

# **A Preliminary Investigation of *Cypripedium parviflorum* (Yellow Lady Slipper) Mycorrhizae at Peninsula State Park**

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## **Abstract**

Orchids are known to require specific associations with mycorrhizal fungi for germination, and the abundance of these fungi may be related to orchid dormancy periods. The photosynthetic orchid *Cypripedium parviflorum* (Yellow Lady Slipper) shows particularly high specificity and is known to associate with mycorrhizae from the Tulasnellaceae family. A previous study identified that orchid fungi is most abundant in the soil within one meter or less from the base of an orchid, and more research is needed to determine if this is a species-specific or orchid-specific trend. The presented study aimed to isolate pure cultures of *C. parviflorum* mycorrhizae with the goal of characterizing the abundance of that mycorrhizae in the soil at sites in Peninsula State Park.

## **Introduction**

In order to germinate, orchid seeds require the presence of particular mycorrhizal fungal symbionts in the soil. Orchid population size has been found to be affected by the abundance of mycorrhizal fungi, and it is thought that related dormancy periods may also occur in response to fungal availability (Rock-Blake et al. 2017). High levels of mycorrhizal fungi in soil tend to indicate locations for successful orchid seed germination, although other environmental conditions such as soil moisture and nutrient availability play a role in determining overall plant success (McCormick et al. 2016). Identification of mycorrhizal fungi may predict which orchid species may certain locations, as many photosynthetic and non-photosynthetic orchids have been found show high fungal specificity (McCormick et al. 2004).

This study aimed to characterize the fungal symbionts of *Cypripedium parviflorum* (Yellow Lady Slipper) at Peninsula State Park by culturing mycorrhizae from root samples. *Cypripedium parviflorum* form endophytic mycorrhizal relationships predominantly with fungi from the Tulasnellaceae family (Shefferson et al., 2005). *Cypripedium* species show high mycorrhizal specificity comparable to that of non-photosynthetic plants (Shefferson et al., 2007), although the orchid is photosynthetic itself. This specificity has motivated many researchers to investigate possible reasons as to why *C. parviflorum* associates with this specific mycorrhizal symbiont.

A previous study identified that orchid mycorrhizal symbionts tend to be most abundant in the soil near the base of the plant and drop off by the distance of one meter away (McCormick et al., 2016). Further research is needed to determine if this pattern in abundance is common to orchids in general, or just the specific orchid species studied by McCormick et al. (2016). For that reason, we decided to collect soil core samples from some of the plants we sampled from in order to quantify the abundance of the *C. parviflorum* mycorrhizae in the soil. With the results of this study we hope to inform Peninsula State Park land managers of the habitat requirements of *C. parviflorum* for future preservation.

## Methods

*Field Methods:* Data collection took place during mid-June and beginning of July when *C. parviflorum* was in bloom at Peninsula State Park. Sampling took place at two main populations of *C. parviflorum* near the park headquarters (n = 6) and the winter water station in Tension Bay (n = 2) (Figure 1). Malcore collected GPS coordinates, general vegetation data, soil type (sandy, loamy, or clay), soil core samples, plant height (cm), and root samples from each plant, and assigned each plant a unique identification number. Soil samples were collected in four cardinal directions up to one meter from the plant (0, 25, 50, 75, and 100 cm) (Figure 2), based on the sampling distances of (McCormick et al., 2016). Not all plants had soil samples taken. Additional notes included observations of other nearby *C. parviflorum* plants that may have been in close proximity to a soil sample.



**Figure 1: Sampling sites at Peninsula State Park.** (A) Samples were taken from two locations: the park headquarters (n = 6) and winter water station (n = 2). (B) Close-up of the sampling sites at the park headquarters. (C) Close-up of the sampling sites at the winter water station. All images were generated from ARCGIS Online, sampling sites are indicated by yellow circles.



*Figure 2: Soil sample collection layout. Orange flags mark 25, 50, 75 and 100 centimeters from the base of the plant in four cardinal directions, and soil samples were taken from each flagged site.*

Laboratory Methods: All culturing was performed under a laminar flow hood following sterile techniques.

