

**Comparing the fungal community in pitcher plant fluid
of *Sarracenia purpurea* and *Nepenthes robcantleyi* individuals**

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Abstract

The microbial makeup of pitcher plant fluid is remarkably diverse. This microorganism-enrich fluid provides an ideal platform to characterize fungal community by using DNA barcode technique. This project employed two DNA barcode locus, ITS and LSU, to characterize fungal communities in two pitcher plant species: *Sarracenia purpurea* and *Nepenthes robcantleyi*. DNA sequence analysis confirmed *Candida palmiolephia* and *Candida pseudoglaebosa* existence in *Sarracenia purpurea* pitcher fluid individuals. However, their description and the role in symbiotic relationship with the *Sarracenia purpurea* need further investigation. ITS phylogenetic tree implied that the three identified fungal species in *Sarracenia purpurea* pitcher fluid were closely related. However, the uncultured fungal species in *Nepenthes robcantleyi* were not related to the identified fungal species in *Sarracenia purpurea*. To further characterize those uncultured fungus, culture-dependent method would be required.

Key word: *Sarracenia purpurea*, *Nepenthes robcantleyi*, Internal Transcribed Spacer, Large Subunit, DNA barcode.

Introduction

The pitcher plant is a carnivorous plant that thrives in low-nutrient areas. In order to supplement nutrients not found in the surrounding soil, the pitcher plant lures insects into the sticky liquid inside its pitfall trap (Koopman, et al., 2010). When the insects die, the plant protease Nepenthesin dissolves the prey to release critical nutrition. Fungi belong to a large group of eukaryotic organism that distinguish themselves from other kingdoms by chitin cell walls and lack of chloroplast. Playing a significant role in ecosystem, fungi contribute to the organic material turnover and food chain maintenance. The microorganism-enrich pitcher fluid constitutes an ideal platform to characterize the fungal community. This project compares the fungal communities in two different pitcher plant species: *Sarracenia purpurea* and *Nepenthes robcantleyi*. *Sarracenia purpurea* originates from North America. It differentiates itself from other pitcher plant species by its leaves directly growing from the root. *Nepenthes robcantleyi*, the notorious tropical pitcher plant species is famous for its colorful trumpet-shape pitchers hanging down from the vines. The aim of the fungal community characterization is to identify the associative fungi that may provide clue to pitcher plant health situation.

DNA barcode technique allows us to characterize fungal species in its native environment directly. Advance in molecular biology confers each species with its own DNA barcode. DNA barcode technique uses a short DNA segment as a biomarker to characterize the unknown species by comparing the amplified DNA

sequence to the sequence data of existing species. This short DNA fragment should cover all taxa of interests and contain enough single nucleotide polymorphism to provide discriminatory power. For instance, 16s rDNAs and RUBISCO are frequently employed as biomarkers in phylogenetic analysis of bacteria and plants respectively. In this project, I used Internal Transcribed Spacer (ITS) and Large Subunit (LSU) to characterize fungal species in pitcher plant fluid. Massive phylogenetic research articles prove that ITS and LSU are reliable and economic DNA barcode genes in specific fungal characterization and frequently used by many scientists.

Methods

1) Sample collection.

The two *Sarracenia purpurea* pitcher samples were gift from Paul Smith's College VIC. All pitchers were excavated frozen solid from sphagnum mat next to the Boreal Life Trail at the Paul Smiths VIC and sent to me in plastic bag with ice pad. The six *Nepenthes robcantleyi* pitcher fluid samples came from Native Exotics Inc, Ithaca. Approximately 5mL of fluid was obtained from each sample and transferred into sterile tubes immediately after the packages were received. Samples were either used immediately or stored in a -20°C freezer for future use.

2) DNA extraction.

To extract DNA from native environment, we used the FastDNA® Spin isolation kit (MP Biomedicals) to extract DNA in accordance with the manufacturer protocol.

3) ITS gene and LSU gene PCR amplification.

