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Culture-Based Analysis of Bacterial Communities within Pitcher Plants (*Sarracenia purpurea*) of Western NC

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Abstract

The ability of the carnivorous pitcher plant (*Sarracenia purpurea*) to digest insect prey depends, in part, on plant-associated microbial communities. However, few studies have addressed diversity, distribution, and function of *Sarracenia*-associated bacterial populations. This study leverages natural populations of *Sarracenia purpurea* at two geographically distinct sites in Western North Carolina. Liquid from within the pitcher was collected aseptically, and culturable bacteria were isolated by plating on solid media. Non-selective medium was used to allow isolation of most environmental oligotrophic bacterial, while a selective medium was used to specifically enrich for antibiotic-producing members of the phylum Actinobacteria. Culturable bacteria were identified by PCR amplification and DNA sequencing of the 16S rRNA gene, confirming that microbial richness within the pitcher is high. Data indicate that bacterial communities differ from plant to plant, even within the same geographic location suggesting that initial colonization may occur at random from the environment. However, specific bacterial phylogroups were present in all pitchers sampled, suggesting that *Sarracenia* may rely on specific microbial associations. Ongoing work will assess the functional diversity of *Sarracenia*-associated bacteria, including ecologically relevant traits such as motility and biofilm formation which aid in plant colonization, exoenzyme production which may aid in prey digestion, and antibiotic production which may aid in bacterial competition in the resource-limited environment within the pitcher.

1. Introduction

Carnivorous plants of the genus *Sarracenia* are clump forming, herbaceous perennials that form modified, tubular leaves that trap rainwater and lure insect prey (Figure 1B). *Sarracenia purpurea*, known commonly as the purple pitcher plant, was originally described by Carolus Linnaeus in "Species Plantarum", and was named specifically for its typical coloration of the flower and foliage [1]. *Sarracenia* utilize the pitfall method of prey capture—insects are attracted to the leaves by the eye-catching coloration and abundant secretions of nectar concentrated around the pitcher opening. Once attracted to the nectar, insects are trapped within the pitcher and unable to exit. Previous work reports that *Sarracenia* produce digestive enzymes in order to obtain nutrients from fallen insects [2], but few studies have explored the role of *Sarracenia*-associated bacteria in this digestion.

Sarracenia represent an excellent model of a well-defined, self-contained ecosystem; the individual pitchers of the plant serve as a microhabitat for a variety of microorganisms and macroorganisms including bacteria, fungi, arthropods, and protozoa. The liquid within each pitcher arises from sterile conditions; thus, the primary mechanism of bacterial introduction is purported to be via insect vectors. Due to this nonuniform method of inoculation, some suggest that *Sarracenia* microbial communities may be nothing more than random assemblages. However, an increasing body of knowledge suggests that *Sarracenia* microbial populations are stable and specific, and play a role in the overall health and productivity of the plant [3]. A similar study, conducted by a team at Louisiana State

University, determined that pitcher microbial communities play a role in the digestion of trapped insects[4]. By comparing bacteria cultured from the plant and comparing it to cultured bacteria from surrounding soil, they suggest that bacteria associated with the pitcher plant were far from random collections of passive organisms.

The current study seeks to advance previous work by not only investigating the composition of the microbial communities found within natural populations of *S. purpurea*, but also to provide a comparison of the microbiota among *Sarracenia* populations growing in distinct geographic locations. We used a culture-based analysis, followed by phylogenetic identification using Polymerase Chain Reaction (PCR) amplification and sequencing of the 16S rRNA gene. Because the DNA sequence of the small-subunit rRNA gene is highly conserved across all kingdoms of life, rRNA sequence is used extensively to determine taxonomy, phylogeny, and to estimate rates of species divergence among bacteria [5]. The bacterial 16S rDNA sequence has hypervariable regions, where sequences have diverged over evolutionary time, flanked by strongly-conserved regions [6]. PCR primers are designed to bind to conserved regions and amplify the intervening variable regions. Subsequent DNA sequencing allows comparison of unknown organisms with sequences stored in publicly available databases. In the current study, PCR amplification of a variable portion (specifically the V1-V3 region), followed by DNA sequencing, enabled genus-level identification of *Sarracenia*-associated bacteria. Aims of this study are to culture bacteria from within the pitfall of *S. purpurea* plants, identify and distinguish characteristics of plant-associated bacteria, and compare the composition of microbial communities from plants at a single site (plant-to-plant variation) versus plants growing in distinct geographic locations (population-to-population variation). Identification of the organisms, followed by functional characterization, will provide information on bacterial community structure and may shed light on the role of these bacterial communities in plant health and prey digestion.

