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# A keystone predator controls bacterial diversity in the pitcher-plant (*Sarracenia purpurea*) microecosystem

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## Summary

**The community of organisms inhabiting the water-filled leaves of the carnivorous pitcher-plant *Sarracenia purpurea* includes arthropods, protozoa and bacteria, and serves as a model system for studies of food web dynamics. Despite the wealth of data collected by ecologists and zoologists on this food web, very little is known about the bacterial assemblage in this microecosystem. We used terminal restriction fragment length polymorphism (T-RFLP) analysis to quantify bacterial diversity within the pitchers as a function of pitcher size, pH of the pitcher fluid and the presence of the keystone predator in this food web, larvae of the pitcher-plant mosquito *Wyeomyia smithii.* Results were analysed at two spatial scales: within a single bog and across three isolated bogs. Pitchers were sterile before they opened and composition of the bacterial assemblage was more variable between different bogs than within bogs. Measures of bacterial richness and diversity were greater in the presence of *W. smithii* and increased with increasing pitcher size. Our results suggest that fundamental ecological concepts derived from macroscopic food webs can also be used to predict the bacterial assemblages in pitcher plants.**

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## Introduction

Bacterial assemblages may be structured by many forces at both local and regional scales. They are integrated into complex food webs and their composition, in terms of species diversity and cell abundance, may be controlled by a combination of 'top-down' factors, such as grazing by predators, and 'bottom-up' factors, such as nutrient availability or other environmental parameters (Moran and Scheidler, 2002). Simple and well-characterized natural model ecosystems can be used to determine how each of these forces function and interact with each other at different scales. However, there is a dearth of such systems available for field studies. That makes the model ecosystem of the carnivorous pitcher-plant *Sarracenia purpurea* (Sarraceniaceae) particularly valuable.

The food web that forms in the water-filled leaves of *S. purpurea* (Sarraceniaceae) has been used as a model system for the study of food webs and ecological dynamics for almost two centuries. The leaves of this long-lived (> 50 years) perennial plant form pitcher-shaped organs which are initially closed but open to fill with rainwater, into which arthropods fall and drown (Arber, 1941). This prey is then mineralized by the food web and provides nitrogen and phosphorous to the plant (Butler *et al.*, 2008). Unlike other North American pitcher plants, *S. purpurea* does not appear to secrete enzymes that degrade chitin (Hepburn and Jones, 1927; Gallie and Chang, 1997). Rather the food web within the pitcher decomposes the prey and provides virtually all of the nutrients for the plant's growth and reproduction (Bradshaw and Creelman, 1984; Butler and Ellison, 2007).

The macroarthropods and protozoa of the pitcher-plant food web are well described (Addicott, 1974; Fish and Hall, 1978; Cochran-Stafira and von Ende, 1998; Bledzki and Ellison, 2003; Buckley *et al.*, 2003). Species include the larvae of three dipterans and one mite, which are obligatorily associated with pitcher plants and occur throughout the broad geographic range of *S. purpurea* (the eastern coast of North America from Florida to Northern Canada, and westward to the Canadian Rocky Mountains) (Buckley *et al.*, 2003). The rotifers and protozoa in the food web are not restricted to *S. purpurea*, but occur in the surrounding peat and ponds of the bogs, fens and seepage swamps in which the pitcher plant grows

(Cochran-Stafira and von Ende, 1998; Bledzki and Ellison, 2003).

Despite their critical role in nutrient cycling – bacteria mineralize the majority of nutrients that the plant derives from its prey (Butler *et al*., 2008) – the structure of the bacterial assemblage in the *S. purpurea* food web has been little studied (Prakevicius and Cameron, 1989; Prankevicius and Cameron, 1991; Cochran-Stafira and von Ende, 1998; Buckley *et al*., 2003). The few studies of bacteria in the pitchers have used morphological and phenotypic descriptions of isolated colonies, and they have reported proteolytic bacteria such as coliforms (Whitman *et al*., 2005). Many questions regarding these communities remain unanswered, including whether the bacterial assemblage is vertically transmitted from the plant to the newly developing pitcher (Hepburn and Jones, 1927).

There is also considerable interest in determining whether conventional ecological concepts, including principles of island biogeography (MacArthur and Wilson, 1967) and the structuring role of keystone predators (Paine, 1966), apply to microbial systems. For example, do bacteria have species–area curves similar to those of larger organisms, for which larger habitats contain more species (Horner-Devine *et al*., 2004; Bell *et al*., 2005; van der Gast *et al*., 2005; 2006)? Classic theory suggests that immigration and extinction rates control the species richness of discrete habitats, such as islands, and numerous studies with plants and animals have confirmed that larger islands typically contain more species (Arrhenius, 1921; Preston, 1962; Hubbell, 2001). Pitcher plants are excellent model systems in which to test this theory for bacteria, as individual pitchers form discrete habitats in bogs, which themselves are discrete, bounded habitats in otherwise upland forested landscapes.

