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

Yayoi Takeuchi[a,1], Samuel Chaffron[b,2], Michaela M. Salcher[c,3], Rie Shimizu-Inatsugi[a], Masaki J. Kobayashi[a,4], Bibian Diway[d], Christian von Mering[b], Jakob Pernthaler[c], Kentaro K. Shimizu[a,*]

[a] *Institute of Evolutionary Biology and Environmental Studies and Institute of Plant Biology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland*
[b] *Institute of Molecular Life Sciences and Swiss Institute of Bioinformatics, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland*
[c] *Limnological Station, Institute of Plant Biology, University of Zurich, Seestrasse 187, 8802 Kilchberg, Switzerland*
[d] *Botanical Research Centre Semenggoh, Km 20 Borneo Height Road, 93250 Kuching, Sarawak, Malaysia*

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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.

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

* Corresponding author at: Institute of Evolutionary Biology and Environmental Studies and Institute of Plant Biology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. Tel.: +41 44 635 6740; fax: +41 44 635 6821.
E-mail address: kentaro.shimizu@ieu.uzh.ch (K.K. Shimizu).
[1] Current address: Center for Environmental Biology and Ecosystem Studies, National Institute for Environmental Studies, Onogawa 16-2, Tsukuba 305-8506, Japan.
[2] Current address: VUB Laboratory of Microbiology and VIB Center for the Biology of Disease, K.U.Leuven – O&N4 Campus Gasthuisberg, 3000 Leuven, Belgium.
[3] Current address: Biology Centre of the Academy of Sciences of the Czech Republic, Institute of Hydrobiology, Na Sadkach 7, 370 05 Ceske Budejovice, Czech Republic.
[4] Current address: Biotechnology Research Institute for Drug Discovery, National Institute of Advanced Industrial Science and Technology (AIST), AIST Tokyo Waterfront Bio-IT Research Building, 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan.

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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

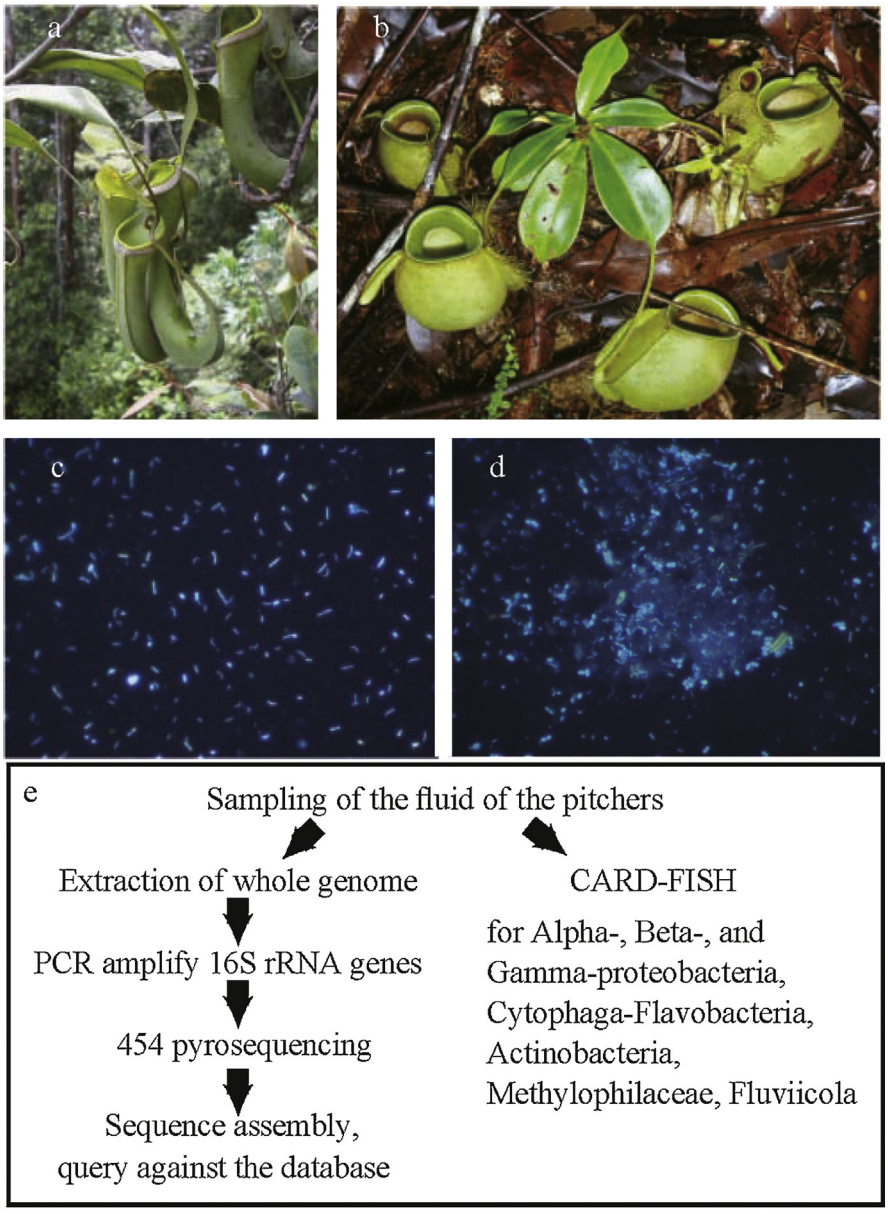

Fig. 1. The photographs show: (a) *Nepenthes albomarginata*; (b) *N. ampullaria*; (c) epifluorescence microscopy of the fluid microbiota of a pitcher from *N. albomarginata* stained with DAPI (see SI Appendix A.2); (d) the fluid of another pitcher from the same individual; (e) schematic diagram of the procedure used to estimate the bacterial diversity and composition of pitcher plant fluids.

bacterial class-level composition [34]. The microbial community compositions were compared from farm soil [67], termite gut [25], coastal seawater [1], human gut [12], phyllosphere of *Arabidopsis thaliana* (which is similar to those of soybean and clover) [11], mouse gut [64], *Sarracenia alata* [30], and the Amazon River [20]. To standardize the sample size (i.e. the number of sequences), 440 sequences were resampled, which was the minimum sample size in all datasets. For each sample, the average Jaccard index (presence/absence) and Bray–Curtis distance (abundance) using 1000 iterations were also computed. NMDS was calculated with the function isoMDS in the R package "MASS" using the default parameters (two-dimensional solution; maximum number of random starts = 50). NMDS was performed using the OTU count data (10,000 resampled sequences from each sample with 1000 iterations) within the *Nepenthes* samples. All statistical analyses and computations were performed using R [51].

Phylogenetic tree reconstruction for common bacteria

All OTUs detected in at least 14 out of 16 samples (see Fig. 2) were used for phylogenetic tree reconstruction, which refined the identification of OTUs and placed them in the context of closely related taxa. Three or more almost full-length reference sequences were aligned for each OTU using the SINA web-aligner (http://www.arb-silva.de) and they were imported into the ARB reference database SILVA SSU108 [39]. Alignments were optimized manually, and sequences were added to the reference maximum parsimony tree. Based on this tree, the closest high-quality related sequences in each OTU (i.e. sequence lengths >1200, pintail value >95, and alignment quality >95) and sequences that were previously obtained from other pitcher plant samples [32,59] were used to reconstruct a bootstrapped maximum likelihood tree (the randomized accelerated maximum likelihood, RAxML, using the GTRGAMMA

Table 1
Summary of taxonomic diversity, total number of sequences, diversity index, microbial density, and fluid pH in 16 samples of *Nepenthes*. The numbers of unique taxa in the samples are shown in brackets. OTUs were defined by 3% dissimilarity.

