Fungal Endophytes Isolated from Norton/Cynthiana grapes of Tennessee, Arkansas, and Missouri

by

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A thesis presented to the Honors College of Middle Tennessee State University in partial Fulfillment of the requirements for graduation from the University Honors College

Spring, 2019

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ACKNOWLEDGEMENTS

I would like to dedicate this thesis to my instructors, Dr. Seipelt-Thiemann, Dr. John D. Dubois, and Dr. Tony Johnston. These individuals have been and will continue to be a source of personal strength and encouragement in my life. Their dedication to the growth of their students is superb. I offer this Honors thesis as a testament to the experiences we have shared.

ABSTRACT

Fungal endophytes inhabiting plant vascular tissue can alter the physiology of their host's. Changes in growth rate, as well as resistance to disease and drought are some of the effects observed in plants whose tissue is influenced by these microorganisms. The Norton/Cynthiana grape is a cultivar of wine grape that has significant resistance to draught, and disease. Fungal endophytes that have been isolated and from Norton/Cynthiana grapes of three different geographic locations, and identified using barcode sequencing techniques are potentially responsible for this. The fungal endophytes found are described species that have been found within other species of plants.

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Introduction

There are many factors that influence the growth, and development of plants. The concentration of nutrients, organic components within soil, sunlight availability, moisture, and herbivory are all critical in the development of plants. All of these factors are important, but far from the only components that affect plant growth (Kohler. 2010). The biotic factors that affect plant development can bring about an even more profound effect on plant physiology in the long run. Rhizobium bacteria, and mycorrhizal fungi will form galls and a complex network of hyphae around the roots of legumes which can enhance the nitrogen fixation ability of their hosts (Korir et al. 2017). Understanding the interaction between plants and their environment has advanced agricultural productivity.

Plant communities are influenced heavily by a variety of abiotic and biotic factors, but how strongly the plants are affected depends on what stage of development that plant is undergoing (Wood et al. 2012). Throughout their evolutionary history, plants have developed intricate mutual relationships with a variety of microorganisms that live within the soil and throughout the air. The many divergent origins of ectomycorrhizal fungi can help explain the many ways in which plants respond to these ectomycorrhizal fungi (Hoeksema et al. 2018). Microorganisms that can be extracted from surface-sterilized plant tissue are defined as fungal endophytes (Hallmann et al. 1997). Fungal endophytes are a diverse group of microorganisms and they can initiate a variety of different physiological effects within their host plant's body. Depending on the species of fungi residing within the plant, however, this can be either problematic to or enhance the quality of the host's physiology. One subset of these microorganisms are necrotrophic

fungi; which may cause serious damage to plant tissue by either destroying leaf tissue or blocking the conduction of nutrients from the soil. The spores of these fungal endophytes deposit themselves within the vascular tissue of plant hosts during nutrient uptake at the site of the host plant's root hairs, and as they mature, their host's roots are destroyed. *Fusarium oxysporum f sp. cubense* on the other hand, is a destructive and infectious plant pathogen that destroys millions of acres of banana farmland globally (Cook et al. 2015). Fungal endophytes that are biotrophic enter into mutual relationships with their host plants. Mycorrhizal fungi can increase root hydraulic conductivity, which may improve the drought tolerance of the host plant (Robert et al. 2008). Some species are classified as hemibiotrophic because they do not destroy their host's tissue outright, but instead reprogram their host's tissues to suppress their immune responses to their presence. *Phytophthora infestan*s is classified as hemibiotrophic, and can become damaging for their host plant as they develop in response to effector proteins that suppress plant cell defense responses (Lee et al. 2010).

Understanding the interaction between host plants and their fungal endophytes can not only open the door to more efficient crop production, but also save billions of dollars in lost produce. Manipulation of the relationship between plant and fungal endophyte may also lend insight into the hardiness of many plant species. Fungal colonies within plants can also enhance adventitious root growth (Contreras et al. 2009). Plants that do contain mutualistic fungal endophyte colonies may also have an improved ability to intake nutrients and defend against harmful pathogens (Zeilinger et al. 2017) among other advantages. The *Vitis aestivalis* Norton/Cynthiana grape is a cultivar of wine grape that has many advantageous attributes including drought tolerance, pest and

disease resistance. Norton/Cynthiana grapes of are confirmed to have fungal endophyte colonies within their bodies. Mycorrhizal fungi may improve the phosphorus uptake and pigment concentration of plants even in harsh dry conditions (Asrar et al. 2010). These mycorrhizal fungi working in concert with a colony of fungal endophytes could be responsible for their resistance to drought. Norton/Cynthiana grape plants have weak reproductive capability, and the success rate for woody stem cutting reproduction is a mere 30% (Bigger et al. 2010). Micro propagation has been unsuccessful thus far, and research has now turned to the fungal endophyte for potential answers to the poor reproductive ability. Auxin suppression via metabolite interaction could be one of the principal causes of this problem. Fungal pathogens do cause a myriad of different chemical changes associated with plant defense, usually to suppress plant immune responses or augment existing methods of fighting back infection.

These fungi will typically target the ethylene, salicylic acid or other chemicals crucial to plant defense (Patkar et al. 2017). Coevolution with plant species has given fungi an expansive chemical tool belt for manipulating their host species. In addition to that benefit, a broad range of new geographic locations can be occupied by well adapted fungal endophytes due to transcontinental shipping of produce and migration of animals. As global temperatures continue to climb, opportunistic fungi have increased their native ranges of influence. Many of these fungi pose a serious health risk to plants and animals; causing concern over food security (Fisher. et al. 2012). The infestation of fungal endophytes within native plants have had a disastrous impact on the production of crops in recent years. *Fusarium Oxysporum cubense sp.* devastated gro Michel banana populations during the 1950's, and because of international trade, the fungus was able to

destroy thousands of acres of cropland in regions it normally would not have been (Dita et al. 2018). Currently a more virulent strain threatens the Cavendish variety as well, and if left unchecked, Panama disease race 4 poses a serious threat to the Cavendish. Technological advances in agriculture will need to increase overall food supply by up to fifty percent within the next decade to keep up with the planet's ballooning population (Haque et al. 2018). Farmers have begun using biocontrol agents to limit herbivory and fungal infections in recent years. Farming sustainably in concert with the local ecosystem may prove to be an effective way of mitigating the effects of climate change on crop production. Obtaining more knowledge about various biocontrol agents can serve that need. Fungal endophytes are just one of many biocontrol agents that can easily fill that role, and the species yet to be discovered have the significant potential of protecting plants from tropical disease. An estimated twenty-five percent of plant species succumb to some sort of plant disease each year (Katoch et al. 2017).

Grape plants are no exception, fungi spreading outside of their native range increases the risk of vulnerable plant species being destroyed. Most crops are uniform in appearance when brought to the marketplace because they are often a monoculture produced by interbreeding, which keeps their fruits uniform in appearance. This sacrifices critical biodiversity and genetic potential. Deforestation claims millions of acres of wild growth every year, compounding that loss. The potential wild relatives of common plants hold is immense, and farmers have begun to recognize the potential of wild plants as a potential breeding stock for their own crops. *Botrytis cinerea* threatens global grape production, but there are wild varieties that are resistant to this necrotrophic fungi's infection, and treatment with bacterial rhamnolipids also reportedly improved

resistance (Wan et al. 2015). While the genetic potential of wild species of plants and fungi is still available to obtain, developing new bioinformatics technology is critical for more efficient DNA analysis. Geneious software allows for streamlined editing of sequenced DNA and its user can search for a given query sequence against an online repository using the BLAST search key (Kearse et al. 2012). Using this technique on the sequenced fungal endophytes of Norton Cynthia grapes of different geographic locations can provide insight into what types of fungi live within the grape tissue. The answer to this question may lead to the discovery of a potential biocontrol for Norton/Cynthiana grapes that can influence its hardiness and improve its reproductive capabilities. The necrotrophic endophytes identified using the Geneious desktop software could also be used as potential bio controls for invasive plant species as well. Hemibiotrophic fungi were also plentiful within these leaves and another source for biocontrol to be utilized in agriculture. The hemibiotrophic fungi begin their lives as biotrophic fungi that may enter into a mutualistic relationship with its host in its early stages of life, but later on, it will become necrotrophic and begin to destroy its host's tissue for nutrients. Hemibiotrophic fungi can also secrete effector proteins into their host's cytosol to alter their response to infections (Koeck et al. 2011). Many surface contaminants inhabit the surface of the selected tissue which posed some complications. These contaminants can become problematic if they enter through the stomata of a plants leaves. Typically plants will attempt to ward off infection by surrounding the contaminant with dead tissue, resulting in rust spotting on leaf tissue. The fungi isolated from Norton grapes of Missouri, Arkansas, and Tennessee came from sterilized leaf tissue. The leaves taken were of reasonable health but exhibited rust spots indicating fungal infection.

The fungal endophytes of Norton Cynthiana grapes have not been isolated from surface sterilized tissue and identified Initial research will require an in depth look at both what kinds of fungal endophytes are present and the geographic locations from which they are most prevalent. For the purposes of this research, we have utilized Geneious software to identify different fungal endophytes from a broad range of geographic locations for future analysis. Filamentous fungi are the most likely candidates for infection of Norton Cynthiana grape vascular tissue infection. Several of the isolates identified are known plant pathogens and if allowed to proliferate through the plant tissue unencumbered; could easily kill their host plant.

In spite of infection from many different types of pathogenic fungi, the Norton Cynthiana grape tissue observed were still of reasonable health before sterilization. The totality of different pathogenic fungi within Norton/ Cynthiana grape plants remains unclear, but the biocontrol factors that have keep them from succumbing to infection. A likely source of protection from infection could be metabolites from other types of fungus that live within and protect their hosts from invasion. Whether or not these microorganisms have some effect on the hardiness against extreme weather conditions or reproductive success is still speculative, but possible. Understanding the relationship between fungal endophytes and their hosts may lie in the identity of the fungal endophytes themselves. A first step in understanding this relationship is isolation and identification of the fungal endophytes. To achieve this, fungal endophytes were isolated from grape tissues from three geographic regions, Missouri, Arkansas, and Tennessee and identified using barcode sequencing.

Materials and Methods

Grape Tissue collection

Vitis aestivalis plant tissues were shipped from Stone Hill Winery, Hermann, MO, Post Family Winery, Altus, AR, and Rutherford County Agricultural Extension/MTSU Vineyard, Murfreesboro, TN. The leaves and stems of Norton grapes placed into a 250ml beaker with 100ml of 70% ethanol bath for approximately three minutes while stirring gently. The process was repeated using a 250 ml beaker filled with 100 ml of 10% sodium hypochlorite solution, a 70% isopropyl alcohol, and the tissue samples were left to dry in an empty 350ml beaker. (Pancher, 2012).

