Microb Ecol (2011) 61:750–758
DOI 10.1007/s00248-011-9832-9

PLANT MICROBE INTERACTIONS

The Microbial *Phyllo*geography of the Carnivorous Plant *Sarracenia alata*

Margaret M. Koopman · Bryan C. Carstens

Received: 6 November 2010 / Accepted: 15 February 2011 / Published online: 24 March 2011

Abstract Carnivorous pitcher plants host diverse microbial communities. This plant–microbe association provides a unique opportunity to investigate the evolutionary processes that influence the spatial diversity of microbial communities. Using next-generation sequencing of environmental samples, we surveyed microbial communities from 29 pitcher plants (*Sarracenia alata*) and compare community composition with plant genetic diversity in order to explore the influence of historical processes on the population structure of each lineage. Analyses reveal that there is a core *S. alata* microbiome, and that it is similar in composition to animal gut microfaunas. The spatial structure of community composition in *S. alata* (*phyllo*geography) is congruent at the deepest level with the dominant features of the landscape, including the Mississippi river and the discrete habitat boundaries that the plants occupy. Intriguingly, the microbial community structure reflects the phylogeographic structure of the host plant, suggesting that the phylogenetic structure of bacterial communities and population genetic structure of their host plant are influenced by similar historical processes.

Electronic supplementary material The online version of this article (doi:10.1007/s00248-011-9832-9) contains supplementary material, which is available to authorized users.

M. M. Koopman · B. C. Carstens (✉)
Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA
e-mail: carstens@lsu.edu

Present Address:
M. M. Koopman
Department of Biology, Eastern Michigan University, Ypsilanti, MI 48197, USA

Introduction

The integration of ecosystem genetics, phylogenetics, and community ecology has provided important insights into the diversity, assembly, evolution, and functionality of communities [1–5]. By exploring ecosystems in an evolutionary framework, investigators can measure genetic interactions across variable temporal and spatial scales and gain insight into fundamental processes such as food web dynamics and nutrient cycling [1, 3, 4]. Studies integrating these fields initially focused on the genetics of plant species that supply a variety of important resources and environmental structure to other organisms in the ecosystem [6]. An intriguing extension of these studies, and an important opportunity for community geneticists, is to further investigate community level responses to host–plant genetic variation. Of particular interest are the associations of plants and their symbiotic microbiota.

The interface between aerial plant surfaces and the environment represents an enormous potential habitat (~1 billion square kilometers of leaves worldwide; [7]) for a variety of organisms and as such represents an extraordinary opportunity to study community diversity and its evolution. Thus, more recent efforts have incorporated intraspecific plant genetic variation with associated community membership [5, 8–11]. Despite the fact that microbes are the most frequent and abundant inhabitants of aboveground leaf surfaces [12, 13], mediate important ecosystem processes [14, 15], and have long played a critical role in ecological research (reviewed in [16]), investigations into complex and species-rich bacterial communities in nature have been hampered, until very recently, by several technical and theoretical limitations [17, 18]. These restrictions have constrained our capability to identify patterns of bacterial community structure as well as

our ability to determine the mechanistic processes that drive these patterns [19]. Recent technological advances, including next-generation sequencing of environmental samples, provide a unique opportunity to investigate the processes that drive biogeographical patterns in microorganisms [20–23]. Here, we exploit these advances and seek to characterize the structure of microbial communities associated with the carnivorous plant *Sarracenia alata* (Sarraceniaceae).

It is clear that microbial populations exhibit spatial patterns [24–26]. Bacterial community membership can be clustered geographically, indicating that dispersal limitation is an important force in structuring communities [27, 28]. Dispersal limitation is a convenient explanation in microbial systems defined by abiotic features, such as deep sea thermal vents or hot springs [25, 27]. When dispersal limitation is not evident, differences in the contemporary environment are thought to be the principal factor in generating spatial discontinuity (i.e., the Baas-Becking hypothesis) [29]. However, many microbial communities are closely associated with eukaryotic species, and in these eukaryotes, generations of biogeographic research indicate that historical processes (e.g., habitat fragmentation, isolation on either side of environmental barriers) play a key role in explaining eukaryotic distributions [30]. Therefore, the historical processes that shape eukaryotic biogeography could influence the biogeographic patterns of their microbial communities [31, 32]. Whereas the role of historical processes in microbial biogeography remains unclear, several researchers have posited that geographic isolation and subsequent neutral divergence could influence microbial systems [27, 33, 34].

By comparing microbial assemblages in *S. alata* across space, we aim to identify the factors that influence their compositional structure. The modified pitcher-shaped leaves of *S. alata* provide an ideal habitat in which to investigate microbial biogeography because each leaf (pitcher) contains a diverse microbial community [35–38] that are restricted to a defined space and are distinct from the surrounding environment [38]. Furthermore, some microbes that inhabit *S. alata* pitchers provide important services to the plant, including prey decomposition [39] and nutrient mineralization and fixation [40]. This intimate plant–microbe association provides an opportunity to test the central concept of microbial biogeography. If microbial communities cluster by habitat, then we anticipate that there will be a broad similarity in the bacterial communities sampled from different populations of plants given that plant populations exhibit no visible signs of local adaptation [41]. If, instead, dispersal limitation is important, then we expect to uncover a clear signature of isolation by distance (IBD), particularly since the eastern and western populations of *S. alata* are separated by the Atchafalaya Basin, one of the largest swamps in the world and a habitat that is substantially different from the pine savannahs that the plants inhabit.