Sample ID	AL1B	AL2B	AL1Z	AL2Z	AM1B	AM2B	AM1Z	AM2Z
Sample code	pp01	pp02	pp03	pp04	pp05	pp06	pp08	pp09
Species	*N. albomarginata*	*N. albomarginata*	*N. albomarginata*	*N. albomarginata*	*N. ampullaria*	*N. ampullaria*	*N. ampullaria*	*N. ampullaria*
Place	Borneo	Borneo	Zurich	Zurich	Borneo	Borneo	Zurich	Zurich
Phylum	6 (0)	7 (0)	13 (0)	16 (1)	7 (0)	11 (0)	8 (0)	8 (0)
Class	11 (0)	12 (0)	17 (0)	24 (1)	12 (0)	16 (0)	14 (0)	13 (0)
Order	18 (0)	16 (0)	22 (0)	37 (1)	23 (0)	26 (0)	25 (1)	18 (0)
Family	31 (1)	26 (0)	37 (0)	61 (5)	38 (2)	49 (2)	49 (2)	27 (1)
Genus	42 (6)	32 (0)	47 (2)	82 (11)	45 (4)	65 (2)	69 (6)	32 (2)
OTUs	407 (45)	400 (35)	590 (111)	875 (198)	484 (119)	622 (56)	566 (176)	238 (18)
Number of sequences	16,292	12,383	23,929	12,605	14,131	13,752	11,599	16,500
ACE (SE)	536.6 (32.3)	523.8 (28.8)	672.3 (18.0)	1087.6 (33.5)	581.9 (22.1)	1022.5 (68.7)	656.3 (20)	339.9 (32.5)
Chao1 (SE)	528.8 (12)	544 (12.4)	711.6 (13.1)	1170.4 (17.9)	619.5 (13)	1080.8 (20.4)	684.8 (12.6)	344 (10.2)
^0D	407	400	590	875	484	622	566	238
^1D	3.77	3.89	3.89	4.67	3.82	4.61	4.24	3.49
^2D	13.13	21.26	16.93	21.84	17.19	30.31	25.43	14.97
Cell density (cells mL^{-1})	1.20E + 08	2.00E + 08	1.45E + 07	2.09E + 05	2.12E + 06	7.38E + 06	4.19E + 07	4.25E + 07
pH	5	5	7	7	6	6	6	5

AMDZ	AMUB	AMUZ	HIB	MI1 C	MI2C	MI3A	MI4A	Total
pp10	pp11	pp12	pp13	pp14	pp15	pp16	pp17	Total
N. ampullaria	*N. ampullaria*	*N. ampullaria*	*N. hirsuta*	*N. mirabilis*	*N. mirabilis*	*N. mirabilis*	*N. mirabilis*	
Zurich dead	Borneo not open	Zurich not open	Borneo	Borneo 24 h	Borneo 24 h	Borneo add ants, 24 h	Borneo add ants, 24 h	
9 (0)	7 (0)	8 (0)	10 (0)	6 (0)	10 (0)	6 (0)	8 (0)	17
14 (0)	12 (0)	14 (0)	16 (0)	10 (0)	15 (0)	10 (0)	14 (0)	25
24 (0)	17 (0)	27 (0)	24 (0)	12 (0)	22 (0)	16 (0)	21 (1)	45
42 (0)	29 (2)	49 (1)	49 (1)	20 (0)	36 (1)	22 (1)	38 (2)	100
55 (1)	36 (2)	75 (11)	66 (10)	22 (1)	41 (1)	26 (2)	50 (5)	195
521 (57)	467 (47)	607 (317)	758 (159)	325 (172)	342 (33)	254 (21)	385 (56)	3260
12,372	15,439	14,347	17,347	15,605	10,248	14,855	11,419	232,823
698 (36.1)	657.1 (38.9)	670.1 (15.3)	981.3 (37.1)	348.3 (8.9)	473.3 (31.9)	327 (23.2)	498.6 (27.8)	
741.1 (14.8)	706.1 (14.9)	691.6 (12.2)	1035.7 (17.4)	355 (8.6)	534.8 (13.2)	330.3 (9.3)	524.4 (12.2)	
521	467	607	758	325	342	254	385	
3.80	3.64	4.26	4.61	2.76	2.99	3.20	2.57	
7.04	10.58	21.00	36.52	3.72	4.79	10.01	3.51	
1.67E + 06	9.63E + 05	1.46E + 06	4.83E + 09	1.87E + 08	2.44E + 07	3.73E + 08	1.82E + 08	
7	6	7	6	–	–	–	–	

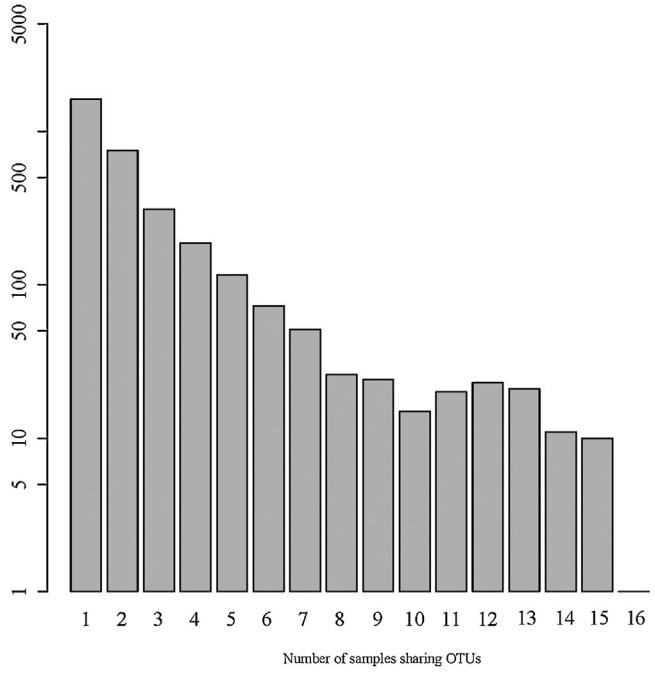

Fig. 2. The numbers of OTUs shared between pitchers. The *x*-axis shows the number of *Nepenthes* samples sharing OTUs and the *y*-axis shows the number of OTUs. OTUs were identified at 3% dissimilarity.

substitution model), which was inferred via a dedicated web server [63].

Fluorescence in situ hybridization followed by catalyzed reporter deposition (CARD–FISH)

To evaluate the distortion of the relative abundances of sequences caused by PCR amplification and sequencing analysis, the bacterial community composition was analyzed in selected samples using CARD–FISH and the results were compared with those from pyrosequencing. In this study, *N. ampullaria* pitcher fluids from Borneo and Zurich were sampled during November 2009 ($n = 12$). The general horseradish peroxidase-labeled oligonucleotide probes used are summarized in Table 3. The probe CF319a targets only *Cytophaga–Flavobacteria*, which comprise only part of all *Bacteroidetes* (44%), so the FISH method might have underestimated the total abundance of *Bacteroidetes*. To overcome this problem, only 454 sequences affiliated with families targeted by the probe (i.e. *Porphyromonadaceae*, *Cryomorphaceae*, *Flavobacteriaceae*, and *Sphingobacteriaceae*) were used for comparison, while *Crenotrichaceae*, *Flexibacteriaceae*, and *Prevotellaceae* were omitted. CARD–FISH was performed as previously described [48] using fluorescently labeled tyramides (Invitrogen, Carlsbad, CA, USA). Filters were counterstained with DAPI and evaluated by automated microscopy, as described by Salcher et al. [57].

The proportions of *Actinobacteria*, *Alpha-*, *Beta-*, and *Gammaproteobacteria*, and *Bacteroidetes* identified by CARD–FISH were

Table 2
Mean percentage of sequences for each bacterial taxon in 16 *Nepenthes* samples and standard error of the mean (S.E.M).