Similarly, many studies have shown that top predators can control the species abundance at lower trophic levels by altering competitive interactions among the latter species (Power *et al*., 1996). Germane to the study presented here is that larvae of the pitcher-plant mosquito (*Wyeomyia smithii* Coq) are thought to be keystone predators (Paine, 1966) with a disproportionately large impact on diversity and abundance of other species in the *S. purpurea* food web (Addicott, 1974; Gotelli and Ellison, 2006). These suspension-feeding mosquito larvae prey on bacteria, protozoa and rotifers, and their presence controls the species diversity and abundance of other arthropods and protozoa within the pitcher-plant food web (Gotelli and Ellison, 2006). Very simplified pitcher-plant food webs, reconstituted in the laboratory with only four bacterial species, indicate that some species can coexist in the presence of *W. smithii* (Cochran-Stafira and von Ende, 1998). Similarly, some field studies suggested an increase in bacterial morphotypes when *W. smithii* was present (Kneitel and Miller, 2002). Whether this result is consistent with more comprehensive measures of bacterial diversity is unknown. As pitcher-plant food webs share many structural attributes with aquatic and terrestrial systems (Gotelli and Ellison, 2006), understanding the microbial ecology of this system may have general implications for understanding a wide range of microbial systems.

The initial aims of this study were to (i) determine whether vertical transmission of bacteria occurs and (ii) characterize the bacterial assemblage within *S. purpurea* pitchers using culture-independent molecular techniques. Additional goals were to determine (i) how the structure of the bacterial assemblage varies within pitchers, within bogs and among bogs, (ii) whether bacterial assemblages follow species–area curves similar to those of plants and animals, and (iii) if the presence or absence of *W. smithii* larvae alters richness of the pitcher-plant bacteria. To address these questions, we used terminal restriction fragment length polymorphism (T-RFLP) signatures/fingerprints as a measure of bacterial species richness and abundance.

## Results

### *No vertical transmission of bacteria to unopened pitchers*

The question of whether pitcher plants are sterile before opening (Hepburn and Jones, 1927) was revisited using more permissive growth conditions and culture-independent molecular techniques. No bacterial colonies were isolated in tryptic soy broth agar plates inoculated with lavages from unopened pitchers. To determine if the pitchers were impermeable to bacteria, unopened pitchers were dipped in *Escherichia coli* expressing a red fluorescent protein (mRFP). The insides of the pitcher were then rinsed and lavaged; these lavages did not yield any mRFP-expressing *E. coli* after incubation on nutrient agar plates. As the majority of bacteria will not grow on laboratory media, we also used polymerase chain reaction (PCR) and microscopy-based culture-independent methods to determine if there were any bacteria present in the unopened pitchers. Only one of the 13 samples from unopened pitchers amplified 16S rRNA gene fragments, and we speculate that this pitcher was torn. Under microscopic observation, the insides of unopened pitchers did not contain structures of the sizes and shapes that would be expected for a broad range of bacteria (data not shown). Thus utilizing modern methods, no evidence was obtained to suggest a consistent bacterial community in the unopened pitchers. From these results we conclude that unopened pitchers of *S. purpurea* are sterile.

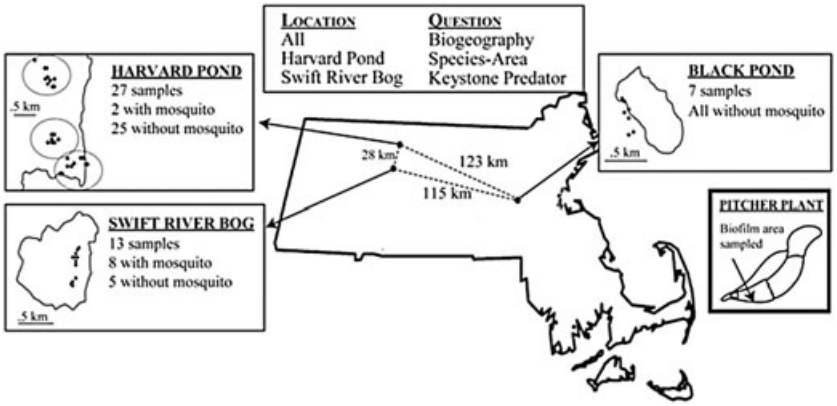


**Fig. 1.** Site map for the spatial analysis of pitcher-plant communities. Samples were harvested from three sites in Massachusetts: Harvard Pond, Swift River Bog and Black Pond Bog. Temporal variation was controlled by harvesting each pitcher 4 weeks after opening. For each pitcher, DNA was extracted from the same area: the biofilms coating the absorptive zone. The Harvard Pond was the main sampling site and circles indicate the small-scale 'regions' used in the biogeographical analysis. For detailed analysis of the effect of the mosquito on community structure, Swift River Bog was used as there were few mosquitoes on the islands sampled at Harvard Pond.

