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The Carnivorous Pale Pitcher Plant Harbors Diverse, Distinct, and Time-Dependent Bacterial Communities[▽][†]

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The ability of American carnivorous pitcher plants (*Sarracenia*) to digest insect prey is facilitated by microbial associations. Knowledge of the details surrounding this interaction has been limited by our capability to characterize bacterial diversity in this system. To describe microbial diversity within and between pitchers of one species, *Sarracenia alata*, and to explore how these communities change over time as pitchers accumulate and digest insect prey, we collected and analyzed environmental sequence tag (454 pyrosequencing) and genomic fingerprint (automated ribosomal intergenic spacer analysis and terminal restriction fragment length polymorphism) data. Microbial richness associated with pitcher plant fluid is high; more than 1,000 unique phylogroups were identified across at least seven phyla and 50 families. We documented an increase in bacterial diversity and abundance with time and observed repeated changes in bacterial community composition. Pitchers from different plants harbored significantly more similar bacterial communities at a given time point than communities coming from the same genetic host over time. The microbial communities in pitcher plant fluid also differ significantly from those present in the surrounding soil. These findings indicate that the bacteria associated with pitcher plant leaves are far from random assemblages and represent an important step toward understanding this unique plant-microbe interaction.

Characterization of the phyllosphere is fundamental to our understanding of the ecology and evolution of plant populations and plant diversity and their interactions with other organisms (46, 64, 66). The carnivorous pitcher plant genus *Sarracenia* is an obvious system to address basic questions in plant-microbe interaction because each pitcher (a modified leaf) of the plant contains a microcosm composed of larval insects, fungi, algae, rotifers, nematodes, and bacteria that, together, ultimately break down nutrients from insect prey for the plant (1, 10, 20, 28, 37). Each pitcher represents a naturally defined and discrete community with a finite volume and a discrete life span (each leaf lasts only one season). Several investigations have explored species interactions within *Sarracenia* pitchers (13, 20, 34, 54), and competition, predation and dispersal frequency appear to be important drivers of community composition in the system (1, 20, 43, 44). Studies involving community patterns on a larger scale within pitchers, however, are few, and the processes that produce these patterns remain unknown (33).

Establishment of bacterial communities. Conventional wisdom posits that the digestion of arthropods, facilitated by species interactions within the pitcher fluid, has allowed pitcher plants to exploit nutrient-poor soils and environments (53). This begs the important question, what is the source of these commensal organisms? Pitchers in *Sarracenia* are sterile before opening (35, 59, 60); thus, the primary establishment of bacterial communities in *Sarracenia* plants is thought to be facilitated by arthropods, either prey species that fall into the pitcher trap (14, 51, 60) or commensal insect larvae that live in the pitcher fluid (34, 37, 63). However, this assumption has never been tested with surveys of the surrounding habitat. What is known is that the bacteria play an important ecological role. The microbial community in *Sarracenia alata* aids in prey decomposition (60), mineralization of nutrients (16), and nitrogen fixation (33, 61). However, the mechanisms of digestion are also probably dependent on the pH of the fluid and age of the plant (35). The addition of bacteria changes the pH of pitcher fluid and initiates ecological succession (28, 60). The bacterial assemblage represents the resource base of a complex food web that is ultimately under the control of a keystone predator (the pitcher plant mosquito, *Wyeomyia smithii* [Culicidae]) (20, 59). Whether these microbial communities or members thereof are passive or have specific associations that have ecological importance is debatable, as all constituents of the complex food web have been called obligate inhabitants, with the exception of protozoa and bacteria (16). Microbial communities within *Sarracenia* pitchers could be drawn at random from the environment, and in this way, identical environments (pitchers) could end up having extremely large differences in metacommunity structure (22). Alternatively, if pitchers developed specific microbial associations, two pitchers could be similar if the same initial microbial species have the same chance of arrival (22, 23) and survival.

Bacterial composition in *Sarracenia*. All previous studies investigating the bacterial composition of *Sarracenia* pitchers, with one exception, have been restricted to culture-based methods. These studies have led to the identification of between 13 and 20 unique morphotypes (15, 47, 59, 67). Additionally, little is known about how this community changes through time. Rates of prey capture and the abundance of bacteria are correlated (44, 55, 56); thus, community succes-

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sional age likely affects community structure (28). In this study, we explore the identity, diversity, and temporal patterns of bacteria associated with pitcher plants in Louisiana and specifically aim to (i) characterize the microbial community in *Sarracenia alata* using three culture-independent methods, (ii) explore how this community changes over the complete growing season and the digestive progression of the plants, and (iii) examine whether or not pitcher plant bacterial communities are different from those found in surrounding soil.

MATERIALS AND METHODS

Bacterial characterization. We used two culture-independent genomic approaches to assess bacterial diversity and community structure in *Sarracenia alata*, as well as evaluate the degree of similarity between the bacterial fauna in the pitchers. Automated ribosomal intergenic spacer analysis (ARISA) (17) and terminal restriction fragment length polymorphism (T-RFLP) (49) methods have proven useful in characterizing bacterial and fungal communities (17, 25), producing rapid, reproducible fingerprints that are useful for comparing samples across time and space (38, 39, 74). In addition to genomic fingerprinting approaches, we generated environmental sequences of the 16S rRNA gene from the bacterial fauna in the pitchers using next-generation sequencing technology. High-throughput sequence-by-synthesis approaches have allowed researchers to characterize microbial diversity with less bias (11, 36) and dramatic decreases in cost and time (52).

Sampling. Pitcher plant fluid was collected from Lake Ramsey Savanna Wildlife Management Area, Saint Tammany Parish, LA (30°31′50.5″N/90°09′38.9″W), during the spring and summer of 2009. Fluid was collected from leaves 1 month after the first pitcher opened, because previous estimates indicate that 4 weeks is required for stabilization of the macroinvertebrate community (54). Fluid was drained from pitchers into sterile collecting tubes and refrigerated until extracted. The contents of 10 pitchers were sampled during the first week of April, May, June, July, and August of 2009. Given the invasiveness of this collection procedure, individual pitchers could not be repeatedly sampled. However, each month pitchers were sampled from the same 10 plants. Soil samples from the base of these same plants were also collected at each time point in a sterile vial and refrigerated until extracted. Our intention was to collect soil from typical habitats that crawling (prey) insects might encounter before falling in a pitcher (our aim was not to sample the rhizosphere).

