Mycorrhizal Fungal Associates of the Eastern Prairie Fringed orchid (*Platanthera leucophaea*)

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Introduction

Platanthera leucophaea (Eastern Prairie fringed orchid or EPFO, Figure 1) is currently listed as a U.S. federally threatened species (U.S. Fish and Wildlife Service, 1989). Conservation efforts for recovery have included habitat restoration, hand pollination, population augmentation, and reintroduction (Lah, 2003). Despite these efforts seedling establishment leading to self-sustaining populations in situ have yet to be verified (Bowles et al. 2005; Zettler and Piskin, 2011).

The levels of genetic diversity of the fungal associates in EPFO populations are unknown. The 2016 EPFO Five Year Review (USFWS, 2016) stated that understanding fungal diversity among prairie sites is important for knowing what strains can be released between sites. Proper identification of the relevant fungi will also prove useful for lab experiments on seed germination.

The purpose of this research was to examine fungal diversity across populations of EPFO in Illinois and Wisconsin using molecular markers (ITS and CETH primers, Figure 2) for

Figure 1. *Platanthera leucophaea* (Crosby, 2017)

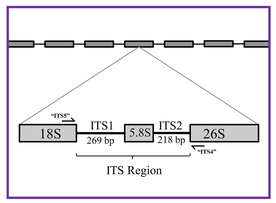

Figure 2. Internal transcribed spacer region (Zhang, Yuan, Yang, Huang, & Huang, 2015)

Methods

Fungi has been isolated from root samples collected between 2018 and 2019 from four EPFO sites in Illinois (including the Nachusa Grasslands) and seven sites in Wisconsin and have been maintained in our lab at SIUE (Figure 3). Fungal DNA isolations were done with the DNA Power PRO soil kit from Qiagen after cultures were grown in potato dextrose broth. PCR ITS reactions were done with the Extract-N-Amp Plant PCR Kit from Sigma Aldrich and products were sequenced by the U of I Core sequencing Facility, Champaign Urbana. Sequence alignments were filtered to exclude regions that typically only included sequence gaps. Nucleotide diversity statistics, including Nei's pi, were calculated using the PopGenome package in R. The seqinr package was used to calculate the distance matrix between isolates, which was used to generate the heatmap and dendrogram (Figure 4). The adegenet and ade4 packages were used to visualize the nucleotide positions with the most genetic variation (Figure 5) and to conduct a principal component analysis (PCA; Figure 6).

Figure 3. Locations of sampled *P. leucophaea* populations at blue dots.

Results

Nucleotide Diversity	Nei's Pi value
Overall	2.75
Within Illinois/Iowa populations	1.44
Within Wisconsin populations	14.57
Between group diversity	8.22

Table 1. Overall nucleotide diversity for this ITS region is low. Fungal sequence diversity within combined Illinois/Iowa sequences (Nei's Pi 1.444) was much lower than within Wisconsin populations (Nei's Pi 14.57). In comparisons among Illinois/Iowa verses Wisconsin fungal ITS sequences, these two groups are quite diverse (Nei's Pi = 8.22).

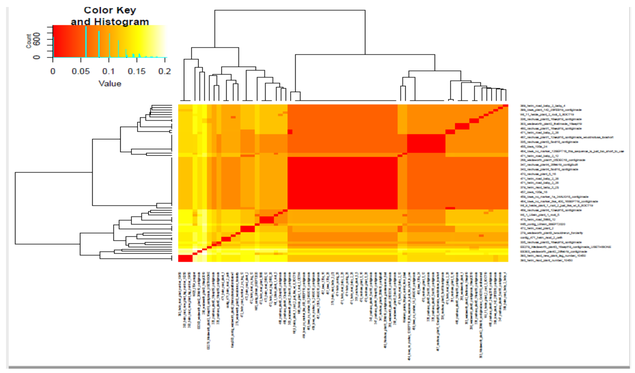

Figure 4. Genetic distance heatmap. Similar colors indicate similar nucleotide sequences. The *Ceratobasdium* isolates examined in this study have high genetic similarity. The red square in the middle represents 33% of the data has genetic identity values ranging between 98.6-99.7% identity. The highest levels of diversity are bright yellow 80-85% and white 80%.