Domain	Phylum	Class	Percentage	S.E.M
Bacteria	*Acidobacteria*	*Acidobacteria*	0.29	0.03
	Actinobacteria	*Actinobacteria*	9.50	0.26
	Bacteroidetes	*Bacteroidetes*	0.55	0.06
		Flavobacteria	6.46	0.23
		Sphingobacteria	6.82	0.20
		Unclassified	1.09	0.09
	Chlamydiae	*Chlamydiae*	0.23	0.02
	Chloroflexi	*Caldilineae*	0.00	0.00
	Cyanobacteria	*Cyanobacteria*	1.10	0.07
	Deinococcus-Thermus	*Deinococci*	0.15	0.02
	Firmicutes	*Bacilli*	2.96	0.14
		Clostridia	11.59	0.13
		Erysipelotrichi	0.01	0.00
		Unclassified	0.08	0.02
	Fusobacteria	*Fusobacteria*	0.05	0.01
	Gemmatimonadetes	*Gemmatimonadetes*	0.04	0.01
	Nitrospira	*Nitrospira*	0.03	0.01
	OD1	OD1 genera *incertae sedis*	0.08	0.01
	OP10	OP10 genera *incertae sedis*	0.11	0.02
	Planctomycetes	*Planctomycetacia*	0.02	0.01
	Proteobacteria	*Alphaproteobacteria*	9.04	0.23
		Betaproteobacteria	9.03	0.27
		Deltaproteobacteria	0.04	0.01
		Epsilonproteobacteria	0.03	0.01
		Gammaproteobacteria	26.66	0.37
		Unclassified	0.78	0.07
	TM7	TM7 genera *incertae sedis*	0.31	0.04
	Verrucomicrobia	*Verrucomicrobiae*	0.21	0.03
	Unclassified		12.75	0.28

compared with the pyrosequencing results using Kruskal–Wallis tests to determine significant differences between the two methods for all samples from *N. ampullaria* ($n = 12$ and $n = 7$ by CARD–FISH and pyrosequencing, respectively), *N. ampullaria* from Borneo ($n = 6$ and $n = 3$ by CARD–FISH and pyrosequencing, respectively) and *N. ampullaria* from Zurich ($n = 6$ and $n = 4$ by CARD–FISH and pyrosequencing, respectively). Similar estimates of the relative abundances of taxa were expected using both methods if there were few artefacts due to PCR and amplicon sequencing.

Results and discussion

Characteristics of the microbial communities in Nepenthes *pitcher fluids*

A total of 232,823 reads were obtained after read-quality filtering and singleton removal, and 105,334 were non-redundant. These sequence data have been submitted to the Short Read Archive (SRA) under BioProject ID PRJNA225539. The frequency of chimeras was 8.29% of the total reads and 11.3% of the non-redundant reads (SI Appendix A.7). Only the quality-controlled reads were aligned, and 17 phyla, 25 classes, 45 orders, 100 families, and 195 genera were identified (excluding unclassified data, Table 1). In general, the bacterial communities in the fluids were dominated by the phyla *Proteobacteria* and *Bacteroidetes* (Table 2), with the exceptions of AM1Z and MI1C, which were dominated by *Firmicutes* (Table A.1). On average, 13% of the sequences were unclassified at the phylum level (Table 2).

Gammaproteobacteria was the most abundant subclass of *Proteobacteria*, possibly because genomes of this class often harbor significantly more copies of the 16S rRNA gene than other proteobacterial genomes [17,69].

In total, 3260 distinct OTUs were identified (3% dissimilarity, Table 1). The nonparametric estimators of species richness, ACE and Chao1, were higher than the total number of OTUs in each sample, which suggested that more OTUs might have existed. All samples contained pitcher-specific OTUs (Table 1), that is, the number of unique OTUs present in only one sample was 1620. The bacterial cell density of samples ranged from 2.09×10^5 to 4.83×10^9 cells mL^{-1}, while the pH values ranged from 5 to 7 (Table 1). No significant correlation was found between cell number and diversity index or between pH and diversity index (GLM, Holm-adjusted P-value <0.05).

Common taxa in Nepenthes

Each OTU was shared by 2.4 pitchers on average, and over half of the OTUs were present in only a single pitcher (Fig. 2). Twenty-two OTUs were detected in at least 14 out of 16 samples (Fig. 2), whereas only one OTU was present in all 16 samples. However, these 22 OTUs were not present in the samples in consistent abundance (Fig. A.1). Most of the core bacterial community in pitcher fluids (14 of the 22 common OTUs, Fig. 3) was affiliated with *Acinetobacter* spp. Close relatives of these OTUs occur in a wide range of habitats, such as sediment, sludge, gut, feces, mycorrhiza, rhizosphere, phyllosphere, soil, compost, biofilm, dust, freshwater, and marine waters. One of the 22 OTUs that affiliated with *Acinetobacter* was most abundant in all samples except for AL2Z, in which it was the second most abundant OTU (Fig. A.1). Three more common gammaproteobacterial OTUs were affiliated with *Pseudomonas* spp., and related genotypes are known from a variety of habitats. *Acinetobacter* spp. and *Pseudomonas* spp. are common on leaf surfaces [70] and some members of these genera are known to be typical copiotrophs (i.e. they grow rapidly in high nutrient and substrate concentrations) but may be susceptible to grazing by bacterivorous protists. Some *Acinetobacter* spp. and *Pseudomonas* spp. have also been detected in dust and clean room samples [36,54,66], so they may be ubiquitous airborne microbes that are dispersed by atmospheric deposition, and they grow rapidly if conditions meet their requirements. Atmospheric deposition was reported previously for microbes living in the air–water interface of lakes [26] and for phyllosphere microbes such as *Pseudomonas syringae* [24]. However, leaf-associated *P. syringae* can also be seed-borne or dispersed via insects [24], which suggests other possible dispersal scenarios for pitcher plant-associated microbes. One core OTU was closely related to *Methylophilus methylotrophus* and *M. rhizosphaerae*, which are methylotrophic bacteria that usually live in various freshwater habitats [57,58], sediments [29], the rhizosphere, and soil [38]. Methylotrophic bacteria are also common in the phyllospheres of different plants, and they can be dominant microbes on leaf surfaces, although these are usually different taxa (i.e. *Methylobacterium* sp., *Alphaproteobacteria*) [70]. *Methylobacterium* spp. were also present in some but not all of the pitchers (i.e. 6/16 samples).

Three OTUs affiliated with *Bacteroidetes* were classified as *Pedobacter* spp. (one OTU), which are typical phyllosphere, freshwater, and soil organisms [4,35,70], or *Fluviicola* spp. (two OTUs), which seem to be restricted to freshwater [46,53,68,71]. High densities of *Fluviicola* spp. were detected in a large lake during a bloom of *Cyanobacteria* [56] or diatoms [13], although they were mostly below the detection limit in non-bloom situations. Thus, these

Table 3
The HRP-labeled oligonucleotide probes used in CARD–FISH.