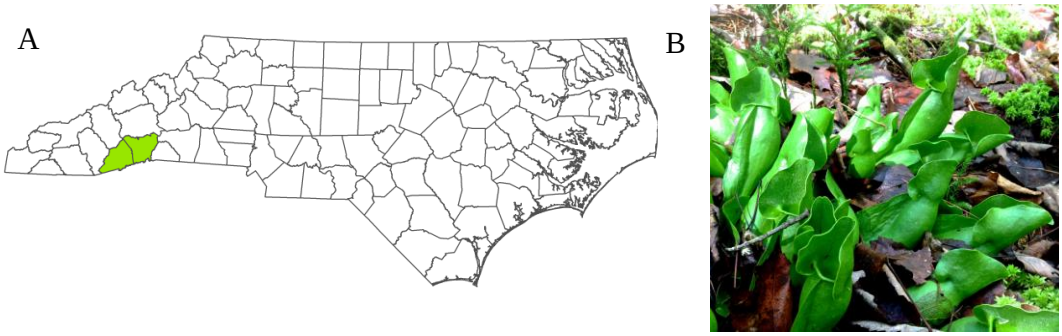

Figure 1. Population sampling locations (A) and *Sarrracenia purpurea* (B) in Western North Carolina.

2. Methods

2.1. Sampling and Cultivation of *Sarracenia*-associated Bacteria

Two geographically distinct, natural populations of purple pitcher plants, *Sarracenia purpurea*, were used in this study (Figure 1A). The first site is a low-lying flood plain area within Dupont State Forest (DF); the second, a protected area within Henderson County (HC). Triplicate samples, representing pitchers from three distinct plants at each site, were analyzed. Liquid from within the pitcher (25-30 ml) was aseptically collected, stored on ice in the field, and processed upon return to the laboratory. Samples were flash frozen at -80°C in 15% glycerol for long-term storage. To isolate culturable bacteria, liquid samples taken from within the pitchers were serially diluted in sterile phosphate buffer and spread-plated on solid bacterial growth medium. Two types of culture medium were used; Dilute Tryptic Soy Agar (DTSA) and *Actinomycete* Isolation Agar (AIA). DTSA is a general purpose, nutrient rich medium commonly employed for the isolation of environmental oligotrophs [7]. AIA is specifically formulated for the isoloation of *Actinomycetes*, a bacterial genus known for anti-biotic production [8]. Plates were incubated at 20°C for 7-10 days. From each sample, all morphologically distinct colony types were selected and streak purified. In total, 73 individual isolates were purified for further study.

2.2. Colony Morphology, Cell Morphology, Gram-staining Technique

Basic colony morphology was determined visually following growth of bacteria on solid medium for 5-7 days. The following colony details were recorded; formation, elevation, margin, surface, opacity, and pigmentation. Cell morphology was determined by staining and direct microscopic visualization, noting cell shape, size, and arrangement. Each bacterial isolate was further characterized by Gram staining, a common stain that distinguishes bacteria based on the composition of their cell wall [9]. Briefly, fresh bacterial cultures are affixed to a microscope slide and subjected to the following procedure: primary staining (crystal violet) for 1 min; mordant (Gram's Iodide) for 1 min; decolorization (95% ethanol); followed by secondary staining (safranin) for an additional 1 min. Cells were visualized directly, at 1000X magnification under oil immersion, using a compound light microscope.