**Roots & Fungi:** Roots were surface sterilized using a 5% ethanol and 5% bleach mixture. Two methods of fungal culturing were then used, and roots were either chopped into small pieces and nutrient media was poured over the root pieces, or pelotons were extracted under a microscope and placed onto nutrient media (Figure 3; Zettler and Corey, 2018). Fungal hyphae growing off root pieces were excised from media and plated onto new agar plates to establish pure cultures. Pure culture fungi were then grown on gel-drying film, scraped off, and placed in 2 microliter plastic DNA isolation tubes with glass beads, which were dried using a freeze drier. Fungal DNA was isolated using a MagJET Plant Genomic DNA Kit (Thermo Scientific, Waltham, Massachusetts, USA) following the manufacturer's protocol. The Ribosomal ITS loci (ITS1F-CNL2F) was amplified by PCR in 25  $\mu$ L reactions composed of 2.5  $\mu$ L DNA, 1x Promega GoTaq® Colorless Master Mix (Promega, Madison, Wisconsin, USA), 0.2  $\mu$ M of each forward and reverse primers specific for each locus, ITS1F and CNL2F. Thermocycler conditions were: 95°C for 2 min (initial denaturation), 94°C for 45 sec (denaturation), 55°C for 45 sec (annealing), 72°C for 2 min (extension), and 72°C for 5 min (final extension) for 35 cycles followed by a 15°C hold. PCR products were visualized using agarose gel electrophoresis following Grubisha et al. (2014) and were cleaned enzymatically using ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA) following the manufacturers' protocol. PCR products were sequenced at the University of Kentucky HealthCare Genomics Core Laboratory. Electropherograms were reviewed and consensus sequences of forward and reverse sequences were assembled using Geneious version R8.1 (Biomatters Inc., Newark, New Jersey, USA).

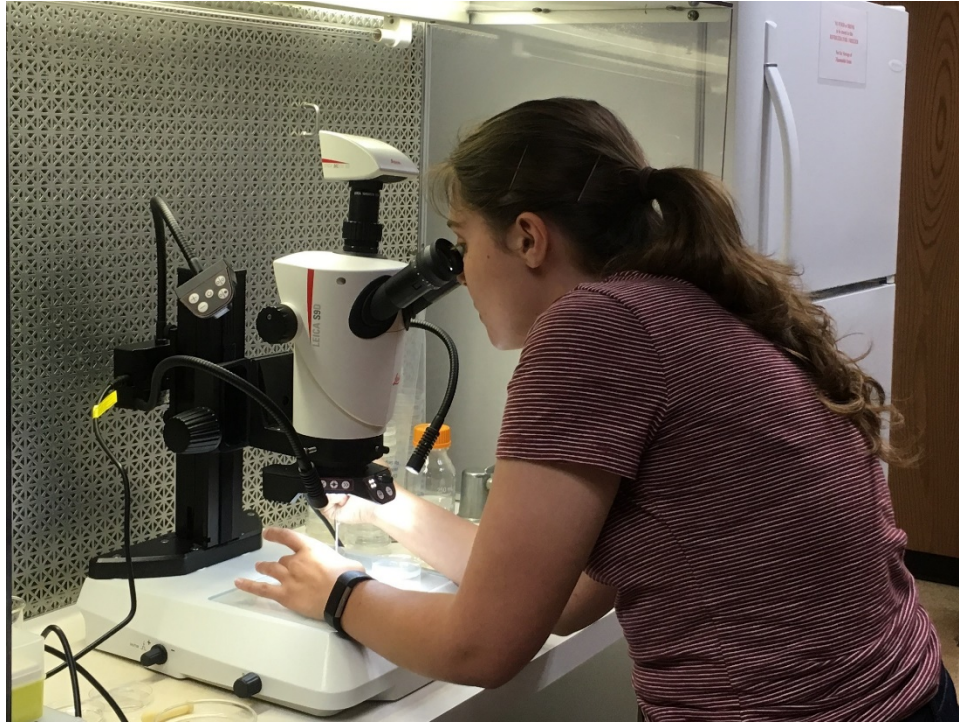


Figure 3: Malcore doing peloton isolation under a microscope from a *C. parviflorum* root.

**Soil:** Soil core samples were sieved using a 4.75 mm followed by a 2.36 mm grate and portioned into 0.25 gram samples in 2 microliter DNA isolation tubes, along with 0.5mm and 0.1 mm glass beads. Soil DNA was isolated using the Quick-DNA Fecal/Soil Microbe Kit (Zymo, Irvine, California, USA) following the manufacturer's protocol.

## Results

Of the pure cultures sequenced thus far, all fungi were opportunistic soil fungi including *Phomopsis spp.* (n = 4; Figure 4) and *Cadophora spp.* (n = 1; Figure 5).