Each extracted DNA sample was quantified on spectrophotometer, and then diluted into 5ng/ μ l solution to set up PCR reaction. The two sets of primers for those genes were designed as below:

LSU primers	Forward 5' -ACCCGCTGAACTTAAGC-3'
	Reverse 5'-TCCTGAGGGAACTTCG-3'
ITS primers	Forward 5'-TCCTCCGCTTATTGATATGC -3'
	Reverse 5'-GGAAGTAAAAGTCGTAACAAGG-3'

The PCR reagents were added according to the protocol below:

DNA template	1 μ l (5ng/ μ l)
Forward primer (10 pmol/ μ l)	1 μ l
Reverse primer (10 pmol/ μ l)	1 μ l
10X PCR Master Mix	5 μ l
DNA Polymerase	1 μ l
dNTP mix	1 μ l
NFW	40 μ l
Total	50 μ l

Run PCR according to the following program:

PCR Program for ITS and LSU locus, 40 cycles		
<i>Step</i>	<i>Temperature (°C)</i>	<i>Time</i>

Denature	95	3 minutes
40 cycles		
Denature	94	30 seconds
Anneal	58	30 seconds
Extend	72	30 seconds
Extend	72	2 minutes
Soak	4	Soak

After PCR was finished, Run 5 μ L of PCR product on 1X TBE, 1% agarose gel. The amplified PCR products were cleaned up by using QIAGEN PCR Miniprep® kit.

4) Ligation and transformation.

Two ITS and two LSU PCR products were inserted into pGEM-T® Easy Vector according to the protocol described below.

Reaction component	<i>Sarracenia</i>	<i>Nepenthes</i>	Negative control
2Xligation buffer	5 μ l	5 μ l	5 μ l
pGEM-T vector (50ng)	1 μ l	1 μ l	1 μ l
PCR product (52ng)	1 μ l	1 μ l	
Insert Control (water)			1 μ l
T4 ligase	1 μ l	1 μ l	1 μ l
NFW	2 μ l	2 μ l	2 μ l

Mix the reactions by pipetting. Incubated the ligation reactions in a 37°C water bath for 2 hours or 4°C overnight. Centrifuge the tubes containing the ligation reactions to collect the contents at the bottom. Add 2 μ l of each ligation reaction to a 1.5 mL sterile

micro-centrifuge tube on ice. Carefully transfer 50µl of competent cells into each tube. Gently mix and incubate them on ice for 20 minutes. Heat-shocked the cells for 45-50 seconds in a water bath at 42°C. Immediately return the tubes to ice for 2 minutes. Added 950µl room temperature LB medium to the tubes containing cells transformed with ligation reactions. Incubate for 2 hours at 37°C with shaking (~150rpm). Set up three parallel LB/ampicillin/IPTG/X-Gal plates for each ligation reaction with different cell concentration. Incubate the plates overnight (16–24 hours) at 37°C. Confirmed correct colonies by picking them onto fresh LB/ampicillin/IPTG/X-Gal plates using sterile pipette tip and plate grids. Pick 30 colonies per site. Grow overnight at 37°C and plates were directly sent out for sequencing.

5) DNA sequences analysis and phylogenetic tree construction.