In Louisiana (U.S.A), historical habitat fragmentation has produced substantial population genetic structure in *S. alata* [42] (Fig. 1a). Koopman and Carstens [42] conducted an extensive phylogeographic investigation of the species using microsatellites and DNA sequence data. STRUCTURE [43] and STRUCTURAMA [44] were used to infer population structure of five plant populations across the state. Both assignment tests indicate that genetic populations largely correspond to sampled populations, and that the deepest level of population genetic differentiation, was identified when populations were divided on either side of the Mississippi River (K=2; using the Δ_k metric [45]). An analysis of molecular variance [46] was consistent with these findings; highly significant (P<0.001) structure was detected at both the regional (i.e., east–west) and local (i.e., population) scale. Tests of IBD indicate a significant positive correlation between geographic distance and genetic differentiation (P= 0.0001). Together, these results indicate that populations of *S. alata* are structured at the deepest level by the Mississippi River and at a finer scale by the boundaries of the distinct habitat occupied by the plant [27]. Given the important role of at least some bacteria for the plant, habitat fragmentation and isolation on either side of the Mississippi River could shape the distribution of bacterial communities associated with *S. alata*. In this case, we predict that bacterial community composition would mirror the pattern of diversification of plant populations. Alternatively, other neutral processes such as dispersal or transmission by arthropod vectors could contribute to the structuring of microbial communities, in which case we would not expect them to reflect the population genetic structure of the plants.

Material and Methods

Sampling

We surveyed pitcher plant-associated microbial communities using a high throughput pyrosequencing approach. Pitcher plants and their fluid contents were collected from four populations throughout the distribution of *S. alata* in Louisiana during the summer of 2009 (Table 1). Plant tissue and associated fluid was obtained from three to six plants per population; two populations on either side of the Mississippi River were sampled (Fig. 1). Samples were collected 3 and 4 months after the opening of leaves to ensure similar leaf developmental stage and microbial community age. These months also harbor the most diverse bacterial communities in *S. alata* [38]. Fluid was drained from pitchers into sterile collecting tubes and refrigerated until extracted. Given the invasiveness of this collection

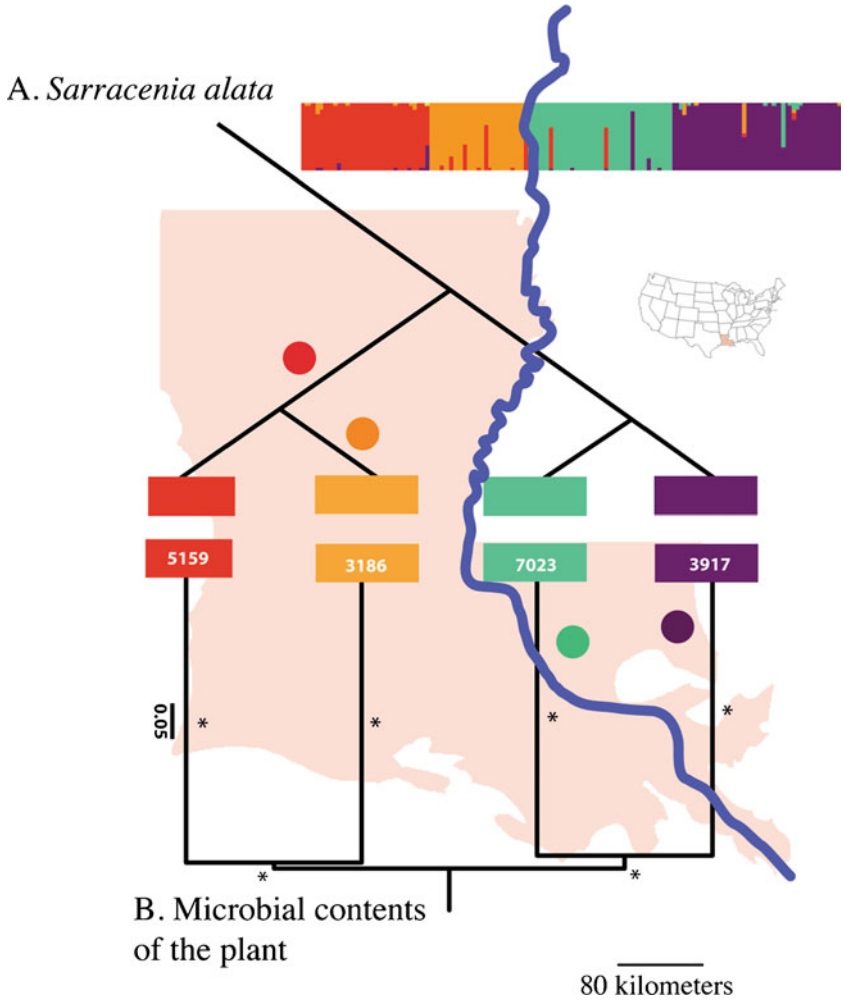

Figure 1 Congruence of population genetic structure between the carnivorous plant *S. alata* and their diverse microbial communities. **a** Population genetic structure of *S. alata* [42] from four populations throughout Louisiana (shown as a cladogram). Genetic structure is organized primarily by the Mississippi River (Evanno's ΔK=2). Consistent with the AMOVA results, Bayesian simulations further assigned individuals to clusters that correspond to sampling sites (K=4). **b** Hierarchical clustering of microbial populations. Numbers in *colored boxes* are the number of unique sequences in the composite population, *bar* represents unweighted UniFrac distance of 0.05, (*asterisk*) jackknife values >99.9%

procedure, individual pitchers could not be repeatedly sampled. All fluid extractions were performed within 24 h of collection following Koopman et al. (2010). Six hundred microliters of well-vortexed pitcher fluid (excluding macroparts of insects) from each sample were centrifuged (to concentrate bacterial cells) into 300-μL aliquots (if less than 600 μL of fluid was available, that volume was used). Extractions were executed using the 2 mL bead-beating tube containing beads from the Powersoil DNA Isolation kit (MoBio; Madison, WI); directions were followed exactly.

