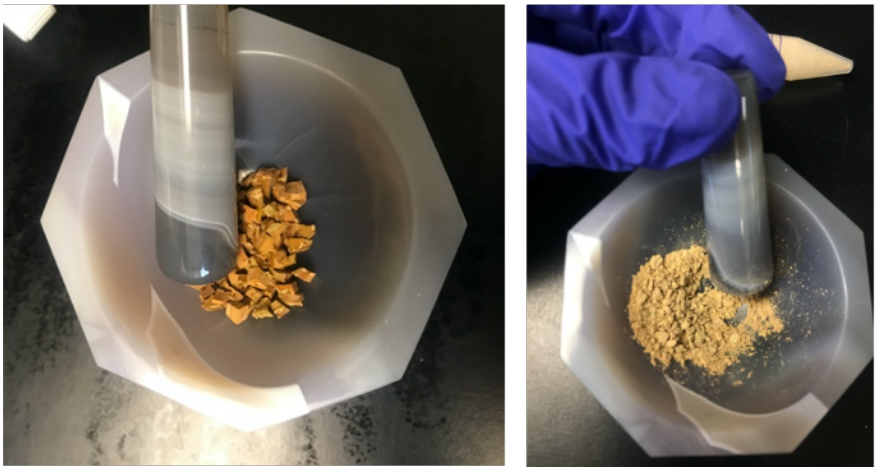
Sword fern - Summary of findings
Mary Ellyn DuPre and Ylva Lekberg
04/28/21

**Introduction/Methods**

To determine if the reason for sword fern mortality in the Seattle, WA area may be due to a pathogen, MPG Ranch used molecular means to characterize fungal and oomycete communities in 24 healthy and 12 symptomatic sword fern rhizome samples and 4 symptomatic leaf samples in a preliminary survey. The rhizomes were predicted to be the main source of potential infection as they are long-lived; thus, rhizomes were the primary focus in DNA characterization. Rhizomes samples, however, were especially difficult to grind during sample preparation for extraction. Samples were ground manually (a mortar and pestle) and using a "1600 MiniG" tissue homogenizer (SPEX SamplePrep) to obtain a fine powder for DNA extractions (Picture 1). Extractions were performed using a DNeasy Plant Pro Kit from Qiagen.



Picture 1: Rhizome samples before homogenization (left) and after (right).

Amplification of the rhizome samples was also difficult as DNA in the extraction template was low relative to what we normally record for roots and soil. We attempted amplification of fungi and oomycetes using the appropriate primers (Table 1). Amplification of fungi was successful, but we failed to amplify oomycetes (although amplification of a positive control was successful, indicating that there was nothing wrong with our DNA extraction and amplification protocol). Thus, it seems unlikely that these samples (both healthy and diseased) harbored oomycetes, which include severe pathogens, such as *Phytophthora* and *Pythium*. However, even with fungi, we had to use twice as much template as we normally use in both PCR reactions to attain visible bands in our gels. That suggests that rhizomes may not harbor a lot of fungi either, possibly due to the woody nature of the tissue. Moving forward, we therefore suggest researchers focus on other tissue, such as roots and leaves.

Out of 36 rhizome samples, only 21 were successfully amplified (14 healthy and 7 symptomatic). All symptomatic leaf samples were amplified without the need to add more template. However, after running the bioinformatics, one leaf sample appeared to not amplify well and was removed after resampling. All samples were resampled to 15,000 sequences during bioinformatics.

Sequences were clustered based on 97% similarity, which we hereafter refer to as operational taxonomic units (OTUs), which is similar to species. OTUs were assigned taxonomy using the UNITE database (https://unite.ut.ee/repository.php) and the FUNGuild database (http://www.funguild.org/) was used to assign potential function based on taxonomic identity.

Table 1: Primers used on the sword fern rhizome and leaf samples in the MPG Ranch lab.

| Region | Forward primer(s) | Reverse primer |
|---|---|---|
| General fungi (ITS2) | fllTS7:fllTS7o (Ihrmark et al., 2012; Kohout et al., 2014) | ITS4 (White et al., 1990) |
| Oomycetes (ITS1) | oom18s (Legeay et al., 2018) | ITS7-ae (Esmaeili Taheri et al., 2017) |

**Results**

Among all amplified rhizome and leaf samples, we identified 348 fungal OTUs. While we only had a few leaf samples and all of them were symptomatic, the fungal communities in leaves were very different from those in rhizomes ($F_{(2,23)}$ =3.36, $R^2$= 0.243, $p$=0.002; Figure 1) based on permutational ANOVA. This test also revealed marginally different communities in healthy and symptomatic rhizomes ($F_{(1,20)}$ =2.069, $R^2$= 0.098, $p$=0.067, Fig. 1).

