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# Archaeal communities discovered in the phytotelmata of *Nepenthes alata* Blco. samples obtained from Mt. Makiling, Philippines as revealed by high-throughput molecular sequencing analysis

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**Abstract** The archaeome in the phytotelmata of the Philippine endemic pitcher plant, *Nepenthes alata* Blco. was investigated. The alpha diversity analyses revealed that the ten most abundant archaeal sequences detected were less than 97% similar to known sequences, and were classified under "Others", indicating the presence of previously uncharacterized archaea. At the genus level, *Methanocorpusculum*, Candidatus_Nitrosopumilus and *Methanimicrococcus* were found to be the most abundant in the phytotelm of young pitchers. In contrast, there were no genera of previously identified archaea that were considered abundant in the phytotelm of mature pitchers. Further analyses of archaeal composition over time showed that of the 560 total phylotypes detected in young pitchers, 269 (~ 48.0%) were consistently present across three monthly sampling periods. On the other hand, of the 570 total phylotypes detected in the mature pitchers, 200 (~ 35.1%) were consistently present across the same sampling periods. This paper establishes that *N. alata* hosts multiple species of archaea regardless of age of the pitchers and implies a high possibility that *N. alata*-archaeome interactions exist which have not been examined previously.

**Keywords:** Archaea, Mt. Makiling Forest Reserve, *Nepenthes alata* Blco., Pitcher plant, Phytotelmata

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## Introduction

The overwhelming majority of microbiome studies are bacteria-centric, and archaea are consistently overlooked (Fischer *et al*., 2016; Pausan *et al*., 2019). In particular, in studies dealing with plant microbiomes, archaea are still underdetected and little-studied (Taffner *et al*., 2018). The potential roles of bacteria in the physiology of pitcher plants have long been recognized (Takeuchi *et al*., 2015). Pitcher plants are phylogenetically unrelated plants that share similar features of plant carnivory, or the ability to attract, catch, kill and digest prey as source of nutrients (Mithofer, 2017). However, very few attempts have been made to examine archaeal communities and their influence in pitcher plants. The only published report, to our knowledge, on the detection of archaea in pitcher plants was by Krieger and Kourtev in 2012 when they examined the sediment from the pitchers of the purple pitcher plant, *Sarracenia purpurea* L. that contained insect cadavers. No additional information on this topic has been reported since. In the Philippines, the archaeal taxa present have not been identified at all. Thus, there are no data on pitcher plant-archaea interactions specifically within the phytotelmata. Phytotelmata are small water-filled cavities in the plant's modified leaves which are commonly called pitchers (Adlassnig *et al*., 2011; Kitching, 2000). The phytotelma is an important part for pitcher plants since here is where their nutrient acquisition occurs. This report provides probably the first data on the presence and patterns of abundance of archaea in any species of *Nepenthes* found in the country. While Krieger and Kourtev (2012) examined sediments, here we focused on the digestive fluids of *N. alata* growing in the Mt. Makiling Forest Reserve, Philippines. We also explored the archaeal community profiles in young and mature pitchers and whether this profile changed over time.

## Materials and methods

### *Sample collection*

Pitcher fluid samples were collected every month from January to March 2017 from the phytotelmata of the pitcher plant *N. alata* growing at Peak 3 of the Mt. Makiling Forest Reserve (MMFR), Philippines (14.14 N; 121.19 E). Sterile RNAse-free, 50 ml centrifuge tubes were used in the collection from pitcher plants classified by age. In this study, pitchers that were succulent, green, relatively smaller, and generally with few insects inside the pitcher were considered as "young", while pitchers which were generally dry, rough, dark green to purple, relatively larger, and generally visited by ants or contained

large amounts of ant cadavers, were considered as "mature". One or two pitchers were selected randomly from a single plant, and 50 ml samples of phytotelmata fluids were pooled by pouring directly onto the sample tubes (Chou *et al*., 2014) to obtain 50 ml samples. Samples collected from mature pitcher phytotelmata in Jan, Feb, and Mar were designated as NBM1, NBM2, and NBM3, respectively; samples collected from young pitcher phytotelmata in Jan, Feb, and Mar were designated NBY1, NBY2, and NBY3, respectively. Samples were transported in coolers with ice and were brought to the laboratory of the Microbiology Division of the Institute of Biological Sciences, CAS, University of the Philippines Los Baños immediately (~ ≤ 4 h from the time of sample collection). These were then stored at -20 $^{o}$C until further processing.