Outgrowth, and Isolation of fungal colonies

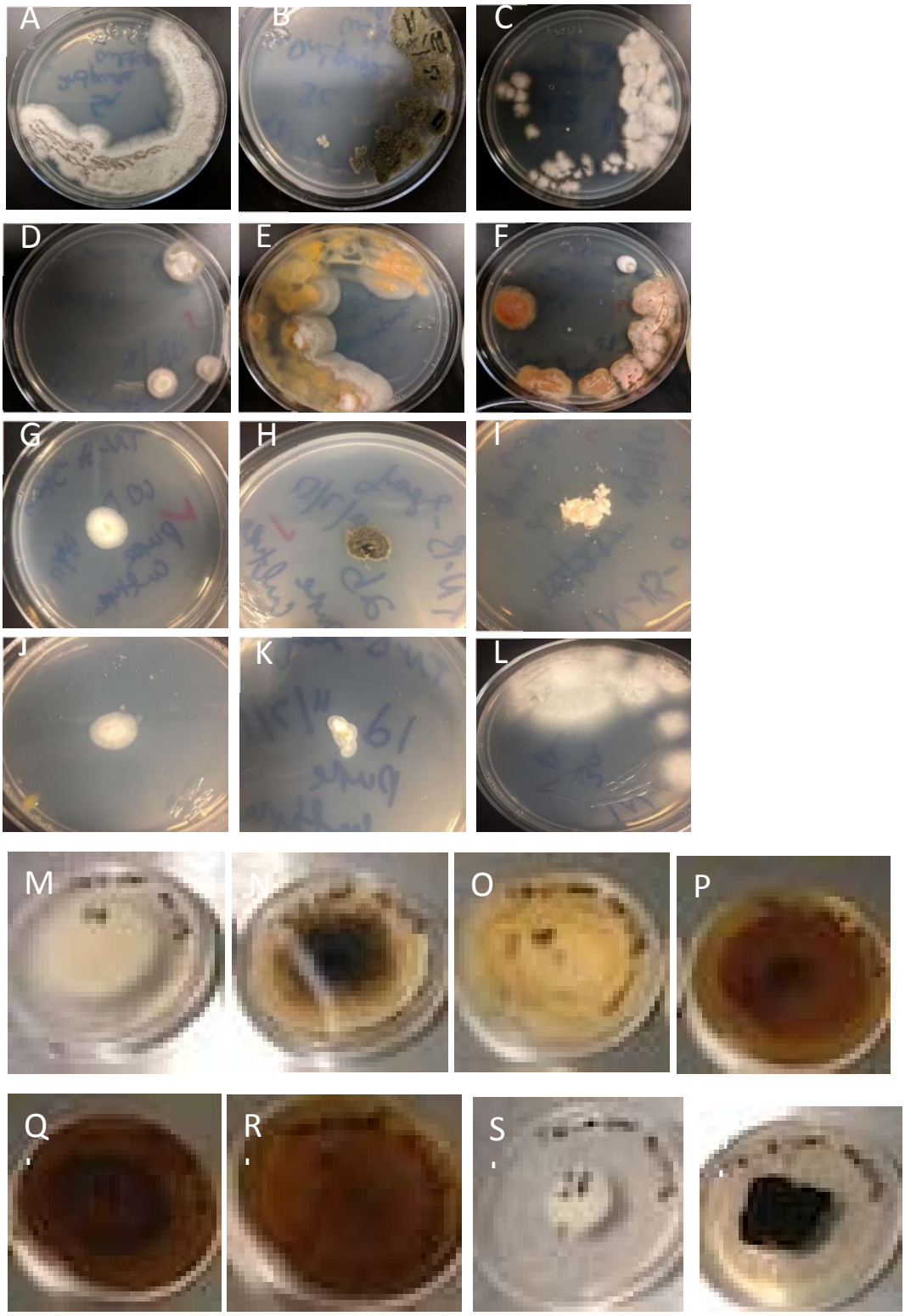
Surface-sterilized plant tissue was cut into 2.5 cm pieces using a sterile scalpel in a tissue culture hood from three different samples. Ten cuttings per sample were then place on malt extract agar (Pancher, 2012) plates containing 1 mg/mL neomycin. Petri dishes were sealed with parafilm and incubated at room temperature for four days. The fungal colonies obtained from this all three geographic locations were visually inspected and those showing a filamentous fungal phenotype were isolated to purity by transferring small portions of fungal outgrowth with sterile lab forceps onto new malt extract agar plates repeatedly until a single phenotype was observed and reached the diameter of a quarter (Figure 1,2,3).

Species Identification by Genetic Methods

Genomic DNA was isolated from each potential endophyte isolate using the thermolysis method (Zhang, et al. 2010). The thermolysis method utilized required initial submersion of a small piece of fungal tissue into 1000 micro liters into a 1.5 ml centrifuge tube followed by discarding the supernatant and replacing it with 100 microliters of DNA Lysis solution. DNA extraction of the fungal endophytes was completed after an incubation period of 20-30 minutes in an 85 degree Celcius water bath. PCR amplification of the ITS barcode region was undertaken using a thermocycler set to run for 19 microliters of ITS4 and ITS1 primers per 380 microliters (Zhang, et al. 2010) of deionized water. The ITS barcode region has been shown to be an effective barcode for fungal identification (Schoch, et al. 2012).

Agarose gel electrophoresis

The DNA of Missouri, Arkansas and Tennessee fungal endophytes that was successfully amplified revealed banding on several electrophoresis gels. Ten microliters of each successfully amplified fragment (approximately 10 ng/uL) were sent for DNA sequencing using the ITS1 (forward) and ITS4 (reverse) primers at 3.2 μM to McLab (South San Francisco, CA). Electopherograms in the abi format were returned, downloaded, and visually inspected using the software program, Geneious (Kearse, et al. 2012). Base-calls were visually verified and ambiguous sequences were trimmed. Overlapping contigs using the forward and reverse sequence results were then generated in Geneious. Each contig was used to search the NCBI non-redundant DNA database to identify putative isolates to a species or genera specific level (Roberttse et al. 2011).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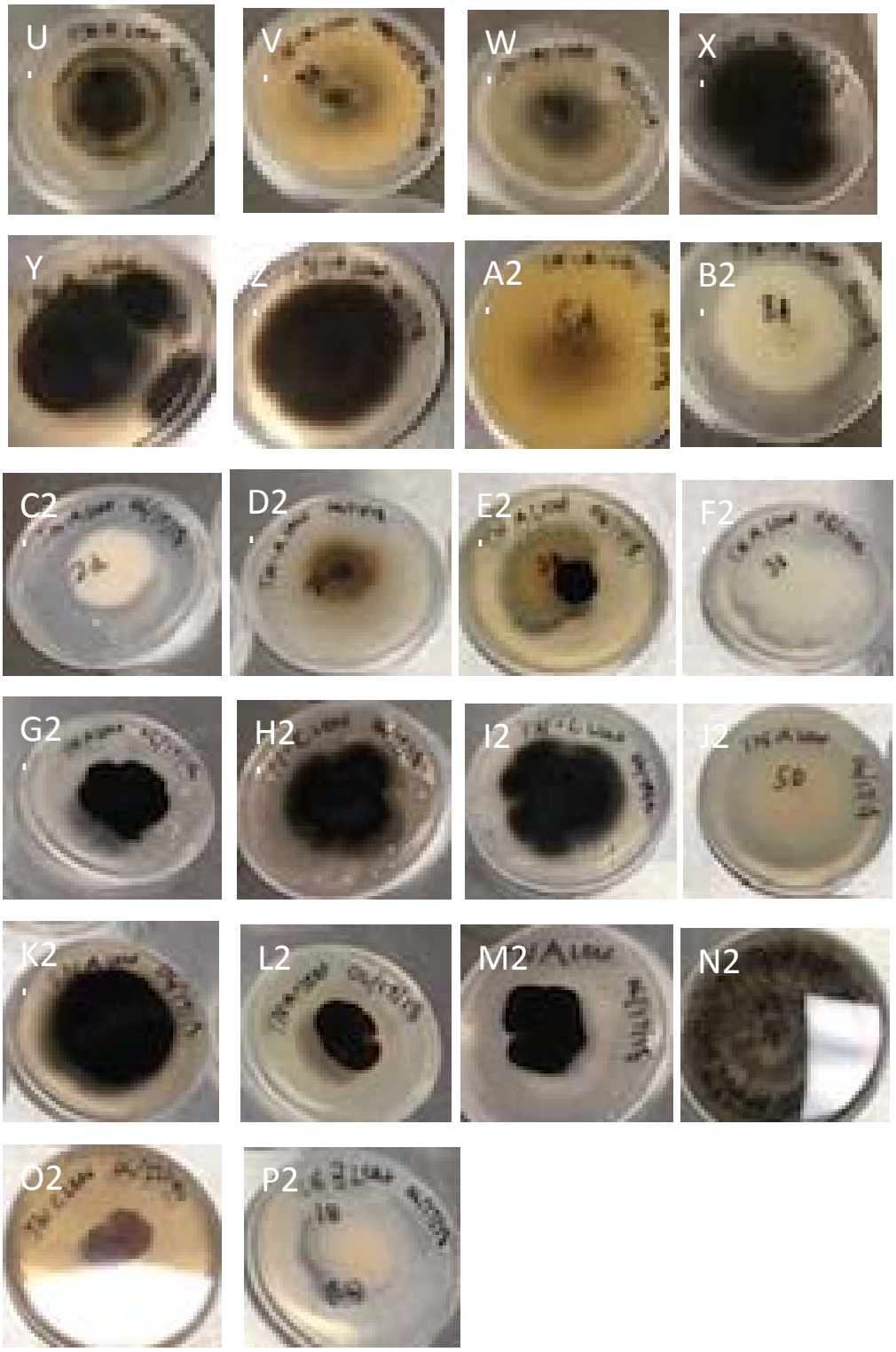
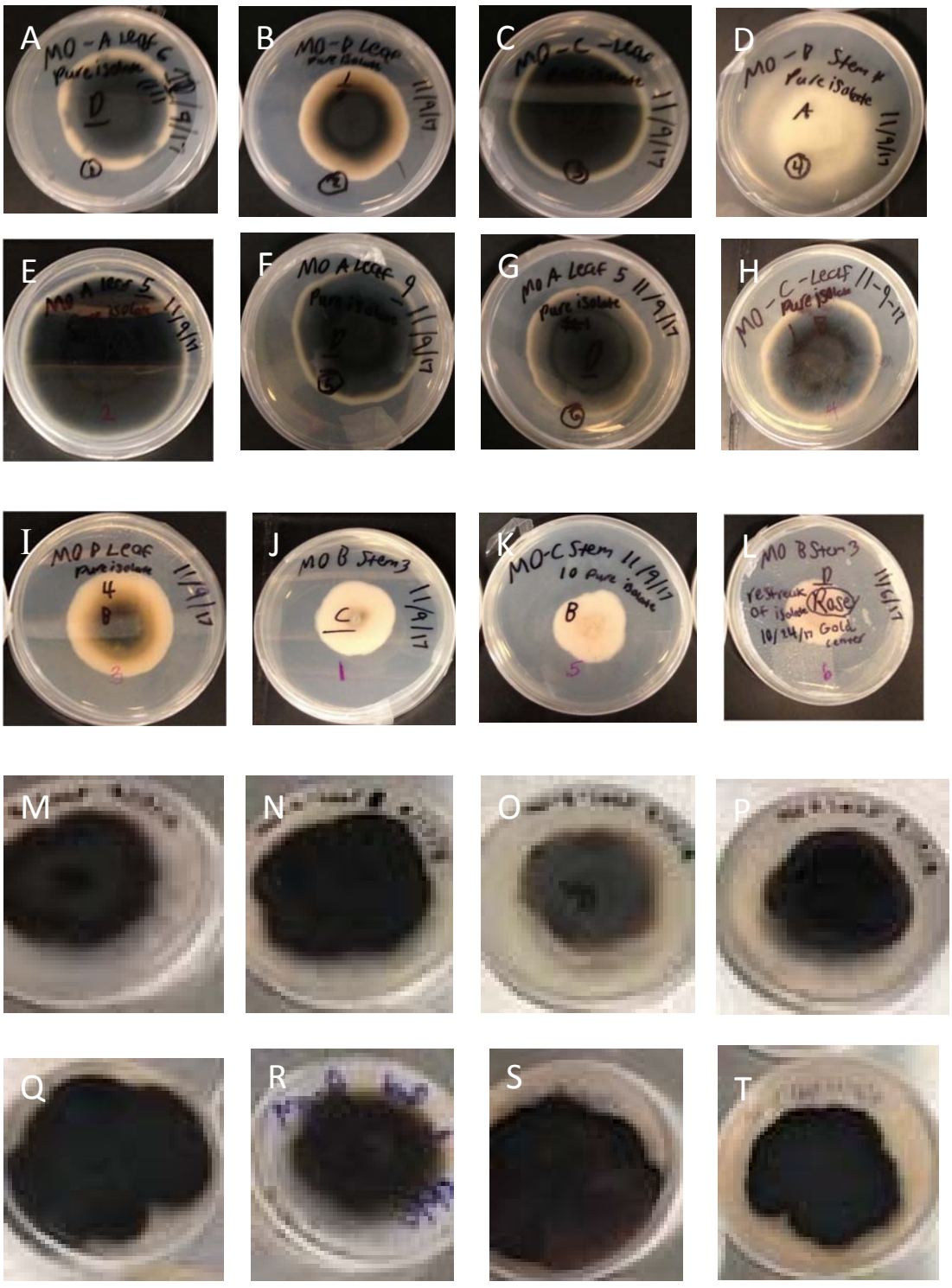