Probe	Target taxa	Reference
HGC69a	*Actinobacteria*	[3,55]
CF319a	*Cytophaga-Flavobacteria*, *Bacteroidetes* (CFB)	[3,45]
ALF968	*Alphaproteobacteria*	[3,44]
BET42a	*Betaproteobacteria*	[3,40]
GAM42a	*Gammaproteobacteria*	[3,40]
MET-1217	*Methylophilaceae*, *Betaproteobacteria*	[19]
Flu-736	*Fluviicola*, *Cryomorphaceae*, *Bacteroidetes*	[56]

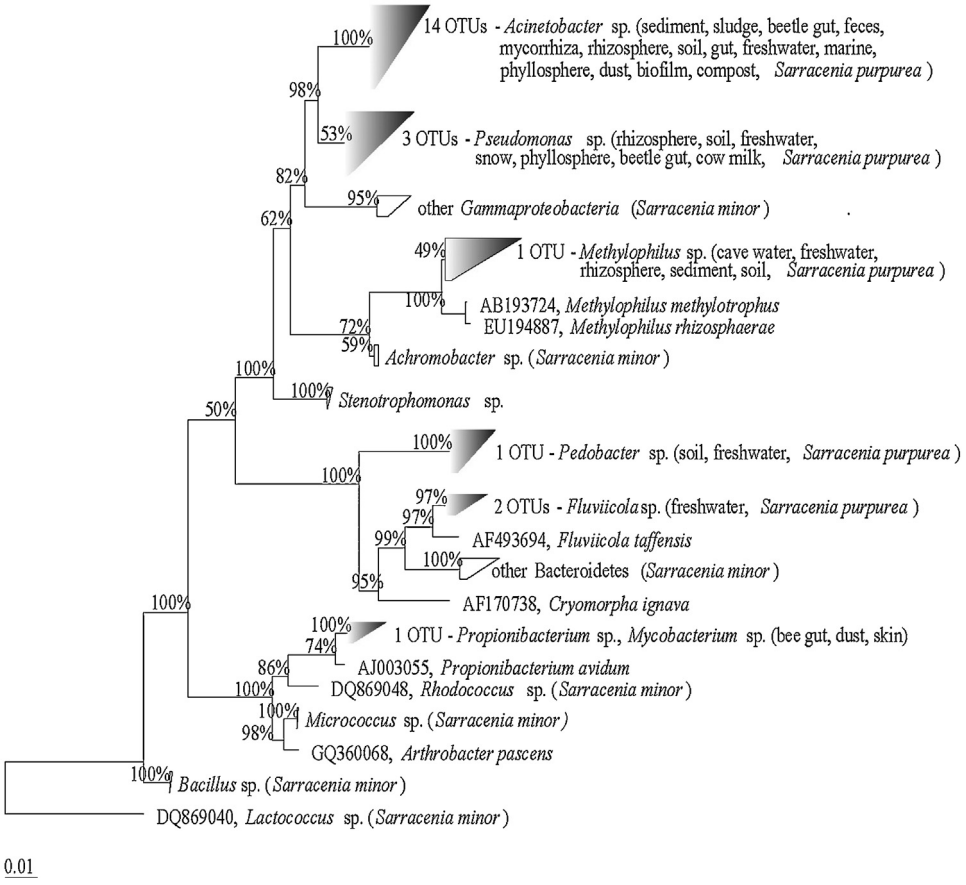

Fig. 3. Bootstrapped maximum likelihood tree for 22 common OTUs found in the fluids of *Nepenthes* spp. The number of common OTUs and habitats of closely related bacteria, including *Sarracenia purpurea* [32] and *S. minor* [59], are given for each cluster. The scale bar at the bottom represents 1% sequence divergence.

microbes appear to be closely associated with primary producers [13]. One OTU was closely related to *Propionibacterium* spp. and *Mycobacterium* spp., both of which are present in insect guts, skin and dust, although they are also known to be typical contaminants in PCR assays (see also SI Appendix A.9).

Diversity and bacterial community composition in Nepenthes

The rarefaction curves indicated that the bacterial diversity of pitcher plants was lower than that in the soil, termite gut, and the ocean, and similar to that in the *Arabidopsis* phyllosphere and mouse gut, but higher than that in the human gut (Fig. 4). The oceanic microbial community is considered to allow free microbial dispersal [18], whereas phyllosphere communities will constrain the colonization or survival of external bacterial species [11]. Similar levels of diversity in the phyllosphere community might be explained by environmental filtering of the bacterial community (i.e. the habitat selects the specific species that are adapted to the local environment) and/or a low microbial migration rate in the pitcher fluid.

The bacterial taxonomic compositions of pitcher plant fluids were compared with other environments at the bacterial-class level using NMDS ordination (Fig. 5a, Fig. A.2a). With the exception of one clear outlier sample (MI1C), all the bacterial communities in *Nepenthes* fluid clustered closely with that of another pitcher plant, *S. alata*, although these genera grow in different environments and have different characteristics, as previously described. Several OTUs of the *Nepenthes* core microbiota were also found in the fluid of *Sarracenia* spp. (e.g. *Acinetobacter* spp., *Pseudomonas* spp., *Fluviicola* spp., *Pedobacter* spp., and *Methylophilus* spp.) (Fig. 3), and these bacteria might be typically associated with different pitcher plant species. In addition, the bacterial communities in the phyllosphere were similar to those in pitcher plants, which suggested that the phyllosphere community might affect the community in

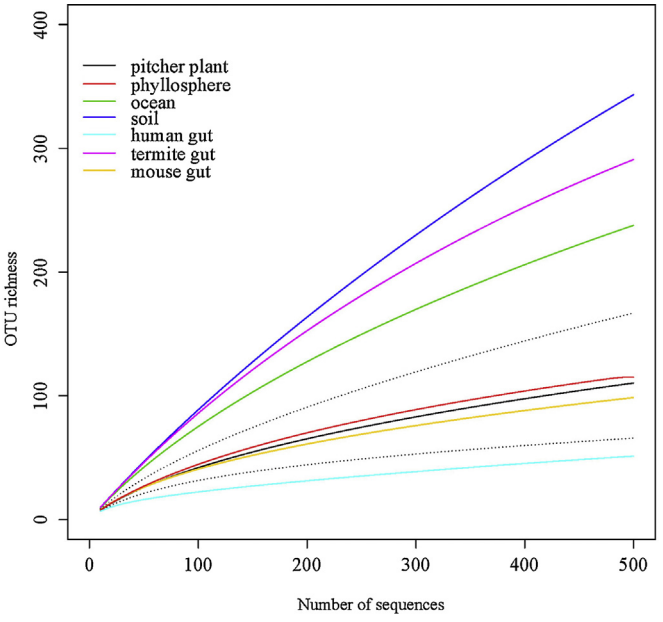

Fig. 4. Rarefaction curves of *Nepenthes* and other environments. The solid black line indicates the average richness, while the black dashed lines indicate the maximum (upper) and minimum (lower) richness for 16 *Nepenthes* samples.

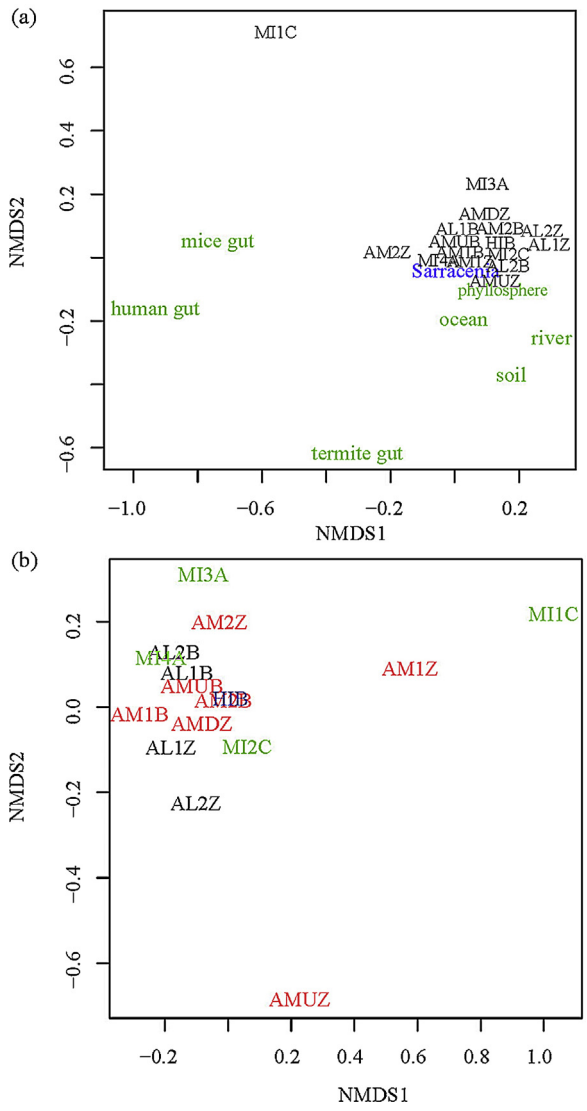

Fig. 5. (a) NMDS ordination of the bacterial-class composition for 16 *Nepenthes* samples, 7 other environmental samples, and another pitcher plant, *Sarracenia*, based on the Jaccard index (presence/absence with 1000 resamplings). The stress value is 11.4, which describes the quality of the solution (i.e. a measure of the mismatch between distance measurements and the distance in ordination space). Stress values <20 generally lead to usable plots and interpretations [34]. (b) NMDS ordination of the OTU composition for 16 *Nepenthes* samples based on the Jaccard index (presence/absence with 1000 resamplings). The stress value is 13.4, and the OTUs were identified at 3% dissimilarity. The frequency of each phylogenetic group was calculated from the abundance of the OTUs that were annotated to the group at the class level.

the pitcher fluid. This similarity between phyllosphere and pitcher microbiota may be reasonable given that developmental studies have shown that pitchers are modified leaves [28].