***DNA extraction, amplicon library construction, sequencing, and bioinformatics analysis***

To remove ants and other debris, liquid samples were filtered using a sterile filter funnel with mesh; samples were then filtered on 0.22 µm cellulose membranes (Millipore, USA) to concentrate the cells. Total DNA was extracted from the membranes using the DNeasy PowerSoil Kit (Qiagen, Germany), following the manufacturer's protocol, with three replicates per sample (n= 3). Extracted total DNA were processed for 16S rRNA meta-amplicon library preparation, sequencing and bioinformatics analysis by Novogene Corporation, Inc. (Chula Vista, CA).

DNA concentration and purity were monitored on 1% agarose gels. DNA was then diluted to 1 ng/µl using sterile water. The 16S rRNA V4 region was amplified using 515F-806R primers (Caporaso *et al*., 2010) with the barcode. All PCR reactions were carried out with PhusionR High-Fidelity PCR Master Mix (Biolabs, New England). DNA was visualized using 2% agarose gel electrophoresis with 1X DNA loading buffer (containing SYBR green), and samples with a bright main strip between 400-450bp were chosen for further experiments.

PCR products were purified using Qiagen Gel Extraction Kit (Qiagen, Germany). Libraries were then generated using NebNextR ultraTM DNA library Prep Kit for Illumina and quantified via Qubit (Thermo Fisher Scientific Inc., USA) and 2100 Bioanalyzer System (Agilent USA). Sample sequences were analyzed using Hi-Seq Illumina platform (Illumina, Inc., San Diego, CA, USA) at Novogene.

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence then merged using FLASH (V1.2.7) (Magoc and Salsberg, 2011). Quality filtering on the

raw tags was performed under specific filtering conditions to obtain high-quality clean tags according to QIIME (V1.7.0) quality control process. The tags were compared with the reference database (Gold database) using UCHIME algorithm to detect chimera sequences. Chimeric sequences were then removed to finally obtain the effective tags.

Sequence analyses were performed using Uparse software (Uparse v7.0.1001). Sequences with greater than 97% similarity were assigned to the same OTUs. Each representative sequence for each OTU was screened for further annotation using Mothur and the SSU rRNA database in SILVA (Wang *et al*., 2007) for species annotation at each taxonomic rank. OTU abundance was normalized to the abundance of the sample with the lowest number of sequences. Multiple sequence alignment was conducted using the MUSCLE software (v. 3.8.31) (Edgar, 2009). Subsequent analysis of alpha diversity and beta diversity were performed based on this output normalized data. Alpha and beta diversity indices were calculated using QIIME (version 1.7.0) and displayed with R software (v 2.15.3). Tree graphs of species annotation were then constructed by GraPhlAn (Asnicar *et al*., 2015, and a taxonomy tree was constructed based on the top 10 genera in high relative abundance by the NAST (Nearest Alignment Space Termination) software (DeSantis *et al*., 2006). For the analysis of significance of difference between groups, T-test, Wilcox (for 2 groups) and Tukey's tests (for > 2 groups) were performed. Analysis of Similarity (ANOSIM) was performed to evaluate variations among and within the young and mature pitcher samples. Non-metric multi-dimensional scaling (NMDS) plot was also constructed to ordinate the similarity data among and between between young and mature samples. All sequences were submitted to GenBank (BioProject Accession Number: PRJNA879260).

## Results

### *Sequence data*

A total of 2,810,047 total paired-end raw reads (raw PE) were generated, with 1,362,995 raw PE reads from young pitcher phytotelmata and 1,447,052 from mature pitcher phytotelmata. After contig assembly and chimera removal, a total of 1,030,635 Effective Tags from young pitcher phytotelmata and 1,071,220 Effective Tags from mature pitcher phytotelmata were obtained, respectively. Finally, a total of 690 operational taxonomic units (OTUs) were obtained from the samples after all the Effective Tags were grouped into OTUs by 97% 16S rRNA sequence similarity. A species accumulation boxplot was

constructed to judge the adequacy of the size of samples from both the young and mature pitcher phytotelmata of *N. alata* (Figure 1).