### *Profiling the bacterial assemblages*

The T-RFLP profile of pitcher-plant bacterial diversity was examined over different spatial scales (Fig. 1) and was found to be very complex. In total, there were 133 gene fragments detected across all the sites (Fig. 2A). Fragment length distribution was dominated by infrequent sizes: it can be seen from Fig. 2A that almost 50% of the gene fragments were present in less than 10% of the samples. None of the fragment peaks were found in all the pitchers. In fact, very few fragments were found in the majority of the plants. Only three fragments were found in > 90% of the plants: size 72, 77 and 119 base pairs (bp). We searched the Ribosomal Database Project (RDP) database to determine if any of these peaks were diagnostic of particular bacterial species or genera. A fragment of 72 bp is diagnostic of *Craurococcus roseus*, a bacteriochlorophyll *a* strain known for its resistance to amoebas (Thomas *et al*., 2006). A fragment of 77 bp could result from the presence of members of at least six different genera (*Azoarcus* sp., *Bordetalla* sp., *Nitrosomonas* sp., *Ralstonia* sp., *Thauera* sp. and *Thiobacillus* sp.). None of the rRNA genes deposited in RDP have a sequence that would predict a T-RFLP peak of 119 bp. In each separate bog, as with the combined samples, most of the peaks were found in very few pitchers (Fig. 2B).

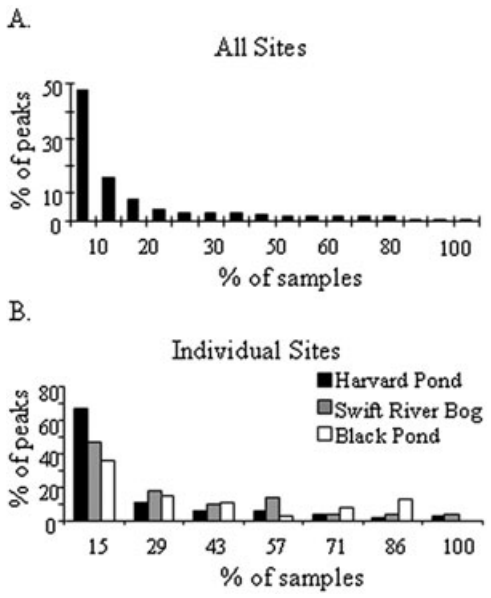


**Fig. 2.** Histograms showing the frequency distributions of fragment lengths in the data set. The *x*-axis is the per cent of pitchers in which fragments were measured. The *y*-axis shows how frequently these classes of peaks were found.
A. Frequency distribution of all T-RFLP bands in this study.
B. Frequency distribution of the T-RFLP bands from each individual site.

A recent report indicated that *S. purpurea* pitchers can contain bacterial species typically found in the intestinal tracts of mammals (Whitman *et al*., 2005). This prompted us to determine if our pitcher-plant samples also contained such enteric bacteria. Indeed, we found *Citrobacter freundii*, *Hafnia alveia*, *Serratia fonticola*, *Rhanella aqualtis*, *Pantoa agglomerans*, *Serratia plymuthica* and an uncultured *Serratia* clone (Fig. 3). However, the majority of other isolates were found only once ('singletons' in Fig. 3).

### *Biogeography of pitcher-plant bacteria*

There were three spatial scales at which bacterial diversity was examined: first, among the three sampled ponds and bogs spread across Massachusetts (from 28 to 123 km apart, Fig. 1); second, between the groups of plants on individual islands at Harvard Pond (from 0.08 km to 0.2 km apart, circles in Fig. 1); and third, between pitchers collected from the same group of plants (less than 100 cm between pitchers). Mean bacterial richness (number of distinct T-RFLP peaks) was similar for the three bogs (22.9 at Harvard Pond, 20.8 at Swift River and 18.7 at Black Pond; $F = 2.27$; $P = 0.109$). But mean bacterial diversity (expressed as the Shannon diversity index $H'$; this index encompasses both richness and evenness) was the highest at Harvard Pond (2.47), intermediate at Black Pond (2.27) and the lowest at Swift River

 

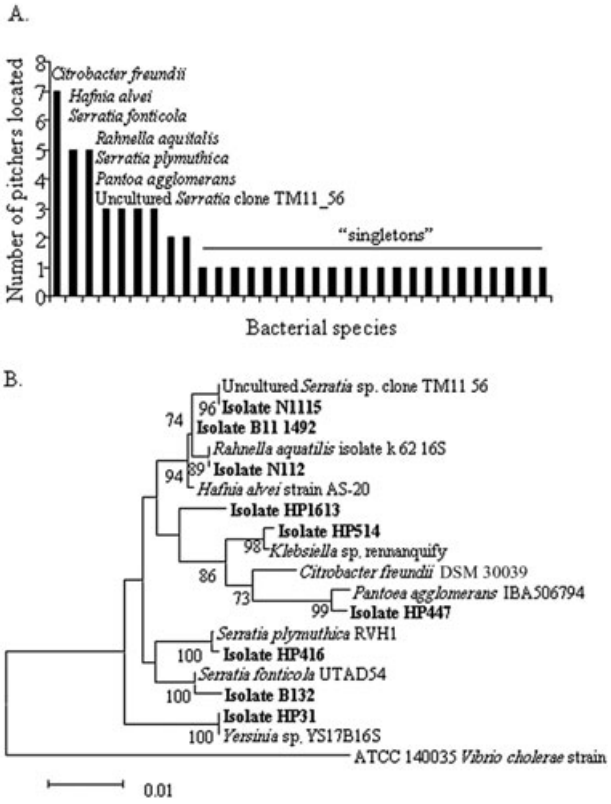


**Fig. 3.** A. Histogram showing the frequency across all sites of isolated species of the genera enteric bacteria.
B. Phylogenetic analysis of enteric bacterial 16S rRNA gene sequences found in more than one pitcher. Phylogenetic relationships were constructed by neighbour-joining analysis of 867 positions of homologous sequence, and rooted to the out-group *Vibrio cholerae* type strain ATCC 14035. Bootstrap values above 50% are shown as a percentage of 1000 replicates and the scale represents the number of mutations per nucleotide position. Sequences obtained in this study are in bold type.