DNA extraction. All extractions were performed within 24 h of collection. Up to 600 μl of well-vortexed pitcher fluid (excluding macro parts of insects) of each pitcher sample was used. If less than 600 μl of pitcher fluid was available, that volume was used. In the event that pitchers were dry (because of dry climatic conditions), the internal surface of the base of the pitcher was swabbed with a sterile toothpick and reconstituted in 300 μl of sterile water. Fluid and soil extractions were executed using a 2-ml bead-beating tube containing beads from a Powersoil DNA isolation kit (MoBio, Madison, WI); the manufacturer's directions were followed exactly.

Fingerprinting methods. T-RFLP–PCR was conducted on a total of 45 pitchers collected from April through August. 16S rRNA was amplified with 3′-end 6-carboxyfluorescein (FAM)-labeled 27F and 1492R primers, Phusion DNA polymerase (Finzyme, Finland), and 1 to 10 ng DNA template, following the method of Peterson et al. (59). To minimize PCR bias, three replicate PCRs were conducted per accession; these triplicate PCR products were combined and then purified with a Qiagen PCR purification kit (Valencia, CA). Twenty microliters of cleaned PCR product was digested using the restriction enzyme HaeIII (10 μl 10× NEBuffer 2, 0.5 μl HaeIII enzyme [New England Biolabs, Ipswich, MA], 69.5 μl water) for 4 h at 37°C. The digestion mixture was heat inactivated for 20 min at 80°C. An amount of 2.0 μl of the digestion mixture was mixed with 7.5 μl Hi-Di formamide (ABI, Foster City, CA) and 0.5 μl ROX500 size standard (ABI, Foster City, CA), heated at 95°C for 3 min, and put on ice until sequencing.

ARISA-PCR was conducted on a total of 51 fluid samples from all sampled months. 16S rRNA was amplified with ITSF and 5′-end phosphoramidite dye HEX-labeled ITSReub primers (17), Phusion DNA polymerase (Finzyme, Finland), and 1 to 10 ng DNA (17). An amount of 1.0 μl of the PCR product was mixed with 0.5 μl ROX500 size standard (ABI, Foster City, CA) in 13.0 μl Hi-Di formamide (ABI, Foster City, CA) and denatured at 95°C for 5 min, followed by 2 min on ice. ARISA-PCR was also performed on soil samples in order to compare the microbial communities from these two spatially close yet nutrient-disparate microhabitats.

Analysis of profiles. T-RFLP and ARISA peaks were visualized on an ABI 3130xl genetic analyzer (Foster City, CA) using the FragmentAnalysis50_POP7_1 run module and DyeSet D. Electropherograms were inspected with Peak Scanner (version 1.0; ABI, Foster City, CA) and uploaded to T-REX (21). Peaks were manually inactivated for lack of length (<50 bp) or supporting peak area (<300) and aligned using a clustering threshold of 0.5.

Assignment tools. (i) T-RFLP peak identification. The web-based Phylogenetic Assignment Tool (PAT) (40) was used to identify and classify members of the microbial community. We used the default tolerance bins and added another bin with a size of 50 bp and tolerance of 1.0.

(ii) ARISA peak identification. Profiles were loaded into the Automated Analysis of ARISA data using the ADAPT system (ADAPT) (R. Schmieder, M. Haynes, E. Dinsdale, F. Rohwer, and R. Edwards, submitted for publication), which filters profiles, transforms the data, and blasts against NCBI and SEED databases. The minimum threshold value for peaks was 50 fluorescence units greater than the baseline, and fragment length binning was reduced to plus-or-minus 1 times the size of the original fragment.

Identifying relationships between communities. We used a suite of multivariate analyses to identify relationships between different pitchers, different time points, and different microhabitats (i.e., fluid versus soil) and to evaluate the variability in microbial communities. A similarity matrix for the T-RFLP data, as well as the pitcher and soil ARISA data sets, was calculated using Bray-Curtis coefficients in Primer-e (19). Nonmetric multidimensional scaling (MDS) (18, 24) was used to ordinate the similarity data, using 999 random restarts for three data partitions, peak presence/absence, peak height, and peak length.

Identifying groups of communities over time. An analysis of similarity (ANOSIM) was implemented in Primer-e (19) and used to summarize patterns in species composition, time, or microhabitat using permutation-based hypothesis testing. This analysis is based on rank similarities between samples using the original Bray-Curtis similarity matrix. We tested the following null hypotheses of community composition: no difference between the 5 sampling months, no difference between pitchers from an individual plant over time, and no difference between soil and pitcher fluid. We report a P value (analogous to a univariate analysis of variance) which tests for differences between groups of (multivariate) samples from different environments. For each data set, similarity dendrograms were also constructed, using Bray-Curtis similarities in Primer-e (19).

454 pyrosequencing. The extracted contents of one pitcher from the first month of sampling were used as template for 454 pyrosequencing. Short fragments of 16S rRNA were amplified according to primer design suggestions for microbial pyrosequencing (27, 32, 50). The forward primer (5′ to 3′) included the 454 Life Sciences primer B (GCCTTGCCAGCCCGCTCAG), the broadly conserved microbial primer 27F, and a 2-base "TC" linker sequence. The reverse primer (GCCTCCCTCGCGCCATCAG**ATATCGCTACTG**CATGCTGCCTCCCGTAGGAGT) contained the 454 Life Sciences primer A (GCCTCCCTCGCGCCATCAG), the bacterial primer 338R (TGCTGCCTCCGTAGGAGT), a "CA" inserted as a linker between the barcode and the rRNA primer, and a unique 12-bp error-correcting Golay barcode (in bold font in the primer sequence). PCRs were performed in 20-μl reaction mixtures containing 1× Phusion high-fidelity buffer (Finzyme, Finland), 2.5 μM Phusion $MgCl_2$ (Finzyme, Finland), 0.25 μM of each primer, 0.5 μM of each deoxynucleoside triphosphate, 0.3 U Phusion high-fidelity *Taq* polymerase (Finzyme, Finland), and 1 to 10 ng DNA. PCR conditions were as follows: 98°C for 2 min; 26 cycles of 98°C for 10 s, 53°C for 10 s, and 53°C for 30 s; and a final extension at 72°C for 10 min. Three replicate PCRs were performed. The samples were pooled and cleaned with a Qiagen PCR purification kit (Valencia, CA). DNA was quantified on an ND 1000 spectrophotometer (NanoDrop, Wilmington, DE). Pyrosequencing was conducted at Engencore (University of South Carolina, Columbia, SC) using their 454 life sciences genome sequencer FLX (Roche) machine. The sequence data set was trimmed to yield sequences of >150 bp with quality scores of >20 using the Ribosomal Database Project (RDP) Pyrosequencing Pipeline's Initial Processing function.