Amplification and Pyrosequencing

The biogeographic pattern of the diverse microbial community associated with *S. alata* was characterized using PCR and deep sequencing of the 16S rRNA gene. Though partial 16S sequences often lack the variation necessary to resolve fine scale biogeographic patterns in microbes (Jaspers 2004) and are not suitable to reconstruct phylogenies at the individual level, these short gene fragments are a valuable tool for accurately identifying community membership to the family level (Cardenas 2008, Ribosomal Database Project (RDP) site). Horner-Devine et al. (2004) argue that if spatial structure is observed using 16S even stronger patterns are likely to be resolved using finer scale markers level while Parnell et al. [47] demonstrated that 16S genes have similar biogeographic patterns to particular genes functioning in methanogenesis across a small spatial area (the Great Salt Lake). The extracted contents of 29 individual pitchers were used as template. The forward primer (5′-3′) included the 454 Life Sciences primer B (GCCTTGCCAGCCCGCTCAG), the broadly conserved microbial primer 27F and a two-base "TC" linker sequence. The reverse primer (GCCTCCCTCGCGCCATCAG **NNNNNNNNNNNN**CATGCTGCCTCCCGTAGGAGT) contained the 454 Life Sciences primer A (GCCTCCCT CGCGCCATCAG), the bacterial primer 338R (TGCTGCC TCCGTAGGAGT), a "CA" inserted as a linker between the barcode and the rRNA primer and a unique 12-bp error correcting Golay barcode (bolded in primer) [48]. PCRs were performed in 20-μL reactions: 1× Phusion High Fidelity Buffer (Finzyme, Finland), 2.5 μM Phusion $MgCl_2$

Table 1 Basic sequence and diversity statistics for the microbial contents of each *S. alata* pitcher

East/west	Time[a]	Population	Pitcher ID	Latitude	Longitude	Total sequences	Avg/bp read	7% Rarefaction	3% Rarefaction	Chao 1 (3%)	Shannon (H′) (3%)
East	1	LR	LR3-258(1)	30.31299	90.09341	15,250	216	173	367.98	606.90476	1.93471
East	1	LR	LR3-263(2)	30.31505	90.09396	12,678	220	193	458.95	739.62921	2.5736
East	1	LR	LR3-260(3)	30.31306	90.09342	12,000	218	134	298	519.80952	3.31472
East	1	A	A3-254(5)	30.30239	89.57586	11,763	216	184	380.95	734.88889	3.16512
East	1	A	A3-256(6)	30.30245	89.58006	31,123	217	187	397.89	608.48649	2.07094
East	1	A	A3-248(7)	30.3444	89.56178	16,696	221	131	308.98	459.96226	2.35249
East	1	A	A3-250(8)	30.34443	89.56173	12,234	206	127	250.99	393.7381	1.75401
East	1	A	A3-252(9)	30.34437	89.56167	12,965	222	161	371.95	535.77465	2.54217
West	1	C	C3-291(10)	30.31388	89.46057	14,778	222	261	461.96	747.33784	3.81589
West	1	C	C3-294(11)	30.01327	92.53269	8,808	220	122	247.92	394.65385	2.54126
West	1	C	C3-296(12)	30.01339	92.53257	11,613	220	190	353.87	621.38182	3.00751
West	1	K	K3-300(13)	32.02413	92.56225	11,331	211	255	507.94	838.11905	3.36119
West	1	K	K3-301(14)	32.02412	92.5622	9,911	219	405	828.96	1,464.11	4.30192
West	1	K	K3-304(15)	32.02414	92.56223	11,127	224	209	430.94	830.01515	3.25306
East	2	LR	LR4-296(16)	30.31299	90.09341	15,168	221	182	360	605.56452	2.32037
East	2	LR	LR4-283(17)	30.31505	90.09396	13,634	227	214	440.81	746.10811	3.10946
East	2	LR	LR4-284(18)	30.31306	90.09342	15,359	216	138	273.88	420.22642	1.84517
East	2	A	A4-274(19)	30.30372	89.57486	14,889	223	136	283.92	485	1.56576
East	2	A	A4-275(20)	30.30359	89.57488	13,943	211	312	653.68	1,247.40	3.46089
East	2	A	A4-278(21)	30.30322	89.57514	11,405	224	150	285.95	413.36842	2.56221
East	2	A	A4-268(22)	30.34452	89.56188	11,585	215	233	458.92	752.83529	3.18815
East	2	A	A4-270(23)	30.34451	89.56181	9,796	213	158	314.9	591.52174	2.42003
East	2	A	A4-273(24)	30.34448	89.56175	12,582	221	187	438.82	709.18293	3.03906
West	2	C	C4-317(25)	30.01327	92.53269	4,341	217	194	331.97	535.06	4.30827
West	2	C	C4-315(26)	30.01339	92.53257	15,699	220	85	204.98	299.52778	1.58373
West	2	C	C4-308(27)	30.31388	92.46057	18,392	225	76	198	277	1.04345
West	2	K	K4-318(28)	31.01337	92.53248	13,561	211	160	350.89	697.07143	2.85603
West	2	K	K4-319(29)	32.02414	92.56217	11,537	211	106	239.95	383.56098	2.63671
West	2	K	K4-322(30)	32.02414	92.56223	9,492	220	290	606.93	1,082.50	3.71138