2.3. Isolation of Genomic DNA

DNA was liberated from pure cultures by boiling or by extraction using the 5-Prime ArchivePure Genomic DNA Isolation Kit, as per the instructions of the manufacturer. Briefly, single colonies were grown overnight in 2 ml Dilute Tryptic Soy Broth (DTSB). After overnight growth, culture fluid was centrifuged to pellet cells, suspended in 300 µl of cell lysis solution and incubated at 80°C for 5 min to facilitate lysis. RNA was removed by the incubation with RNaseA (37°C for 30 minutes). Cellular proteins were then precipitated by addition of 100 µl of Protein Precipitation Solution followed by centrifugation. The DNA-containing supernatant was then recovered and washed with isopropanol (100%) and ethanol (70%). DNA was rehydrated overnight in 50 µl of DNA Rehydration Solution (TE buffer, pH 7.2).

2.4. PCR Amplification of 16S rRNA Gene

DNA was then used as the template in the PCR reaction to amplify the bacterial 16S rRNA gene. Universal 16S rRNA primers, 27F (AGA GTT TGA TCM TGG CTC AG) and 594R (CGG TTA CCT TGT TAC GAC TT) anneal to conserved regions of the bacterial 16S rRNA gene and amplify the intervening variable regions [10]. Each PCR sample contained the following; Taq polymerase (10 units), 10X reaction Buffer (1X), $MgCl_2$ (2.5mM), Forward Primer (0.1 µM), reverse primer (0.1 µM), dNTPS (100 µM), and dH_2O (to a final volume of 50 µl). Thermocycling conditions were as follows: initial denaturation (95 °C for 3 min), 30 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 45 sec, followed by a final extension at 72°C for 5 min. PCR products were analyzed by size separation on a 1% agarose gel stained with ethidium bromide to allow visualization under UV light. Presence of a single band, corresponding to approximately 550 bp in size, indicated successful amplification of the desired region (Figure 2).

2.5 16S rDNA Sequencing

Purified PCR samples were sent to GENEWIZ for DNA sequencing, using the sequencing primer, 27F (AGA GTT TGA TCM TGG CTC AG). rRNA gene sequences were then compared to publicly available sequences using the National Center for Biotechnology Information (NCBI) and Ribosomal Database Project (RDP) databases.

3. Results

3.1 Overall Bacterial Community Structure

Analysis of 16S rRNA gene sequence allowed genus-level identification of over 70 *Sarracenia*-associated bacteria. 3 isolates did not yield sufficient amplification products to allow sequencing, and thus could not be identified. Of the sequenced isolates, approximately 80% of identified bacteria (61 out of 70) were classified as Gram-stain negative, and predominantly consisted of members of the phylum *Proteobacteria* (Figure 3A, 3B). The identified *Proteobacteria* fell within three major classes; Gamma, Beta, and Alpha (Figure 3C). Other abundant Phyla include the Gram-negative Bacteroidetes (8 of 70) and the Gram-positive Actinobacteria. Of note, members of the

Enterobacteriaceae, *Neisseriaceae* and *Pseudomonaceae* were dominant overall; members of the *Neisseriaceae* were present in all pitchers sampled.

3.2 Geographic Differences in Bacterial Community Composition

Overall diversity was higher in pitcher fluid from plants in DF; however striking overlap in the bacterial phylogroups is apparent. All identified bacterial families were present in plants at both locations, with the exception of *Deinococcus/Thermus* which was only cultured from the DF samples. Members of the *Deinoccocus*, *Rahnella*, *Flavobactrium*, *Aquitalea*, *Curtobacterium*, *Curvibacter*, *Cedecea*, and *Unibacterium* genera were exclusive to the DF location, while *Novosphingobium* and *Pantoea* were exclusive to the HC location (Table 1).