RDP Aligner data set. Sequences were aligned using the data processing RDP Aligner function and classified using RDP Classifier, a Bayesian rRNA classifying algorithm (72), at an 80% confidence rate.

RDP Clustering data set. We clustered our aligned data set hierarchically using the RDP Clustering function. This data set was then used to calculate rarefaction and the Chao 1 index. Since RDP Classifier automatically bins unclassified data (<80% confidence) without sequence taxonomic identification, one cannot determine how many higher taxonomic ranks are within an unclassified bin. We were interested in how many phylogroups were comprised by our full data set and within the family *Enterobacteriaceae* in particular. We used a genetic divergence of 3% to represent distinct phylogroups and searched one sequence from each of these phylogroups against the NCBI BLAST database.

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TABLE 1. Summary of microbial diversity present in *Sarracenia alata* pitcher fluid in April

Taxon	454 Pyrosequencing result		ARISA result[c]
	Aligned[a]	Clustered[b]	
Domain	1 (2)	1 (1)	1
Phylum	5 (15)	4 (158)	4
Class	9 (7)	9 (3)	7
Order	15 (78)	16 (6)	18
Family	23 (12)	27 (4)	33
Genus	29 (10,749)	45 (67)	45
Phylogroup (species)		652	182
Total	31,373	652	288

[a] Number of sequences identified at each taxonomic rank (number of sequences not identified at that rank).
[b] Number of phylogroups identified at each rank (number of phylogroups not identified at that rank).
[c] Number of taxa represented in the ARISA fragment data set for each taxonomic rank.

We then assigned that phylogroup to genus or the highest taxonomic rank possible, leaving further hierarchical taxonomy unidentified. For searches that resulted in more than one equally likely taxonomic identification (by percent match), the most abundant named taxon was chosen. All matches were between 95 and 100% identical and had a minimum length of 180 bp. In the event that no named taxon was offered (i.e., "uncultured bacterium" was the only choice), no identification was given. In this way, we were able to count how many phylogroups were within each taxonomic rank.

Experimental setup. We used the three aforementioned culture-independent methods to characterize the microbial community in *Sarracenia alata* plants from 1 month of sampling early in the season (April). Genomic fingerprinting methods were further used to assess changes in the community over time and to determine whether or not the microbial communities in pitcher plants are different from those in associated soil.

RESULTS

Bacterial characterization of *Sarracenia* pitcher fluid in April. Fingerprinting. In total, 193 unique gene fragments were recovered in April. In general, ARISA data produced more fragments than T-RFLP data and were more easily identified with the databases given above.

ARISA. The 10 pitchers sampled from April contained a total of 288 peaks by ARISA, representing 4 phyla, 7 classes, 18 orders, 33 families, 45 genera, and 182 unique phylogroups (Table 1 and Fig. 1). Pitchers had between 11 and 49 peaks, with an average of 28.8 peaks present in each pitcher. A plethora of the fragments were found at a low frequency, 10% of which were found only once. One fragment size (69 bp), however, was found in 90% of pitchers.

T-RFLP. In the T-RFLP analysis, the contents of the seven pitchers sampled in April that met our peak calling standards

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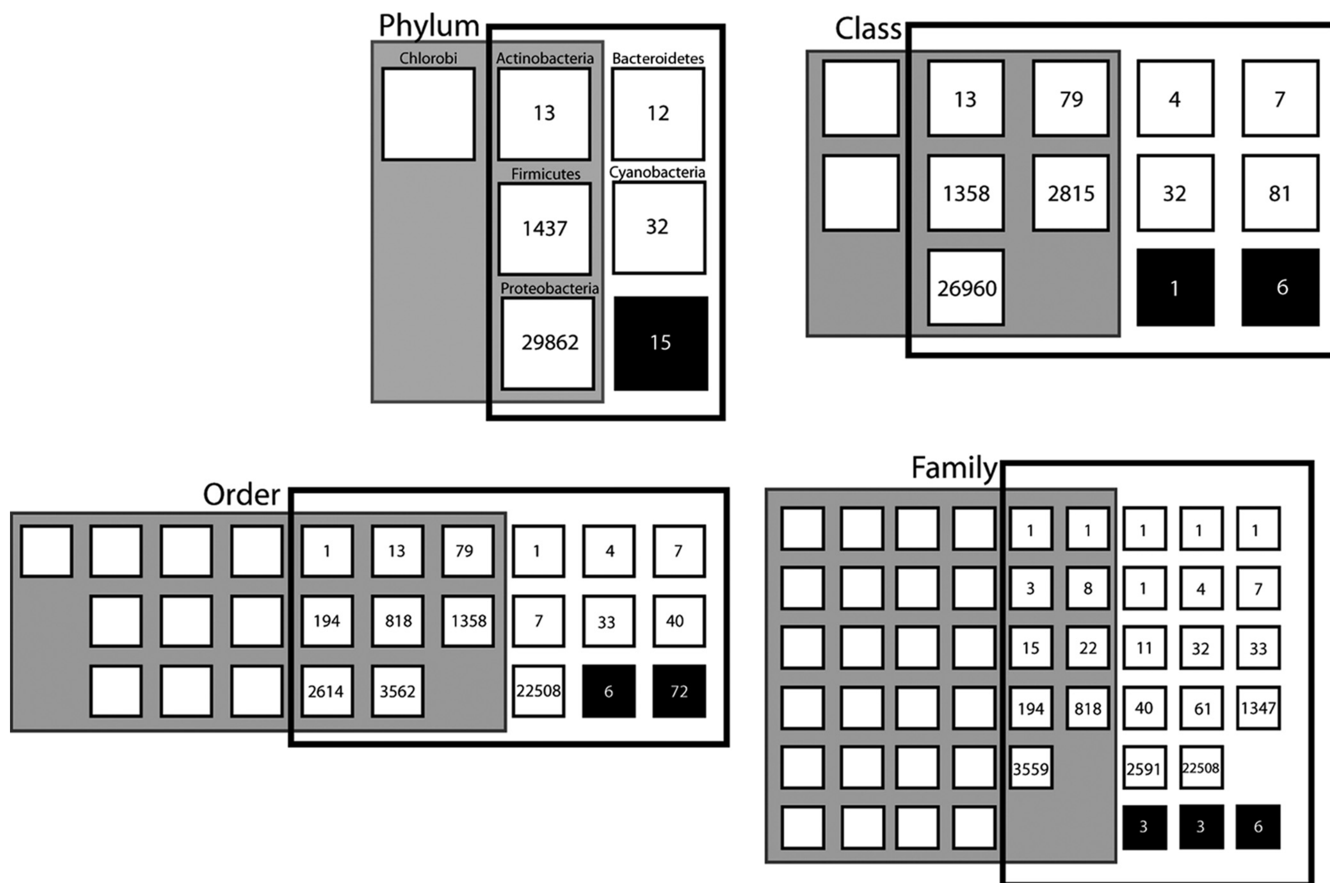