Figure 1. *V. aestivalis* Fungal Isolates from Tennessee Grape Vines. Fungi were outgrown from plant tissue at room temperature on malt extract agar medium. Distinct organisms were patched to purity.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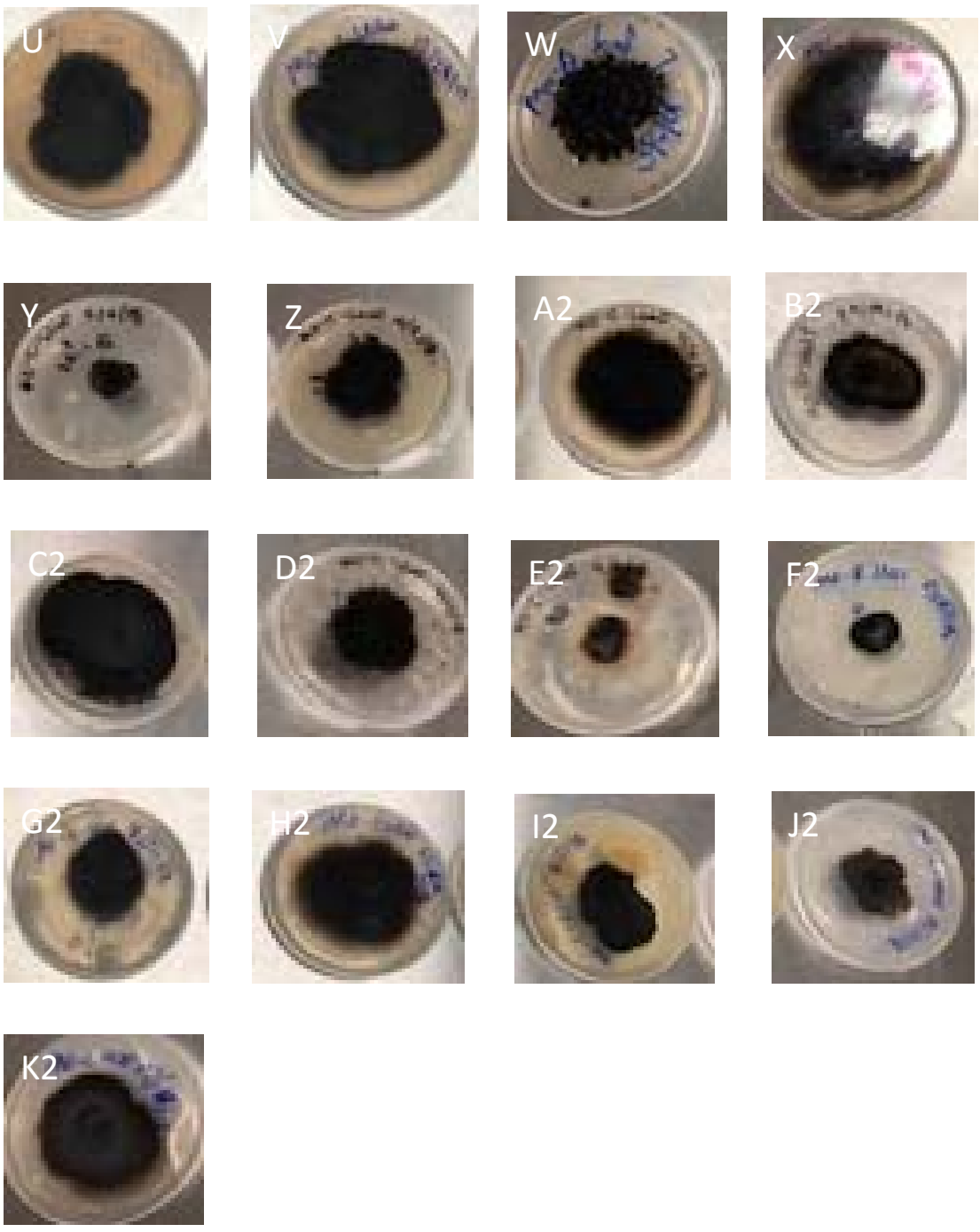
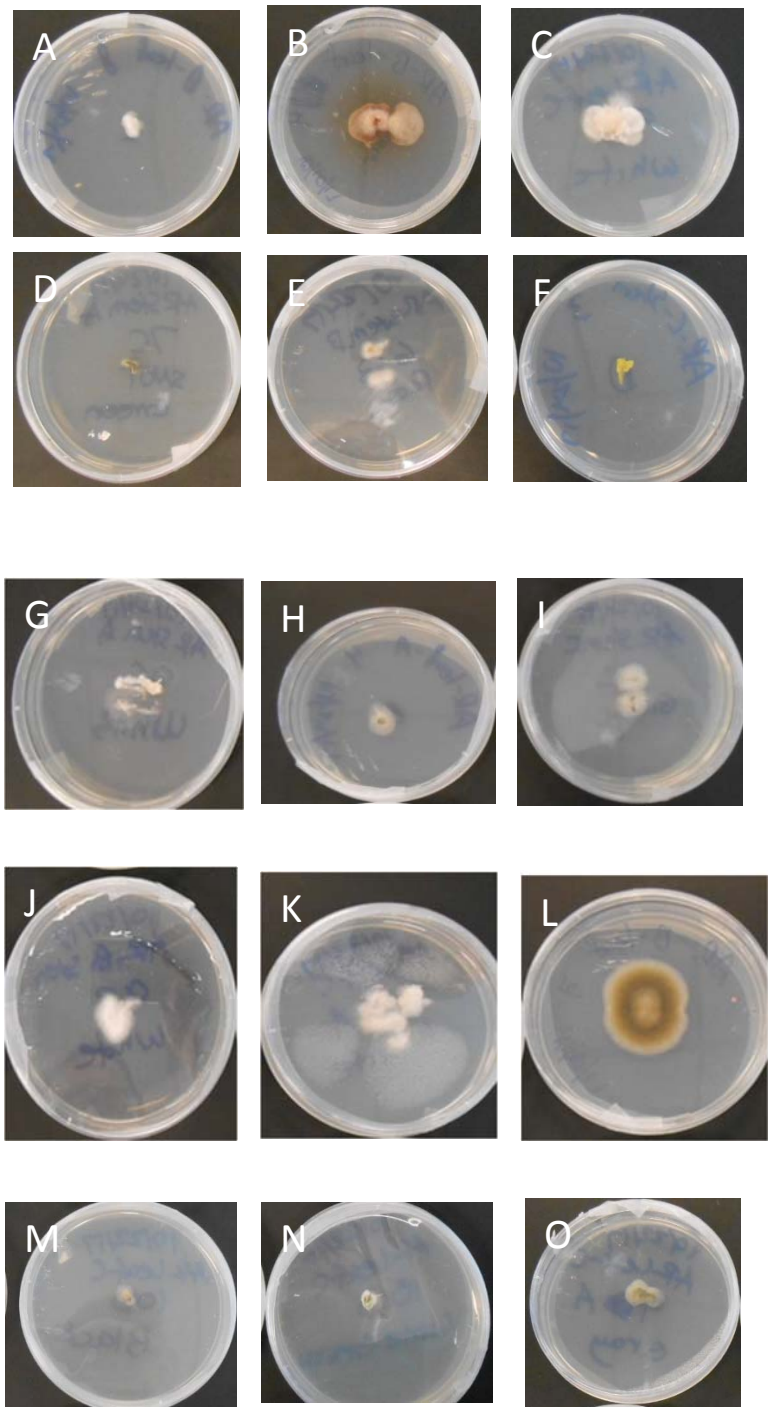


Figure 2. *V. aestivalis* Fungal Isolates from Missouri Grape Vines. Fungi were outgrown from plant tissue at room temperature on malt extract agar medium. Distinct organisms were patched to purity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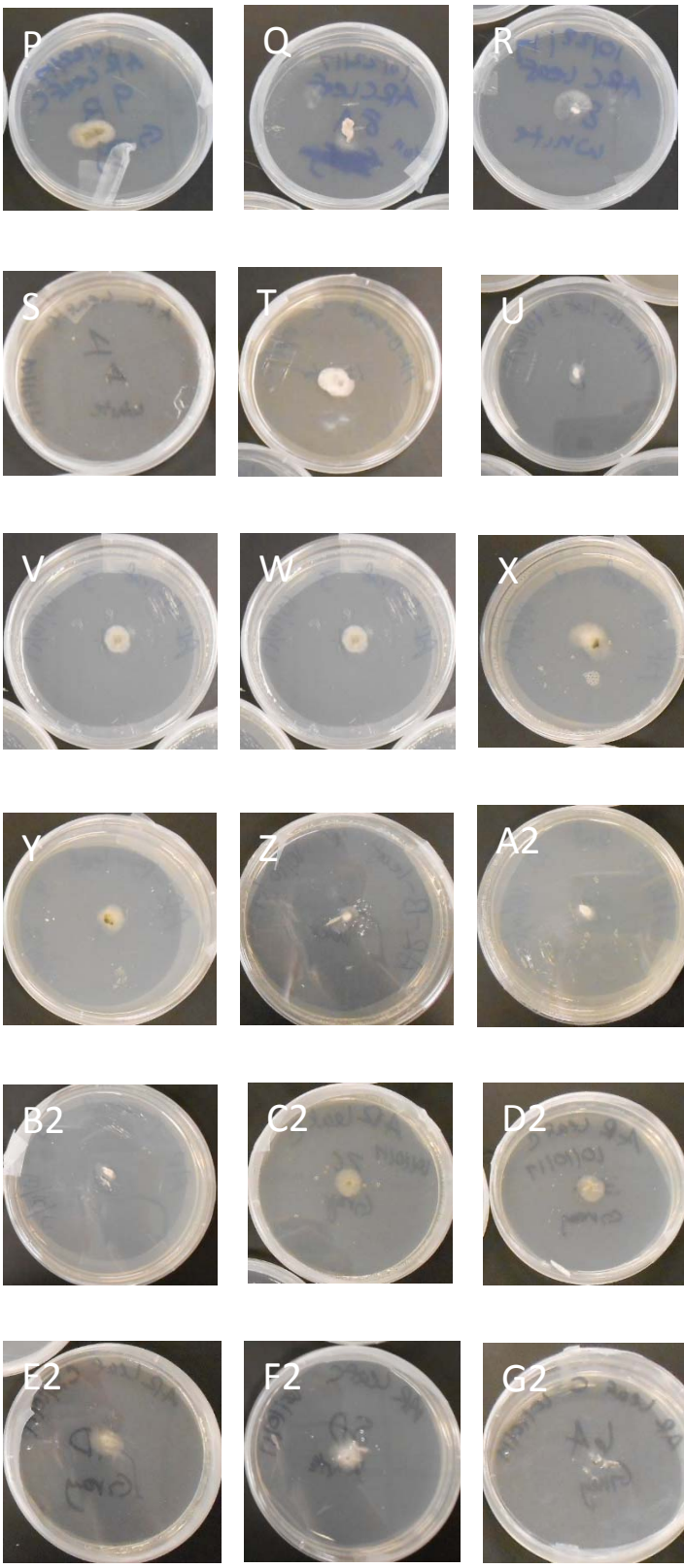
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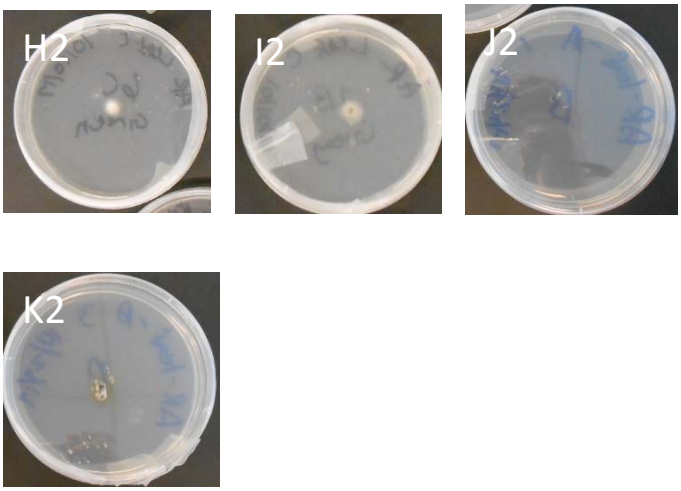


Figure 3. *V. aestivalis* Fungal Isolates from Arkansas Grape Vines. Fungi were outgrown from plant tissue at room temperature on malt extract agar medium. Distinct organisms were patched to purity

Results

Most fungal endophytes isolated from the three geographic locations studied were not successfully amplified and sequenced. Only 111 fungal isolates were successfully sequenced of the 243 the fungal endophytes that were successfully amplified using pcr analysis. The amplified ITS barcode region of fungal DNA appeared reveals that amplification of that barcode region was successful for each of the first three groups of twelve initial isolates sequenced (Figures 4A, 4B, 4C). Identification of the first three groups of twelve fungal endophytes served as a proof of concept for isolation and identification of the remaining seventy-five isolates. Each of the three groups contained isolates from Missouri Arkansas and Tennessee respectively. These isolates which were numbered on each gel according to their placement on each lane; whether or not the ITS barcode region was successfully amplified. Most of the fungal isolates were identified as being from the genus *Alternaria*, which was found in grapes from all three states, but other species were also found (Table 6). Missouri fungal isolates showed the least variety, while Arkansas and Tennessee showed the most variety of species.