Rarefaction (conducted at 3% and 7% sequence similarity, species and genus level divergence, respectively), Shannon and Chao 1 indices were computed for each pitcher using the analysis tools available from RDP

LR Lake Ramsey Savanna Wildlife Management Area, *A* Abita Springs Flatwoods Preserve, *C* Cooters Bog, Kisatchie National Forest; *K* Goldana, Kisatchie National Forest

[a] Sampling time, 1=third month, 2=fourth month after pitcher opening

(Finzyme, Finland), 0.25 μM each primer, 0.5 μM each dNTP, 0.3 U Phusion High-Fidelity Taq Polymerase (Finzyme, Finland), and 1–10 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, 53°C for 30 s; with a final extension at 72°C for 10 min. Three replicate PCRs were performed. Replicate reactions were pooled and cleaned with a Qiagen PCR Purification Kit (Valencia, CA). DNA was quantified on a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). Sequencing was conducted at ENGENCORE (University of South Carolina, Columbia) using their 454 Life Sciences Genome Sequencer FLX (Roche) machine.

Sequence Analysis and Tree Construction

The sequence dataset was trimmed to yield sequences >150 bp with quality scores >20 using the RDP's Pyrosequencing Pipeline's *initial processing* function. Filtered sequences were assigned to pitcher by their unique barcode. Within each pitcher, all sequences were aligned using the data processing *RDP Aligner* function. Sequences were clustered at a minimum distance (0.01 dissimilarity; max distance, 1.0; step size, 0.2) in order to remove identical sequences using the *RDP Dereplicate* function. All representative operational taxonomic unit (OTU) sequences were renamed with a pitcher ID, pooled, aligned, and clustered with a maximum distance of 93% sequence similarity. FastTree v. 2.1.1 [49] was used to construct an approximate maximum likelihood phylogenetic tree for this reduced dataset (19,285 sequences; identical sequences removed within pitchers) using a GTR model of sequence evolution and a single rate for each site, this tree was rooted with several archaeal sequences. All sequences were classified using the classification algorithm [50] in *RDP Classifer* at an 80% confidence rate and *RDP* taxonomic nomenclature.

Quantifying Diversity

Alpha diversity within communities was evaluated with rarefaction (Table 1), Analyses of Similarity (ANOSIM) and phylogenetic methods. ANOSIM analyses were implemented in PRIMER-E [51] to summarize patterns in species composition (and abundance) at four taxonomic ranks (class, order, family, genus) using permutation-based hypothesis testing and a Bray–Curtis similarity matrix (Table 2). This analysis statistically tests whether two or more sample groups are statistically different; if two groups differ in their species composition then the similarities within groups should be larger than between them. The significance of R is assessed by permuting group assignment to obtain a distribution of R under the null model. We tested the following null hypotheses of community composition: no

Table 2 Analyses of microbial community structure at four taxonomic ranks using Mantel and ANOSIM tests

	Mantel Test		ANOSIM				
Taxonomic Rank	A	B	Population	Pop+time	E/W	E/W+time	Time
Class	r = 0.0090 (p = 0.5500)	r = -0.2829 (p > 0.9990)	-0.025 -0.011	-0.027 0.137	-0.011 **0.204***	0.038 **0.245****	0.064 -0.024
Order	r = -0.3477 (p > 0.9999)	r = -0.3278 (p = 0.9998)	-0.018 -0.019	0.022 **0.186***	-0.018 **0.115***	0.054 **0.233****	0.041 -0.021
Family	r = -0.3415 (p > 0.9999)	r = -0.3095 (p = 0.9998)	0.076 -0.049	0.053 **0.166***	0.08 0.103	**0.188**** **0.233****	0.013 -0.019
Genus	r = -0.3088 (p = 0.9997)	r = -0.2473 (p = 0.9991)	0.029 0.17	0.128 **0.167****	0.099 0.076	**0.246**** **0.166****	0.056 0.041
UniFrac distance	r = 0.0101 (p = 0.588)	r = -0.2734 (p = 0.9990)					

Mantel tests A correlation between microbial community structure and geographic distance (isolation by distance) and B correlation between plant genotype and microbial community structure (presence/absence matrix using a Bray–Curtis similarity index) and UniFrac distance (pairwise distance matrix prepared in Fast UniFrac). Ten thousand permutations were conducted for each comparison. ANOSIM results: permutation-based hypothesis testing and Bray–Curtis similarity matrices were used to compare microbial communities over several categorical divisions from two data partitions: presence/absence (above the line) and abundance (below the line). A higher R value indicates that two communities are more differentiated. Outlined box and shaded box indicate significance using only "rare" (non-ubiquitous) phylogroups for abundance and presence/absence datasets, respectively

*P<0.05; **P<0.009, the p value is analogous to a univariate ANOVA and was assessed with 999 permutations

difference between populations, between time points, between populations at each time point, between the east and west, and between east and west at each time point. ANOSIM tests were repeated with ubiquitous OTUs (those present in every sample) removed in order to investigate whether these frequent sequences were biasing the results (Table 2).

