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Bacterial diversity and composition in the fluid of pitcher plants of the genus *Nepenthes*

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ABSTRACT

Pitchers are modified leaves used by carnivorous plants for trapping prey. Their fluids contain digestive enzymes from the plant and they harbor abundant microbes. In this study, the diversity of bacterial communities was assessed in *Nepenthes* pitcher fluids and the composition of the bacterial community was compared to that in other environments, including the phyllosphere of *Arabidopsis*, animal guts and another pitcher plant, *Sarracenia*. Diversity was measured by 454 pyrosequencing of 16S rRNA gene amplicons. A total of 232,823 sequences were obtained after chimera and singleton removal that clustered into 3260 distinct operational taxonomic units (OTUs) (3% dissimilarity), which were taxonomically distributed over 17 phyla, 25 classes, 45 orders, 100 families, and 195 genera. Pyrosequencing and fluorescence in situ hybridization yielded similar estimates of community composition. Most pitchers contained high proportions of unique OTUs, and only 22 OTUs (<0.6%) were shared by ≥14/16 samples, suggesting a unique bacterial assemblage in each pitcher at the OTU level. Diversity analysis at the class level revealed that the bacterial communities of both opened and unopened pitchers were most similar to that of *Sarracenia* and to that in the phyllosphere. Therefore, the bacterial community in pitchers may be formed by environmental filtering and/or by phyllosphere bacteria.

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Introduction

Carnivorous pitcher plants, such as those in the genera *Nepenthes* and *Sarracenia*, have fascinated biologists for centuries because of their unique characteristics [10,28]. The pitchers contain fluid to drown insects and other small invertebrates, which are then digested by the action of secreted enzymes [28]. The pitcher fluid of *Nepenthes* is acidic, generally ranging from pH 2 to 6 depending on the species [65]. The fluid contains high concentrations of digestive enzymes derived from the plant [21,28], as well as debris from prey [62], which may result in anaerobic conditions at the bottom of the pitcher [43] and determine nutrient availability. Thus, the fluid appears to be a complex and extreme habitat, which has also been reported to harbor a high density of bacteria (i.e. up to $\sim 10^8$ cells mL^{-1}) in mature pitchers [62,65]. It is suggested that the microbes present in the fluid could contribute significantly to the quantities of enzymes produced and might play a role in prey digestion and nutrient mineralization [65]. Recent molecular-based

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studies have revealed a diverse and complex microbial community assemblage in the fluid of pitchers of *Nepenthes* [8] and *Sarracenia* [30–33]. However, the full picture of the community, including minor species, is still unclear because of sample size limitations (i.e. the low number of sequences identified).

The pitchers of *Nepenthes* and *Sarracenia* show striking functional similarity [28], although they originated independently in the evolution of angiosperms [15]. However, we hypothesized that *Nepenthes* and *Sarracenia* could have different microbial biota because of certain specific characteristics. First, the fluid in unopened *Nepenthes* pitchers may already contain microbes ([8,62]; but see [6]), the origin of which is still uncertain, although they may come from the phyllosphere. In contrast, it was reported that *Sarracenia* pitchers are sterile before opening [22,49,50], so prey and/or debris falling into the pitchers may facilitate the initial microbial colonization after pitcher opening. Second, the fluid in *Nepenthes* contains proteins with antibacterial effects [21] and high concentrations of various digestive enzymes [2,65]. In *Sarracenia*, the efficiency of digestive enzymes in the fluid may be limited [2,61], although their activity remains unclear. Therefore, the factors that shape the microbial communities are likely to differ between *Nepenthes* and *Sarracenia*, which may result in the dissimilar composition of their microbial communities.

Since the pitchers of carnivorous plants are responsible for digestion of prey, they function like the stomach in animals. Thus, a functional analogy between the pitchers and animal guts has been proposed [10,42]. Plant pitchers and animal gut (e.g. human [16] and mouse [41]) may both contain similar microbiota because they share an acidic environment where nutrients are abundant. Koopman and Carstens [30] recently highlighted the similarity between the human gut flora and that of *Sarracenia* in which four abundant bacterial phyla are shared (*Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*), although the similarity was not assessed quantitatively.