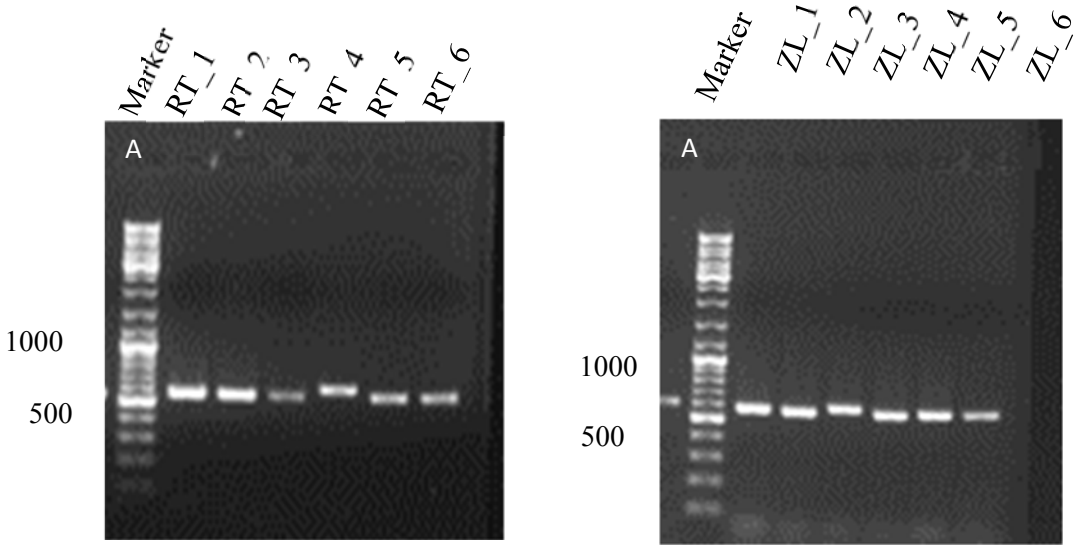


Figure 4A. Agarose Gel Electrophoresis Results of the Amplified ITS Barcode Regions for Samples Isolated from Missouri. DNA fragment sizes in the marker are noted at the left of each gel. A. Samples RT MO 1-6. B. Samples ZL MO 1-6

Individually isolated endophytes of Tennessee, and Missouri had the ITS barcode region amplified and each fungus successfully amplified was prepared for sequencing (Figures 4B, 4C, 5B, 6B, 6C,6D). Many fungal endophytes could not have their DNA extracted and successfully amplified despite repeating the systematic workflow of steps necessary to do so (Figure 8). In fact fewer endophytes were able to have their ITS region amplified as the extractions progressed. (Figures, 7A, 7B, 7C, 7D). Most endophytes located within the three geographic locations specified can be identified using the non-redundant DNA database of NCBI with a 97% match rate or higher. (Tables 1,2,3).

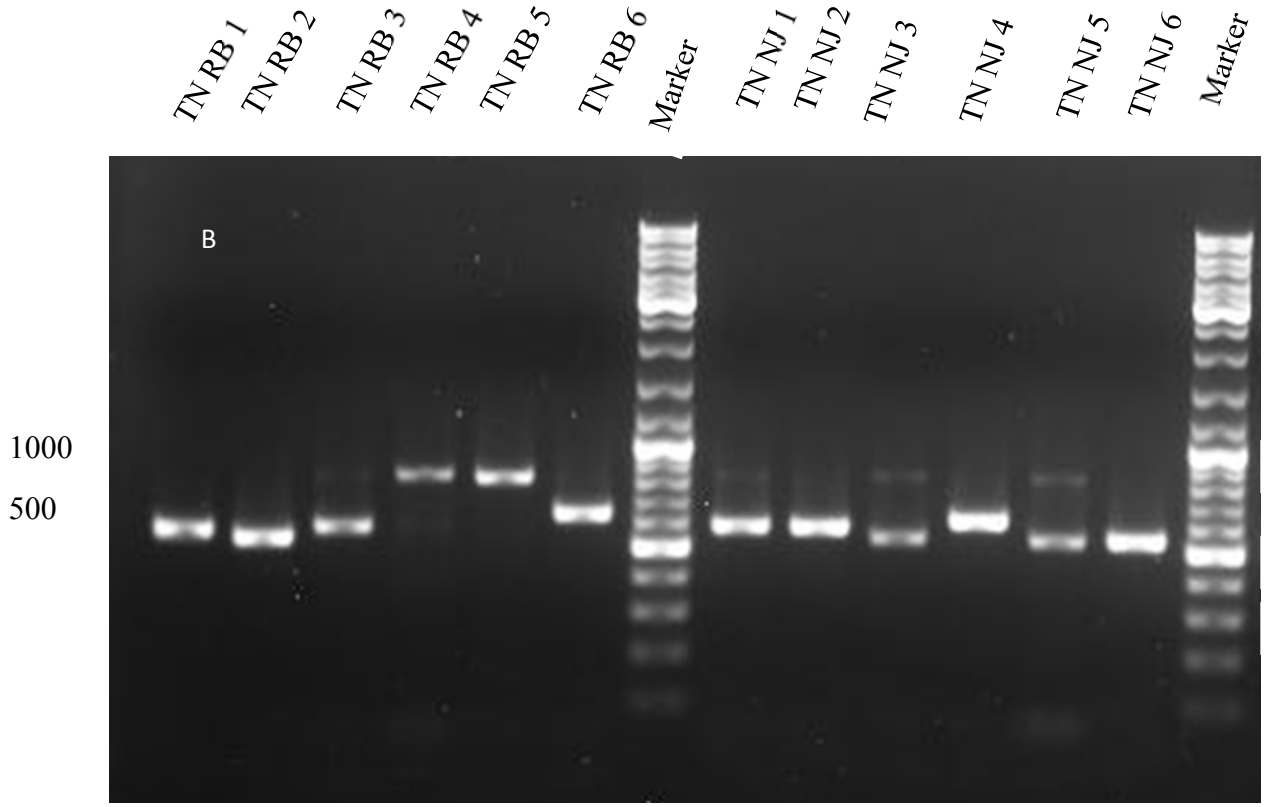


Figure 4B. **Agarose Gel Electrophoresis Results of the Amplified ITS Barcode Regions for Samples Isolated from Tennessee.** DNA fragment sizes in the marker are noted at the left of each gel. L-R Samples NJ TN 1-6. RB TN 7-12.

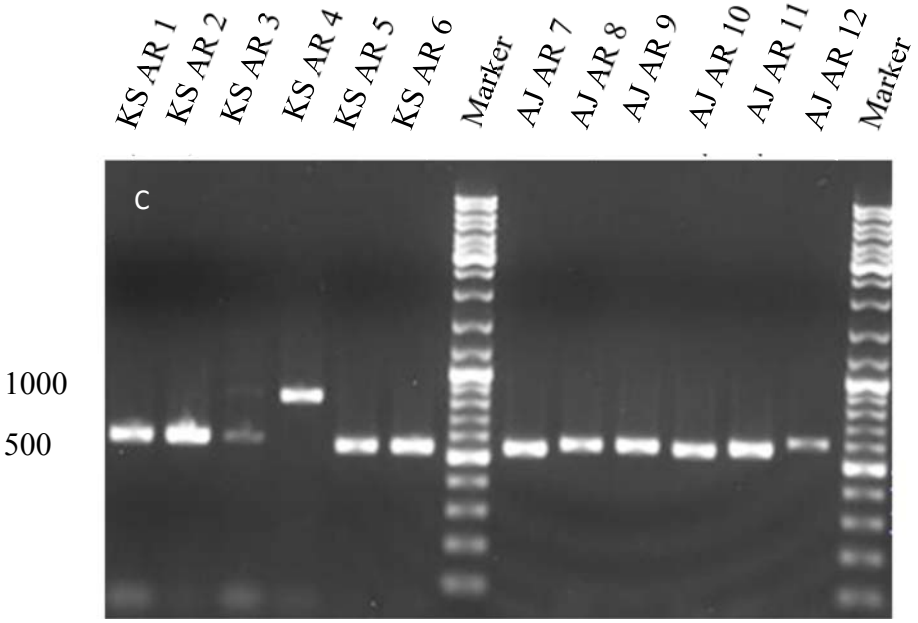


Figure 4C. Agarose Gel Electrophoresis Results of the Amplified ITS Barcode Regions for Samples Isolated from Arkansas. DNA fragment sizes in the marker are noted at the left of each gel. KS AR 1-6, AJ 7-12

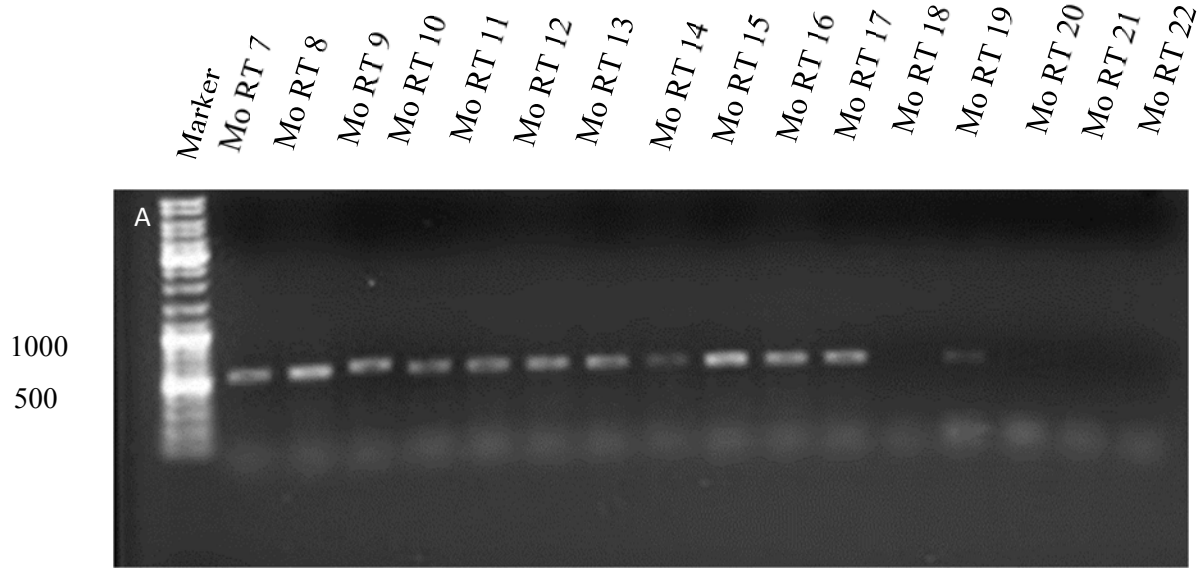


Figure 5A. **Agarose Gel Electrophoresis Results of the Amplified ITS Barcode Regions for Samples Isolated from Missouri.** DNA fragment sizes in the marker are noted at the left of each gel. C. Samples RT MO 7-22

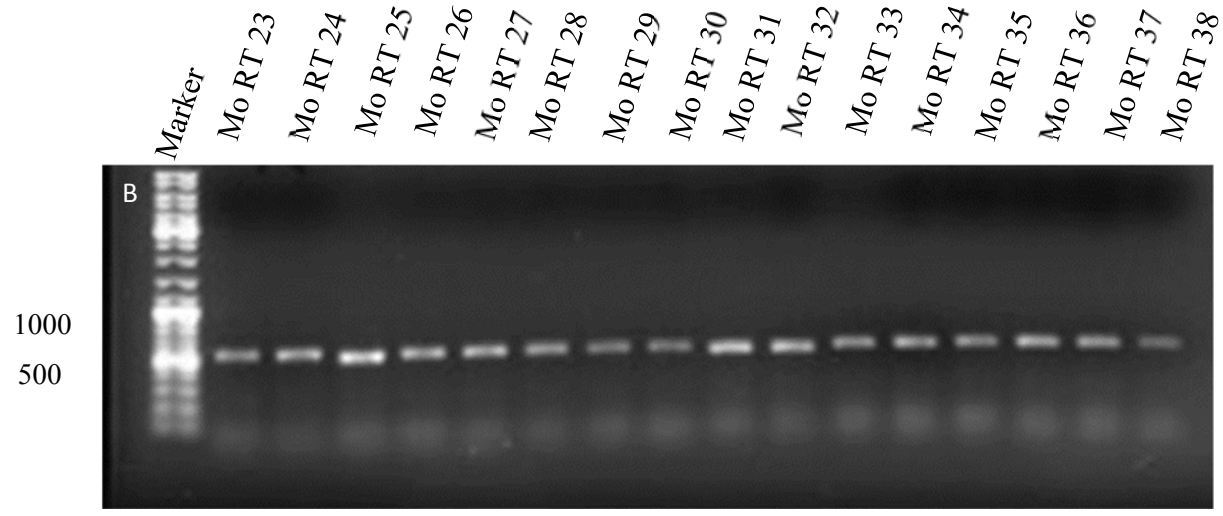


Figure 5B. **Agarose Gel Electrophoresis Results of the Amplified ITS Barcode Regions for Samples Isolated from Missouri.** DNA fragment sizes in the marker are noted at the left of each gel D. Samples RT MO 23-38.