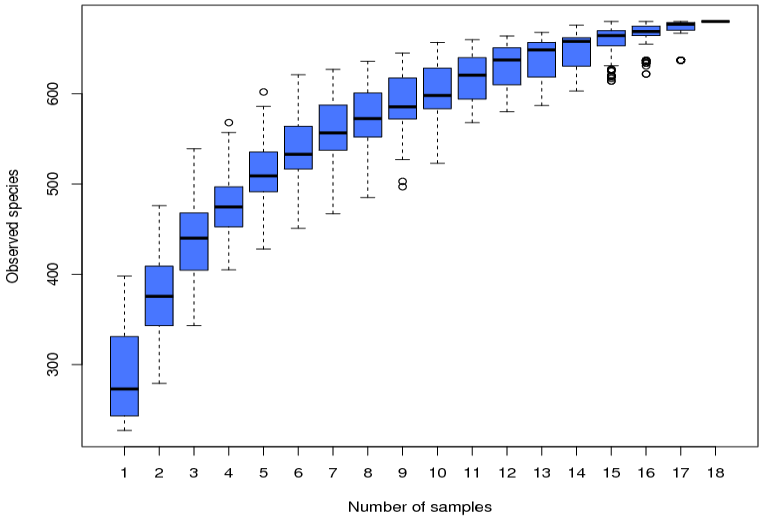


**Figure 1.** Boxplot of the species accumulation of young and mature pitcher phytotelmata of *N. alata*

With increasing sample size, the trend in the number of observed species flattened, indicating adequate sample number for species richness analysis.

***Pattern of archaeal abundance at the phylum level***

After analysis, all ten most abundant species for all types of samples (from young and mature pitcher phytotelmata) were classified as "others" as none of the sequences were at least 97% similar to known sequences in the SILVA database. These sequences may highly indicate unique and/or unreported archaeal taxa. The comparison to bacteria was surveyed more widely, archaea was less studied and only few well-characterized archaea sequences are available from databases. Additionally, alternatives for a better marker other than the commonly used 16S V3-V4 hypervariable region are limited. Tree graphs of species annotation were then constructed by GraPhlAn (Asnicar *et al*., 2015) Three main clusters were formed: a cluster for *Methanomicrobia* (red), for unclassified archaeon, "SCG" (blue) and for unidentified *Thaumarcheota* (green) (Figure 2). The data further support the findings of high numbers of unclassified archaea from *N. alata* pitcher phytotelmata.