In general, the bacterial communities in pitcher plants were similar at the bacterial-class level to environmental samples from the ocean, a river, and soil, whereas they were very different from the flora of human, mouse and termite guts (Fig. 5a). This could be due partly to the pH value in our samples (pH 5–7), which was higher than that in human (pH 1–2.5 [16]) and mouse (pH 3–4 [41]) gut, and lower than that in termite gut (pH 11–12.5 [5]). Koopman and Carstens [30] reported a similarity between human gut flora and that of *Sarracenia* pitchers, which had an acidity similar to our samples (pH 5–7 [2]), based on the presence of four abundant shared bacterial phyla (*Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*). However, our analysis based on the bacterial class (i.e. a higher taxonomic resolution than the phylum) showed that the microbial communities shared little similarity (Fig. 5a, Fig. A.2a), although these four phyla were also detected (Table 2).

As mentioned at the end of the Introduction, it was anticipated that specific bacteria would be abundant in the fluid (e.g. some *Acidobacteria* genera known to live in acidic environments), but none of these expected taxa were found in the samples (Table 2, Table A.1). This may be due partly to the acidity of the sample not being very high. It was also expected that bacteria involved in the nitrogen (N) cycle (e.g. ammonia-oxidizing or nitrite-oxidizing species) would be abundant because N sources (ammonia, nitrite, or nitrate) may be rapidly oxidized to nitrate in an N-rich environment. However, nitrite-oxidizing bacteria (*Nitrospira*) were only present at low levels in some samples. The anaerobic bacteria *Clostridium* (*Clostridia*) were highly abundant only in AM1Z and MI1C, at 15% and 25%, respectively (Table A.1), while in other samples they were not abundant or were not detected. It was reported that this genus was also dominant in the traps of other carnivorous plants, *Utricularia* and *Genlisea* [7], in which the environments can be anoxic. *Utricularia* also dominantly harbored the facultative anaerobic bacteria *Aeromonas* (*Gammaproteobacteria*), but it was not abundant in our samples (Table A.1). In *Sarracenia*, Paisie et al. [47] reported that the aerobic bacteria *Comamonadaceae* (*Betaproteobacteria*) were abundant, and these bacteria were also detected in all samples in our study. Thus, anaerobic and aerobic environments may coexist in one pitcher. A plausible explanation for this would be as follows: at least the surface of the fluid would be oxygenated because a pitcher is always open, unlike sealed traps such as *Utricularia* and *Genlisea*. In addition, the pitcher is a modified leaf and has a photosynthetic function [2], thus the fluid would be aerobic. However, accumulated debris [43] and/or poor circulation of the fluid at the bottom of the pitcher could result in local anoxic conditions. Therefore, both aerobic and anaerobic conditions could coexist in the pitcher fluid, although this could be influenced by the amount of prey present and the metabolism of the plant.

The diversity indices ${}^1D$ and ${}^2D$ were similar among samples (Table 1). The NMDS analysis distinguished no clear groups in the bacterial communities of the different *Nepenthes* samples (Fig. 5b, Fig. A.2b). The results showed that the community was sample-specific, and not dependent on the location or host species. In addition, no clear trends based on the developmental stages were found (i.e. there was no clear grouping of unopened, mature, and dead pitchers). It has been suggested that the microbial community in the fluid may vary over time because of changes in the metabolic systems of plants and/or their environment [30,31,49,59].

The results of this study suggested that the fluid of unopened pitchers contained a high density of bacteria (Table 1), which was consistent with previous studies of *Nepenthes* [8,62] (but see [6]). Surprisingly, the diversity indices did not differ between unopened and opened pitchers. The diversity of the bacterial communities in unopened pitchers was similar to, or higher than, that in opened pitchers (Table 1). *Alphaproteobacteria* dominated the microbial communities in unopened pitchers (~30%) (Table A.1), a situation similar to that reported in phyllosphere assemblages [11]. The unopened pitcher from Borneo was dominated by *Rhodobacteraceae*, whereas the one from Zurich was populated mainly by *Methylobacteriaceae* and *Sphingomonadaceae* (Table A.1), which are microbes typically present in the phyllosphere [70]. Moreover, other typical phyllosphere bacteria (e.g. *Sphingobacteria*, especially *Flexibacteriaceae*) [11,70] were highly abundant in the unopened pitcher from Zurich (AMUZ, Table A.1). Thus, the microbial community in unopened pitchers may be derived to some extent from the phyllosphere (Fig. 5a), which is consistent with a recent study by Chou et al. [8] that demonstrated endophytic bacteria in the unopened pitchers of *Nepenthes*. These authors also showed that the composition of the microbial community in the pitcher fluids of

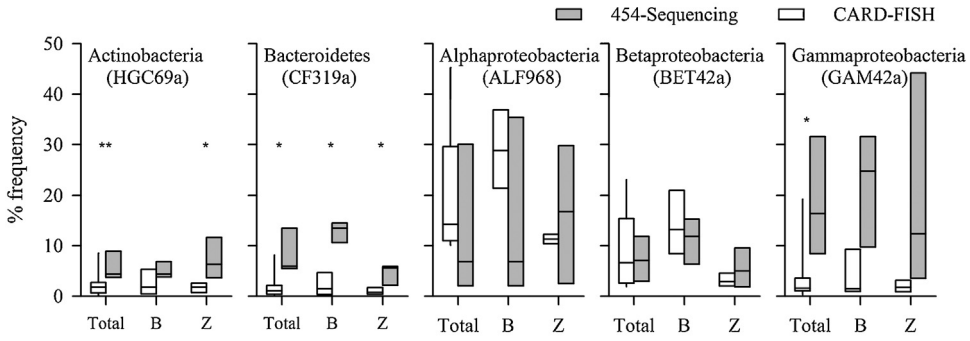

Fig. 6. Methodological comparison of the frequencies of five phylogenetic groups generated by 454 pyrosequencing and CARD–FISH in Borneo (B) and Zurich (Z). OTUs were identified at 3% dissimilarity. The frequency of each phylogenetic group was calculated from the abundance of the OTUs that were annotated to the group at the class level. Significant differences between the methods are marked by asterisks (Kruskal–Wallis test: $^{*}p < 0.05$; $^{**}p < 0.005$).

three different *Nepenthes* species was sample-specific, and showed no clear difference between unopened and opened pitchers. Most of their samples were strongly dominated by *Alphaproteobacteria*, but only a few taxa were shared between these pitcher plants and some of our samples (i.e. *Xanthobacter* spp. were present in 2, *Sphingomonas* spp. in 12, and *Novosphingobium* spp. in 4 out of 16 samples; see Table A.1).