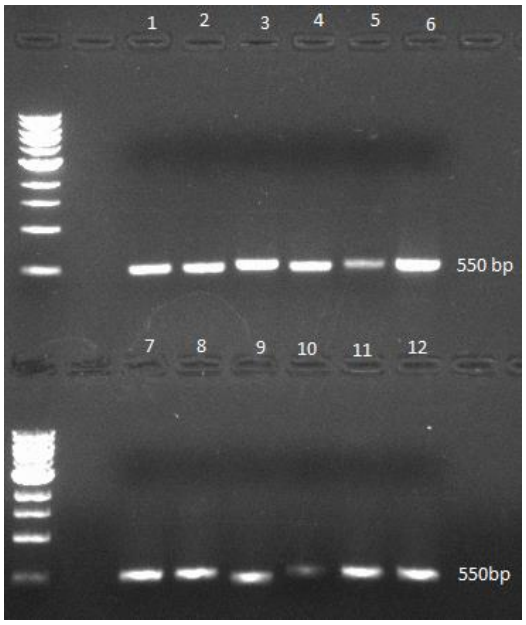

Figure 2. PCR amplification of 16S rRNA gene. A representative gel highlighting PCR results from a subset of the bacterial isolates. Top row (from left to right); DNA size standard; DF2-3; DF3-6; DF3-11; DF1-5; DF1-2; HC2-11. Bottom row (from left to right) DNA size standard; HC2-2; HC1-8; HC1-2; HC3-1; HC3-3; HC1-4. Products were separated on a 1% agarose gel stained with ethidium bromide.for visualization.

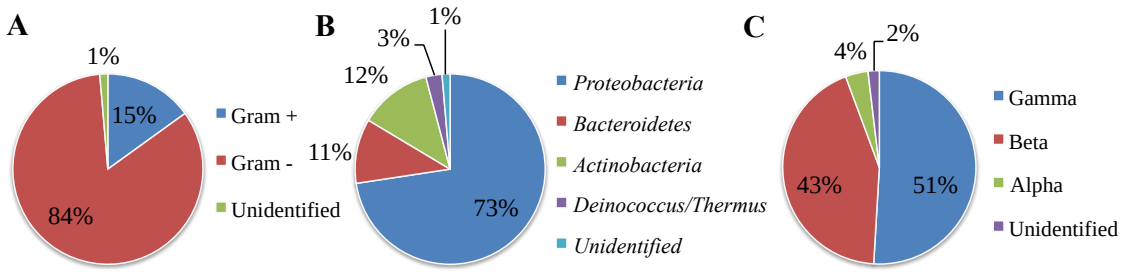

Figure 3. General classification of *Sarracenia*-associated bacteria. (a) Isolates organized by Gram-type (b) Distribution of bacteria within different Phyla (c) Distribution of the classes represented within the phylum *Proteobacteria*.

Table 1. Phylogenetic analysis of *Sarracenia*-associated bacteria. DNA sequence analysis of 16S rRNA gene sequence allowed Phylum-, Family-, and Genus-level identification of bacteria isolated from *Sarracenia* pitcher fluid. Triplicate plants (1, 2, and 3) were analyzed from two geographically separate locations, Henderson County (HC) and Dupont Forest (DF). ND; *not determined*.

HC1	**Phylum**	**Family**	**Genus**	**Gram-Type**
1	Proteobacteria	Neisseriaceae	*Chromobacterium*	-
2	Proteobacteria	Sphinogomonadaceae	*Novosphingobium*	-
3	Bacteroidetes	Sphingobacteriaceae	*Pedobacter*	-
4	Proteobacteria	Comamonadaceae	*Variovorax*	-
5	Proteobacteria	Comamonadaceae	*Variovorax*	-
6	Proteobacteria	Comamonadaceae	*Variovorax*	-
7	Proteobacteria	Comamonadaceae	*Variovorax*	-
8	Proteobacteria	Comamonadaceae	*Variovorax*	-
9	Proteobacteria	Oxalobacteraceae	ND	-
HC2	**Phylum**	**Family**	**Genus**	**Gram-Type**
1	Proteobacteria	Neisseriaceae	*Chromobacterium*	-
2	Proteobacteria	Neisseriaceae	*Chromobacterium*	-
3	Actinobacteria	Microbacteriaceae	*Microbacterium*	+
4	Proteobacteria	Oxalobacteraceae	ND	-
5	Proteobacteria	Oxalobacteraceae	ND	-
6	Proteobacteria	Enterobacteriaceae	*Pantoea*	-
7	Proteobacteria	Enterobacteriaceae	*Pantoea*	-
8	Proteobacteria	Enterobacteriaceae	*Pantoea*	-
9	Proteobacteria	Neisseriaceae	*Chromobacterium*	-
10	Proteobacteria	Enterobacteriaceae	*Pantoea*	-
11	Proteobacteria	Enterobacteriaceae	*Serratia*	-
HC3	**Phylum**	**Family**	**Genus**	**Gram-Type**
1	Proteobacteria	Neisseriaceae	*Chromobacterium*	-
2	Bacteroidetes	Sphingobacteriaceae	*Pedobacter*	-
3	Bacteroidetes	Sphingobacteriaceae	*Pedobacter*	-
4	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	-
5	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	-
6	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	-
7	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	-
8	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	-
9	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	-
10	Proteobacteria	Enterobacteriaceae	*Serratia*	-
DF1	**Phylum**	**Family**	**Genus**	**Gram-Type**
1	Proteobacteria	Neisseriaceae	*Aquitalea*	-
2	Proteobacteria	Neisseriaceae	*Chromobacterium*	-
3	Actinobacteria	Microbacteriaceae	*Curtobacterium*	+
4	Proteobacteria	Comamonadaceae	*Curvibacter*	-
5	Deinococcus/Thermus	Deinococcaceae	*Deinococcus*	+
6	Bacteroidetes	Flavobacteriaceae	*Flavobacterium*	-
7	Actinobacteria	Microbacteriaceae	*Microbacterium*	+
8	Bacteroidetes	Sphingobacteriaceae	*Pedobacter*	-
9	Bacteroidetes	Sphingobacteriaceae	*Pedobacter*	-
10	Bacteroidetes	Sphingobacteriaceae	*Pedobacter*	-
11	Bacteroidetes	Sphingobacteriaceae	*Pedobacter*	-
12	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	-
13	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	-