FIG. 1. Microbial diversity in *Sarracenia alata* pitcher fluid at different taxonomic ranks. Degree of overlap between 454 sequence data (large white boxes) and ARISA fragment data (large gray boxes) at the level of phylum, class, order, and family. The number in each small box indicates the number of sequences in that particular group, and black boxes indicate that the group was unidentifiable at that rank.

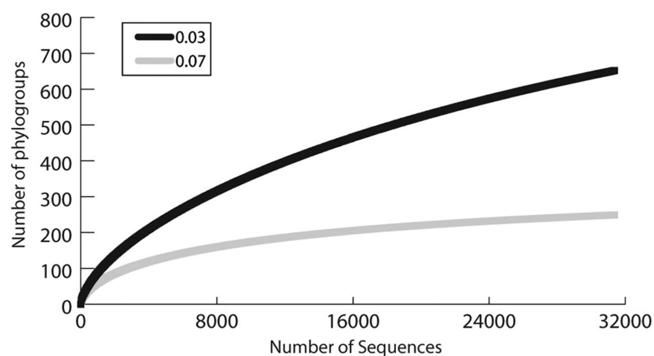

FIG. 2. Rarefaction analysis of 454 sequence data at 3 and 7% divergence. Chao 1 estimates suggest 1,033.94 phylogroups at 0.03 divergence and 298.76 at 0.07 divergence.

contained 10 unique peaks. Only one peak was found in more than one pitcher. Fragment lengths are not exclusive to one taxon in the databases used; each of the 10 fragments identified are associated with between 3 and 16 known bacteria. Identification of bacteria for this data set was therefore not attempted.

454 pyrosequencing. In the 454 pyrosequencing analysis, high-throughput sequencing conducted on the contents of one pitcher collected in April contained a total of 31,373 usable sequences after quality was considered. The lengths of the sequences ranged from 150 to 316 bp (mean, 214.83 bp).

RDP Aligner data set. Five phyla, 9 classes, 15 orders, 23 families, and 29 genera were represented in the sequences aligned using the RDP Aligner function (not including unclassified data) (Table 1 and Fig. 1; see Table S1 in the supplemental material). Two sequences could not be assigned to domain and 15 could not be assigned to phylum (Fig. 1; see Table S1 in the supplemental material). The most abundant sequences assigned to phylum (*Proteobacteria*), class (*Gammaproteobacteria*), order (*Enterobacteriales*), and family (*Enterobacteriaceae*) were represented in approximately 95%, 86%, 72%, and 72% of the total sequences, respectively. Of the 22,508 total sequences in the family *Enterobacteriaceae*, 10,122 were placed into a single unclassified bin (in other words, we know the sequences belong to the family but know nothing of their richness, diversity, or identity). Other well-represented genera outside the *Enterobacteriaceae* include *Herbaspirillum* (2,568 sequences) and *Pseudomonas* (3,119 sequences). Nearly half of the named genera (14 of 29) were represented by fewer than 10 sequences (see Table S1 in the supplemental material).

RDP Clustering data set. The results of rarefaction analysis performed at a genetic distance that approximates genus level divergence (7%) suggest that diversity may be beginning to plateau; the Chao 1 index predicts 298.76 generic groups, while clustering identified 249 (Fig. 2). Rarefaction analysis performed at a genetic distance that approximates species level divergence (3%) indicates that diversity is still increasing; the Chao 1 index estimates 1,033.94 groups rather than the 652 phylogroups identified using the clustering method (Fig. 2).

Within the 652 phylogroups, 4 phyla, 9 classes, 16 orders, 27 families, and 45 genera were identified (Table 1; see Table S2 in the supplemental material) when representative sequences were searched against NCBI BLAST. Other levels of sequence divergence (0.00 to 0.10) and their associated richness are reported in Table 2. Similar to the results with RDP Aligner, the most abundant sequences assigned to phylum (*Proteobacteria*), class (*Gammaproteobacteria*), order (*Enterobacteriales*), and family (*Enterobacteriaceae*) were representative of a large number of the unique phylogroups (70%, 58%, 41%, and 40%, respectively). Two hundred sixty-one unique phylogroups were assigned to the *Enterobacteriaceae* and encompassed 14 named genera and two unnamed genera (see Table S2 in the supplemental material). Between 2 and 86 unique phylogroups were identified within each named genus in the family. Eighty-six unique phylogroups (representative of 11,048 sequences; 13% of all sequences) were classified as genus *Enterobacter*. By blasting phylogroups, 8 additional genera were recovered in the *Enterobacteriaceae* that were absent in the RDP Aligner data set. However, our RDP Clustering data set also identified 158 phylogroups as unassignable to phylum. Discrepancies between the data set clustered with the RDP Clustering function and that aligned with the RDP Aligning function could be attributed to differences in database content.

Bacterial community structure of *Sarracenia* pitcher fluid over time. In total, the fingerprinting approaches recovered 541 unique fragments from the point of initial pitcher opening (sterile environment) to pitcher death over the course of 5 months (Table 3). The species accumulation curve for ARISA data shows signs of plateau, and the Chao1 index predicts 574 phylogroups (Fig. 3). When this curve is compared with the observed curve for sampling in April alone, it is clear that not nearly all of the diversity is present in the first month of pitcher opening (Fig. 3). Both data types resulted in a high number of fragments that were found at a low frequency and, often, only once (Fig. 4).