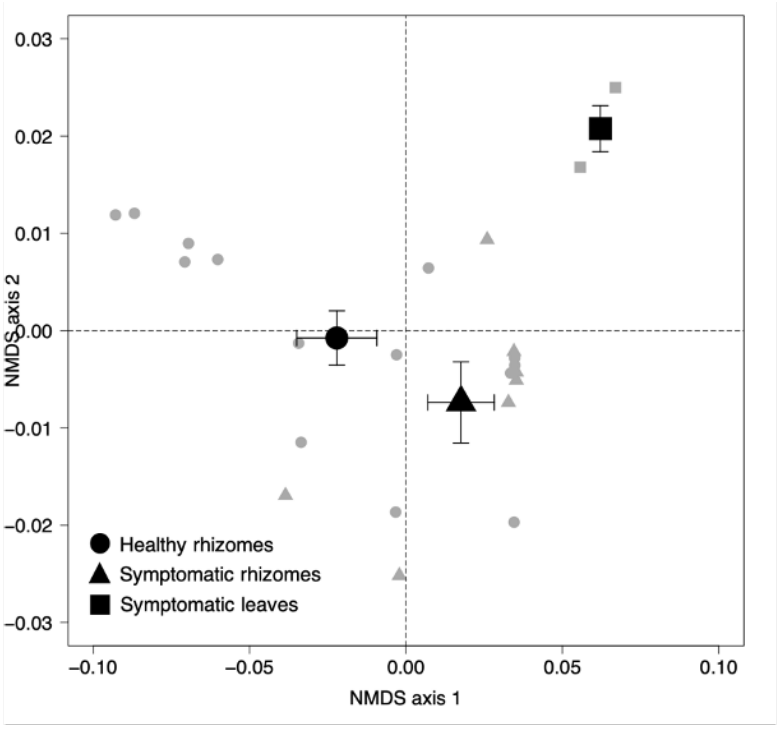



Figure 1: Variation of fungal OTU composition in healthy and symptomatic rhizomes and symptomatic leaf samples. Error bars represent the standard errors of centroids.

In all amplified rhizome samples, 226 OTUs were identified by the UNITE database. Since we were interested in identifying a prevalent plant pathogen in these samples, our analysis focused on abundant OTUs hereafter. Eighteen OTUs were identified as genus *Cladosporium* accounting for 25% of all rhizome sequences. Species within the *Cladosporium* genus are known as molds

that can occur on different plant structures. This particular genus was not assigned a guild in the FUNGuild database, indicating that its function is not well known. When all *Cladosporium* OTUs sequences were combined, there was no significant difference in mean sequence abundance among healthy and symptomatic fern samples ($F_{(1,19)}$= 2.25, , $p$=0.1499), suggesting that this group is an unlikely candidate to cause the disease observed.

One of the most reoccurring "plant pathogens" assigned by FUNGuild was *Cronartium ribicola* (1.8% of all rhizome sequences, 90% of all 'plant pathogen' sequences). After a quick search of this species, there is a *Cronartium* species that can infect ferns (https://tidcf.nrcan.gc.ca/en/diseases/factsheet/23). Like with *Cladosporium*, however, there was no significant difference in the number of *C. ribicola* OTU sequences between healthy and symptomatic samples ($F_{(1,18)}$=0.097, $p$=0.758). One healthy sample was removed as an outlier before this analysis because *C. ribicola* OTUs amplified better than all other rhizome samples.

In the three symptomatic leave samples, 128 OTUs were identified. The most abundant OTU was identified as order *Capnodiales* (56% of all leaf sequences) by the UNITE database, but we found no match in FUNGuild, most likely because the match in UNITE was only down to order, which is too broad in most cases to assign a potential function. Fungi in the *Capnodiales* are known as "sooty molds" which appear to be generally harmless to plants. Several other abundant OTUs were identified but none appeared abundant in all three samples. Similar to the rhizome samples, 15 OTUs were identified as *Cronartium ribicola* in the leaf samples (3.6% of all leaf sequences). Several other OTUs were identified as plant pathogens by FUNGuild but were very low in abundance. No statistics were conducted on these samples as we did not have any healthy samples for comparison.

**Conclusions**

Overall, the rhizome samples produced some challenges, but we were successful in amplifying specific species of fungi that occurred in both rhizomes and leaves. Unfortunately, this preliminary screening did not present a "smoking gun" as to what may cause the decline in the sword fern populations. The rhizomes in healthy and diseased plants differed marginally in fungal communities, and no oomycetes amplified in any samples, suggesting that the group is rare if present at all. We recommend that future investigations focus on comparisons of fungal and possibly oomycete communities between healthy and diseased leaves or roots in a balanced sampling design.