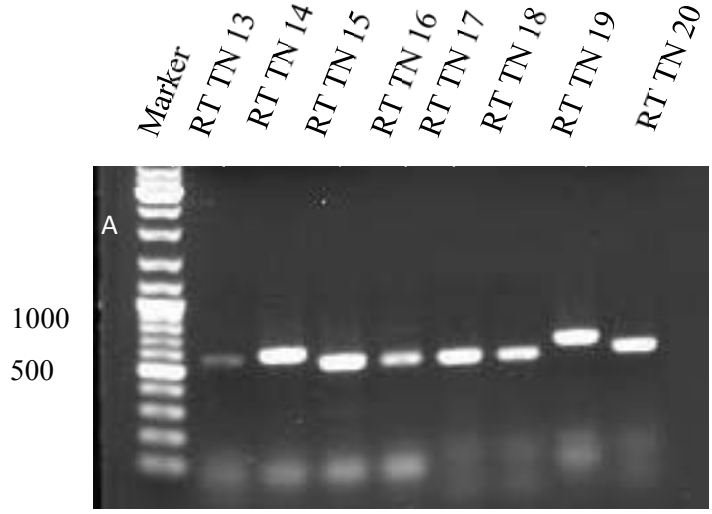


Figure 6A. **Agarose Gel Electrophoresis Results of the Amplified ITS Barcode Regions for Samples Isolated from Tennessee.** DNA fragment sizes in the marker are noted at the left of each gel. Samples RT TN 13-20

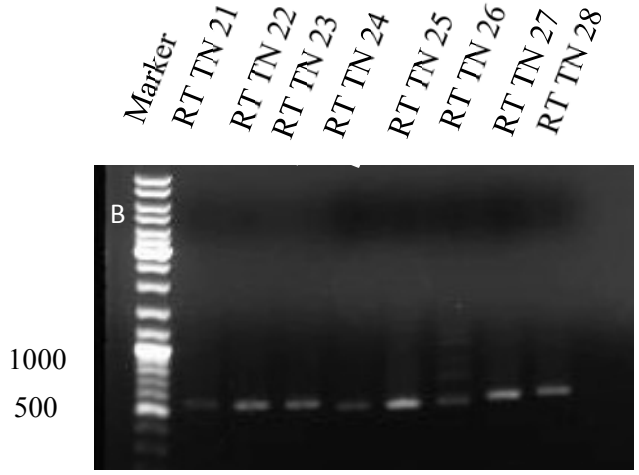


Figure 6B. **Agarose Gel Electrophoresis Results of the Amplified ITS Barcode Regions for Samples Isolated from Tennessee.** DNA fragment sizes in the marker are noted at the left of each gel. Samples RT TN 21-28

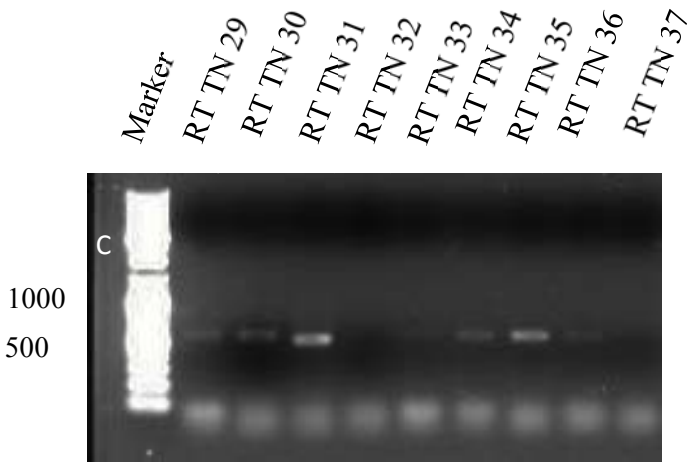


Figure 6C. Agarose Gel Electrophoresis Results of the Amplified ITS Barcode Regions for Samples Isolated from Tennessee. DNA fragment sizes in the marker are noted at the left of each gel. Samples RT TN 29-37

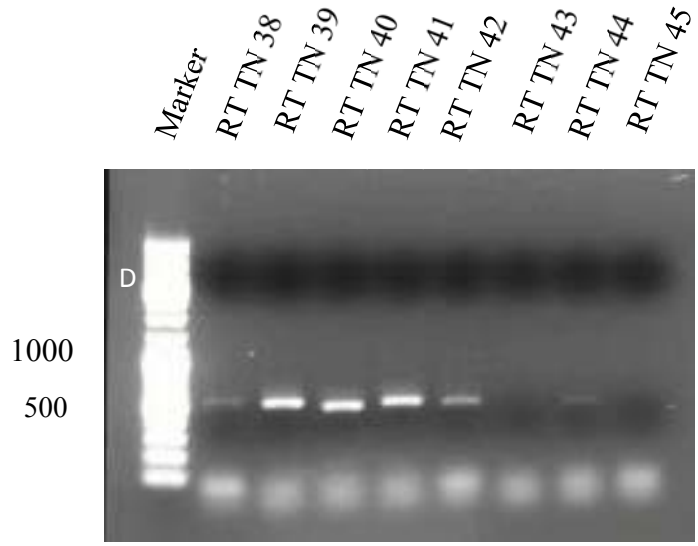


Figure 6D. Agarose Gel Electrophoresis Results of the Amplified ITS Barcode Regions for Samples Isolated from Tennessee. DNA fragment sizes in the marker are noted at the left of each gel. RT TN 38-45.

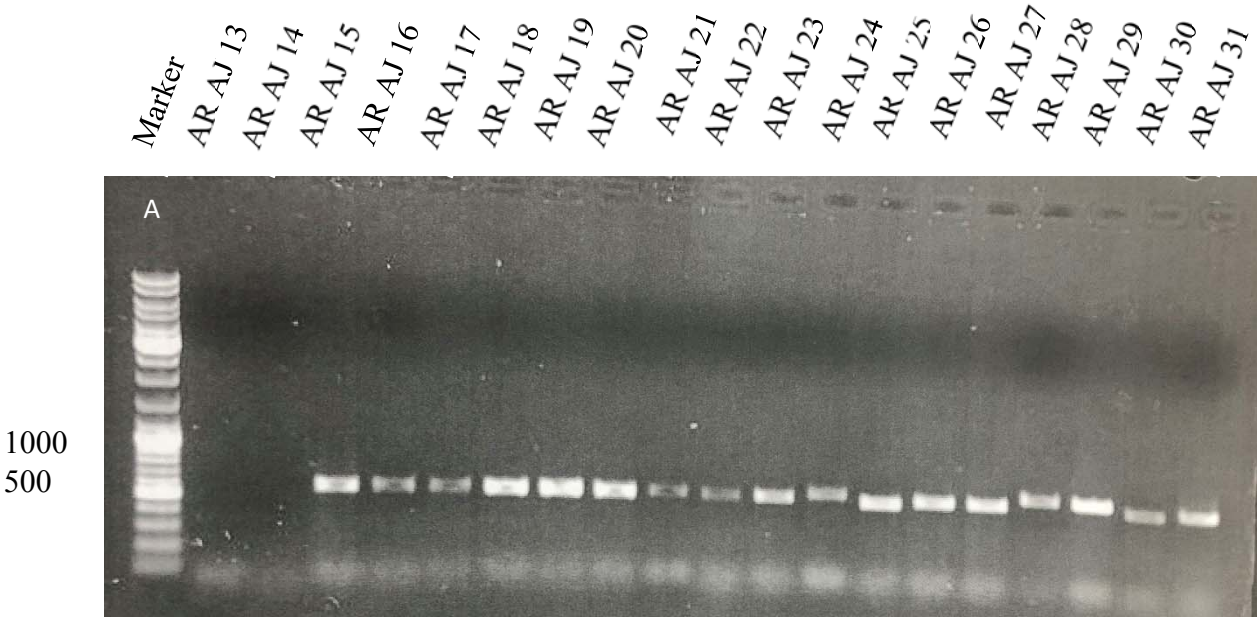


Figure 7A. **Agarose Gel Electrophoresis Results of the Amplified ITS Barcode Regions for Samples Isolated from Arkansas.** DNA fragment sizes in the marker are noted at the left of each gel. Samples AN AR 13-31

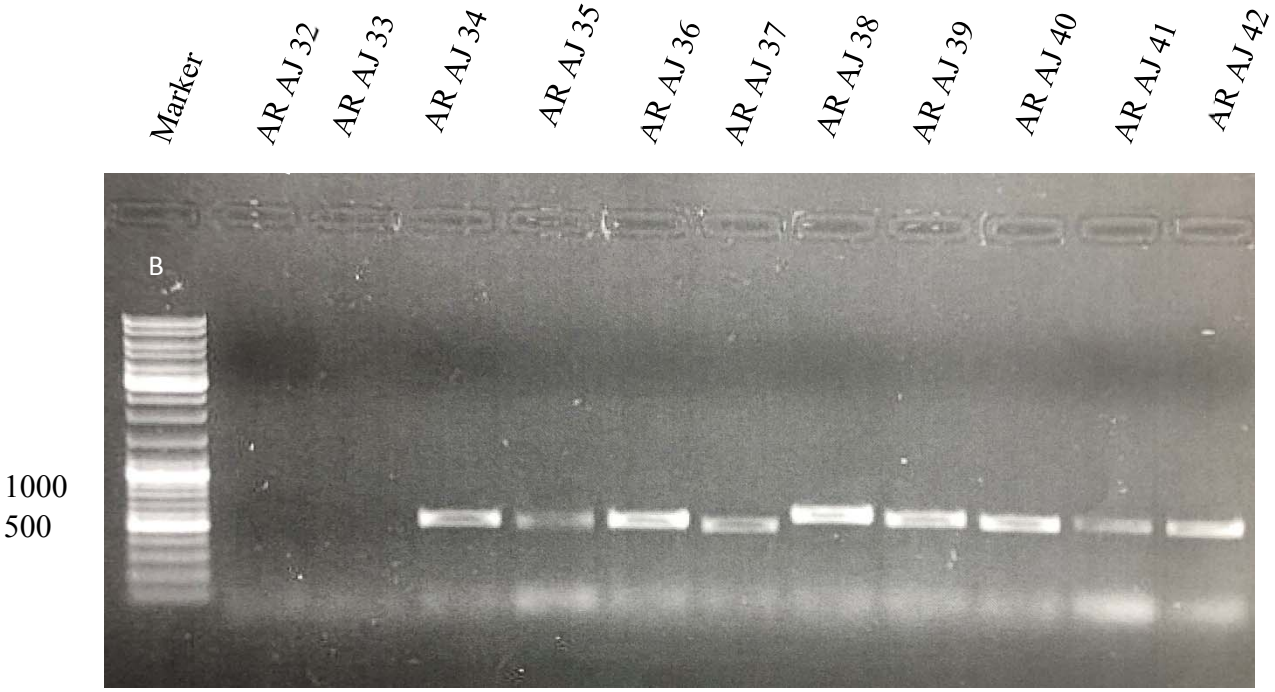


Figure 7B. **Agarose Gel Electrophoresis Results of the Amplified ITS Barcode Regions for Samples Isolated from Arkansas.** DNA fragment sizes in the marker are noted at the left of each gel. Samples AN AR 32-42