The phylogeny-based metric UniFrac [52], implemented in Fast UniFrac (http://bmf2.colorado.edu/fastunifrac/index.psp), was used to compare microbial communities in a phylogenetic framework. Randomizations of population assignment were used for each significance test. Groups were defined as pitchers (29 sample units; not shown), populations (four sample units) and populations together with time (eight sample units). The probability (assessed as UniFrac significance) that each population has more unique branch lengths than expected was measured for each population assignment using 1,000 permutations (Table 3). In order to determine the probability that population structure on the tree differs significantly from chance, tips were randomly reassigned to populations and the degree of branch lengths unique to one environment versus the degree of shared branch lengths was compared in a pairwise fashion (Table 3). *P* values for UniFrac significance and the *p* test were corrected for multiple comparisons using Bonferroni correction.

Similarity (presence/absence) matrices of microbial composition were produced at four taxonomic ranks (class, order, family, genus) across all pitchers. A fifth matrix was produced using UniFrac distances. IBD as well as the significance of UniFrac distance on geographic distance between sample pairs was tested using a Mantel test [53] with 10,000 permutations. These same microbial matrices were further tested against host–plant genetic similarity [42].

Results

We generated a dataset of 383,660 high-quality microbial 16S rRNA gene sequences with a mean of 13,230±4,406 (SD) sequences per pitcher (N=29) and an average read length of 218 bp (Table 1). Eleven phyla were recognized across all pitchers; however, three dominate and represent 97% of total sequences: Firmicutes (11.1%), Bacteroidetes (21.6%), and Proteobacteria (64.3%). Representatives of these phyla and Actinobacteria, were present in every pitcher sampled (SI Table 1). These four phyla are among the most prevalent phyla in the human gut [54], and likely play a role in mineral and nutrient assimilation for the plant. Sixty-five percent and 46% of sequences assigned to class and order, respectively, were found in all pitchers, and three families (Enterobacteriaceae, Comamondaceae, Pseudomonadaceae) were present in every sample (SI Table 1). Despite these commonalities, phylotype diversity varied between individual pitchers as well as populations (Table 1). Between 198 and 829 phylotypes (defined as ≤3% sequence divergence) were identified in each pitcher (mean phylotypes/pitcher=383). The average number of phylotypes per population ranged from 299 to 494 (Table 1).

Mantel tests were used to examine the correlation of geographic distance on microbial community composition at four taxonomic levels (class, order, family, genus). Similar tests were employed to test for correlations between plant genetic diversity and microbial community composition. In no case were results significant (P>0.5 in all tests; Table 2). An ANOSIM was implemented to summarize patterns in species composition at four taxonomic ranks (class, order, family, genus). The global R statistic from ANOSIM was never significant when partitioned by population or time alone indicating that there were no significant community compositional differences between populations or sampling month at any taxonomic level (Table 2). Together with Mantel tests, these data strongly suggest that dispersal limitation cannot explain the observed pattern and that there are no substantial compositional differences among populations. Rather than revert to the Baas-Becking explanation, we explored the relationship between plant population structure and bacterial community composition in a phylogenetic framework.

The UniFrac metric [52] was used to compute the phylogenetic distance between microbial pitcher communities measured as the proportion of branch lengths that lead to descendants in one population but not the other. Randomizations of population assignment were used to test for significance. Within populations, community composition did not vary significantly between pitchers or between time points (Fig. 2). Phylogenetic distance between each pair of populations, however, differed significantly (Bonferroni-corrected UniFrac significance and P test <0.001 for each comparison; Table 3) and microbial composition varied more between populations than within them (Fig. 2). Furthermore, plant populations on either side of the Mississippi River harbor microbial

Table 3 Results from UniFraq analyses for microbial community comparisons from the four distinct pitcher plant populations (Population) or for samples defined as populations from each time point (Time 1, Time 2). Values that are significant after Bonferroni correction are shown as bold text.

	Population	Time 1	Time 2
A	<1.0e-03	0.761	<1.0e-03
L	**0.001**	0.032	0.206
C	**<1.0e-03**	0.218	**0.002**
K	**0.001**	0.032	0.206

A Abita Springs Flatwoods Preserve, *L* Lake Ramsey Savanna Wildlife Management Area *C* Cooters Bog, Kisatchie National Forest; *K Goldana, Kisatchie National Forest*

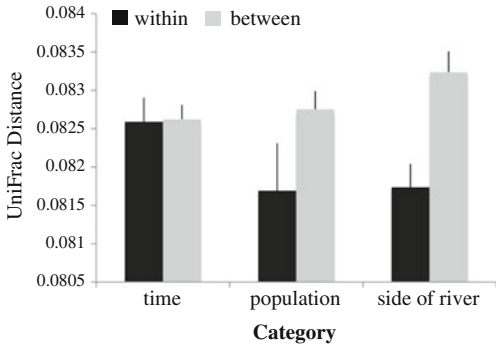

Figure 2 Mean (±SEM) unweighted UniFrac distance between communities from three different categories

communities that differ significantly in abundance at the class and ordinal delineation (Table 2; Fig. 2). A distance matrix was calculated for all pairwise combinations of populations on a tree from which hierarchical clustering of UniFrac distances with UPGMA produced a well-supported tree (Fig. 1b). The microbial contents of pitchers exhibit clear biogeographic patterns are clustered to either side of the Mississippi River and are congruent with that of the host plant's current population structure. The probability of this degree of similarity in two rooted phylogenetic trees is <0.005 [55].