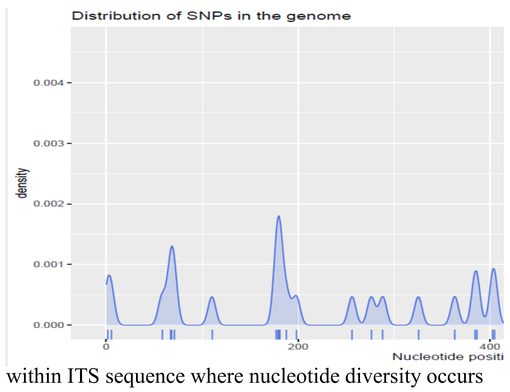

Figure 5 Areas within ITS sequence where nucleotide diversity occurs

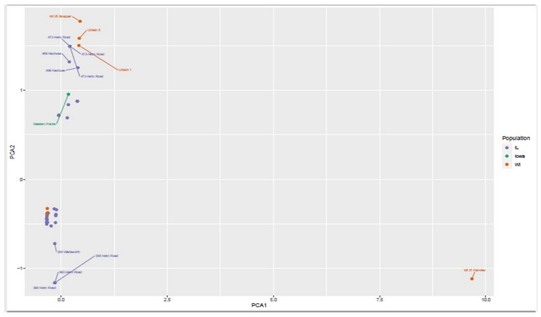

Figure 6. Figure 6. PCA analysis of nucleotide diversity and geographic location. There is an association between geographical site and the clustering of isolates based on the PCA. Sites that are close together are not necessarily genetically more similar. One Wisconsin isolate (W 31 Fairview) was vastly genetically different from all other isolates. Two other clusters were also identified based upon their PCA2 values. These clusters contained both Illinois/Iowa and Wisconsin isolates. Overall, Wisconsin isolates are more diverse than Illinois. In Illinois, the Helm Road fungal population is the most genetically unique in comparison to the other Illinois sites.

Discussion

Previous research has determined that the mycorrhizal fungus *Ceratorhiza goodyeraerepentis* a strain of *Ceratobasdium*, promotes the germination of eastern prairie fringed orchid seed, and this fungus has been found in adult plants (Zettler et al. 2005). Why does EPFO associate so commonly with *Ceratobasdium? Ceratobasdium* may be favored over other orchid fungi, such as *Tulasnella* because it has genes that can utilize both nitrate as well as ammonium, while *Tulasnella* only utilizes ammonium (Fochi 2017). The ability of *Ceratobasdium* to utilize more forms of nitrates could facilitate orchid survival in more environments.

Why are these fungi so genetically similar over long range geographic areas and sometimes very diverse, even within the same collection location? The Illinois/Iowa fungal populations all exhibit high genetic similarity, and the similarity may be explained by high nutrient levels at these sites and the differences in the ability of the fungi to tolerate high nitrate environments (Beyrle et al. 1995). Orchid seed germination is inhibited by high nitrate levels, which may be an adaptation to occupy nutrient poor areas and reduce competition with other species (Figura et al. 2021). Figura et al. (2021) hypothesize that the genetic similarity in orchid mycorrhizal strains is due to the select genotypes that that can survive in higher nitrate environments and are able to form the symbiotic relationships necessary for seed germination. Nutrient poor sites would select for more fungal diversity in order to utilize as many nutrient sources as possible. The mechanisms behind the selective survival remain unknown. The higher fungal diversity seen in the Wisconsin populations and the Illinois Helm road site may reflect more nutrient poor sites where more root mycobionts are needed for orchids to access more nutrients. In terms of conservation an understanding of nutrient levels (nitrates, potassium, etc.) at sites may help in identifying fungi that are capable of seed germination at higher nutrient levels and could be mixed with seed sources to more successfully augment population numbers.

Future Work

We would like to examine fungal diversity across a larger the range of the species, including more fungal isolates form Wisconsin and from Michigan and Ohio. Fungal diversity has not yet been extensively surveyed in Michigan and Ohio and fungi collected now will provide a record of what was beneath the soil in light of climate change.

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Thank you to the Friends of Nachusa Grasslands for funding this research!