(2.17) ($F = 4.74$; $P = 0.014$). There was no difference in bacterial richness ($F = 1.39$; $P = 0.283$) or diversity ($F = 0.872$; $P = 0.431$) among regions or clusters of pitchers within the Harvard Pond site, or among pitchers collected from the same cluster of plants.

The structure of the bacterial assemblage was also analysed at the three different spatial scales. It varied more among different bogs than within the bogs ($A = 0.098$; $P = 0.000015$) when the binary data were analysed using Multiple Response Permutation Procedure (MRPP), which tests whether there is a significant difference between groups of pitchers based on the values of the Sørensen similarity index (Dunbar *et al*., 2001; McCune and Grace, 2002). Assemblages from pitchers in neighbouring bogs (Swift River and Harvard Pond) were not more similar to each other than assemblages from pitchers in the distant bog (Black Pond). Although they were still significantly different, the two sites that were the most similar were Harvard Pond and Black Pond ($A = 0.053$; $P = 0.002$). These sites were the furthest apart, but both contain open water. Samples from Swift River Bog, which has no open water, differed from the other two samples, especially from the Black Pond samples ($A = 0.144$; $P = 0.0022$). A neighbour-joining dendogram of the binary profiles also revealed modest clustering among the sites (Fig. 4), with some segregation between the Swift River Bog and Black Pond samples. As Swift River samples also had the highest abundance of *W. smithii* (see below), the MRPP analysis was repeated without the samples containing *W. smithii* larvae. This second analysis confirmed a small but significant difference between the sites ($A = 0.063$; $P = 0.0024$). However, within the Harvard Pond site alone there was no relationship among pitchers or regions of pitchers in the composition of the bacterial assemblages. Moreover, there was no significant correlation between the composition of the community and the geographic distance between the pitchers in the Harvard Pond as determined by a Mantel test ($r_{Mantel} = 0.043$, $P = 0.192$; for log transformed data, $r_{Mantel} = 0.073$, $P = 0.070$). Thus a spatial structuring of the assemblages occurred between the different sites across Massachusetts but not within the sites, and was not dependent on geographic distance at this smaller scale.

### *Ecological predictors of bacterial diversity*

At our focal site at Harvard Pond, we found that greater bacterial richness was associated with taller pitchers and

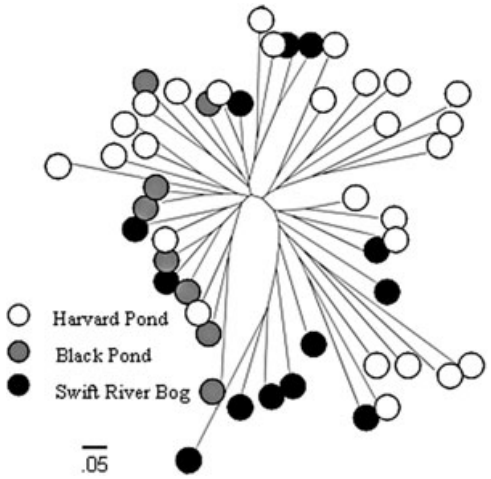


**Fig. 4.** Cluster analysis of T-RFLP peaks profiles shows modest clustering of bacterial assemblages according to their location ($A = 0.098$; $P = 0.000015$). The two locations that were the most similar were Harvard Pond and Black Pond Bog ($A = 0.053$; $P = 0.002$), which both have open water. The locations that were the least similar, with modest overlap on the dendogram, were Swift River Bog and Black Pond Bog ($A = 0.144$; $P = 0.022$). The dendograms are neighbour-joining dendograms obtained from a similarity matrix ($C_S$) from binary data. A distance of 0.05 indicates a 5% difference between samples.