Whereas a significant pattern of IBD was recovered between plant populations in our study of the plants [42], we find no evidence for IBD (Table 2) in associated microbial communities, although the limited geographic distances in this study likely reduce the power to discern such associations. We hesitate to rely on environmental selection alone to explain the spatial patterns we observe. While local adaptation could be a factor, there are no fixed morphological differences between sampled populations of *S. alata* [41], and it is difficult to isolate and identify selective pressures for hundreds of bacterial lineages. Rather, we propose that historical events are at least partially responsible for observed differences in microbial composition. Microbial populations associated with *S. alata* share a remarkably similar microbial fauna dominated by three phyla (SI Table 1), but the collective microbial community contained within each plant population differ significantly (Fig. 1; Tables 2 and 3). The probability that population structure on the tree is significantly different than chance using UniFrac analyses for samples defined as four distinct pitcher plant populations (or as populations from each time point) is $P<0.001$ for every comparison (not shown). Microbial communities are structured primarily across the Mississippi River (Figs. 1b and 2; Table 2). Plant genetic data [42] indicate that the dominant pattern of microbial community diversity in the pitchers reflects that of the plant genetic diversity (Fig. 1).

Discussion

The Baas-Becking hypothesis is difficult to test [56]. Microbes can influence their local environment [57] and respond to its inherent selective pressures. For any specific bacterium, it is difficult to isolate environmental variation that influences abundance. For bacterial communities within a pitcher, with hundreds of bacterial phylotypes, this endeavor would prove challenging. Consequently, it is difficult to distinguish between environmental effects on the community and the reverse. If IBD can be rejected, and the Baas-Becking explanation is difficult to falsify, then the challenge for microbial ecologists is to identify other factors that influence microbial diversity. In *Sarracenia*, bacterial abundance has not been correlated with specific environmental predictors [58], and species interactions likely account for some of the spatial variation among microbial communities [35–37]. Further, interactions within the pitcher extend far beyond bacterial–bacterial interaction because pitchers also contain living arthropod, yeast, protozoan, nematode, and algal populations [35, 59, 60] that each differ in terms of their abundance, ecological role, generation time, and mode of dispersal. While we acknowledge that chance, geography and some degree of biologically linked dispersal limitation are likely drivers of microbial community structure in *S. alata*, these additional options do not detract from the fact that the community phylogeographic structure of the microbiome mirrors that of its host plant. This is remarkable given that each member of the microbial community has its own unique evolutionary history, genetic architecture, and response to host–plant genetic variation.

Though our sampling represents a snapshot of community structure in contemporary populations (single aliquots from each leaf in a single season) the results reflect evolutionary processes acting over a substantial time period [3, 61]. Historical processes are important determinants of biogeographic patterns for eukaryotes; here, we argue that habitat fragmentation and subsequent isolation can similarly influence bacterial communities. These processes are most easily recognized for microbial communities that are associated with eukaryotic species; in *S. alata*, isolation on either side of the Atchafalaya basin and the Mississippi River [42] represents a key driver of bacterial community structure. Plant genetic variation can significantly affect communities [1, 5, 11, 62–64], and the genetic pattern of pitcher plant population divergence accurately predicts the microbial community's biogeographic structure. While there is no evidence that genetic diversity in individual plants is significantly correlated to microbial composition (Table 2), cluster analyses indicate that the pattern of variation in bacterial community composition mirrors that of its host plant differentiation (Fig. 1a, b; Table 3) and

suggests that both are influenced at the deepest level by the Mississippi River. This work demonstrates that biogeographic analyses, when they are not limited to particular taxonomic groups, can identify landscape processes that are important in shaping the evolution of all members of a community.

Pitcher plants are excellent model systems [65] for studying community genetics and metacommunity structure of microbial diversity due to the taxonomic diversity of contained within pitcher plants [66], the small size of their microcosms, and the diverse communities with several trophic levels that can repeatedly be sampled across time and space [35, 36, 38]. This complex plant–microbe interaction provides a unique opportunity to further develop hypotheses about the causal mechanisms that influence the biogeographic structure of microbial communities. Furthermore, we have only begun to describe the microbes that inhabit carnivorous plants, and decipher their role in the evolution of plant carnivory. Our findings offer a unique perspective into the evolutionary necessity and functional roles of microbes in eukaryotic digestive systems and to the essence of these symbioses. However, since Enterobacteria represent the majority of the bacteria in pitchers from across the range of *S. alata* (SI Table 1) and dominate the bacterial community that is ubiquitous to *S. alata*'s pitchers (SI Fig. 1), we strongly suspect that our understanding of community composition and function will be incomplete until we investigate the bacterial communities associated with the arthropods that interact with *S. alata* as prey, parasites, or symbionts.

Acknowledgments We thank members of the Carstens laboratory, especially Sarah Hird and Daniel Ence for assistance with bioinformatics processing. We thank Brent Christner, Gary King, and Kyle Harms for valuable comments on this project and manuscript. This work has been supported by grants from the LSU Board of Regents Research Competitiveness Grant, the LSU Faculty Research Program, the LSU Pfund program, and the National Science Foundation (DEB 0956069). Sequences are deposited in the NCBI Genbank (accession numbers XXX).