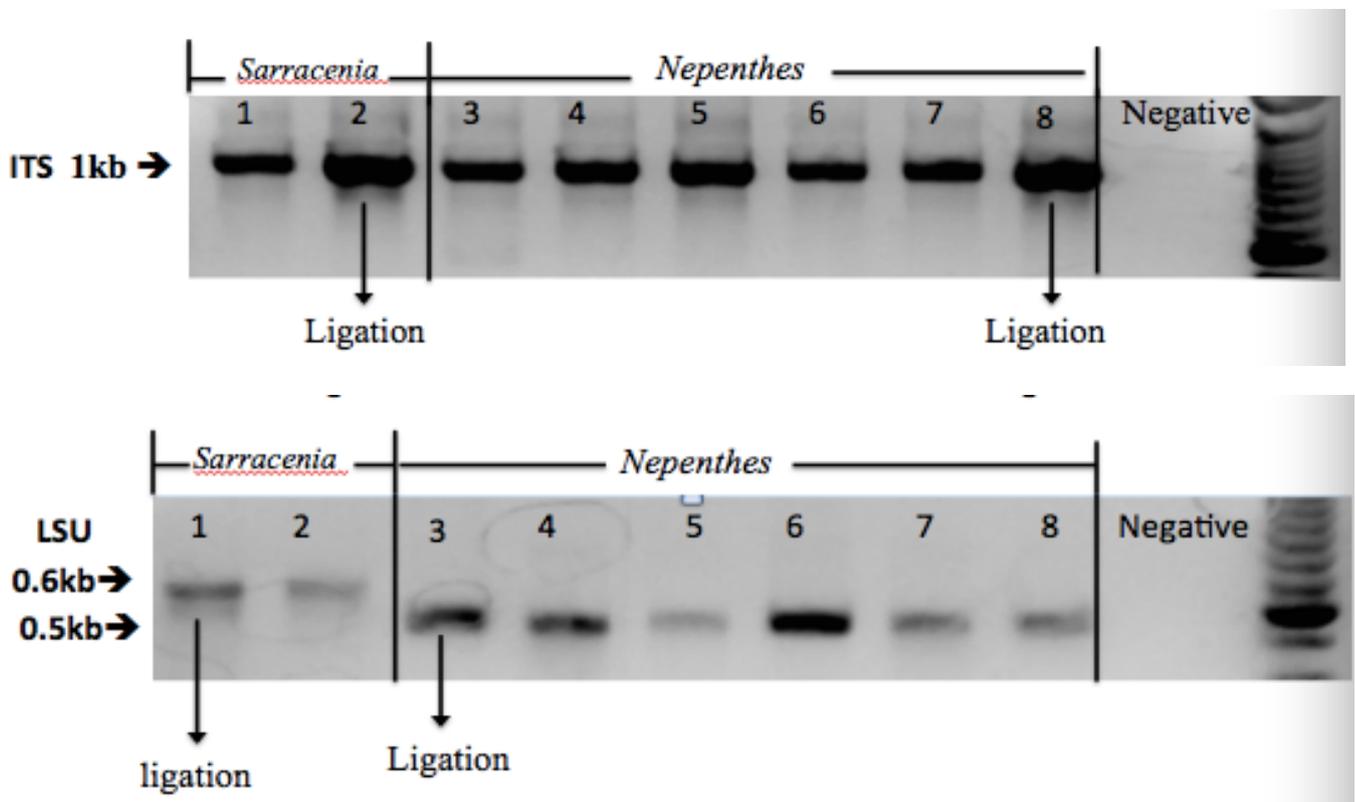
DNA sequence data were “BLAST” to determine species. To measure the relationship of the uncultured fungus to the fungal species identified in *Sarracenia purpurea* pitcher fluid individuals, I aligned the 3 identified fungal sequences, 30 uncultured fungal sequences and 21 primer referential fungal sequences in ApE® software. Extra sequences were trimmed away so that every sequence contained the same leading and ending sequence. The multiple trimmed sequences were then aligned in Clustal Omega® to create a phylogenetic tree.

Result

1) Amplification of ITS and LSU locus.

ITS and LSU locus were successfully amplified in two *Sarracenia purpurea* samples and six *Nepenthes robcantleyi* samples. Upon amplification, a band of 1kb was observed for ITS gene and 500b/600b for LSU gene (Figure1).

Figure1



Top panel shows the ITS locus amplification after 40 cycles. Lane 1, 2 were samples (1kb) from *Sarracenia* and lane 3,4,5,6,7,8 were from *Nepenthes*. Lane 9 is the negative control. The bottom panel shows the LSU locus amplification after 40 cycles. Lane arrangement is the same as the top panel.