The present study aimed to characterize the diversity and composition of bacterial communities in the pitcher fluids of *Nepenthes* species (Fig. 1). To explore the range of diversity, four *Nepenthes* species were examined using various samples, such as pitchers from natural (Borneo) and cultivated (Zurich) environments and those at different developmental stages. It was expected that the pitcher fluid would contain bacterial taxa that would be able to survive in acidic, nutrient-rich, and partially anaerobic environments. The microbial community composition was investigated by 454 pyrosequencing of bacterial 16S rRNA gene amplicons. Fluorescence in situ hybridization (FISH) was also used to estimate the abundance of specific taxa, and the results were compared with the 454 pyrosequencing results. The level of operational taxonomic unit (OTU) diversity and the class-level composition were compared with comparable published data from other habitats. In particular, we examined whether the independently evolved pitchers of *Nepenthes* and *Sarracenia*, as well as animal guts, harbored a similar bacterial composition. Phyllosphere bacteria were also considered from the viewpoint of development because pitchers are modified leaves.

Materials and methods

Sample collection and preparation

A total of 16 samples were used from four *Nepenthes* species: *N. albomarginata* T. Lobb ex Lindl., *N. ampullaria* Jack, *N. mirabilis* var. *echinostoma* Adam and Wilcock (hereafter, *N. mirabilis*), and *N. hirsuta* Hook.f. Samples were given four-character identifiers, with the first two characters representing the species: "AL," "AM," "HI," and "MI" for *N. albomarginata*, *N. ampullaria*, *N. hirsuta*, and *N. mirabilis*, respectively. The third represented a replicate of the mature (opened) sample, 1–4, if any, or the developmental stages, "U" and "D" for unopened and dead, respectively. The last character represented the location: "B" and "Z" for Borneo and Zurich, respectively, except for *N. mirabilis* (see Table 1). For example, one mature pitcher of *N. albomarginata* from Borneo was identified as "AL1B." The samples were collected in or around Lambir Hills National Park (LHNP), Sarawak, Malaysia, Borneo (4°2′N, 113°50′E; 150 m above sea level). Of the four *N. mirabilis* samples collected in LHNP, two were supplied with ants and left for 24 h in the laboratory, whereas the other two served as negative controls without ants. The sample ID of the last character is "A" or "C" for adding ants or negative control, respectively. The goal of this experiment was to analyze the effect of prey addition on the bacterial community, although a recent report by Sirota et al. [60] suggested that 24 h could represent only a very early response (see SI Appendix A.10). Fluid samples were collected from each pitcher in the field using a pipette (see details in SI Appendix A.1). Fluid was also collected from cultivated *N. albomarginata* and *N. ampullaria* plants in a greenhouse in the Botanical Garden at the University of Zurich, Zurich, Switzerland (47°21′N, 8°33′E; 440 m above sea level). The bacterial density was determined using 4′,6-diamidino-2-phenylindole (DAPI) staining and the pH of the fluids was also measured (SI Appendix A.2; Fig. 1c and d).

Culture-independent approaches were used to determine the bacterial diversity and community structures of the samples (Fig. 1e). After extracting DNA from the fluid (SI Appendix A.3), DNA in the V5–V6 16S rRNA hypervariable regions (length in *Escherichia coli* ~280 bp) was amplified using the general primers for bacteria, according to the method described by Stecher et al. [64] (see also SI Appendix A.4). Pyrosequencing was conducted using a 454 Life Sciences Genome Sequencer FLX (Roche, Basel, Switzerland). Details of the methods used for DNA extraction, purification, polymerase chain reaction (PCR), and pyrosequencing are provided in SI Appendixes A.3 and A.4.

OTU definition and taxonomic assignment

OTUs were defined by using read-quality filtering to avoid inflating the diversity, which is caused partially by sequencing errors, and putative chimeric sequences were detected using a sequence similarity approach. All the non-redundant reads were aligned to a reference alignment in the RDP database [9] using the Infernal aligner [14]. OTUs were identified by hierarchical cluster analysis at various distances (0.01, 0.03, 0.05, and 0.10) using complete linkage clustering, and singleton OTUs were removed from subsequent analyses as these may have resulted from sequencing errors. A consensus taxonomy was assigned to each OTU using the prediction of the RDP classifier and based on reference sequences if present in a given OTU. Full details of the bioinformatics analysis methods are described in SI Appendices A.5–A.7.

Data analysis

The bacterial taxonomic diversity was estimated using Hill's numbers (i.e. the orders of diversity (D), ${}^{q}D$) or effective numbers of species [23]. D is generally a useful diversity index if $q = 0$, 1, or 2 [27] (i.e. $q = 0$ indicates species richness, $q = 1$ represents the exponentially transformed Shannon–Weaver index, and $q = 2$ corresponds to the reciprocal Gini–Simpson index). The bacterial diversity was also compared to that of other environments using rarefaction curves (see details in SI Appendix A.8).

Non-metric multidimensional scaling (NMDS)

NMDS was used to compare the similarity between *Nepenthes* samples and those from other environments based on their

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