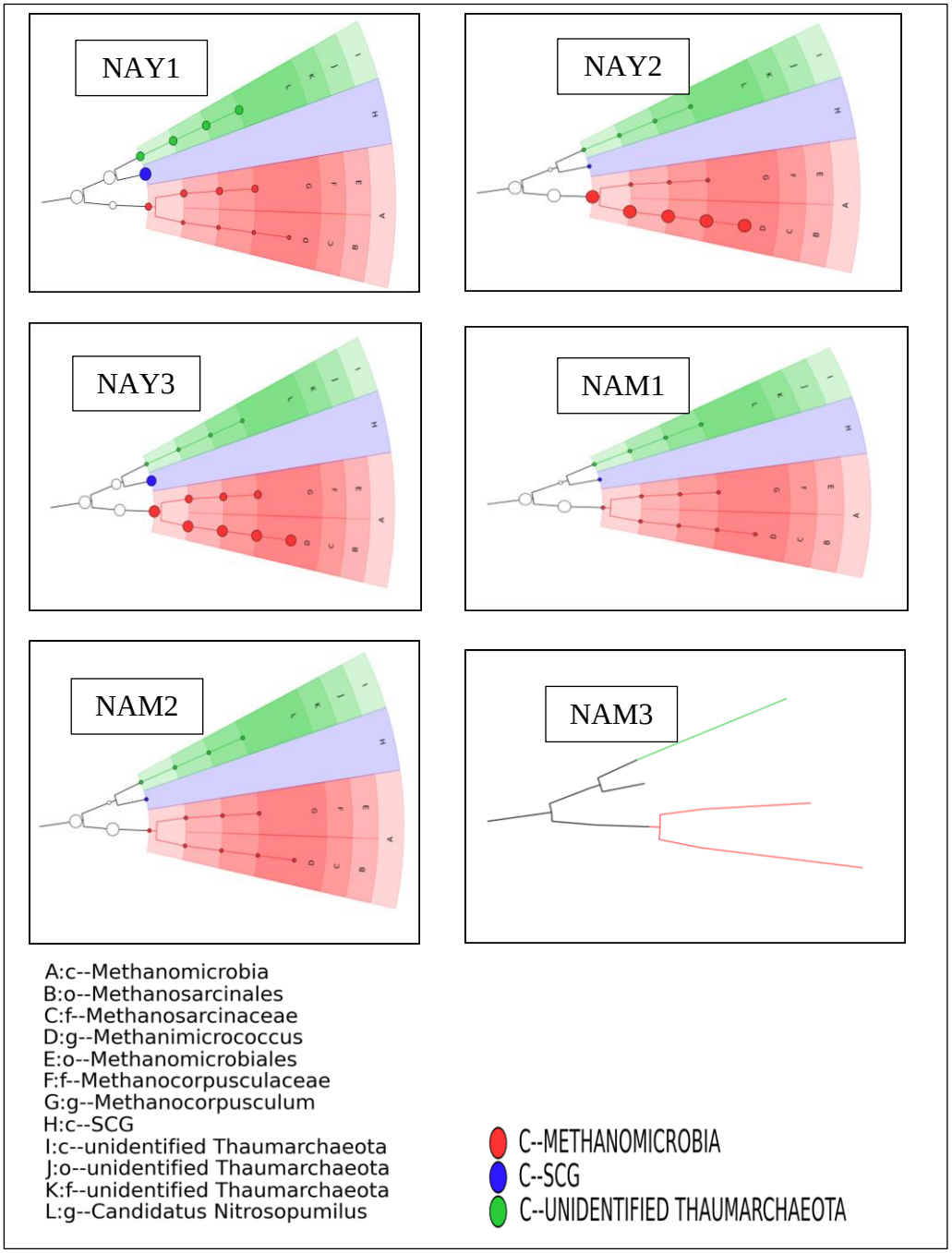


**Figure 2.** OTU annotation trees for young (NAY1, NAY2 and NAY3) and mature (NAM1, NAM2, and NAM3) pitcher phytotelmata of *N. alata*. Different colors stand for different phyla and solid circles stand for the top 40 species with high abundance

A taxonomy tree was further constructed which based on the top 10 genera in high relative abundance by an independent R&D software, NAST (Nearest Alignment Space Termination) (Figure 3).

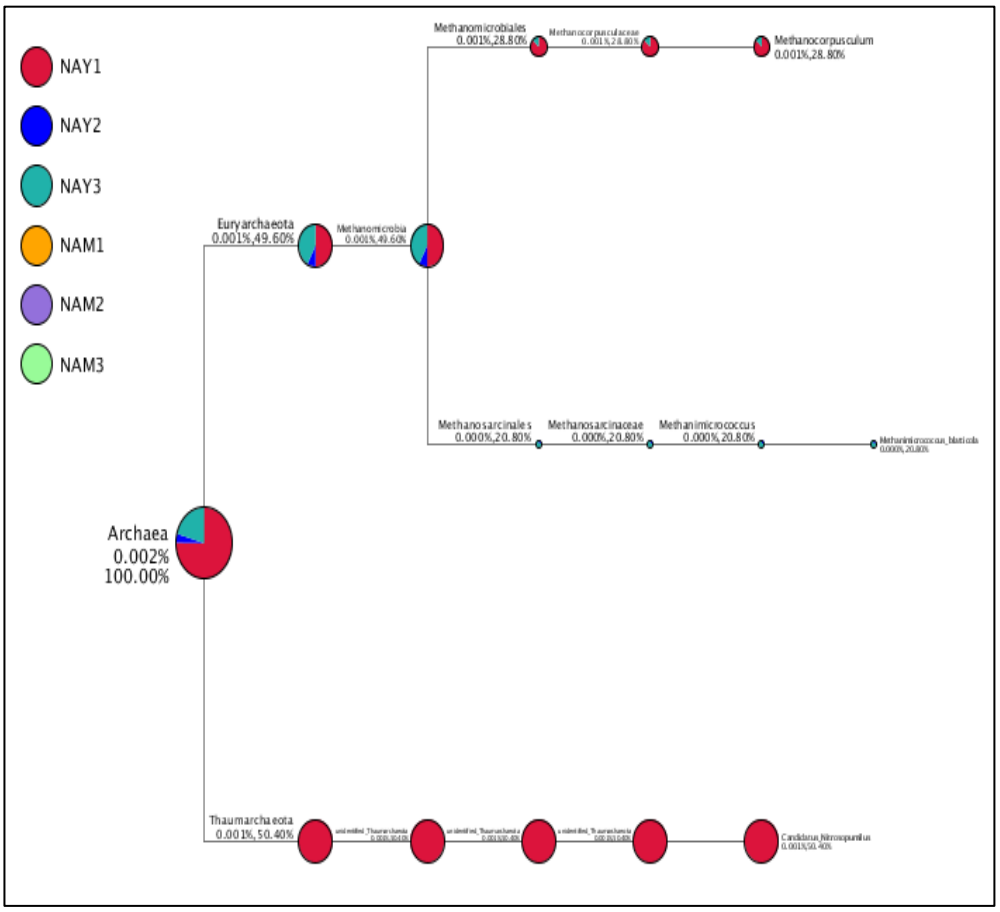


**Figure 3.** Taxonomy tree of archaeal 16S rRNA OTUs associated with the young (NAY1, NAY2 and NAY3) and mature (NAM1, NAM2, and NAM3) pitcher phytotelmata of *N. alata*