 

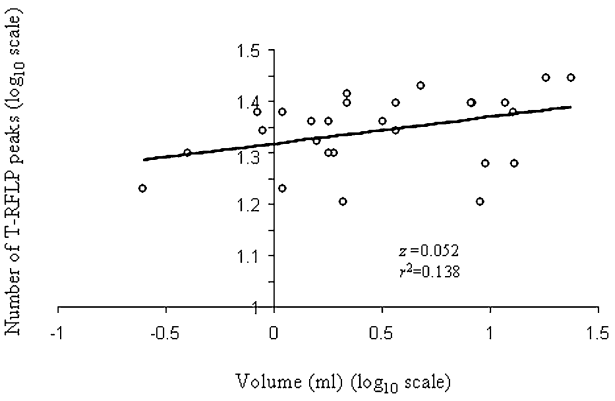


**Fig. 5.** Mean bacterial richness is weakly correlated with habitat volume with a *z*-value of 0.052 (d.f. = 26; $F = 3.993$; $P = 0.057$). Bacterial richness was estimated from the number of T-RFLP bands present in any single pitcher and habitat volume was measured as volume of liquid inside the pitcher.

the presence of the mosquito larvae [general linear model (GLM), d.f. = 24; $F = 4.254$, $P = 0.026$] and that Shannon diversity ($H'$) and species richness were very well correlated ($r^2 = 0.62$, $P = 1.2 \times 10^{-6}$). Together these two variables (height and mosquito presence) accounted for 26% of the variation in bacterial richness. The presence of mosquito larvae had only a marginally significant effect on bacterial richness or diversity at Harvard Pond ($P = 0.09$; Table 1) but there were only two pitchers with the predator there (the role of the mosquito larvae was explained more formally with data from the Swift River Bog; see below). Pitcher height was significantly associated with bacterial richness ($P = 0.026$; Table 1). There was no gain in explanatory power when a model incorporating pH or pH*height interactions was tested.

There was a significant association between pitcher height and pitcher volume ($r^2 = 0.73$, $P = 1.4 \times 10^{-8}$). Microbial alpha diversity analyses are often expressed as a species–volume relationship, using the volume power law $S = cV^z$ (where $S$ = number of species estimated by the number of T-RFLP peaks, $c$ = constant depending on the taxon and location, $V$ = volume, and $z$ is the slope of the log–log line) For the bacterial species–volume relationship in the pitcher plants, the *z*-value was 0.052 (d.f. = 26; $F = 3.993$; $P = 0.057$; Fig. 5).

In other analyses of the pitcher-plant communities, the keystone predator (*W. smithii* larvae) controls community assemblage of macroorganisms (Gotelli and Ellison, 2006). Because *W. smithii* larvae were only abundant in pitcher plants at Swift River Bog, we analysed the bacterial assemblages from this site for their association with this putative keystone predator in pitchers of approximately the same size. Bacterial richness increased when *W. smithii* larvae were present (d.f. = 12; $F = 7.713$; $P = 0.018$; Fig. 6A). Pitcher plants with *W. smithii* larvae averaged 23.6 peaks whereas plants without *W. smithii* larvae averaged only 16.4 peaks. MRPP analysis demonstrated a difference in bacterial composition between pitchers with and without *W. smithii* larvae ($A = 0.133$; $P = 0.022$). The ordination method non-metric multidimensional scaling (NMS) separated bacterial assemblages of pitcher plants with and without *W. smithii* (Fig. 6B). The key indicator fragments for this difference were 64, 73, 195 and 226 bp. According to the RDP database, both fragment sizes 64 and 67 are indicative of *Chloroflexi* spp., fragment size 195 is indicative of any of seven different bacterial genera (*Bradyrhizobium* sp., *Mortella* sp., *Nitrobacter* sp., *Psychromas* sp., *Rhodopseduomonas* sp., *Shewanella* sp. and *Vibrio* sp.) and fragment size 226 is indicative of four genera (*Desulfotomaculum*, *Paenibacillus*, *Streptomyces* and *Thermobispora*). Thus, the keystone predator appears to

**Table 1.** Individual statistics of the general linear model of parameters affecting bacterial richness.

| | Estimate | Standard error | *t*-value | Pr(> [*t*]) |
|---|---|---|---|---|
| Intercept | 17.526 | 2.019 | 8.682 | $7.2 \times 10^{-9}$ |
| Mosquito larvae | 4.037 | 2.314 | 1.745 | 0.094 |
| Height | 0.975 | 0.411 | 2.375 | 0.026 |

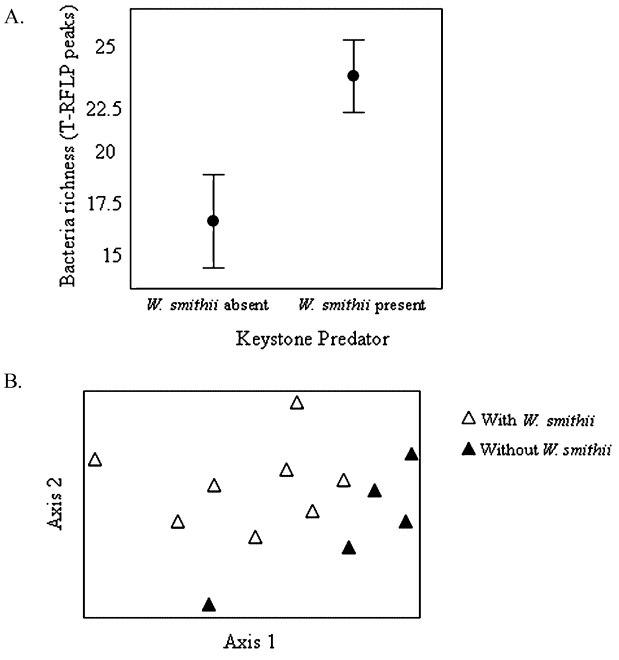


**Fig. 6.** A. Bacterial richness correlates with presence of the keystone predator (d.f. = 12; $F = 7.13$; $P = 0.018$). Bacterial richness was estimated by the number of T-RFLP peaks measured and the presence of the mosquito larvae was determined by examining the pitcher fluid ($n = 13$; 8 with mosquito; 5 without). Error bars indicate standard error of the means.
B. A non-metric multidimensional scaling (NMS) analysis of T-RFLP peaks profiles based on the Sørensen similarity index ($C_S$) from binary data demonstrates significant clustering of plants containing the keystone predator ($A = 0.133$; $P = 0.023$).