Comparison of methodologies: 454 pyrosequencing versus CARD–FISH

The results obtained with pyrosequencing and CARD–FISH were compared in order to assess the likelihood of artefacts related to the PCR-based method and their possible effects on the community composition analysis. Different *Nepenthes* samples were used in this analysis, but there were no significant differences between pyrosequencing and CARD–FISH with respect to the two most abundant classes, *Alpha-* and *Betaproteobacteria* (Fig. 6), which demonstrated the agreement of the two methods. *Alphaproteobacteria* were the most abundant bacteria in all pitchers analyzed by CARD–FISH (20.6% of the total cell numbers), although their abundances were highly variable (range = 9.9–50.9% of the total cell numbers). *Beta-* and *Gammaproteobacteria* also varied greatly between pitchers (1.9–24.6% and 0.3–25.8% of the total cell numbers, respectively). *Actinobacteria* and *Bacteroidetes* were only present at relatively low levels in the pitchers ($2.4\% \pm 2.8\%$ and $1.9\% \pm 2.8\%$ of the total cell numbers, respectively, $n = 12$). The slight but insignificant overestimation of *Alphaproteobacteria* by CARD–FISH might have been caused by a high number of out-group hits targeted by the general probe, ALF968 (14% of all hits, RDPII probe match; [3]), mainly with *Delta-* and *Gammaproteobacteria* but also with the genus *Sulfurimonas* (*Epsilonproteobacteria*; RDPII probe match), which was not present in the 454 sequences. All other probes applied in this study had very few out-group hits [3]. *Actinobacteria*, *Gammaproteobacteria*, and *Bacteroidetes* were significantly less frequent with the CARD–FISH approach (Fig. 6). However, *Actinobacteria*, *Bacteroidetes*, and *Gammaproteobacteria* include taxa with more than one 16S rRNA operon in their genomes [37,52]. For example, *Acinetobacter* spp. and *Pseudomonas* spp. (*Gammaproteobacteria*) were highly abundant in the pyrosequencing dataset. Cultivated representatives of these groups possess 5–7 and 4–7 16S rRNA operons, respectively [52], which might explain these discrepancies. Similarly, the highly abundant orders *Actinomycetales* (*Actinobacteria*), *Flavobacteriales*, and *Sphingobacteriales* (*Bacteroidetes*) have an average of three copies of the 16S rRNA gene. Bacteria affiliated with *Fluviicola* spp. (probe Flu-736) and *Methylophilaceae* (probe MET1217) were present in all pitchers, although mainly at very low densities (0.1–5% of DAPI, data not shown). Two OTUs of *Fluviicola* spp. were present in 15 pitcher samples (range: 0.1–2.5% of sequences), and one *Methylophilaceae* OTU was present in 14 pitchers (range: 0.01–3.3%). Therefore, the results obtained by pyrosequencing and CARD–FISH were comparable for small well-defined populations (i.e. the genus to family levels).

Substantial differences were also found in the community compositions of samples analyzed by CARD–FISH, which were consistent with the 454 pyrosequencing results. There were no specific trends associated with location or species-specific bacteria, with the exception of *N. ampullaria* from Zurich, which contained a high number of bacteria that did not hybridize with any of the probes (i.e. only ~20% of all bacteria could be assigned to the sum of all probes). Moreover, the community compositions of the bacteria assigned were similar in the six samples of *N. ampullaria* from Zurich. The class *Alphaproteobacteria* was the only abundant bacteria in *N. ampullaria* from Zurich ($11.3\% \pm 1.1\%$ of the total cell numbers, $n = 6$), whereas all other bacteria analyzed were present at very low levels (i.e. 0.3–5.3% of the total cell numbers). This trend was not found in samples of the same species from Borneo (i.e. the standard deviation of the six pitcher samples ranged from 3.9% to 11.7%).

To the best of our knowledge, this is the first report that compares estimations of bacterial diversity obtained by pyrosequencing and FISH in the fluid of pitcher plants. Although the microbial communities in the same samples were not compared, the community compositions did not differ greatly from the phylum level to the class level using the two methods. This suggests that the pyrosequencing bias was low, at least for *Alpha-* and *Betaproteobacteria*. However, more replicates are needed in order to allow a general conclusion to be drawn concerning the agreement of the two methods.

Conclusion

A comparison of the results of 454 pyrosequencing and CARD–FISH showed that both methods yielded similar estimates of community composition from the phylum to the class level of bacteria. This suggested that pyrosequencing was used successfully to characterize the bacterial taxonomic compositions of the pitcher fluids of *Nepenthes* species. The results of comparisons between *Nepenthes*, *Sarracenia*, and other environments showed that the bacterial communities were similar to those found in the pitchers of *Sarracenia* and the phyllosphere but not to those in animal guts. The pitchers of *Sarracenia* and *Nepenthes* evolved independently during angiosperm history; thus, the similarity of their bacterial communities cannot be derived from ancestral pitcher communities. To explain this similarity, two explanations, which are not mutually exclusive, are proposed. First, plant pitchers have convergent unique characteristics, such as the acidic digestive

environment. Thus, similar bacterial communities would survive under this environmental condition. Second, pitchers are modified leaves from the viewpoint of development. Thus, phyllosphere (or leaf-surface) bacteria would be transmitted from the leaf surface into the pitcher, and both may provide similar environmental niches for these bacteria. Similarities in the phyllosphere bacteria of three diverse angiosperm species have been reported [11]. Here, similarity between bacterial communities was shown in the phyllosphere and in pitchers from *Nepenthes* and *Sarracenia*. These data suggested that phyllosphere bacterial communities shared between plant species contributed to the composition of those in *Nepenthes* and *Sarracenia* pitchers, and thus the two pitcher plant species had similar bacterial composition. The study of more species and replications would be critical for confirming this hypothesis.

Although similarity was evident at the class level, it was found that few OTU was shared between the 16 *Nepenthes* samples and OTU diversity was high. Enzymes derived from microbes in the fluid are usually found in most samples [65], so the microbial communities in pitchers might differ taxonomically but be functionally similar to some extent. In other words, environmental filtering might work mainly at the functional repertoire level of the microbiota rather than at its compositional level. Functional gene analyses using proteomics and/or transcriptomics, in combination with taxonomic profiling, will be required to test this hypothesis and to determine the functional convergence of microbial communities. Future studies using more replicates should also test community ecology hypotheses in order to understand the mechanisms that control pitcher-specific microbial communities, as well as investigating the effects of interspecific interactions with insect prey and mosquito larvae living in the pitchers, which may be transporters that introduce bacteria into the fluid [31,49,59].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2015.05.006