Different colors in the pie charts represent different groups and the size represents relative abundance. The first number below the taxon represents the percentage in the whole taxon, while the second number represents the percentage in the selected taxon. A higher percentage of previously classified archaea were detected in the young pitcher phytotelmata relative to the mature pitcher phytotelmata of *N. alata* (Figure 3).

***Pattern of archaeal abundance at the genus level***

The abundance distribution of the most dominant genera among all samples (Figure 4) indicated no previously identified archaeal species among mature pitchers. On the other hand, Candidatus_Nitrosopumilus and *Methanocorpusculum* were strongly dominant/abundant in the first sampling time, *Methanimicrococcus* was moderately dominant/abundant in the second sampling time and was the most abundant species in the third sampling time. *Methanocorpusculum* was also moderately abundant in the third sampling time.

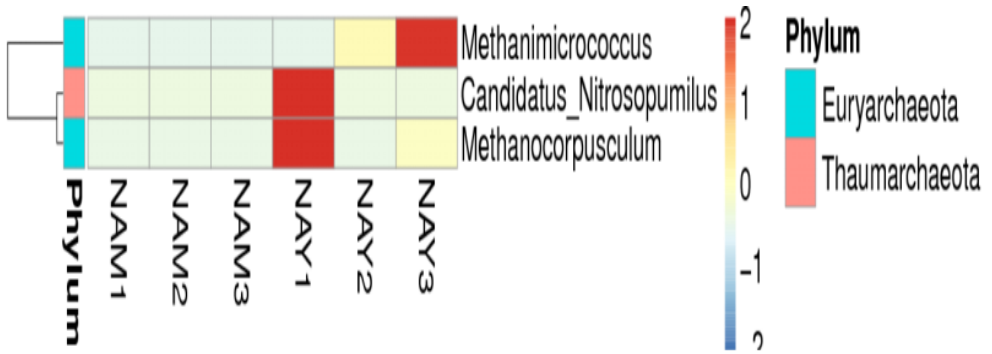


**Figure 4.** An abundance distribution heatmap showing the most dominant archaeal genera from the pitcher phytotelmata of *Nepenthes alata* Blco

***Richness and alpha diversity analysis of archaeal community composition among young and mature pitchers of N. alata***

A decreasing trend of species richness was observed in the young pitchers (NAY1, NAY2, and NAY3) as the time progressed although the difference in the observed species was not statistically significant (Figure 5A). On the other hand, no trend was seen on the observed species in the mature pitchers (NAM1, NAM2 and NAM3) as time progressed, and the observed species number was not significantly different (Figure 5A). Similarly, the Shannon Index did not differ significantly among samples of young pitchers (NAY1, NAY2, and NAY3) and among samples of mature pitchers (NAM1, NAM2 and NAM3) across the sampling periods. There was also no significant difference between the young and mature pitchers (Figure 5B). T-test, Wilcox (for 2 groups) and Tukey's tests (for > 2 groups) were performed for the analysis of significance of difference between groups.

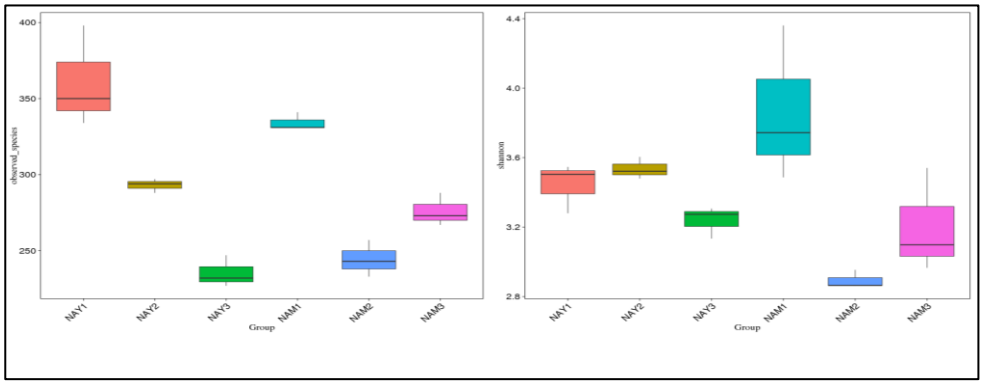


**Figure 5.** The richness (number of observed species) (5A) and Shannon Index (5B) in young and mature pitchers of *N. alata* samples