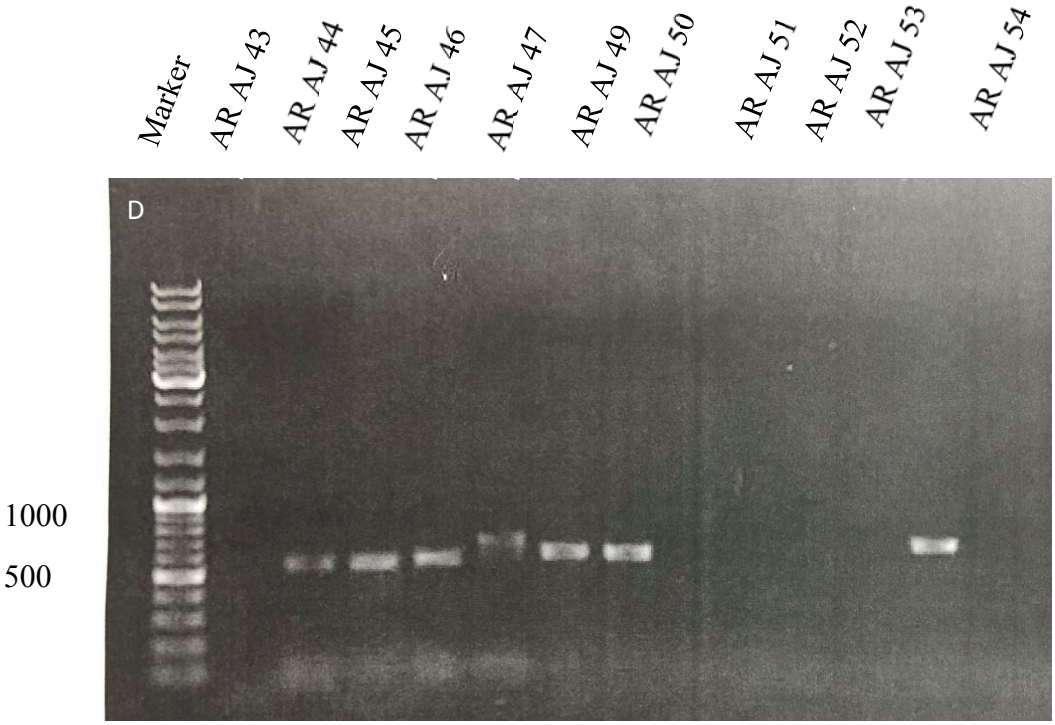


Figure 7C. **Agarose Gel Electrophoresis Results of the Amplified ITS Barcode Regions for Samples Isolated from Arkansas.** DNA fragment sizes in the marker are noted at the left of each gel. Samples AN AR 43-54

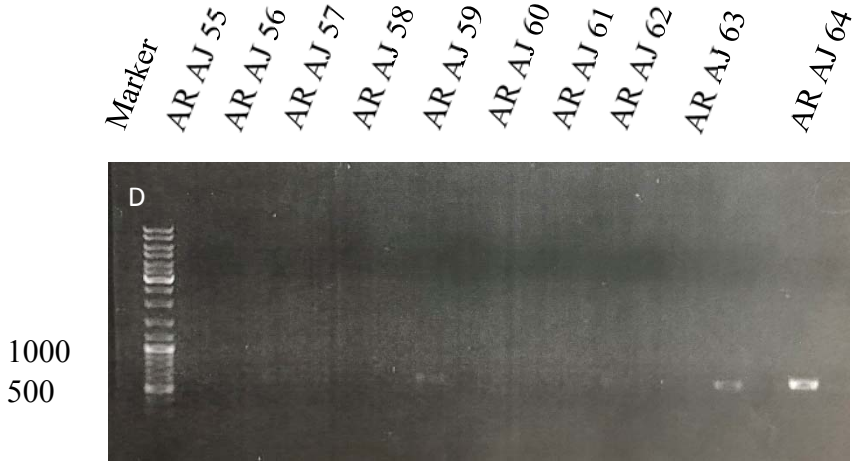


Figure 7D. Agarose Gel Electrophoresis Results of the Amplified ITS Barcode Regions for Samples Isolated from Arkansas. DNA fragment sizes in the marker are noted at the left of each gel). Samples AN AR 55-6

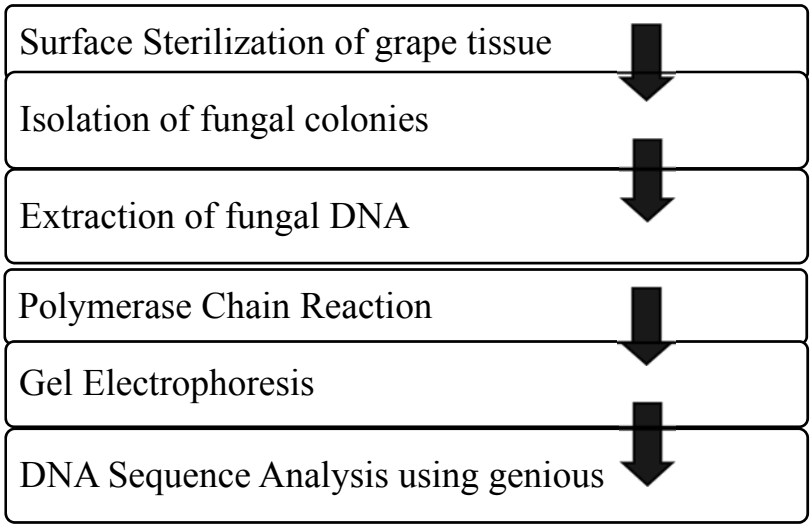


Figure 8. Simple workflow from sterilization to Sequence Analysis

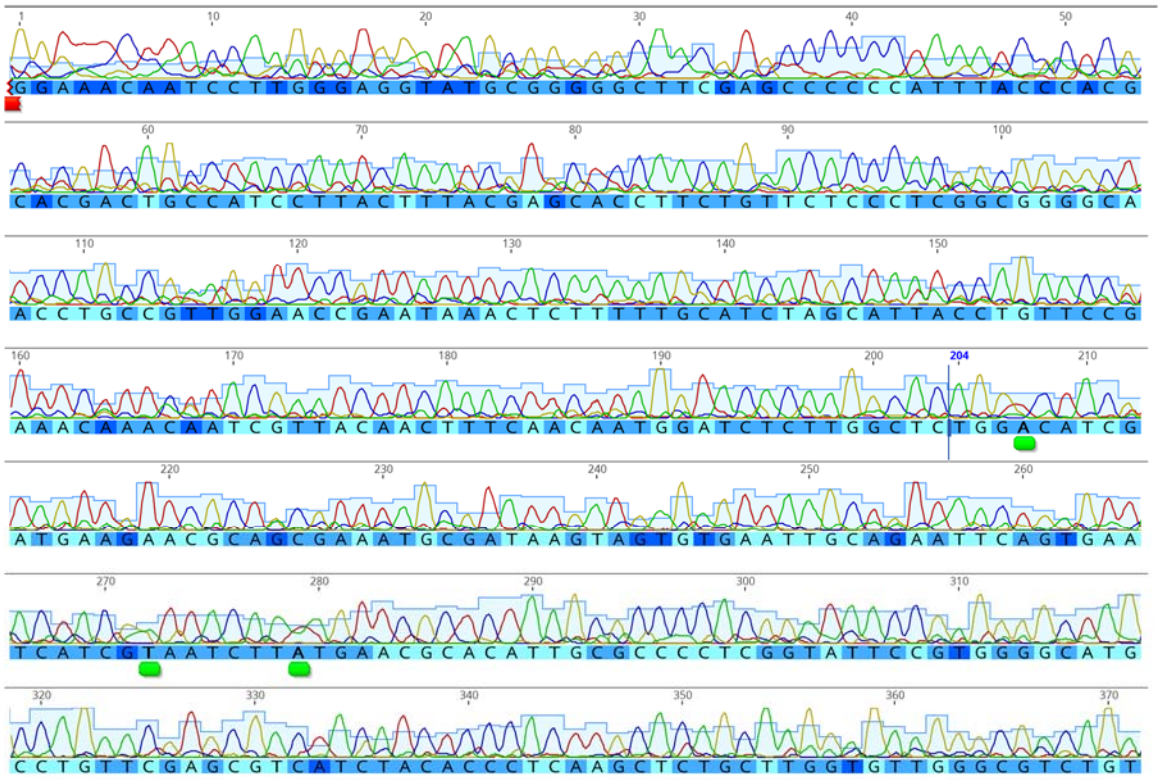


Figure 9. Eletrohpherogram displaying sequenced and trimmed DNA of endophytes

After editing the electropherograms generated by the genious software, the reverse complimented sequence of DNA was generated, and compared to the NCBI DNA data base (Figure 9). Each endophyte had a specific data table and the most likely identity of each fungi was determined based on the grade percentage and hit end value (Figure 10).

Bit-Score	E Value	Grade	Hit start	Hit end	Name	Description	Sequence
1,162.66	0	83.9%	128	876	KU937315	Alternaria tenuissima isolate 4 18S ribos...	755

Figure 10. Sample NCBI data table used to identify fungal endophytes to the species level.

Table 1. Missouri Isolates Identified

Likely Identity (species)	Gel name	% idetitiy
Alternaria alternata	MO_ZL1	100.0%
Alternaria tenuissima	MO_ZL2	98.9%
Alternaria alternata	MO_ZL3	98.9%
Trametes versicolor	MO_ZL4	99.4%
Alternaria tenuissima	MO_ZL5	99.9%
Alternaria alternata	MO_ZL6	99.6%
Aureobasidium pullulans	MO_RT 1	99.8%
Alternaria alternata	MO_RT 2	99.9%
Alternaria infectoria	MO_RT 3	99.9%
Aureobasidium pullulans	MO_RT 5	99.8%
Aureobasidium pullulans	MO_RT 6	99.8%
Corynespora sp.	MO_RT 7	86.5%
Alternaria tenuissima	MO_RT 8	95.1%
Alternaria infectoria	MO_RT 9	90.4%
Alternaria alternata	MO_RT 10	100%
Alternaria alternata	MO_RT 11	100%
Alternaria alternata	MO_RT 12	100%
Alternaria alternata	MO_RT 13	100%
Alternaria alternata	MO_RT 14	99.8%
Alternaria alternata	MO_RT 15	100%
Alternaria alternata	MO_RT 16	100%
Alternaria alternata	MO_RT 19	99.7%
Alternaria alternata	MO_RT 20	99.5%
Didymella pinodella	MO_RT 21	100%
Alternaria alternata	MO_RT 22	100%
Alternaria alternata	MO_RT 23	99.6%
Alternaria alternata	MO_RT 24	99.7%
Alternaria tenuissima	MO_RT 25	100%
Alternaria tenuissima	MO_RT 26	100%
Didymella pinodella	MO_RT 27	99.8%
Cercospora capsici	MO_RT 28	99.4%
Alternaria alternata	MO_RT 29	99.2%
Alternaria alternata	MO_RT 30	98.8%
Alternaria alternata	MO_RT 31	98.8%
Alternaria alternata	MO_RT 32	99.4%
Alternaria tamaricis	MO_RT 33	94.4
Alternaria tenuissima	MO_RT 34	100%

Table 2. Tennessee Isolates Identities

Likely Identity(species)	Gel name	% idetitiy
Fusarium equiseti	TN_RB_2	100%
Curvularia clavata	TN_RB_3	99.70%
Saccharomyces uvarum	TN_RB_4	99.60%
Xylaria sp.	TN_RB_5	98.30%
Agricomycete sp.	TN_RB_6	99.00%
Colletotrichum acutatum	TN_NJ_2	99.70%
Fusarium incarnatum	TN_NJ_3	99.70%
Epicoccum sorghinum	TN_NJ_6	99.80%
Alternaria longissima	TN_RT 7	93.4%
Pestalotiopsis microspora	TN_RT 8	100%
Pestalotiopsis microspora	TN_RT 9	74.4%
Epicoccum nigrum	TN_RT 10	99.5%
Epicoccum nigrum	TN_RT 11	92.4%
Epicoccum nigrum	TN_RT 12	90.7%
Stereum complicatum	TN_RT 13	99.1%
Alternaria alternata	TN_RT 14	92.2%
Alternaria longissima	TN_RT 15	88.5%
Colletotrichum kahawae	TN_RT 16	97.2%
Colletotrichum gloeosporioides	TN_RT 17	99.9%
Cladosporium anthropophilum	TN_RT 18	95.7%
Nigrospora oryzae	TN_RT 19	76.1%
Cladosporium cladosporiodes	TN_RT 20	99.7%
Diaporthe Phaseolorum	TN_RT 21	98.8%
Colletotrichum boninense	TN_RT 22	99.4%
Alternaria longissima	TN_RT 23	83.1%
Diaporthe gulyae	TN_RT 24	91.6%
Cercospora beticola	TN_RT 25	96.01%
Alternaria tenuissima	TN_RT 27	81.4%
Alternaria alternata	TN_RT 28	89.3%
Alternaria alternata	TN_RT 29	91.7%
Alternaria longissima	TN_RT 30	88.6%
Alternaria alternata	TN_RT 31	84.2%
Cercospora beticola	TN_RT 32	91.6%
Alternaria tenuissima	TN_RT 33	99.8%
Nigrospora oryzae	TN_RT 34	98.4%
Epicoccum nigrum	TN_RT 35	98.5%
Alternaria longissima	TN_RT 36	95.4%
Alternaria longissima	TN_RT 37	98.7%