References

1. Whitham TG, Bailey JK, Schweitzer JA, Shuster SM, Bangert RK, LeRoy CJ, Lonsdorf E, Allan GJ, DiFazio SP, Potts BM, Fischer DG, Gehring CA, Lindroth RL, Marks J, Hart SC, Wimp GM, Wooley SC (2006) A framework for community and ecosystem genetics: from genes to ecosystems. Nat Rev Genet 7:510–523
2. Whitham TG, DiFazio SP, Schweitzer JA, Shuster SM, Allan GJ, Bailey JK, Woolbright SA (2008) Extending genomics to natural communities and ecosystems. Science 320:492–495
3. Johnson MTJ, Stinchcombe JR (2007) An emerging synthesis between community ecology and evolutionary biology. Trends Ecol Evol 22:250–257
4. Cavender-Bares J, Kozak KH, Fine PVA, Kembel SW (2009) The merging of community ecology and phylogenetic biology. Ecol Lett 12:693–715
5. Crutsinger GM, Cadotte MW, Sanders NJ (2009) Plant genetics shapes inquiline community structure across spatial scales. Ecol Lett 12:285–292
6. Ellison AM, Bank MS, Clinton BD, Colburn EA, Elliot K, Ford CR, Foster DR, Kloeppel BD, Knoepp JD, Lovett GM, Mohan J, Orwig DA, Rodenhouse NL, Sobczak WV, Stinson KA, Stone JK, Swan CM, Thomspon J, Von HOlle B, Webster JR (2005) Loss of foundation species: consequences for the structure and dynamics of forested ecosystems. Front Ecol 3:479–486
7. Delmotte N, Knief C, Chaffron S, Innerebner G, Roschitzki B, Schlapback R, von Mering C, Vorholt JA (2009) Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. Proc Natl Acad Sci USA 106:16428–16433
8. Schweitzer JA, Bailey JK, Bangert RK, Hart SC, Whitham TG (2007) The role of plant genetic variation in determining above- and belowground microbial communities. In: Bailey MJ, Lilley AK, Timms-Wilson TM, Spencer-Phillips PTN (eds) Microbial Ecology of Arial Plant Surfaces. CABI Publishing, Wallingford pp 109–119
9. Crutsinger GM, Souza L, Sanders NJ (2008) Intraspecific diversity and dominant genotypes resist plant invasions. Ecol Lett 11:16–23
10. Lankau RA, Strauss SY (2007) Mutual feebacks maintain both genetic and species diversity in a plant community. Science 317:1561–1563
11. Johnson MTJ, Agrawal AA (2005) Plant genotype and environment interact to shape a diverse arthropod community on evening primrose (*Oenothera biennis*). Ecology 86:874–885
12. Lindow SE, Brandl MT (2003) Microbiology of the phyllosphere. Appl Environ Microbiol 69:1875–1883
13. Bailey JK, Lilley AK, Timms-Wilson TM, Spencer-Phillips PTN (2006) Microbial ecology of aerial plant surfaces. CABI, Wallingford
14. Falkowski PG, Fenchel T, Delong EF (2008) The microbial engines that drive Earth's biogeochemical cycles. Science 320:1034–1039
15. van der Heijden MGA, Bardgett RD, van Straalen NM (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol Lett 11:296–310
16. Jessup CM, Kassen R, Forde SE, Kerr B, Buckling A, Rainey PB, Bohannan BJM (2004) Big questions, small worlds: microbial model systems in ecology. Trends Ecol Evol 19:189–197
17. Brock TD (1987) The study of microorganism in situ: progress and problems. Symp Soc Gen Microbiol 41:1–17
18. Achtman M, Wagner M (2008) Microbial diversity and the genetic nature of microbial species. Nat Rev Microbiol 6:431–440
19. Horner-Devine MC, Carney KM, Bohannan BJM (2004) An ecological perspective on bacterial biodiversity. In: Proceedings of the Royal Society of London Series B-Biological Sciences 271:113–122
20. Cardenas E, Tiedje JM (2008) New tools for discovering and characterizing microbial diversity. Curr Opin Biotechnol 19:544–549
21. Mardis E (2008) The impact of next-generation sequencing technology on genetics. Trends Genet 24:133–141
22. Rothberg JM, Leamon JH (2008) The development and impact of 454 sequencing. Nat Biotechnol 26:1117–1124
23. Keller M, Hettich R (2009) Environmental Proteomics: a paradigm shift in characterizing microbial activities at the molecular level. Microbiol Mol Biol Rev 73:62–70
24. Cho JC, Tiedje JM (2000) Biogeography and degree of endemicity of fluorescent *Pseudomonas* strains in soil. Appl Environ Microbiol 66:5448–5456
25. Papke RT, Ramsing NB, Bateson MM, Ward DM (2003) Geographical isolation in hot spring cyanobacteria. Environ Microbiol 5:650–659
26. Hughes Martiny JB, Bohannan BJM, Brown JH, Colwell RK, Fuhrman JA, Green JL, Horner-Devine MC, Kane M, Krumins JA, Kuske CR, Morin PJ, Naeem S, Øvreås L, Reysenbach A,