ARISA results. In total, 2,007 fragments were recovered from the contents of 51 pitchers over 5 months of sampling. These correspond with 485 unique taxon peaks, resulting in an average of 39.35 peaks per individual and 222.8 peaks per month (between 32 and 68 peaks were restricted to 1 month) (Table 3). Peak abundance, however, was not constant over time. The total fragment number ranged from 288 (representing 182 unique peaks) in April to 570 (284 unique) in July. Peak abundance and peak diversity (Fig. 5) increased from April to July and then declined in August. The most abundant peak over the sampling period was found in 30 pitchers

TABLE 2. Rarefaction results from the RDP Clustering data set for various levels of sequence divergence (0.00 to 0.10) and their associated richness for 454 pyrosequencing data

Sequence divergence	No. of phylogroups
0	1,793.45
0.01	1,274.67
0.02	853.81
0.03	651.87
0.04	490.92
0.05	382.95
0.06	300.97
0.07	248.97
0.08	194.98
0.09	159.99
0.1	131.99

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TABLE 3. Summary of genomic fingerprinting data over all months for T-RFLP data set and ARISA fluid and soil data sets[a]

Data set	n	P	P^*	P_m	P_i	NID	Most abundant
ARISA fluid	51	2,007	485	222.8	39.35	145	484 (58.8)
T-RFLP fluid	45	139	56	27.8	18		204 (36.7)
ARISA soil	40	950	302	97	19.27	77	70 (100)

[a] n, total number of pitchers sampled; P, total number of peaks; P^*, total number of unique peaks; P_m, average number of unique peaks per month; P_i, average number of peaks per individual; NID, total number of peaks not identified in database; Most abundant, most abundant fragment length (bp) over sampling period (percentage of pitchers in which the peak was present is in parentheses).

(58.8%) and is identified in the RDP database as *Nitrosomonas eutropha*. Three additional peaks were found in more than half of the pitchers. Each month, at least one fragment size was very common (found in 90 to 100% of sampled pitchers); the identity of the abundant gene fragment, however, changed over time. In 3 sampled months, at least one fragment was found in 100% of pitchers, but the identity of the ubiquitous fragment was never the same between months. Between 5 and 22 unique fragments were found in 50% or more of pitchers across all months, and the number of peaks shared among 50% of pitchers increased with time and precipitation (Fig. 6). Of the identified peaks, 3.5% were present in all months, 11% were found in 4 of the 5 months, 20% were found in 3 months, 25% were found in 2 months, and 385 were found in only 1 month (summarized in Fig. 7).

Multidimensional scaling analyses of the three data sets (presence/absence, peak height, and peak area) demonstrate that microbial pitcher composition changes over time and that the bacteria from pitchers sampled in 1 month are likely to be more similar in composition than those in pitchers from the same plant host over several months (Fig. 8 and Table 4). The three data sets produced very similar results, and only the presence/absence results are presented here. Pitcher communities in April are the most dissimilar, while bacterial communities in later months tend to be more similar (Fig. 8).

T-RFLP results. The contents of 45 pitchers that contained T-RFLP fragments and could be scored to our peak standards included a total of 139 peaks, of which 56 were unique (Table 3). One fragment was found in 37% of all pitchers over the 5-month sampling, corresponding with a fragment size of 204 bp. The total fragment number ranged from 11 (representing 10 unique peaks) in April to 40 (29 unique) in August. Peak abundance was not constant over time but did not show a clear pattern of diversity increasing with time as in the ARISA data set (Fig. 5). The presence of abundant peaks ranged from one particular fragment in 20% of pitchers in April to a fragment that was present in 89% of pitchers in May. Multidimensional scaling analyses of the three data sets assigned to month resulted in one undifferentiated cluster (not shown).

Identifying relationships and groups between communities. The global R statistic from ANOSIM analyses for T-RFLP data, partitioned by month, was 0.159, which is indicative of some community differences between months (Table 4). Pairwise comparisons of months ranged from R values of -0.026 between May and June to 0.385 between May and August, illustrating that May and June have almost identical community compositions, while May and August differ the most. When these data are partitioned by individual plant over all months, the global R value is negative, indicating that the communities residing in one plant host are not more similar to one another over time than communities found in a different plant (Table 4).

Similar to the T-RFLP data, the global R value for ARISA data partitioned by plant of origin (individual) is a negative value (Table 4). The global R statistic for ARISA data, partitioned by month, was 0.448, which is demonstrative of significant differences between community structures over time (Table 4). All pairwise comparisons of months resulted in positive R values; we therefore reject the null hypothesis of no difference across months. Pairwise comparisons of

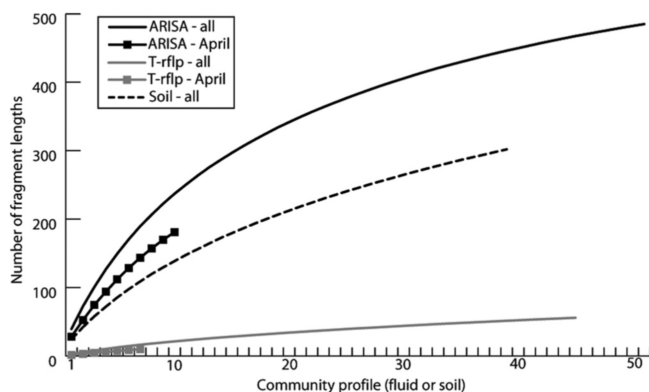

FIG. 3. Species accumulation curves for ARISA and T-RFLP data for all pitchers over 5 months (smooth lines) and for April only (lines with symbols). A curve is also estimated for fragments from soil over 5 months (broken line). ARISA data from fluid indicate 485 unique fragment sizes; the Chao 1 estimate for these data estimates a total of 574. All T-RFLP data indicate a total of 56 unique peaks; Chao 1 for these data estimates a total of 112.

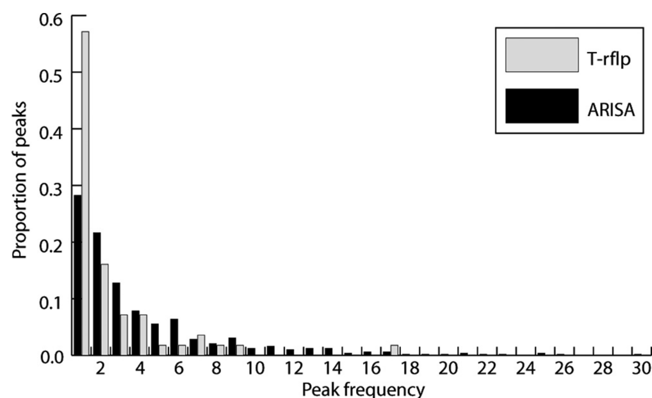

FIG. 4. Frequency distribution of fragment lengths in the ARISA and T-RFLP pitcher fluid data sets over all sampling time points.