2) Sequence analysis of ITS and LSU confirmed that *Candida palmiolephia* and *Candida pseudoglaebosa* probably existed and predominated in fungal community of *Sarracenia purpurea* pitcher fluid individuals.

For each pitcher plant species, one PCR product was used to do ligation and transformation. After re-streaking white colonies onto the plate, 30 colonies were formed for each pitcher plant species. Every colony may represent one fungal species. Totally 120 colonies were sent out for sequencing for two barcode genes. The identified fungal species were listed in figure 2. The majority of colonies for *Sarracenia purpurea* discovered *Candida* species. However, 59 colonies for *Nepenthes robcantleyi* identified the uncultured fungus in “BLAST” results.

Figure 2.

Plant species	DNA barcode	Fungal species identified
<i>Sarracenia purpurea</i>	LSU	<i>Candida palmiolephia</i> (80%)
	ITS	<i>Candida pseudoglaebosa</i> (66%) <i>Phoma herbarum</i> (10%) <i>Lambertella advenula</i> (10%)
<i>Nepenthes robcantleyi</i>	LSU	Uncultured fungus (100%)
	ITS	Uncultured fungus (100%)

For LSU in Sarracenia purpurea, 24 colonies confirmed the existence of Candida palmiolephia; 5 colonies are empty vectors.

For ITS in Sarracenia, 20 colonies confirmed the existence of Candida palmiolephia; 3 colonies identified Phoma herbarum; 3 colonies identified Lambertella advenula; 4 colonies were empty vectors.

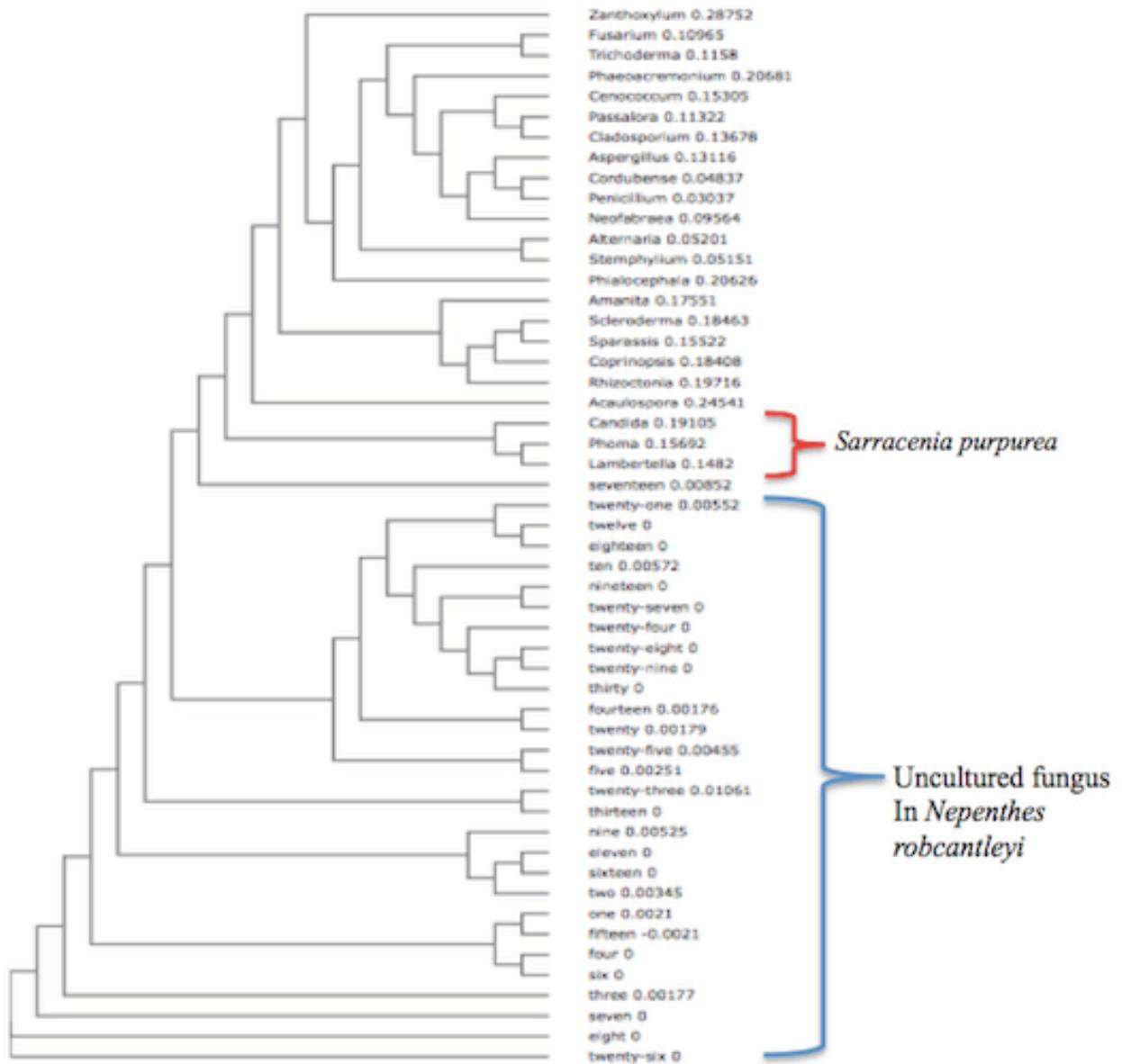
For LSU in Nepenthes, all colonies identified uncultured fungus.