***Temporal changes in archaeal composition in the young and mature pitcher phytotelmata***

In total, there were 269 phylotypes common among young pitcher phytotelmata across all sampling times representing 48.0% of the total archaeal phylotypes (Figure 6). Being consistently present in all phytotelmata, these 269 phylotypes are likely to be the core archaeal biome in young pitcher plants. Furthermore, based on Analysis of Similarity (ANOSIM), variation among all samples is not statistically significant.

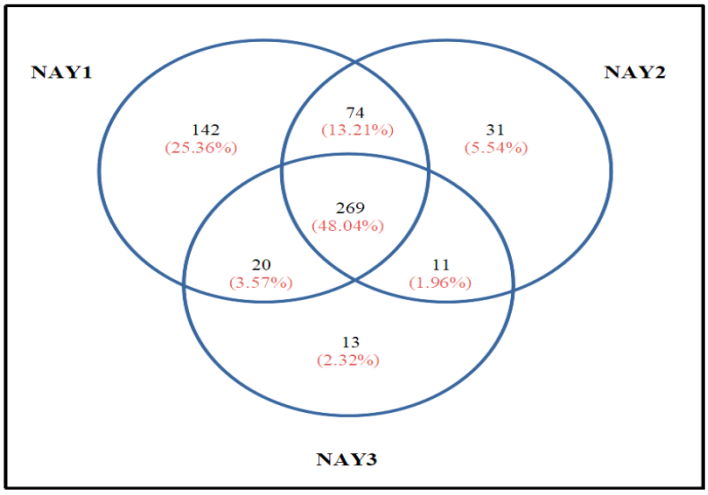


**Figure 6.** Venn Diagram showing the number of shared and unique archaeal phylotypes as Operational Taxonomic Units (OTUs) among young pitchers of *Nepenthes alata* Blco. during the first sampling time (NAY1), second sampling time (NAY2), and third sampling time (NAY3)

There were 200 OTUs common among mature pitcher phytotelmata across all sampling times representing 35.1% of the total phylotypes (Figure 7). Being consistently present in all phytotelmata, these 200 phylotypes likely form the core archaeal biome in mature pitcher plants. Based also on ANOSIM, variation among each samples was not statistically significant. The possible core microbiome of mature plants represented a lower percentage than that of the core microbiome of young pitcher phytotelmata. However, the variation among all samples regardless of the age of pitchers was also not statistically significant based on ANOSIM.

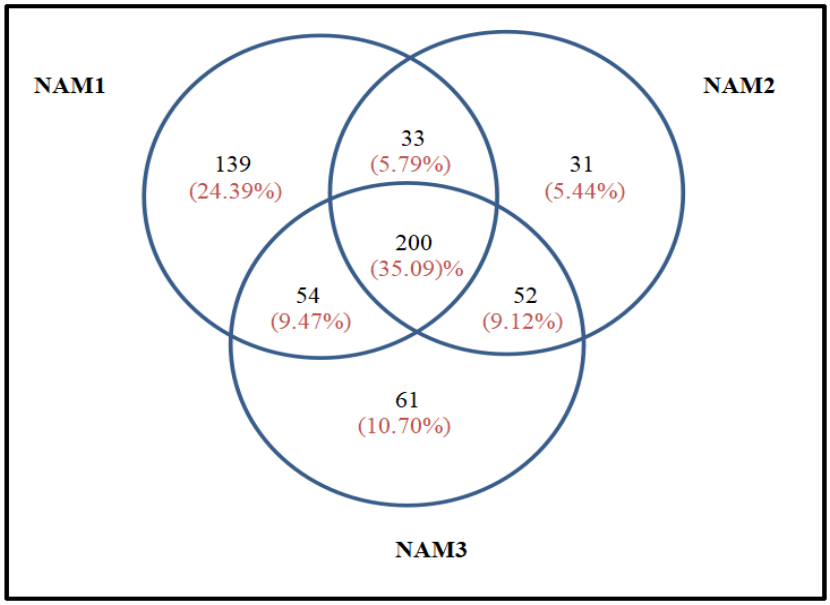


**Figure 7.** Venn Diagram showing the number of shared and unique archaeal phylotypes (OTUs) among mature pitchers of *Nepenthes alata* Blco. during the first sampling time (NAM1), second sampling time (NAM2), and third sampling time

***Non–metric multi-dimensional scaling (NMDS) analysis of archaeal community structure***

Overall, samples from young and old pitchers from the same sampling time clustered with each other, suggesting similar archaeal communities in the pitchers collected at the same sampling site and sampling time (Figure 8). Thus, over randomly selected pitchers at the sampling site, the archaeal community

appeared to be similar, regardless of possible micro-changes in environmental conditions.