14	Proteobacteria	Comamonadaceae	*Variovorax*	-
DF2	**Phylum**	**Family**	**Genus**	**Gram -Type**
1	Proteobacteria	Neisseriaceae	*Aquitalea*	-
2	Proteobacteria	Neisseriaceae	*Aquitalea*	-
3	Actinobacteria	Microbacteriaceae	*Curtobacterium*	+
4	Proteobacteria	Comamonadaceae	*Curvibacter*	-
5	Actinobacteria	Microbacteriaceae	*Microbacterium*	+
6	Actinobacteria	Microbacteriaceae	*Microbacterium*	+
7	Proteobacteria	Sphinogomonadaceae	ND	-
8	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	-
9	Proteobacteria	Enterobacteriaceae	*Rahnella*	-
10	Proteobacteria	Enterobacteriaceae	*Rahnella*	-
11	Proteobacteria	Enterobacteriaceae	*Serratia*	-
12	Proteobacteria	Enterobacteriaceae	*Serratia*	-
13	Proteobacteria	Enterobacteriaceae	*Serratia*	-
DF3	**Phylum**	**Family**	**Genus**	**Gram-Type**
1	Proteobacteria	Neisseriaceae	*Aquitalea*	-
2	Proteobacteria	Enterobacteriaceae	*Cedecea*	-
3	Proteobacteria	Enterobacteriaceae	*Cedecea*	-
4	Proteobacteria	Enterobacteriaceae	*Cedecea*	-
5	Actinobacteria	Microbacteriaceae	*Curtobacterium*	+
6	Actinobacteria	Microbacteriaceae	*Curtobacterium*	+
7	Actinobacteria	Microbacteriaceae	*Microbacterium*	+
8	Proteobacteria	Enterobacteriaceae	ND	-
9	Proteobacteria	Enterobacteriaceae	ND	-
10	Proteobacteria	Enterobacteriaceae	ND	-
11	Proteobacteria	Oxalobacteraceae	ND	-
12	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	-
13	Deinococcus/Thermus	Thermaceae	*Thermus*	+
14	Proteobacteria	Oxalobacteraceae	*Undibacterium*	-

4. Discussion

Within the domain Bacteria, the Proteobacteria constitutes the largest and most phenotypically diverse phylogenetic lineage; thus, it is perhaps not surprising that this Phylum dominates the culturable bacteria of pitcher plant communities (Figure 3B; Table 1). Our results also align with data from previous studies of the culturable microbial community within the pitcher fluid of *Sarracenia.* For example, Siragusa *et al* report that *Proteobacteria* were the most abundant taxa of culturable bacteria within the pitcher fluid of 93 *Sarracenia minor* pitchers in Northern Florida, USA[11]. Of the cultured *Proteobacteria* in our analyses, only three classes were represented: α-, β-, and γ-proteobacteria. β-proteobacteria are often associated with plants, likely due to the capability of many members of this class to fix nitrogen[12]. In general, α-, β-, and γ-proteobacteria have similar metabolic profiles and nutritional requirements, making it possible for them to simultaneously inhabit the same environmental niche, as has previously been reported [13,14].