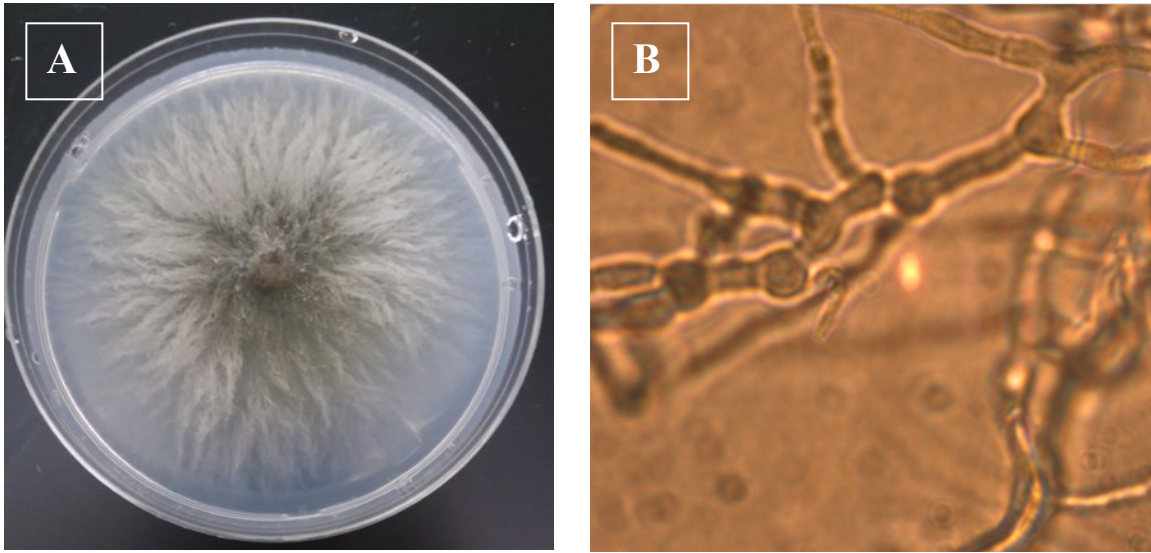


Figure 4: (A) *Phomopsis spp.* from one of the root samples on B.S. media. (B) Microscope image of the monilloid cells of *Phomopsis* hyphae.

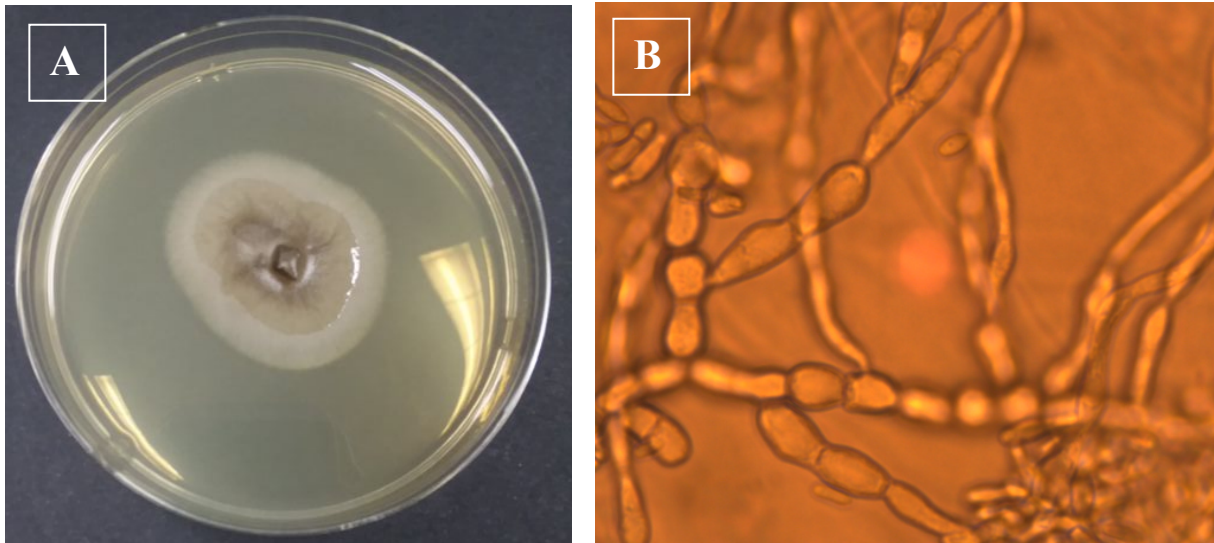


Figure 5: (A) *Cadophora spp.* from one of the root samples on PDA media. (B) Microscope image of the monilloid cells of *Cadophora* hyphae.

## Discussion & Conclusion

As previously stated, two different opportunistic fungi were identified from the root samples: *Phomopsis spp.* and *Cadophora spp.* *Phomopsis spp.* are a common endophytic fungus that associate with many plants and plays a significant role in decomposition (Chen et al., 2013). *Cadophora spp.* are also a common general endophytic mycorrhiza (Knapp et al., 2018). However, neither *Phomopsis spp.* or *Cadophora spp.* are known as *C. parviflorum* mycorrhizae.

Unfortunately, no pure cultures of Tulasnellaceae mycorrhizae have been obtained thus far, and we were unable to use qPCR to quantify the amount of mycorrhizae in the soil samples taken at different distances from the *C. parviflorum* plants we sampled from. Isolating and establishing pure cultures of orchid mycorrhizae is a challenging process because orchid fungi is typically slow-growing, while opportunistic fungi is typically fast-growing and overtakes the plate it is growing on. As far as we know, pure cultures of *C. parviflorum* mycorrhizae have yet to be isolated and obtained by researchers in this field. In the summer of 2019 and likely 2020, Dr. Lisa Grubisha's graduate student Jason Miller hopes to follow up on this project, building off of the methods we developed, and may sequence more pure cultures obtained from the sampling done by Malcore.

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