Table 3. Arkansas Isolates Identified

Likely Identity(species)	Gel Name	% Match
Alternaria tenuissima	AJ2	100.0%
Fusarium proliferatum	AJ4	100.0%
Pestalotiopsis microspora	AJ6	99.8%
Xylaria sp.	KS04	97.4%
Epicoccum nigrum	KS05	83.9%
Fusarium equiseti	KS06	92.4%
Alternaria longissima	AR_AJ_7	88.4%
Alternaria citri	AR_AJ_8	99.8%
Alternaria tenuis	AR_AJ_9	99.7%
Alternaria alternata	AR_AJ_10	84.5%
Alternaria brassicicola	AR_AJ_11	99.9%
Alternaria tenuissima	AR_AJ_12	100.0%
Alternaria tenuissima	AR_AJ_13	97.2%
Coniothyrium sp.	AR_AJ_14	100.0%
Trametes versicolor	AR_AJ_15	99.9%
Trametes versicolor	AR_AJ_16	99.9%
Alternaria alternata	AR_AJ_18	100.0%
Fusarium oxysporum	AR_AJ_19	95.1%
Pestalotiopsis microspora	AR_AJ_20	98.5%
Alternaria alternata	AR_AJ_21	100.0%
Alternaria porri	AR_AJ_22	99.3%
Alternaria tenuissima	AR_AJ_24	100.0%
Coniothyrium sp	AR_AJ_25	83.4%
Corynespora sp.	AR_AJ_26	95.8%
Cercospora beticola	AR_AJ_27	97.9%
Trametes conchifer	AR_AJ_28	99.9%
Curvularia sp.	AR_AJ_29	87.8%
Cercospora lactucae-sativae	AR_AJ_31	90.6%
Cercospora cocciniae	AR_AJ_32	85.9%
Didymella pinodella	AR_AJ_33	89.6%
Androdia sinuosa	AR_AJ_34	92.8%
Colletotrichum acutatum	AR_AJ_35	99.0%
Alternaria tenuissima	AR_AJ_36	95.3%
Trametes versicolor	AR_AJ_37	99.1%
Coniothyrium sp	AR_AJ_40	94.1%
Alternaria porri	AR_AJ_43	99.0%
Leptosphaerulina chartarum	AR_AJ_44	90.6%

Table 4. Common Fungal Species

Common to All States	Common to TN-AR	Common to AR-MO
Alternaria alternata	*Alternaria longissima*	*Corynespora*
Alternaria tenuissima	*Cercospora beticola*	*Didymella pinodella*
Fusarium equiseti	*Colletotrichum acutatum*	
	Curvularia clavata	
	Epicoccum nigrum	
	Pestalotiopsis microspora	
	Xylaria sp.	

Table 5. Unique Fungal Species

TN	MO	AR
Agricomycete sp.	*Alternaria infectoria*	*Alternaria brassicola*
Cladosporium anthropophilum	*Alternaria tamaricis*	*Alternaria citri*
Cladosporium cladosporiodes	*Cercospora capsici*	*Alternaria porri*
Colletotrichum boninense	*Cercospora lactucae-sativae*	*Alternaria tenuis?*
Colletotrichum kahawae	*Uncultured organism*	*Antrodia sinuosa*
Diaporthe gulyae		*Coniothyrium*
Diaporthe phaseolorum		*Fusarium oxysporum*
Epicoccum sorghinum		*Fusarium proliferatum*
Fusarium incarnatum		*Leptosphaerulina chartarum*
Nigrospora oryzae		*Trametes conchifer*
Stereum complicatum		*Trametes versicolor*

Tennessee and Arkansas are within the same region of the United States, so they shared a large number of fungal endophytes. Missouri shared the fewest number of endophytes with the other two states, most likely due to the colder climate (Table 4). The number of fungi unique to Tennessee and Arkansas was also the highest, which may speak to their cache of biodiversity due to the warmer temperatures (Table 5 and Figure 11). A higher number of green plants also provides increased opportunities for endophytes to develop. The most common within these three geographic locations was Alternaria. Missouri contained the most Alternaria fungi of all three states, most likely because more well-adapted fungi of the south outcompete it for host plants (Figure 12).

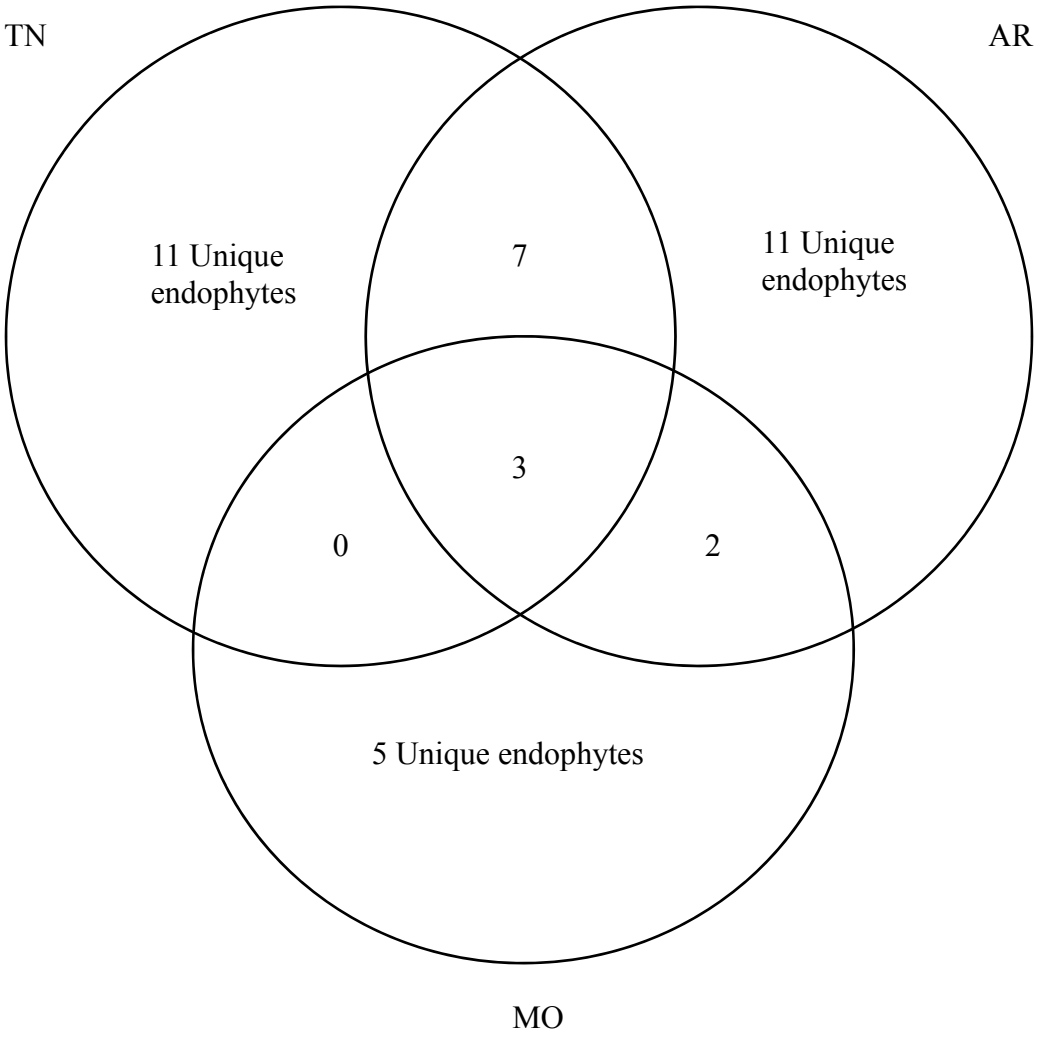
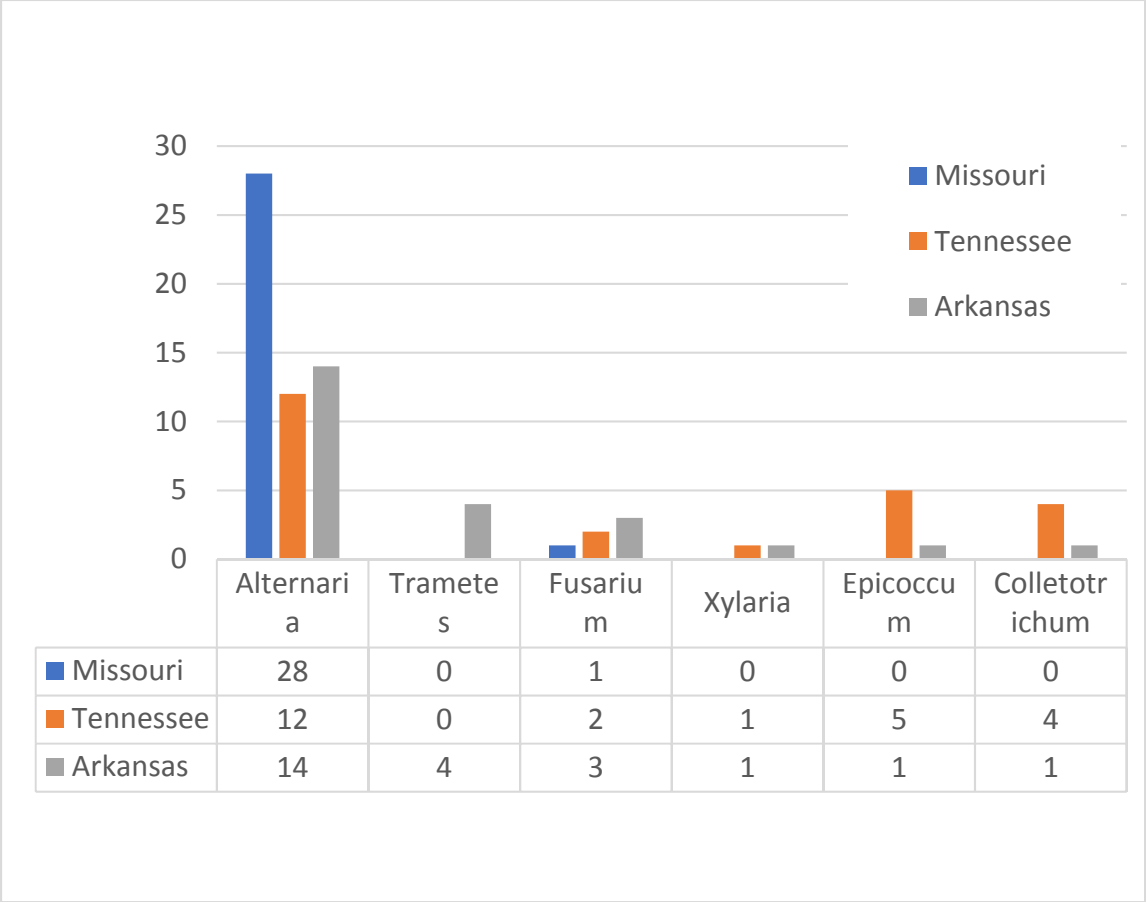


Figure 11. Distribution of unique fungal isolates among three geographic locations



	Alternaria	Trametes	Fusarium	Xylaria	Epicoccum	Colletotrichum
Missouri	28	0	1	0	0	0
Tennessee	12	0	2	1	5	4
Arkansas	14	4	3	1	1	1

Figure 12. Distribution of the Five Most Prevalent Genera

Table 6. Distribution of Fungal Species by State

	TN count	TN %	AR count	AR %	MO count	MO %
Alternaria alternata	4	10.5%	3	8.1%	21	58.3%
Alternaria infectoria	0	0.0%	0	0.0%	2	5.6%
Atlternaria longissima	6	15.8%	1	2.7%	0	0.0%
Alternaria porri	0	0.0%	2	5.4%	0	0.0%
Alternaria tenuissima	2	5.3%	5	13.5%	5	13.9%
Cercospora beticola	2	5.3%	1	2.7%	0	0.0%
Coniothyrium	0	0.0%	3	8.1%	0	0.0%
Corynespora	0	0.0%	1	2.7%	1	2.8%
Didymella pinodella	0	0.0%	1	2.7%	2	5.6%
Epicoccum nigrum	4	10.5%	1	2.7%	0	0.0%
Fusarium esqueti	1	2.6%	1	2.7%	1	2.8%
Nigrospora oryzae	2	5.3%	0	0.0%	0	0.0%
Pestalotiopsis microspora	2	5.3%	2	5.4%	0	0.0%
Trametes versicolor	0	0.0%	3	8.1%	0	0.0%
Xylaria	1	2.6%	1	2.7%	0	0.0%
unique	14	36.8%	12	32.4%	4	11.1%

Conclusions

The fungal endophytes isolated from Norton Cynthiana grapes of Missouri, Tennessee, and Arkansas are both unique to individual states and shared between these three states. Two thirds of all of the Norton/Cynthiana grape tissue sterilized came from warmer regions of the country. The warmer temperatures of the southeastern United States are most likely responsible for the diverse number of fungal isolates obtained from Arkansas and Tennessee (Rather et al. 2018). *Alternatia alternata*, a common irritant and plant pathogen was found within each of the three states. Alternaria alternata has been linked to several pathogenic diseases in plants caused by the host-selective toxins it secretes (Takashi et al 2013). *Alternaria tenuissima* is another fungal endophyte of three common to all three states. *Alternaria tenuissima* is an opportunistic fungal endophyte that has been linked to improving growth and Selenium accumulation in *Astragalus bisulcatus (Lindblom et al. 2018).* The pathogenicity of *Fusarium equiseti*-another endophyte common to all three states observed- is high in legume plant species.