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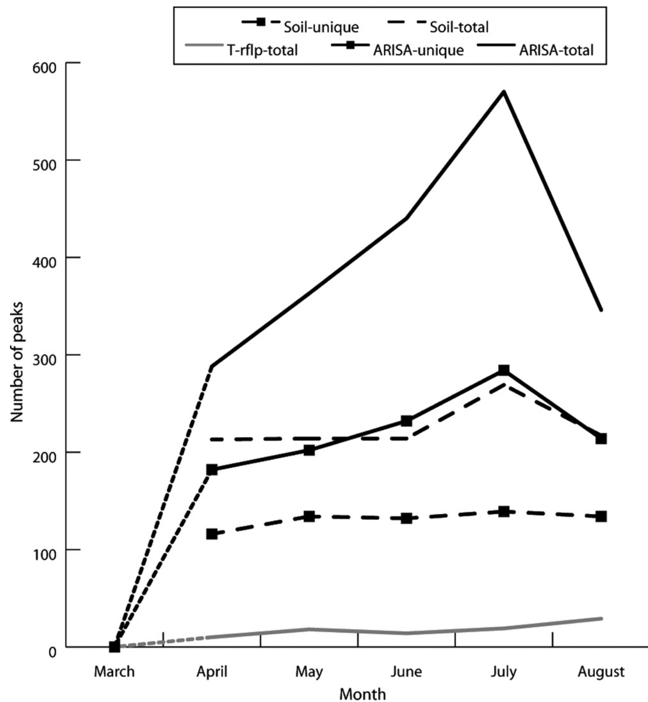

FIG. 5. Abundance and diversity in peak number for fingerprinting data over sampling season. Peak abundance (total) is provided for ARISA (fluid and soil) and T-RFLP data, and peak diversity (unique) is provided for ARISA data (fluid and soil). Pitcher fluid was not sampled in March, since pitchers opened in March and were sterile (0 bacteria) at the time of opening (dotted lines originating at 0 in March represent an increase in bacteria since opening).

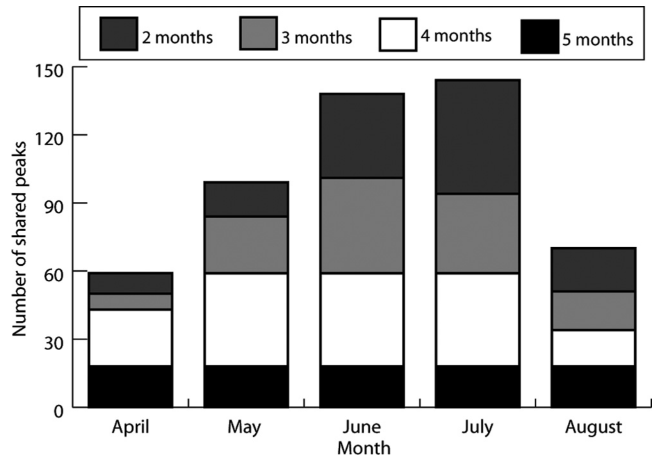

FIG. 7. Distribution of shared ARISA peaks in pitcher fluid over time. Colors indicate the number of shared peaks over a range of consecutive months. For example, 18 peaks are shared in all 5 months. The most shared peaks are found in months with the highest bacterial abundance and diversity (June and July).

months ranged from R values of 0.213 between June and July and 0.653 between April and August, illustrating that June and July (consecutive months) have the most similar community compositions, while April and August differ the most (Table 4 and Fig. 7). Each of these comparisons is highly significant.

Bacterial community structure of soil associated with *Sarracenia* over time. ARISA analyses for 40 soil samples associated with pitchers resulted in 302 unique peaks, with an average of 19.27 peaks per individual and 97 unique peaks per month (Table 3). Peak abundance ranged from 214 (representing 116 unique peaks) in April to 269 (139 unique) in July. One fragment size (70 bp) was found in 100% of samples, and two additional prominent fragment sizes (51 and 79 bp) were found in 95% of all soil samples. ANOSIM analyses for ARISA data for soil communities over time suggest that we cannot reject the null hypothesis of no difference (Table 4). Likewise, no significant difference in community structure is detected between individual plants over time. MDS analyses of soil ARISA data suggested that there is no visible change in composition over time and that most soil samples are quite similar (not shown).

Comparison of the bacterial composition of pitcher and soil microhabitats. Fluid samples from pitcher plants harbor more-diverse bacterial communities than associated soil samples (Fig. 5 and 6). Over the 5 months of sampling, more than twice the number of peaks were identified in fluid samples as in soil

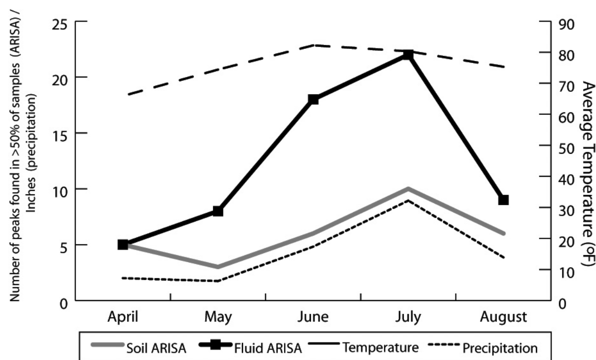

FIG. 6. Number of peaks found in more than 50% of sampled pitchers within each month according to ARISA (fluid and soil) data. Two environmental measures (average temperature by month and precipitation in inches) are also plotted.

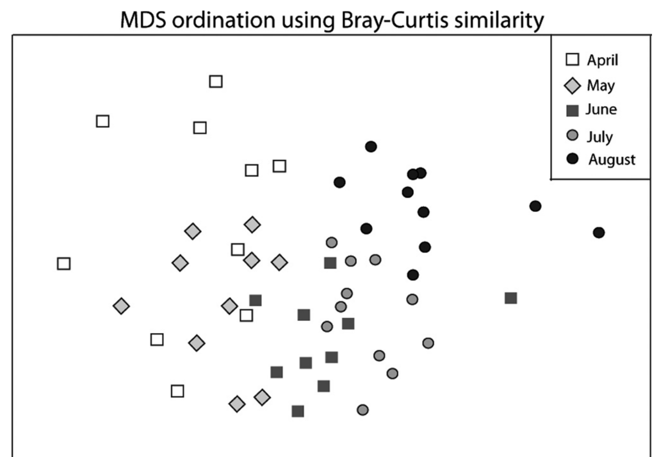

FIG. 8. Multidimensional scaling ordination of ARISA fluid data coded by month. Each point represents the bacterial community from one pitcher.