 

determine both the richness and community of the bacteria within the pitchers.

## Discussion

We sought to characterize and understand the forces controlling the structure of the bacterial assemblages that occur within pitchers of the carnivorous plant *S. purpurea*. In brief, we found that the unopened pitchers are sterile and the bacterial assemblages of each pitcher establish *de novo* as pitchers open. Although most fragments resulting from the T-RFLP analyses were singletons, measures of similarity suggested that bacterial assemblages from different pond sites were distinct from each other, but that those from different regions within a pond were not distinct from each other. Overall, the bacterial richness of the individual pitchers was determined by both the size of the pitcher and the presence of a keystone predator, but not by pH. The patterns observed here were similar to those described for macroscopic food webs. This suggests that many of the ecological principles that apply to macroorganisms also apply to bacteria, despite their small size.

Two fundamental tenets of community ecology posit that islands size and the presence or absence of keystone predators influence species diversity and the structure of food webs and ecological communities (Paine, 1966; MacArthur and Wilson, 1967). Our data support the hypothesis that pitcher-plant food webs are structured by the keystone predator *W. smithii* (Addicott, 1974; Gotelli and Ellison, 2006); the presence of the mosquito larvae is a good predictor of bacterial diversity within pitchers. It influences both the richness and the structure of bacterial communities. These results corroborates prior studies performed using only bacterial colony morphologies (Kneitel and Miller, 2002). However this molecular confirmation is important, as other field reports found that the trophic cascades were attenuated before they reached the bacterial level, both in this microecosystem (Gray *et al*., 2006) and in other ecosystems (Pace and Funke, 1991; Pace, 1993). It is possible that the effect of the *W. smithii* larvae could be direct as larval faeces contribute nutrients to the system, which are food for the bacteria. It is more likely that the effect of the predator is indirect through its effect as a predator on protozoa. Protozoan species richness declines with increasing density of mosquito larvae (Cochran-Stafira and von Ende, 1998) and protozoa often prey on bacteria (Matz *et al*., 2005; Kneitel, 2007). Experiments performed in the laboratory to directly manipulate protozoan assemblages in pitcher plants will help to clarify the role of *W. smithii* on pitcher-plant bacteria.

Larger pitchers have greater bacterial diversity and evenness of their distributions, and follow typical species–area curves. This trend is similar to what has been demonstrated for bacteria living in oil-filled sump tanks and within water-filled tree holes in the buttress root systems of European beech trees (Bell *et al*., 2005; van der Gast *et al*., 2005). The *z*-value in species–volume relationship recorded for the pitcher plants (0.052) was much lower than in the other studies of bacteria in 'islands' (~0.2) (Bell *et al*., 2005; van der Gast *et al*., 2005) but was higher than the value reported from continuous swamp marshes (0.04) (Horner-Devine *et al*., 2004). There could be several reasons why this *z*-value is lower than other values reported. First, it has been shown that islands subject to large stochastic effects have smaller *z*-values (Losos, 1998; Lomolino and Weiser, 2001). Migration of the bacteria into the pitchers may be more punctuated than continuous, especially if most of the bacteria are introduced by insects. Second, different methods were used in each study to measure species richness, and the method can affect *z*-values (Woodcock *et al*., 2006). We chose a T-RFLP approach which does not detect rare samples and can underestimate diversity; the other studies used denaturing gradient gel electrophoresis (DGGE), which is also a coarse method, and 16S rRNA clone libraries, which represent a small sample of the entire population (Ranjard *et al*., 2000). Nevertheless, the general trend is for bacterial T-RFLP fragment richness in isolated pitchers to follow the same species–area relationships as have been found for macroscopic organisms occurring on isolated islands.