For ITS in Nepenthes, 29 colonies identified uncultured fungus, 1 colony was empty vector.

3) ITS phylogenetic tree implied that the uncultured fungus recovered from *Nepenthes robcantleyi* were probably not related to the identified fungal species of *Sarracenia purpurea*.

For ITS, three identified fungal species sequence from *Sarracenia purpurea*, thirty uncultured fungal sequence from *Nepenthes robcantleyi* and 21 primer referential sequences were trimmed and aligned together in “Clustal Omega®” to create a phylogenetic tree. ITS phylogenetic tree implied that the three identified fungal species in *Sarracenia purpurea* pitcher fluid were closely related. The 30 uncultured fungus of *Nepenthes robcantleyi* were also related to each other. However, the uncultured fungal species in *Nepenthes robcantleyi* were not related to the identified fungal species in *Sarracenia purpurea*. Further culture-dependent method characterization would be required.

Figure3.



ITS phylogenetic trees indicated that the uncultured fungus recovered from Nepenthes robcantleyi were probably not related to fungal species discovered from Sarracenia purpurea. 21 ITS primer referential fungal sequences were added.

Discussion

ITS gene PCR amplification showed a band of 1kb for each sample that was in accordance with the ITS band size in reference (Schoch. et al. PNAS 2012). LSU PCR amplification showed a band of 0.6kb for *Sarracenia purpurea* and a 0.5kb band for *Nepenthes robcantleyi*. This band size difference indicated that different fungal species probably exist in those two pitcher plant species. ITS gene identified more fungal species than LSU in this project because the amplified ITS gene size was much larger and contained more variations than LSU to provide discrimination. Therefore when using the DNA barcode technique to characterize species in an unknown community, it is preferential to use combination of different DNA barcode genes. The common discovery of *Candida* species by ITS and LSU suggested that *Candida* species probably existed and predominated in *Sarracenia purpurea* pitcher fluid. However, their functional description and the reason why *Candida* species are so adapted to *Sarracenia purpurea* are still largely unknown. *C. palmioleophila* was reported in 1991 as an opportunistic pathogen causing intravenous catheter-associated fungemia (Rasmus H. Jensen, et al., 2011). The ITS-based phylogenetic tree implied that the uncultured fungus recovered from *Nepenthes robcantleyi* were probably not related to the fungal species discovered from *Sarracenia purpurea*. To further determine those uncultured fungus, culture-dependent method would be required.

Reference

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