Of the predominant groups of bacteria identified, many are prevalent members of the rhizosphere and a number have previously been reported as plant-associated. These plant-associated bacteria range from plant-growth promoting (members of genera including *Pseudomonas*, *Novosphingobium*, and *Microbacterium*) to plant-pathogenic (genus *Curtobacterium*). In particular, *Pseudomonas*, *Chromobacterium*, *Rahnella*, and *Microbacterium* species are all well-known for their plant growth promoting or biological control activities, thus highlighting the possibility that *Sarracenia*-associated bacteria may positively impact the health of the plant. Of note, several isolates from this study are from genera known to fix atmospheric nitrogen (*Serratia Novosphingobium*, and *Pantoea*), which may potentially provide additional forms of nitrogen for plant growth [15,16]. In addition, an increasing body of evidence points to a direct role for *Sarracenia*-associated bacteria in decomposition of trapped insect prey. While it

has long been known that digestive enzymes are produced by the plant, bacterial-produced enzymes are also likely to aid in prey digestion. In our study, a number of isolates typically encode chitinases, which may help facilitate insect exoskeleton degradation [10,17,18]. Potential chitinolytic organisms identified in the current study include *Serratia*, *Pedobacter*, *Flavobacterium*, and *Pantoea* species, which were abundant in all pitchers sampled (Table 1). An increasing body of evidence suggests that a structured, complex bacterial community is likely of great importance to nutrient cycling and metabolism in these, and other, carnivorous plants.

Members of the genera *Chromobacterium* and *Pseudomonas* are well-known for their capacity to produce antibiotics and other bioactive compounds, and these organisms have been touted for their potential to control plant disease through pathogen suppression (biological control) [19,20]. A role for such biocontrol bacteria in the health of carnivorous plants such as *Sarracenia* has not been determined; however, the dominance of *Pseudomonas* species across all plants sampled raises the question of the potential role of these organisms in maintaining pitcher plant health Of the cultured isolates, many are dominant members of plant and rhizosphere communities and thus the association of these organisms with *Sarracenia* is predictable; however, organisms within the genera *Rahnella*, *Deinococcus*, and *Undibacterium* are relatively rare and not often associated with the plant environment. Members from these genera were cultured only from the DF location. Reasons underlying the presence of rare genera at the DF location cannot be determined definitively, but could be related to the interesting history of the site. During the 1950's, the Dupont Corporation purchased what was then the Coxe property (now Dupont State Forest, DF) in order to control the supply of clean water for their manufacturing processes. The land upon which these samples were taken, contained a manufacturing facility that produced silicon chips and later transitioned to the production of x-ray film [21]. It is unconfirmed if the DF soil contains residual contamination from the manufacturing facility; however, heavy metal contamination or other industrial waste can dramatically alter soil microbial communities even decades after clean up.

It has been estimated that more than 99% of the microorganisms in nature are unculturable [22]. Our analysis of *Sarracenia* bacterial communities was culture-based, requiring growth of the organisms in laboratory culture prior to identification and analysis. While these methods yielded a species-rich and diverse microbiota, future culture-independent metagenomic techniques could be used to access the genetic resources of the unculturable microbial species. For instance, a recent metagenomics-based study estimated the microbial richness associated with pitcher plant fluid could contain more than 1,000 unique phylogroups [4]. This suggests that the number of microbial species estimated by our culture-dependent techniques is likely a gross underestimate of the true diversity, and further analysis is likely to yield additional organisms of interest. In conjunction with this culturing technique, future research should record more data concerning details specifically concerning plant morphology. A study specifically on *S. purpurea* examined the relationship between the microbial richness and the size of the pitcher plant itself. It found that as the size of the plant increased, the microbial community became richer [23]. This could have been an important factor in our study because the size of the plants sampled was not recorded nor did it play any significance in the selection process of plants to be sampled. By sampling from larger pitcher plants we may have been more successful it cultivating more diverse communities, thus resulting in a broader spectrum of genera.

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