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TABLE 4. Results from analysis of similarity (ANOSIM) tests comparing microbial communities in a pairwise fashion by month for all T-RFLP data and ARISA data from pitcher fluid and soil

Pairwise comparison	*R* value for method, microhabitat(s)[d]		
	T-RFLP	ARISA	
		Fluid	Soil
Monthly[a]			
1,2	0.331*	0.261**	−0.014
1,3	0.14*	0.445**	0.012
1,4	0.253*	0.543**	0.079
1,5	0.064	0.653**	−0.09
2,3	−0.026	0.331**	0.024
2,4	0.185*	0.492**	0.086
2,5	0.385**	0.684**	−0.25
3,4	0.063	0.213*	0.08
3,5	0.139*	0.604**	−0.035
4,5	0.081	0.496**	−0.277
Global	0.159*	0.448**	0.012
Individual (global)[b]	−0.026	−0.137	0.085
Microhabitat[c]	Month	Fluid, soil	
	April	0.942**	
	May	0.958**	
	June	0.929**	
	July	0.952**	
	August	0.387	
	Global	0.698**	

[a] 1, April; 2, May; 3, June; 4, July; 5, August.
[b] An *R* value is given for individual plant pairwise comparisons for the three data sets over all time points. A global *R* value is provided for each data set.
[c] Microhabitat pairwise comparisons evaluate how similar ARISA data from fluid and soil communities are by month.
[d] The larger the *R* statistic, the more different two communities are. Significance was assessed using 999 permutations; * $P \leq 0.05$, ** $P \leq 0.001$.

samples using ARISA methods (2,007 versus 950 peaks, respectively). These correspond to 485 (fluid) and 302 (soil) unique peaks. Clustering (Fig. 9) and MDS analyses (not shown) of fluid and soil samples together demonstrate that the microbial communities from these spatially linked microhabitats are definitively different in composition. Though there is substantial overlap of individual fragments (225 fragments are shared between soil and fluid over the course of sampling) between the two microhabitats, the communities remain distinct through time (Fig. 9). For example, one peak that is found in all soil samples is absent from pitchers in every month except August. The most common fragment found in pitchers is absent in all soil samples.

R statistics from ANOSIM analyses for ARISA data partitioned by microhabitat (fluid versus soil) are all positive, and for 4 months, values approach 1.0 ($P = 0.001$). This suggests that the microhabitat communities are significantly different from one another and that communities are most similar to one another in August ($R = 0.387$).

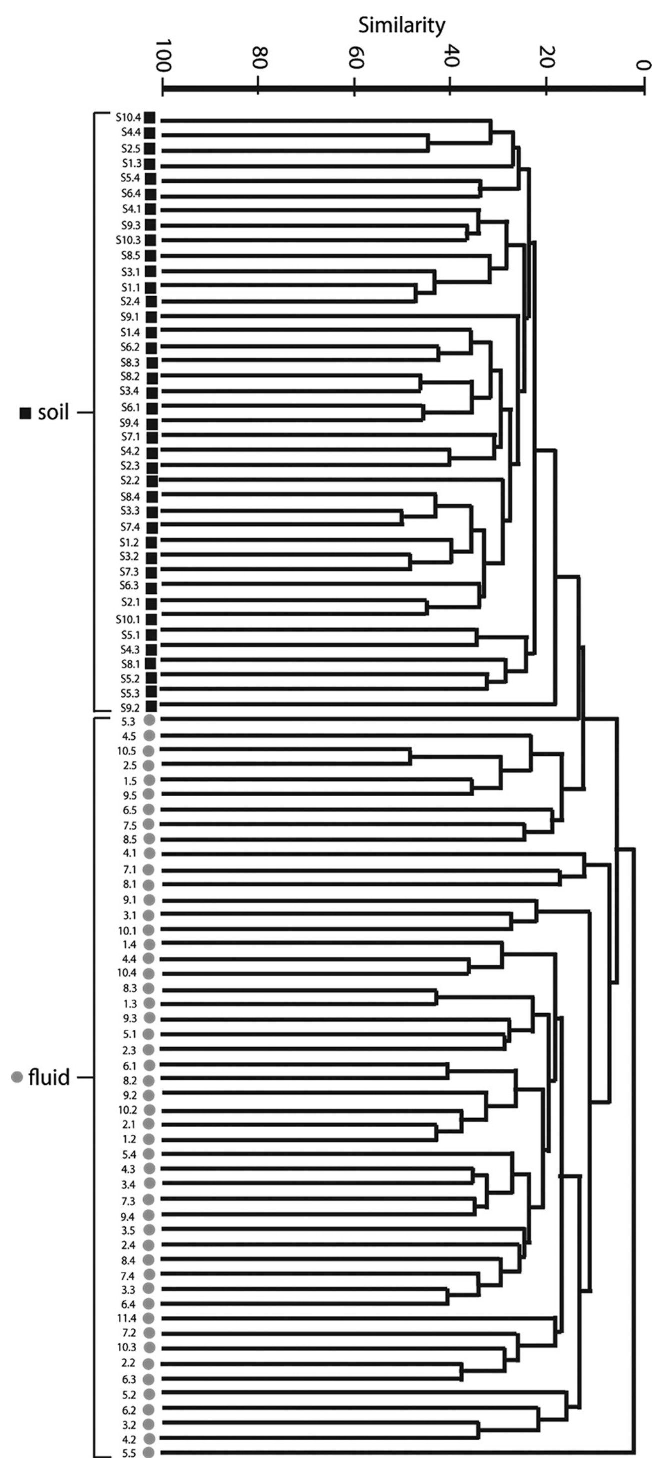

FIG. 9. Similarity dendrogram using Bray-Curtis similarities, highlighting the difference in composition of the microbial communities present in soil samples and pitcher samples over the 5-month sampling regimen.