The samples from the young pitchers also clustered together along one axis, suggesting a relatively high level of similarity in archaeal composition in young pitcher phytotelmata over the sampling period (3 months) (Figure 8). In contrast, the archaeal composition in mature pitchers appeared to have a larger shift from the first sampling time to the second and third. This change in archaeal communities from the mature phytotelmata over time could be likely correlated with the possible changes in environmental conditions during the sampling period.

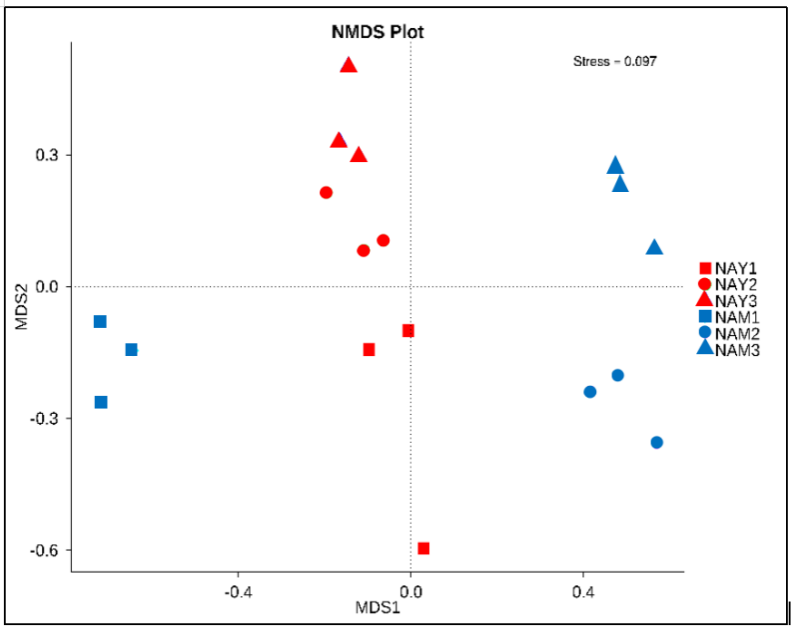


**Figure 8.** Non-metric multi-dimensional scaling (NMDS) plot showing the relative similarities of archaeal communities among and between young (NAY1, NAY2, NAY3) and mature (NAM1, NAM2, NAM3) pitchers of *Nepenthes alata* across three sampling periods

## Discussion

For archaeal communities in *Nepenthes*, the sequences of the 10 most abundant phyla could not be mapped to both the Euryarchaeota and the Thaumarchaeota. Based on the taxonomic tree constructed, high number of OTUs clustered to unclassified archaeon “SCG” and as well as to unclassified Thaumarcheota. The phytotelmata of the samples, therefore, harbored high

abundance of previously uncultured archaea; known phyla with cultivable or sequenced species/genera were not actually present in the phytotelmata. This result is in contrast with the findings of Taffner *et al.* (2018) when they surveyed the rhizosphere of alpine vegetation and found that the Euryarcheota was the dominant group. *Candidatus_Nitrosopumilus*, *Methanocorpusculum* and *Methanimicroccus* were the most abundant genera from young pitchers with the latter two being methanogens. This finding is in agreement with the results by Krieger and Kourtev (2012) where all of the archaeal DNA sequences obtained from the pitcher plant *Sarracenia* were closely related to methanogenic genus *Methanobrevibacter*.