The radicles of chickpeas and other legumes developed reddish-brown lesions during germination which eventually lead to the deaths of the young seedlings *(*Rubella et al. 2010). The fungal endophytes found within the surface-sterilized tissue of grape plants from Tennessee and Arkansas were the most diverse colonies observed. Seven unique isolates were found within grapes from both of these states. *Alternaria longissimi* was another isolate found that has not been observed extensively as an endophyte, and should be further studied. *Cercospora beticola* is responsible for leaf spot in beets and spinach (Weiland J, et al. 2004). *Colletotrichum acutatum* was another isolate common to both Tennessee and Arkansas. Other species of endophytes from the same genus have been

responsible for spotting in the leaves of other plants like *Anemone coronaria* L (Freeman et al. 2000). Through genetic analysis of the internal transcribed spacer region, *Curvularia clavata* was the fungal endophyte responsible for the severe leaf spotting in pineapple plants of china, (Zhong et al 2016). *Epicoccum Nigrum,* is an endophyte that may be one of several fungi isolated from the Norton grapes of Tennessee and Arkansas that can improve reproductive capability by secreting fungal metabolites that increase root biomass, and defend their hosts from other fungal endophytes (Léia et al. 2012).

Pestalotiopsis microspora would not be a viable candidate for improving hardiness of Norton grapes because this endophyte also causes leafspot and kills its host plant, oil palm *Elaeis guineensis (Shen et al. 2014).* A member of the *Xylaria* genus was also discovered, which is the primary cause decline in the health of soybean taproots (Sharma et al. 2018). *Corynespora sp.* is a pathogen, endophyte, and saprophyte that is found within over 580 different species of within southeastern America. The Cornyespora species isolated is most likely of the *Cassicola* species, as a single clonal lineage is the predominant lineage in the southeastern United States (Shresthsa et al. 2017). *Didymella pinodella* is a relative of *Didymella* pinodes which is the attacker of leguminous crops worldwide (Barilli et al 2016). *Cladosporium anthropophilum* is one of many Ascomycota which may function as biocontrol agent for plant diseases (Köhl et al. 2015). *Cladosporum cladosporiodes* is responsible for blossom rot in strawberries, as such, it may be one of several isolates which contributes to Norton grape leafspot (Nam et al 2015). *Colletotrichum boninense* is also a suspected member of a group of fungi likely causing stress in Norton Cynthiana grapes of Tennessee. Members of the *Colletotrichum* genus cause anthracnose disease in over 197 different plant species (Oo et al 2017).

Colletotrichum Kahawae is an endophyte that destroys coffee berries, but is restricted to the areas that grow coffee (Batista et al. 2017). Given that this is a species far outside of its native range, it is most likely a misidentified relative within the *Colletotrichum* genus. *Diaporthe gulyae* is responsible for glycine max which is responsible for stem cankers in sun flowers *(Mathew et al. 2014).* This endophyte was most likely responsible for the small dark patches of crust on the stem tissue of the Norton grape plants it was extracted from. *Diaporthe phaseolorum* is another endophyte found within Norton grape plant tissue thought to have been responsible for the destruction of soybean plants *of* Argentina (Grijalba et al. 2012). *Epicoccum sorghinum* has been isolated as a factor contributing leafspot in *Bletilla Striata (Zhou et al. 2018). Fusarium incarnatum* has been associated with fruit rot in Bell peppers (*Capsicum annuum L.)* of trinidad (Ramdial et al. 2015).

Additional observations of this fungi within Norton grapes will be informative for future analysis. *Nigrospora oryzae,* another endophyte typically found within *Combretum dolichopetalum*, emits metabolites within its host's body that reveal a potential mutualistic relationship (Uzor et al. 2015). *Stereum complicatum* also known as crowded parchment, an edible surface contaminant that lives on oak trees, was also isolated from Norton grape tissue of Tennessee. This is most likely a surface contaminant that was not fully removed; giving it the opportunity to reproduce. Missouri contained the fewest number of unique fungal isolates, the first of which was *Alternaria infectoria. A. infectoria* is a common opportunistic filamentous fungi much like many of those found within all Norton grape tissue selected for isolation (Silva et al. 2013). *Alternaria tamaricis* has not been studied as extensively as its relatives, and must be re-isolated for further analysis. Individuals of the *Cercospora lactucae-sativae* species that struck

Thailands lettuce with leaf spot may be resistant to Carbendazim; a widely used fungicide (Suwan et al. 2012). *Alternaria Brassicicola* are necrotophic endophytes that kill a wide variety of plant species and absorbs nutrients from their destroyed hosts (Cho, 2015). The Norton grapes of Arkansas were most likely fighting off this infection, as the leaves this fungi were extracted from were covered with brown circular patches of dead tissue. *Alternaria citri* is the cause of Alternaria brown spot in citrus fruits using host selective toxins to cause necrotic spots to appear (Isshiki et al. 2001). *Alternaria porri* is responsible for purple leaf spotting in onion leaves. *Antrodia sinuosa* is responsible for the wood rot within lemon trees (Matheron et al. 2006). An individual endophyte of the *Coniothyrium* genus was found within a Norton grape plant of Arkansas.

Coniothyrium minitans can act as a biological control for *Sclerotinia sclerotiorum, or cottony rot (*Gerlagh et al 2007). The Coniothyrium fungi found was most likely a surface contaminant, as there were no traces of cottony rot within the grape tissue sample it was extracted from. *Fusarium Oxysporum* is another opportunistic isolate found within Norton grape plant tissue of Arkansas which have clonal lineages in numerous locations (Koenig et al. 2007). *Fusarium proliferatum* on the other hand, infects soybeans and is another factor in glycine max disease (Diaz et al. 2011). The fungal endophyte *Leptosphaerulina chartarum* produces xylanolic enzymes which can improve plant immunity, so additional study of this endophyte within the grape plant itself will be valuable (Wu et al. 2013). *Trametes conchifer* was one of the few endophytes found within the Norton grapes of Arkansas that had not been extensively researched as an endophyte. Additional observations within endophyte free grapes will be needed in future studies. *Trametes versicolor* is an edible surface contaminant that has

not been studied extensively as an endophyte.

The fungal endophytes found within the leaf tissue of *Vitis aestivalis* Norton Cynthiana grapes of Missouri, Tennessee, and Arkansas are a diverse group. The poor reproductive success of the Norton grape could be attributed to necrotrophic endophytes within the plant's tissue. Necrotrophic fungal endophytes secrete mycotoxins into a plant's tissue; slowly killing their host in order to extract nutrients (Gunther et al. 2017). The fungal endophytes extracted from these Norton grape tissue samples are overwhelmingly harmful to the growth and development of the grape plant. Even though fungal endophytes limit the flavonoid and sugar content of grape berries by modifying the metabolic activity of their hosts this is not without associated costs to the longevity of the grape vine (Yang et al. 2016). One of the most common species of fungal endophyte isolated from *Vitis aestivalis* grape leaves of Missouri was *Alternaria alternata.* This particular fungus has been associated with bunch rot in other grape species, and because this fungi appears within the grape leaves of Missouri at such a high rate, the subsequent health of those grapes is quite poor. Alternaria alternata accounted for half of the fungi found within leaves of the Missouri grape plants, which would explain why their leaves were peppered with rust spotting from this particular fungus.

Flavonoid concentration also plays a large role in that spotting when the concentration of plant pathogens becomes higher or environmental conditions change (Cantín et al. 2007). Unlike Missouri, a much more diverse community of fungal endophytes was isolated from the grape leaves of Tennessee and Arkansas. The number of isolates found was so large, a method of colony PCR using non-purified fungal mycelial lysates may be useful for future researchers (Michael et al. 2012). Within

Missouri, Tennessee, and Arkansas, uncommon fungal endophytes were found that may hold the answer to why *Vitis aestivalis* is so difficult to propagate. Two fungi of the genus Diaporthe were found within the leaf tissue of the *Vitis aestivalis* grape plants located in Tennessee. Diaporthe is a very common pathogenic fungi in other species of plants, unsurprisingly, at least some population of *Vitis aestivalis* grape vines contains it as well (Botella & Diez 2011). At least two species of fungi of the genus Cercospora were isolated from the tissue of Missouri grape leaves. This is another species that could be responsible for the leaf spot disease observed. One species of fungi affecting wood rot in lemon trees is *Androdia sinuosa* (Matheron et al. 2006) which was found within grape leaf tissue of Arkansas. It is important to remember that while there are many endophytes within the *Vitis aestivalis* grape plants of Missouri, Tennessee, and Arkansas that have already been identified from other sources, understanding the interaction with each of these fungi have with their hosts is vital. Most of the species identified in this study parasitize their plant hosts in ways that may or may not hinder their growth in the long term. In the future, obtaining fungi from young cuttings will give some insight into what pathogenic factors are at play during ideal periods of propagation.

Necrotrophic fungal endophytes could be interrupting the growth factors and hormones that regulate lateral root growth of young grape cuttings, but additional research is needed to determine whether these lesser-known fungi isolated have necrotrophic or bio trophic effects on *Vitis aestivalis* Norton grape plants. Understanding the interface between fungal pathogen and plant may improve the Norton grape's reproductive potential by discovering how the chemical controls governing root growth in cuttings are disrupted by the fungal endophytes. Fungi belonging to the Alternaria

genus tend to cause damage to cereal grain, fruit, and vegetable crop production. While one individual fungi may not be responsible for the hardiness of the Norton/Cynthiana grapevine health, large communities of fungi living within grape tissue are more likely to be responsible for the condition of their host plant. If most fungi residing within the vascular tissue of a plant host are pathogenic, the overall health of the host plant will reflect that. While a comprehensive interaction of Norton/Cynthiana grapes is still far out of reach, a survey of different endophyte communities is still necessary to understand the climatic factors that affect the proliferation of these endophytes. Fungal endophytes adapt to habitat related stress under various environmental conditions (Rodriguez et al. 2008).

Identification of the fungal endophytes within Tennessee, Missouri, and Arkansas Norton/Cynthiana grapes could have far-reaching implications for the future of agricultural science as well. In addition to the benefits provided, fungal endophytes provide their hosts with protection from parasitic infection by acting as a supplemental immune system. Fungal endophytes may have antagonistic effects against pathogenic fungi that can harm their host's tissue; such is the case with *Ustilago maydis* and *Fusarium verticillioides*. *F. verticillioides* is thought to secrete metabolites that suppress *U. maydis* development within *Zea mays* (Rodriguez et al. 2012). Without the fungal endophytes inhabiting the tissue of plant hosts, their bodies would be susceptible to infection, or be significantly smaller due to inefficient nutrient absorption. Raising fungal endophyte-free grape plants that will eventually be inoculated with the fungal endophytes previously isolated to determine their role within the plants vascular tissue.

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