DISCUSSION

Diversity of *Sarracenia*-associated microbes. The microbial richness associated with pitcher fluid from *Sarracenia alata* is high, with more than 1,000 phylogroups identified across at least seven phyla and over 50 families (Fig. 1). By comparison, approximately 20 cultured isolates have been previously documented from the entire genus over the past 35 years (15, 47, 59, 67). One hundred thirty-three T-RFLP gene fragments

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were recovered from 47 *Sarracenia purpurea* pitchers in three bogs across Massachusetts (59, 67). Our results, sampled from one population and only 10 plants, indicate that either microbial diversity in *Sarracenia* genus pitchers has previously been underestimated or there is considerably more diversity in *S. alata* pitchers than in those of previously studied species. Alternatively, our sampling efforts could assess diversity more adequately or the greater diversity in our results is a combination of some or all of these factors. Furthermore, a large proportion of the phylogroups identified in this study are novel. Thirty-seven percent of sequenced phylogroups did not BLAST to any known bacterium, and 30% of ARISA gene fragments were not matched using ADAPT. The high levels of sequence and taxon novelty are not surprising; studies investigating previously unexplored microbial communities find exceptional levels of previously uncharacterized diversity (45, 65).

It is difficult to compare our results with the only previous culture-independent molecular bacterial characterization of the *Sarracenia* phyllosphere, as a majority of individual fragment lengths were not listed (59), but for the most abundant peaks reported there, we see little overlap with T-RFLP fragments in our study. Lack of overlap between microbial communities of different pitcher plant species could be due to several factors but could indicate that these species use and harbor different bacterial communities. Future studies are necessary to demonstrate whether *S. alata*, which grows at more southern latitudes than *S. purpurea* and *Sarracenia minor* (59, 67), harbors greater prokaryotic diversity than its northern relatives and to properly test findings that suggest that bacterial species richness in *Sarracenia* increases with increasing latitude (15).

The microbial communities in *S. alata* pitcher plant fluid are clearly different in several ways from those present in surrounding soil. Microbial communities in pitchers are more abundant and more diverse than in soil (Table 3 and Fig. 5). The microbial community composition differs significantly between these distinct microhabitats (Table 4 and Fig. 9). The pitcher bacterial community also changes dramatically over time, while the soil community remains more stable (Fig. 5, 6, and 8). These findings provide evidence that microbes within *Sarracenia* pitchers are not exclusively drawn at random from the soil environment.

Different methods capture different diversities. It is clear that genomic fingerprinting methods will underestimate actual richness (7, 8); however, these data, when augmented with 454 sequences, have detected more bacterial diversity in *Sarracenia* than has previously been identified (59, 67). Of the seven phyla indentified in pitcher plants in this study, one is unique to the ARISA data set and three are restricted to the 454 sequence set. The family *Enterobacteriaceae* is represented by 71% of the 454 sequences (Fig. 1) and is obviously an important component of the community in the sampled pitcher. Surprisingly, however, no gene fragment in the ARISA data set matched any of the more than 600 gene fragments linked to *Enterobacteriaceae* in the ADAPT database. Thirty-two sequences were identified as autotrophic (*Cyanobacteria*) in the aligned data set, yet all gene fragments in the ARISA data set that could be classified to a trophic category were designated heterotrophic. (Taxonomy note: RDP Classifier binned all of the sequences of *Cyanobacteria* into family *Chloroplast*, genus *Streptophyta*, and omits ordinal classification [see Table S1 in the supplemental material]; our RDP Clustering data set BLAST results do not assign any sequences to class *Cyanobacteria* [see Table S2 in the supplemental material].) Also of note, of the 50 families identified using fingerprinting and sequencing methods, only 9 overlap (Fig. 1).

Community composition over time. Many factors will determine how and which microbes are introduced to a leaf surface; these include leaf age, host selectivity, resource availability, humidity, climate, and season (3–6, 12, 42, 58, 73). Little is known about how seasonal patterns will affect community composition (9, 71), and where this has been investigated, samples have been taken at four or fewer time points (48, 62, 68). Consequently, investigators have little idea as to what forces direct compositional change in microbial communities (41).

Before they open, the pitcher-shaped leaves of *Sarracenia* are sterile; however, upon opening, the phyllosphere is initiated and continues to diversify over the course of leaf growth, prey accumulation, and prey digestion. The early-arriving bacteria in pitchers (within the first month of pitcher opening) comprised the most disparate communities among the 10 sampled plants. As the season progressed, however, the pitcher communities became more similar to one another at a given time point (Table 4 and Fig. 8). It is all the more interesting that while bacterial communities in every pitcher in a given sampling month converged, community composition changed significantly between time points (Table 4 and Fig. 6 and 8). Thus, pitcher communities from different plants are more similar within 1 month than communities coming from the same genetic host over time (Table 4). This pattern has been demonstrated in three other studies (26, 62, 69) and shows that bacterial communities associated with leaf surfaces need not be random assemblages. In contrast to studies that have found no discernible pattern in bacterial community richness over time (62, 70), our data indicate an increase in diversity and abundance with time until the latest stages of prey digestion. Our data clearly show that in a protected environment (high in resources, low UV exposure, etc.) and within a discrete time period, the phyllosphere can achieve a predictable and repeatable pattern and peak in diversity and abundance.

To understand community structure at the local level, one must understand how the community is assembled (23). The potential role of insects as vectors for microbe transportation throughout nature was identified early (30, 31). Bacterial introduction into *Sarracenia* plants is likely facilitated by insect prey (14, 51, 60) and by commensal insect larvae (34, 37, 63) that die and live, respectively, in the pitcher fluid. Our investigations support these ideas, as the bacterial community associated with *S. alata* is not random and differs significantly from that of the soil.

Do *Sarracenia alata* pitchers harbor specific microbes? Bacterial communities have been demonstrated to be specific to their plant hosts (45), possibly because plants can exert strong selection on microbial communities (2, 29, 57). Because pitcher plant-bacterium interactions are distributed throughout the genus *Sarracenia*, one might suspect that symbiotic interactions have developed between the two. The plants could assert strong selection on the microbial community, and this community could be host specific. If bacteria routinely form pitcher-specific associations and are necessary for prey diges-

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tion, we would expect that such associations would be maintained over space and time. Here, phylogroups are shared in multiple individuals, months, and sampling methods (Fig. 1, 7, and 8). Community profiles of pitchers on different plants are more similar to one another in each sampled month, changes in microbial community from month to month are congruent across pitchers, and pitcher communities are consistently more similar to one another than to communities in surrounding soils. Understanding of the bacterial community's composition and its role and function represents some of the last missing pieces in this ecologically well-studied system.

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