Smith VH, Staley JT (2006) Microbial biogeography: putting microorganisms on the map. Nat Rev Microbiol 4:102–112
27. Whitaker RJ, Grogan DW, Taylor JW (2003) Geographic barriers isolate endemic populations of hyperthermophilic archaea. Science 301:976–978
28. Telford RJ, Vandvik V, Birks HJB (2006) Dispersal limitations matter for microbial morphospecies. Science 312:1015
29. Baas-Becking LGM (1934) In: van Stockum WP, Zoon NV (eds) Geobiologie of Inleiding Tot de Milieukunde. The Hague, The Netherlands
30. Lomolino MV, Riddle BR, Brown JH (2005) Biogeography. Sinauer, Sunderland
31. Biek R, Drummond AJ, Poss M (2006) A virus reveals population structure and recent demographic history of its carnivore host. Science 311:538–541
32. Wirth T, Myer A, Achtman M (2005) Describing host migrations and origins by means of their microbes. Mol Ecol 14:3289–3306
33. Zhou J, Xia B, Treves DS, Wu LY, Marsh TL, O'Neill RV, Palumbo AV, Tiedje JM (2002) Spatial and resource factors influencing high microbial diversity in soil. Appl Environ Microbiol 68:326–334
34. Treves DS, Xia B, Zhou J, Tiedje JM (2003) A two-species test of the hypothesis that spatial isolation influences microbial diversity in soil. Microb Ecol 45:20–28
35. Buckley HL, Miller TE, Ellison AM, Gotelli NJ (2003) Reverse latitudinal trends in species richness of pitcher-plant food webs. Ecol Lett 6:825–829
36. Peterson CN, Day S, Wolfe BE, Ellison AM, Kolter R, Pringle A (2008) A keystone predator controls bacterial diversity in the pitcher-plant (*Sarracenia purpurea*) microecosystem. Environ Microbiol 10:2257–2266
37. Harvey E, Miller TE (1996) Variance in composition of inquiline communities in leaves of *Sarracenia purpurea* L on multiple spatial scales. Oecologia 108:562–566
38. Koopman MM, Fuselier D, Hird S, Carstens B (2010) Bacterial characterization of the carnivorous Pale Pitcher Plant reveals diverse, distinct and time-dependent communities. Appl Environ Microbiol 76:1851–1860
39. Plummer GL, Jackson TH (1963) Bacterial activities within the sarcophagus of the insectivorous plan, *Sarracenia flava*. Am Midl Nat 69:462–469
40. Prankevicius AB, Cameron DM (1991) Bacterial dinitrogen fixation in the leaf of the northern pitcher plant (*Sarracenia purpurea*). Can J Bot Rev Canadienne De Botanique 69:2296–2298
41. Sheridan PM (1991) What is the identity of the West Gulf Coastal pitcher plant, *Sarracenia alata*? Carnivorous Plant Newsletter 20:102–110
42. Koopman MM, Carstens BC (2010) Conservation genetic inferences in the carnivorous pitcher plant *Sarracenia alata* (Sarraceniaceae). Conservation Genetics (in press)
43. Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959
44. Huelsenbeck JP, Huelsenbeck ET, Andolfatto P (2007) Structurama: Bayesian inference of population structure. Bioinformatics (in press)
45. Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. Mol Ecol 14:2611–2620
46. Excoffier L, Smouse P, Quattro J (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 136:343–359
47. Parnell LD, Rompato G, Latta L, Pfrender M, van Nostrand J, He Z, Zhou J, Anderson G, Champine P, Ganesan B, Weimer B, Aziz R (2010) Functional biogeography as evidence of gene transfer in hypersaline microbial communities. PLoS ONE 5:e12919
48. Hamady M, Walker J, Harris J, Gold N, Knight R (2008) Error-correcting barcoded primers allow hundreds of samples to be pyrosequenced in multiplex. Nat Meth 5:235–237
49. Price MN, Dehal PS, Arkin AP (2010) FastTree 2—approximately maximum-likelihood trees for large alignments. PLoS ONE 5: e9490. doi:9410.1371/journal.pone.0009490
50. Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267
51. Clarke KR, Gorley RN (2006) PRIMER v6: user manual/tutorial. PRIMER-E Ltd., Plymouth Marine Laboratory, Ivybridge, UK
52. Lozupone C, Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol 71:8228–8235
53. Mantel NA (1967) The detection of disease clustering and a generalized regression approach. Cancer Res 27:209–220
54. Dethlefsen L, McFall-Ngai M, Relman DA (2007) An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature 449:811–818
55. Felsenstein J (2004) Inferring phylogenies. Sinauer Sunderland, Mass
56. Foissner W (2006) Biologeography and dispersal of microorganisms: a review emphasizing protists. Acta Protozoologica 45:111–136
57. Fierer N (2008) Microbial biogeography: patterns in microbial diversity across space and time. ASM Press, Washington
58. Buckley HL, Burns JH, Kneitel JM, Walters EL, Munguia P, Miller E (2004) Small-scale patterns in community structure of *Sarracenia* purpurea inquilines. Community Ecol 5:1588–2755
59. Addicott JF (1974) Predation and prey community structure-experimental study of effect of mosquito larvae on protozoan communities of pitcher plants. Ecology 55:475–492
60. Kneitel JM, Miller TE (2002) Resource and top-predator regulation in the pitcher plant (*Sarracenia purpurea*) inquiline community. Ecology 83:680–688
61. Webb CO, Ackerly DD, McPeek MA, Donoghue MJ (2002) Phylogenies and community ecology. Annu Rev Ecol Syst 33:475–505
62. Wimp GM, Whitham TG (2001) Biodiversity consequences of predation and host plant hybridization on an aphid-ant mutualism. Ecology 82:440–452
63. Schweitzer JA, Bailey JK, Fischer DG, LeRoy CJ, Lonsdorf EV, Whitham TG, Hart SC (2008) Heritable relationship between plant genotype and associated soil microorganisms. Ecology 89:773–781
64. Shuster SM, Lonsdorf E, Wimp GM, Bailey JK, Whitham TG (2007) Community heritability measures the evolutionary consequences of indirect genetic effects on community structure. Evolution 60:991–1003
65. Srivastava DS, Kolasa J, Bengtsson J, Gonzalez A, Lawler SP, Miller TE, Munguia P, Romanuk T, Schneider DC, Trzcinski MK (2004) Are natural microcosms useful model systems for ecology? Trends Ecol Evol 19:379–384
66. Ellison AM, Gotelli NJ (2009) Energetics and the evolution of carnivorous plants—Darwin's 'most wonderful plants in the world'. J Exp Biol 60:19–42