*Nitrosopumilus* is an autotrophic ammonia-oxidizing archaeal genus and is the dominant chemolitotroph in pelagic waters (Madigan *et al*., 2019). Despite recent enrichment of both mesophilic and thermophilic ammonia-oxidizing archaea (AOA), the first successful isolation of AOA happened only in 2005 when Konneke and colleagues isolated *N. maritimus* (Walker *et al*., 2010; Konneke *et al*., 2005). Ammonia oxidation is the rate-limiting step in nitrification (He *et al*., 2018), and is a fundamental core process in the global biogeochemical cycle of nitrogen (Lehtovirta-Morley, 2018). Prior to the discovery of ammonia-oxidizing archaea (AOA) in 2004-2005, ammonia oxidizing bacteria (AOB) were always considered to be the sole players in this process (He *et al*., 2018) and so, the discovery of AOA prompted a reassessment of the paradigm for ammonia oxidation (Lehtovirta-Morley, 2018). The detection of Candidatus_Nitrosopumilus in our study implies the presence of both ammonia and nitrite in the phytotelmata. As early as 1885, ammonia was already considered to be omnipresent in leaves of pitcher plants (Higley, 1885; as cited by Bradshaw and Creelman, 1984). Previous studies on measurement of ammonia on leaves and inquilines of pitcher plants have also been reported (Bradshaw and Creelman, 1984). Nitrous oxide ($N_2O$), a trace gas formed during the nitrification-denitrification process, has also been previously detected in *Nepenthes* (Baby *et al*., 2017). Because of its ability to oxidize ammonia, *Nitrosopumilus* may be a contributor to the nitrogen cycling for use by the pitcher plant. In their work, Takeuchi *et al*. (2015) expected that bacteria involved in the nitrogen cycle would be abundant in the fluid of *Nepenthes* but surprisingly, their result showed that bacteria involved in N cycle like *Nitrospira* were only present at low levels in some of their samples. Our data supports the possibility that archaea such as *Nitrosopumilus* are a major contributor to the N cycle in the phytotelmata of *Nepenthes*. The exact benefit of the pitcher plant from AOA is yet to be discovered but it is well-known that nitrogen limits growth in most terrestrial ecosystems (Vitousek *et al*., 1997). This is particularly true for pitcher plants which are generally

restricted to extremely N-limited habitats and have developed carnivory as an alternative pathway for obtaining nutrients (Karagatzides *et al*., 2009). Furthermore, the northern pitcher plant *Sarracenia purpurea* was reported to receive nitrogen from multiple sources including $NH_4$ and $NO_3$ (Butler and Ellison, 2007) and this can also be true for *N. alata.*

Both *Methanocorpusculum* and *Methanimicrococcus* are irregular coccoid methanogens. The former uses $H_2$+$CO_2$, formate, and alcohols as substrate for methanogenesis while the latter uses methanol and methylamines in the presence of $H_2$ (Madigan *et al*., 2019). Their growth in the young pitchers likely indicate hydrogenotrophic methanogenesis as the dominant pathway for methane generation, as opposed to acetogenic methanogenesis. Additionally, this result implies the presence of their substrates and metabolic products in the pitcher phytotelmata of *N. alata.* For instance, Baby *et al*. (2017) found that opened pitchers of *N. khasiana* are constant emitters of $CO_2$ (476.75 +/- 59.83 ppm). Ambient levels of methane were also detected. According to Baby *et al*. (2017), dissolved $CO_2$ in *Nepenthes* pitcher fluid instantaneously forms an equilibrium with its hydrated form $H_2CO_3$ which dissociates into $H^+$ and $HCO3^-$ . On the other hand, production of methanol in leaves is very common, and is formed by plants by demethylation of macromolecules (Dorokhov *et al*., 2018). Formate in turn, can be formed from methanol (Dorokhov *et al*., 2018). It is possible that the metabolic by-products of *Nepenthes alata* are the key sources of substrates of the methanogens detected in our study.

Additionally, of the 560 total phylotypes detected, 269 (~48.0%) and 200 (35.1%) were consistently present across the sampling periods in young and mature pitchers, respectively. In their study on natural vegetation of alpine bogs, Taffner *et al.* (2018) showed that archaea are a substantial component of the plant microbiome and are able to fulfill functions for the host. In the same manner, pitcher plant-archaea interactions could also exist, based on the data gathered in this study.

NMDS analyses of the archaeal communities showed that the composition of the young pitcher phytotelmata were more clustered while those of the mature pitcher phytotelmata were more dispersed across time. This finding, therefore, indicates relative greater similarity of archaeal community composition in the former and relative greater dissimilarity in the latter.

Considering that many of the genera detected in the phytotelmata were only described in the last 15 years or so, and some genera currently contain only a limited number of species, the possibility of isolating novel microorganisms in this environment is still very high. Interestingly, many of the earlier archaeal taxa described were more commonly associated with extreme environments. The potential discovery of previously undescribed archaea in

non-extreme environments such as pitcher plant phytotelmata is possible and worth investigating in the future.

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