References

[1] Acinas, S.G., Klepac-Ceraj, V., Hunt, D.E., Pharino, C., Ceraj, I., Distel, D.L., Polz, M.F. (2004) Fine-scale phylogenetic architecture of a complex bacterial community. Nature 430, 551–554.
[2] Adlassnig, W., Peroutka, M., Lendi, T. (2010) Traps of carnivorous pitcher plants as a habitat: composition of the fluid, biodiversity and mutualistic activities. Ann. Bot. 107, 181–194.
[3] Amann, R., Fuchs, B.M. (2008) Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. Nat. Rev. Microbiol. 6, 339–348.
[4] Bai, Y., Yang, D., Wang, J., Xu, S., Wang, X., An, L. (2006) Phylogenetic diversity of culturable bacteria from alpine permafrost in the Tianshan Mountains, northwestern China. Res. Microbiol. 157, 741–751.
[5] Brune, A., Kühl, M. (1996) pH profiles of the extremely alkaline hindguts of soil-feeding termites (Isoptera: Termitidae) determined with microelectrodes. J. Insect Physiol. 42, 1121–1127.
[6] Buch, F., Rott, M., Rottloff, S., Paetz, C., Hilke, I., Raessler, M., Mithofer, A. (2013) Secreted pitfall-trap fluid of carnivorous *Nepenthes* plants is unsuitable for microbial growth. Ann. Bot. 111, 375–383.
[7] Caravieri, F.A., Ferreira, A.J., Ferreira, A., Clivati, D., de Miranda, V.F.O., Araújo, W.L. (2014) Bacterial community associated with traps of the carnivorous plants *Utricularia hydrocarpa* and *Genlisea filiformis*. Aquat. Bot. 116, 8–12.
[8] Chou, L., Clarke, C., Dykes, G. (2014) Bacterial communities associated with the pitcher fluids of three *Nepenthes* (Nepenthaceae) pitcher plant species growing in the wild. Arch. Microbiol. 196, 709–717.
[9] Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M. (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37, D141–D145.
[10] Darwin, C. 1875 Insectivorous Plants, John Murray, London.
[11] Delmotte, N., Knief, C., Chaffron, S., Innerebner, G., Roschitzki, B., Schlapbach, R., von Mering, C., Vorholt, J.A. (2009) Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. Proc. Natl. Acad. Sci. U.S.A. 106, 16428–16433.
[12] Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E., Relman, D.A. (2005) Diversity of the human intestinal microbial flora. Science 308, 1635–1638.
[13] Eckert, E.M., Salcher, M.M., Posch, T., Eugster, B., Pernthaler, J. (2012) Rapid successions affect microbial *N*-acetyl-glucosamine uptake patterns during a lacustrine spring phytoplankton bloom. Environ. Microbiol. 14, 794–806.
[14] Eddy, S.R. (2002) A memory-efficient dynamic programming algorithm for optimal alignment of a sequence to an RNA secondary structure. BMC Bioinf. 3, 18.
[15] Ellison, A.M., Gotelli, N.J. (2009) Energetics and the evolution of carnivorous plants – Darwin's 'most wonderful plants in the world'. J. Exp. Bot. 60, 19–42.
[16] Evans, D.F., Pye, G., Bramley, R., Clark, A.G., Dyson, T.J., Hardcastle, J.D. (1988) Measurement of gastrointestinal pH profiles in normal ambulant human subjects. Gut 29, 1035–1041.
[17] Farrelly, V., Rainey, F.A., Stackebrandt, E. (1995) Effect of genome size and rrn gene copy number on PCR amplification of 16s ribosomal-RNA genes from a mixture of bacterial species. Appl. Environ. Microbiol. 61, 2798–2801.
[18] Finlay, B.J. (2002) Global dispersal of free-living microbial eukaryote species. Science 296, 1061–1063.
[19] Friedrich, U., Van Langenhove, H., Altendorf, K., Lipski, A. (2003) Microbial community and physicochemical analysis of an industrial waste gas biofilter and design of 16*S* rRNA-targeting oligonucleotide probes. Environ. Microbiol. 5, 183–201.
[20] Ghai, R., Rodríguez-Valera, F., McMahon, K.D., Toyama, D., Rinke, R., Cristina Souza de Oliveira, T., Wagner Garcia, J., Pellon de Miranda, F., Henrique-Silva, F. (2011) Metagenomics of the water column in the pristine upper course of the Amazon River. PLoS ONE 6, e23785.
[21] Hatano, N., Hamada, T. (2008) Proteome analysis of pitcher fluid of the carnivorous plant *Nepenthes alata*. J. Proteome Res. 7, 809–816.
[22] Hepburn, J., Jones, F. (1927) The enzymes of the pitcher liquor of *Sarraceniaceae*. Trans. Wagner Free Inst. Sci. 11, 49–68.
[23] Hill, M.O. (1973) Diversity and evenness: a unifying notation and its consequences. Ecology 54, 427–432.
[24] Hirano, S.S., Upper, C.D. (2000) Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* – a pathogen, ice nucleus, and epiphyte. Microbiol. Mol. Biol. Rev. 64, 624–653.
[25] Hongoh, Y., Deevong, P., Inoue, T., Moriya, S., Trakulnaleamsai, S., Ohkuma, M., Vongkaluang, C., Noparatnaraporn, N., Kudol, T. (2005) Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. Appl. Environ. Microbiol. 71, 6590–6599.
[26] Hortnagl, P., Perez, M.T., Sommaruga, R. (2010) Living at the border: a community and single-cell assessment of lake bacterioneuston activity. Limnol. Oceanogr. 55, 1134–1144.
[27] Jost, L. (2006) Entropy and diversity. Oikos 113, 363–375.
[28] Juniper, B.E., Robbins, R.J., Joel, D.M. 1989 The Carnivorous Plants, Academic Press, London.
[29] Kalyuzhnaya, M.G., Lapidus, A., Ivanova, N., Copeland, A.C., McHardy, A.C., Szeto, E., Salamov, A., Grigoriev, I.V., Suciu, D., Levine, S.R., Markowitz, V.M., Rigoutsos, I., Tringe, S.G., Bruce, D.C., Richardson, P.M., Lidstrom, M.E., Chistoserdova, L. (2008) High-resolution metagenomics targets specific functional types in complex microbial communities. Nat. Biotechnol. 26, 1029–1034.
[30] Koopman, M., Carstens, B. (2011) The microbial *phyllo*geography of the carnivorous plant *Sarracenia alata*. Microb. Ecol. 61, 750–758.
[31] Koopman, M.M., Fuselier, D.M., Hird, S., Carstens, B.C. (2010) The carnivorous pale pitcher plant harbors diverse, distinct, and time-dependent bacterial communities. Appl. Environ. Microbiol. 76, 1851–1860.
[32] Krieger, J., Kourtev, P. (2012) Bacterial diversity in three distinct sub-habitats within the pitchers of the northern pitcher plant, *Sarracenia purpurea*. FEMS Microbiol. Ecol. 79, 555–567.
[33] Krieger, J.R., Kourtev, P.S. (2012) Detection of methanogenic archaea in the pitchers of the Northern pitcher plant (*Sarracenia purpurea*). Can. J. Microbiol. 58, 189–194.
[34] Kruskal, J.B. (1964) Nonmetric multidimensional scaling: a numerical method. Psychometrika 29, 115–129.