Biogeographic patterning was found for the bacterial assemblages across sites but not within them. This result likely reflects local mixing of assemblages of bacteria that enter pitchers from the surrounding environment, by wind, water, or on insects. Our 16S rRNA analyses suggest that the bacterial populations present in the pitcher contain mostly generalist species associated with other habitats, similar to the other small organisms in the plant such as the rotifers and protozoa but in contrast to the macroarthropods, which specialize in the pitcher-plant environment (Cochran-Stafira and von Ende, 1998; Bledzki and Ellison, 2003). Given the broad distribution of types of bacteria found in the pitchers, it is possible that the plant does not exert any strong selection for any particular species. In systems where there are strong selective forces on community assembly, such as sponges secreting secondary metabolites, the symbioses are specific and only selected bacteria are recovered from the habitat (Taylor *et al*., 2004). When there is no selection, community composition may be extremely variable and follow patterns similar to those predicted by neutral community models (Sloan *et al*., 2006; Woodcock *et al*., 2007). In fact, six of the taxa found in the *S. purpurea* pitchers were also identified in a recent survey of 13 bacterial isolates from a different species of pitcher plant, *Sarrancia minor*

(Siragusa *et al*., 2007) – *Rhodococcus* (including *Rhodococcus equi*), *Serratia* (including *Serratia marcescens*), *Pantoea*, *Bacillus*, *Lactococcus* (including *Lactococcus lacti*) and *Chryseobacterium*. This suggests that similar bacteria are capable of inhabiting different pitcher-plant species, although at low frequencies in both cases.

In conclusion, the pitcher plant offers an attractive model system for theoretical and experimental work integrating bacterial ecology into food web concepts derived from studies of macroscopic organisms (Srivastava *et al*., 2004). The pitchers themselves can serve as 'natural replicates' and are simple enough to allow for direct manipulations of their volume and constituents. Moreover, nearly every other component of the pitcher food web has been extensively studied; characterization of the bacteria can help eliminate the 'black box' effect often found in food web studies, and allow for simultaneous study of every component of the system. Recent work shows that bacteria are responsible for the majority of nitrogen mineralization by the pitcher-plant food web (Butler *et al*., 2008). Future studies should involve experiments aimed at understanding the role that specific bacteria play in cycling the nutrients using functional gene approaches.

## Experimental procedures

### *Study area and sampling design*

Pitcher-plant samples were collected from three bogs in Massachusetts, USA. The most extensive sampling (26 pitchers) was performed at Harvard Pond in Petersham, MA. The second site (13 pitchers) was 28 km south at Swift River Bog in Belchertown, MA. The third site (seven pitchers) was at Black Pond Bog in Norwell, MA, 123 km east of Harvard Pond (Fig. 1). Detailed site descriptions are published elsewhere (Ellison and Gotelli, 2002).

Our sampling scheme was designed to examine spatial relationships while controlling for temporal variations. Newly opened pitchers were tagged and mapped in June 2006, and harvested 4 weeks later. These four weeks correspond to the approximate time it takes for the macroinvertebrate community to stabilize within a pitcher (Miller *et al*., 1994). Pitchers were selected for their spatial location and in the case of the Swift River Bog, for a size $> 15$ cm, as larger pitchers are more likely to host *W. smithii*. The distance between the pitchers was determined using a tape measure for distances less than 10 m and calculated from global positioning system (GPS) coordinates measured by a hand-held GPS unit with an accuracy of $\pm 3$ m. At harvest, the liquid from all the pitchers was removed and examined by eye for the presence of *W. smithii*. The pH and the volume of this liquid were noted and the length of the pitcher was measured from the bottom of the opening to the tip of the leaf. The pitchers were sliced open with a sterile razor blade, the prey was removed and the biofilm in the absorptive zone at the bottom of the pitcher was scraped with a sterile spatula (Fig. 1). Samples were then re-suspended in 20% glycerol and immediately frozen.

### *Assays of unopened pitchers*

Unopened pitchers were collected from all three sites in May and June 2006 by cutting the stem below the pitcher. We used both culture-based and molecular techniques to assay for bacteria growing inside the unopened pitchers. The inside of 14 pitchers was lavaged by inserting a needle into the pitcher and rinsing the inside with sterile $H_2O$. Lavages were plated on TSB (tryptic soy broth) agar and incubated at 30°C for 3 days. Aliquots (1 μl) were also used for PCR with 16S universal primers (see below for details). To confirm that the pitchers were impermeable to bacteria and that the lavage technique was sterile, the exteriors of additional pitchers were dipped in *E. coli* expressing mRFP from a plasmid and then lavages were plated on TSB plates. For culture-independent approaches, the lavages were used as template for 16S PCR (see below). For microscopy, unopened pitchers were sliced open vertically and the inside tissue was scraped out, stained with Syto9 which binds to membranes, and examined under a Nikon fluorescent microscope at 40× for cells that were the size and shape of bacteria.

### *Isolation of enteric species*

Samples from 15 pitchers were plated on MacConkey lactose agar plates and grown at 30°C for 3 days. Seven colonies from each plate were purified and their 16S gene was PCR amplified (see below) and sequenced. The 16S rRNA sequences of the isolates were compared with sequences in the NCBI database using the BLASTN algorithm and grouped based on 99% similarity. Sequences were aligned using the CLUSTALW program in the Alignment Explorer module of Mega 4.0 (Molecular Evolution Genetics Analysis Software, version 4; The Biodesign Institute, Tempe, AZ). Neighbour-joining trees were constructed using the Mega 4.0 Phylogeny construction program and the reliability of the tree branch points was assessed by bootstrap analysis of 1000 replicates.

