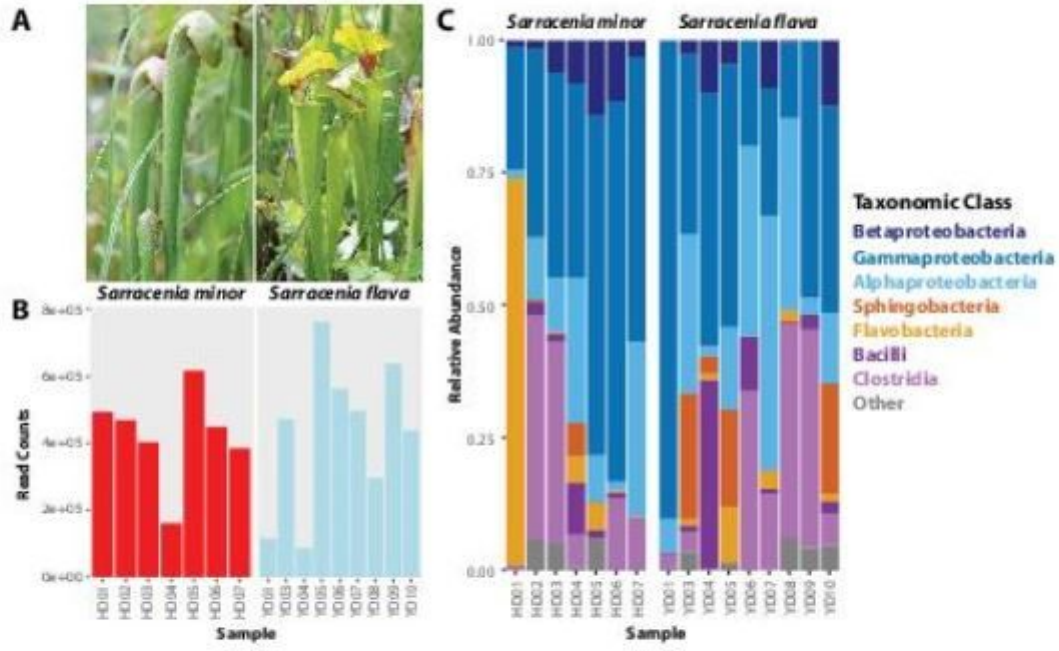

Figure 1

Initial sampling and community profiling of pitcher plant detritus. After sampling the detritus of both (A) Sarracenia minor (left) and Sarracenia flava (right), the bacterial communities were profiled using the 16S rRNA gene with (B) the number of reads collected from each sample ranging between 1x105 – 8x105 reads per pitcher. The 16s rRNA reads were then compared to known genetic sequences to determine (C) the relative abundance of different bacterial phyla within each pitcher.

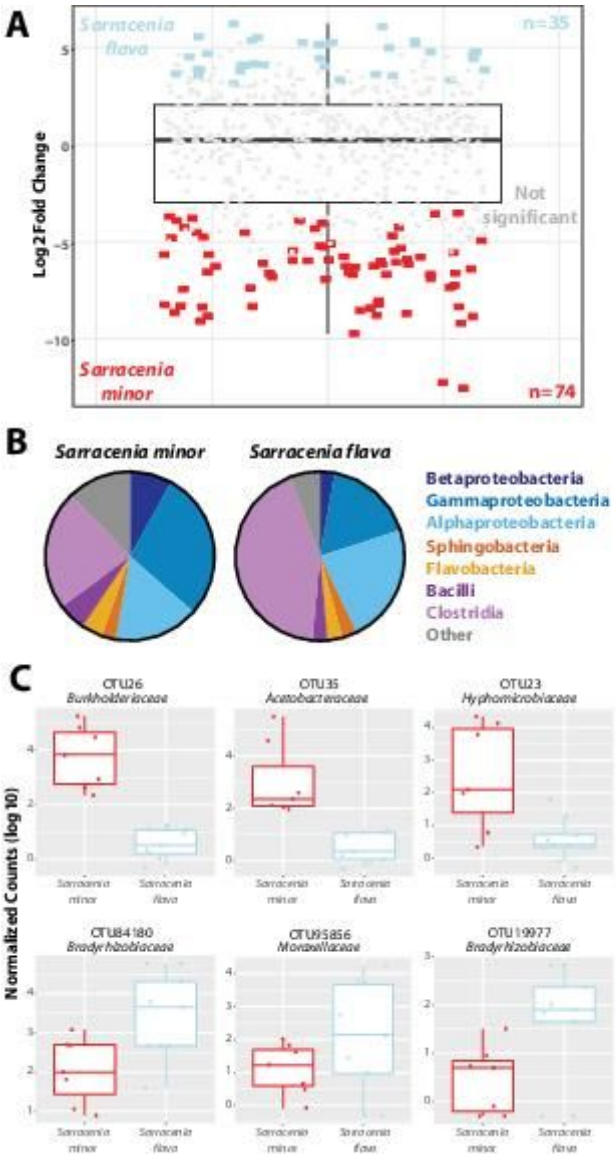

Figure 2

Constrained Analysis of Principal Coordinates (CAP) indicates a bacterial community difference based on plant species. A Beta diversity CAP analysis, providing the plant species metadata, was performed on the community profiling sequence data indicating that 10.24% of the variance between the samples is due to the pitcher species alone.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- additionalfile4CAPanalysis.eps
- additionalfile3alphadivtest.png
- additionalfile2otufiltering.png
- additionalfile1rarefaction.png