[35] Kwon, S.-W., Son, J.-A., Kim, S.-J., Kim, Y.-S., Park, I.-C., Bok, J.-I., Weon, H.-Y. (2011) *Pedobacter rhizosphaerae* sp. nov. and *Pedobacter soli* sp. nov., isolated from rhizosphere soil of Chinese cabbage (*Brassica campestris*). Int. J. Syst. Evol. Microbiol. 61, 2874–2879.
[36] La Duc, M.T., Osman, S., Vaishampayan, P., Piceno, Y., Andersen, G., Spry, J.A., Venkateswaran, K. (2009) Comprehensive census of bacteria in clean rooms by using DNA microarray and cloning methods. Appl. Environ. Microbiol. 75, 6559–6567.
[37] Lee, Z.M.P., Bussema, C., Schmidt, T.M. (2009) rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. Nucleic Acids Res. 37, D489–D493.
[38] Lesaulnier, C., Papamichail, D., McCorkle, S., Ollivier, B., Skiena, S., Taghavi, S., Zak, D., Van Der Lelie, D. (2008) Elevated atmospheric CO_2 affects soil microbial diversity associated with trembling aspen. Environ. Microbiol. 10, 926–941.
[39] Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K.H. (2004) ARB: a software environment for sequence data. Nucleic Acids Res. 32, 1363–1371.
[40] Manz, W., Amann, R., Ludwig, W., Wagner, M., Schleifer, K.-H. (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. Syst. Appl. Microbiol. 15, 593–600.
[41] McConnell, E.L., Basit, A.W., Murdan, S. (2008) Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments. J. Pharm. Pharmacol. 60, 63–70.
[42] Morrissey, S. (1955) Chloride ions in the secretion of the pitcher plant. Nature 176, 1220–1221.
[43] Mouquet, N., Daufresne, T., Gray, S.M., Miller, T.E. (2008) Modelling the relationship between a pitcher plant (*Sarracenia purpurea*) and its phytotelma community: mutualism or parasitism? Funct. Ecol. 22, 728–737.
[44] Neef, A. 1997 Anwendung der in situ-Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen, Univ. Munich, Munich.
[45] Neef, A., Zaglauer, A., Meier, H., Amann, R., Lemmer, H., Schleifer, K.H. (1996) Population analysis in a denitrifying sand filter: conventional and in situ identification of *Paracoccus* spp. in methanol-fed biofilms. Appl. Environ. Microbiol. 62, 4329–4339.
[46] O'Sullivan, L.A., Rinna, J., Humphreys, G., Weightman, A.J., Fry, J.C. (2005) *Fluviicola taffensis* gen. nov., sp. nov., a novel freshwater bacterium of the family *Cryomorphaceae* in the phylum '*Bacteroidetes*'. Int. J. Syst. Evol. Microbiol. 55, 2189–2194.
[47] Paisie, T.K., Miller, T.E., Mason, O.U. (2014) Effects of a ciliate protozoa predator on microbial communities in pitcher plant (*Sarracenia purpurea*) leaves. PLoS ONE 9, e113384.
[48] Pernthaler, A., Pernthaler, J., Amann, R. (2002) Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. Appl. Environ. Microbiol. 68, 3094–3101.
[49] Peterson, C.N., Day, S., Wolfe, B.E., Ellison, A.M., Kolter, R., Pringle, A. (2008) A keystone predator controls bacterial diversity in the pitcher-plant (*Sarracenia purpurea*) microecosystem. Environ. Microbiol. 10, 2257–2266.
[50] Plummer, G.L., Jackson, T.H. (1963) Bacterial activities within the sarcophagus of the insectivorous plant, *Sarracenia flava*. Am. Midl. Nat. 69, 462–469.
[51] R Development Core Team, 2014 R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org/
[52] Rastogi, R., Wu, M., DasGupta, I., Fox, G.E. (2009) Visualization of ribosomal RNA operon copy number distribution. BMC Microbiol. 9, 208.
[53] Rensing, S.A., Lang, D., Zimmer, A.D., Terry, A., Salamov, A., Shapiro, H., Nishiyama, T., Perroud, P.F., Lindquist, E.A., Kamisugi, Y., Tanahashi, T., Sakakibara, K., Fujita, T., Oishi, K., Shin-I, T., Kuroki, Y., Toyoda, A., Suzuki, Y., Hashimoto, S., Yamaguchi, K., Sugano, S., Kohara, Y., Fujiyama, A., Anterola, A., Aoki, S., Ashton, N., Barbazuk, W.B., Barker, E., Bennetzen, J.L., Blankenship, R., Cho, S.H., Dutcher, S.K., Estelle, M., Fawcett, J.A., Gundlach, H., Hanada, K., Heyl, A., Hicks, K.A., Hughes, J., Lohr, M., Mayer, K., Melkozernov, A., Murata, T., Nelson, D.R., Pils, B., Prigge, M., Reiss, B., Renner, T., Rombauts, S., Rushton, P.J., Sanderfoot, A., Schween, G., Shiu, S.H., Stueber, K., Theodoulou, F.L., Tu, H., Van de Peer, Y., Verrier, P.J., Waters, E., Wood, A., Yang, L.X., Cove, D., Cuming, A.C., Hasebe, M., Lucas, S., Mishler, B.D., Reski, R., Grigoriev, I.V., Quatrano, R.S., Boore, J.L. (2008) The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. Science 319, 64–69.
[54] Rintala, H., Pitkaranta, M., Toivola, M., Paulin, L., Nevalainen, A. (2008) Diversity and seasonal dynamics of bacterial community in indoor environment. BMC Microbiol. 8, 56.
[55] Roller, C., Wagner, M., Amann, R., Ludwig, W., Schleifer, K.-H. (1994) In situ probing of Gram-positive bacteria with high DNA G + C content using 23S rRNA-targeted oligonucleotides. Microbiology 140, 2849–2858.
[56] Salcher, M.M., Pernthaler, J., Frater, N., Posch, T. (2011) Vertical and longitudinal distribution patterns of different bacterioplankton populations in a canyon-shaped, deep prealpine lake. Limnol. Oceanogr. 56, 2027–2039.
[57] Salcher, M.M., Pernthaler, J., Zeder, M., Psenner, R., Posch, T. (2008) Spatio-temporal niche separation of planktonic *Betaproteobacteria* in an oligo–mesotrophic lake. Environ. Microbiol. 10, 2074–2086.
[58] Shabarova, T., Pernthaler, J. (2010) Karst pools in subsurface environments: collectors of microbial diversity or temporary residence between habitat types. Environ. Microbiol. 12, 1061–1074.
[59] Siragusa, A.J., Swenson, J.E., Casamatta, D.A. (2007) Culturable bacteria present in the fluid of the hooded-pitcher plant *Sarracenia minor* based on 16S rDNA gene sequence data. Microb. Ecol. 54, 324–331.
[60] Sirota, J., Baiser, B., Gotelli, N.J., Ellison, A.M. (2013) Organic-matter loading determines regime shifts and alternative states in an aquatic ecosystem. Proc. Natl. Acad. Sci. U.S.A. 110, 7742–7747.
[61] Smith, J.G. (1893) Recent studies of carnivorous plants. Am. Nat. 27, 413–420.
[62] Sota, T., Mogi, M., Kato, K. (1998) Local and regional-scale food web structure in *Nepenthes alata* pitchers. Biotropica 30, 82–91.
[63] Stamatakis, A., Ludwig, T., Meier, H. (2005) RAxML-II: a program for sequential, parallel and distributed inference of large phylogenetic trees. Concurr. Computat. Pract. Exper. 17, 1705–1723.
[64] Stecher, B., Chaffron, S., Käppeli, R., Hapfelmeier, S., Freedrich, S., Weber, T.C., Kirundi, J., Suar, M., McCoy, K.D., von Mering, C., Macpherson, A.J., Hardt, W.-D. (2010) Like will to like: abundances of closely related species can predict susceptibility to intestinal colonization by pathogenic and commensal bacteria. PLoS Pathog. 6, e1000711.
[65] Takeuchi, Y., Salcher, M.M., Ushio, M., Shimizu-Inatsugi, R., Kobayashi, M.J., Diway, B., von Mering, C., Pernthaler, J., Shimizu, K.K. (2011) In situ enzyme activity in the dissolved and particulate fraction of the fluid from four pitcher plant species of the genus *Nepenthes*. PLoS ONE 6, e25144.
[66] Täubel, M., Rintala, H., Pitkäranta, M., Paulin, L., Laitinen, S., Pekkanen, J., Hyvärinen, A., Nevalainen, A. (2009) The occupant as a source of house dust bacteria. J. Allergy Clin. Immunol. 124, 834.e847-840.e847.
[67] Tringe, S.G., von Mering, C., Kobayashi, A., Salamov, A.A., Chen, K., Chang, H.W., Podar, M., Short, J.M., Mathur, E.J., Detter, J.C., Bork, P., Hugenholtz, P., Rubin, E.M. (2005) Comparative metagenomics of microbial communities. Science 308, 554–557.
[68] Van den Wyngaert, S., Salcher, M.M., Pernthaler, J., Zeder, M., Posch, T. (2011) Quantitative dominance of seasonally persistent filamentous cyanobacteria (*Planktothrix rubescens*) in the microbial assemblages of a temperate lake. Limnol. Oceanogr. 56, 13.
[69] Větrovský, T., Baldrian, P. (2013) The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. PLoS ONE 8, e57923.
[70] Vorholt, J.A. (2012) Microbial life in the phyllosphere. Nat. Rev. Microbiol. 10, 828–840.
[71] Xing, P., Hahn, M.W., Wu, Q.L. (2009) Low taxon richness of bacterioplankton in high-altitude lakes of the eastern Tibetan plateau, with a predominance of *Bacteroidetes* and *Synechococcus* spp. Appl. Environ. Microbiol. 75, 7017–7025.