### *DNA extraction*

DNA was extracted from the biofilms of open pitchers using a bead-beating protocol modified from the MoBio Soil DNA Isolation kit. Briefly, 300 μl of each biofilm sample was added to a 2 ml bead-beating tube containing beads from the MoBio Soil Kit (Madison, WI) and 60 μl of solution C1. Samples were bead beat for 30 s, followed by 30 s on ice and the process was repeated. The mixture was then centrifuged for 1 min at 10 000 *g*. The supernatant was collected and 750 μl of solution C2 was added to the supernatant; the mixture was incubated at 4°C for 5 min The mix was centrifuged again for 1 min at 13 000 r.p.m., the supernatant was collected again, and DNA was precipitated with ice-cold ethanol and 3 M sodium acetate. After centrifugation, the pellet was washed with 70% EtOH and re-suspended in TE.

### *Polymerase chain reaction*

Hot-start reactions were used for all PCR runs. For the 16S PCR, universal primers 27F (5′-AGAGTTTGATCMTG GCTCAG-3′) and 1492 (5′-TACGGYTACCTTGTTACGACTT-

3′) were used. Phusion DNA polymerase (Finnzyme, Finland) was used at the following cycles: denaturing for 10 s at 98°C, followed by annealing for 10 s at 53°C, and then extension for 30 s at 72°C. To avoid PCR bias in the T-RFLP analysis, each reaction was carried out in eight replicates for only 25 cycles; replicates were subsequently combined for further processing. Polymerase chain reaction product was purified using the Qiagen PCR purification kit.

### *Terminal restriction fragment length polymorphism*

T-RFLP of 16S rDNA PCR amplicons was performed. Briefly, 16S rDNA PCR was carried out as described above using a modified 27F primer with a 3′ FAM group. After replicates were combined, PCR products (~75 ng) were digested using the restriction enzyme HaeIII. MspI and RsaI (NEB, Beverly, MA) enzymes were also tested but it was found that HaeIII digestion resulted in the largest number of peaks and best resolution of the community structure. The labelled fragments were separated and detected with an ABI 3700 96 capillary sequencer running in GeneScan mode. T-RFLP electropherograms were inspected with the software Genemapper (version 4.0, Applied Biosystems, Foster City, CA). Only peaks of 50–510 bp were used in the multivariate statistical analysis. Two thresholds cut-offs were used to determine the significance of the peaks: a variable percentage threshold method (0.075) which calculated the threshold for each sample individually based on its total fluorescence (Osborne *et al*., 2006) and a flat 1% cut-off that applied the same threshold value to all samples. Both approaches gave the similar results. Peaks with < 0.5 bp differences were considered to be of the same length, due to slight differences in running times on the capillary sequencer, with the Microsoft Excel macro Treeflap (http://www.sci.monash.edu.au/wsc/staff/walsh/treeflap.xls) (Rees *et al*., 2004).

### *Statistical analyses*

In order to understand how species richness varied, T-RFLP data were transformed to a binary set encompassing the presence/absence data for each reproducible peak in every community (Dunbar *et al*., 2001). The richness index of each sample was calculated by summing of all the peaks in each sample profile. To calculate the Shannon diversity index $H'$, raw T-RFLP data were used with the height of the peaks as a measure of abundance. A Kolmogorov–Smirnov test was used to determine that data fit a normal distribution. Height and volume were found to be correlated ($r^2 = 0.73$, $P = 1.4 \times 10^{-8}$) as were richness and Shannon diversity ($r^2 = 0.62$, $P = 1.2 \times 10^{-6}$). Correlations between richness and different observation groups (presence/absence of the keystone predator, pH and height of pitcher) were analysed with a general linear model that was selected by minimizing Akaike's Information Criterion (AIC) on a step-wise regression (function stepAIC in the MASS library of the R software package, version 2.6.1: http://www.r-project.org/), which started with the full model of richness = mosquito + pH + height + pH*height. Volume was not included in this model, as it is highly correlated with pitcher height [log(volume) = –1.9 + 0.67*height ; $r^2 = 0.73$]. The best model proved to be richness = mosquito + height (reduction of AIC from 147.05 in the full model to 143.4 in the reduced model). Additional linear regressions and ANOVA were carried out in SPSS (SPSS, Chicago, IL).

Multidimensional community structure data were analysed with the binary sets (Dunbar *et al*., 2001). Profiles were analysed using PC-ORD version 5.0 (MJM software, Oregon). Profiles were transformed to achieve normality and analysed with MRPP to determine if there were significant differences between the groups of the different sites and the presence/absence of the keystone predator (McCune and Grace, 2002). To graphically assess the differences in community composition with the keystone predator, the ordination method of NMS was applied (McCune and Grace, 2002). The Mantel test was also conducted in PC-ORD, using Monte Carlos tests (999 randomized runs) to determine significance. The MRPP, NMS and Mantel test all used the Sorensen distance index; $C_S = 2N_{ab}/(N_a + N_b)$ where $N_{ab}$ is the number of shared peaks and $N_a$ and $N_b$ are the number of total peaks in each sample A and B respectively. Data from the different sites were exported into MEGA 4.0 (Molecular Evolution Genetics Analysis Software, version 4; The Biodesign Institute, Tempe, AZ) to generate neighbour-joining dendograms (Wawrik *et al*., 2007).

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