A. Investigating the growth, performance, and mycorrhizal communities in seedlings of American, Chinese, and hybrid chestnuts planted in stands of different chestnut types

B. Summary (<100 words)

This pilot project explores the mycorrhizal relationships in wild-type American, Chinese and backcross hybrid chestnuts. It addresses TACF Priority Research Area 2. *Studies which compare ecological functionality, local adaptation, and/or stress tolerance of backcross or other hybrid American chestnuts to wild type American chestnuts*. We will assess mycorrhizal communities in monotypic stands of American, Chestnut, and backcross hybrids and assess mycorrhizal colonization and performance of seedlings established in each stand. Mycorrhizal relationships are crucial for successful seedling establishment and an understanding of how genetic makeup of seedlings influence colonization is important for successful reintroduction of American chestnut hybrids.

C. Principle Investigator:

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D. Duration of Project : Nov. 1, 2024 – Oct. 31, 2024

E. Total Amount Requested; $10,000

Matching Funds: Horton summer salary and uncollected F&A from this proposed grant

F. Project Goals

Short-term Goals:

1. Characterize patterns of survivability, growth, and physiology of chestnut seedlings of different genetic lineage (American, Chinese, and backcross hybrids) planted in monotypic stands of chestnuts (American, Chinese, and backcross hybrids)
2. Determine if there are different ectomycorrhizal communities in monotypic stands of different chestnut lineages (American, Chinese, and backcross hybrids)
3. Determine if there are differences in ectomycorrhizal colonization among chestnut seedlings of different lineages out-planted into monotypic stands of different lineages.
4. Train undergraduate students in research methods and educate them about American chestnut, the restoration efforts of TACF, and potential ecological interactions of out-planted chestnuts of different lineages.

Long-term Goal:

1. Help to facilitate the successful deployment of trees improved through genomic selection and other tree improvement strategies for ***establishment and restoration*** of

blight-resistant chestnuts in different silvicultural scenarios. Use the data generated from this project to investigate the ecological role of ectomycorrhizae and common mycorrhizal networks in the success of out-planted chestnut seedlings of different lineages to support reintroduction of blight-resistant American Chestnuts.

2. Use the data generated from this project to seek additional funding to explore the mycological ecology of chestnuts to help with restoration efforts.
3. Train the next generation of researchers working toward the successful ecological restoration of chestnut to intact forests of eastern North America.

G. Narrative

Background

Successful reintroduction of blight-resistant chestnuts will require effective seed production and dispersal and successful natural regeneration (Jacobs et al. 2012). Little is known about natural regeneration dynamics in American chestnut (*Castanea dentata* (Marshall) Borkh.). One important component of successful seedling establishment is the development of beneficial fungal symbioses. Most plant species form mycorrhizal symbioses with fungi, that aid in nutrient acquisition and can protect plants from drought and pathogens (Horton and van der Heijden 2008). These relationships can be especially important during seedling establishment. Many mycorrhizae are not host-specific forming symbioses with multiple plants from a range of host species, often creating mycorrhizal networks. Seedlings can become integrated into these hyphal networks maintained by the surrounding vegetation. Early establishment in these networks can be greatly beneficial for seedling growth and survival, especially in nutrient-poor sites (van der Heijden and Horton 2009).

Unfortunately, little is known about the fungal symbionts associated with American chestnut before its effective extinction in natural forest ecosystems from its native range. There have been some studies investigating these symbionts, particularly ectomycorrhizal fungi (ECM), but these were either conducted in locations outside of American chestnut's native range (e.g. Palmer et al. 2008) or on seedlings that had not established under natural conditions (e.g. Dulmer et al. 2014).

American chestnut is a member of the Fagaceae family, along with oaks (*Quercus* sp.). It is likely that they shared mycorrhizal symbionts in natural ecosystems where they cooccurred. Dulmer et al 2014 showed that American chestnut seedlings can tap into existing ECM networks in forests in the former range of American chestnut. They found that four month old seedlings had similar ECM hosts as corresponding red and white oak, American beech, black birch, hop hornbeam, hemlock and white pine, and that the highest number of shared hosts were with red oak and white pine. They determined that there was inoculum potential in soils in the former range of American chestnut for colonization of chestnut seedlings. They also found that using soil from intact forests in chestnut's former range was an effective way of inoculating laboratory

or greenhouse grown seedlings. However, the ECM species found in laboratory seedlings were not in high frequency or abundance in field planted seedlings, a pattern often seen in studies comparing lab and field soil bioassays (e.g. Taylor and Bruns 1999; Cline et al. 2005). Dulmer et al. 2014 found 14 different ECM taxa on lab grown seedlings and found that field-grown seedlings had an average of 17 ECM root tups belonging to 71 ECM taxa. Another study found that the ECM community collected from naturally occurring chestnut saplings in a second growth forest in Tennessee consisted of 18 ECM taxa with very little overlap with previous studies (Stephenson et al. 2017). A recent study by Reazin et al. (2019) on chestnut seedlings bred for blight resistance and planted in plant nursery beds found that seedlings from different families (including pure American, Chinese, and backcross) developed different fungal communities in the rhizobiome, and that rhizobiome communities differed from the fungal communities in the bulk soil of the nursery bed, suggesting some selection for fungal communities associated with the different lineages. However, more needs to be known about ECM communities supported by different chestnut breeding lines and how these colonize seedlings from different lines.

The goal of this study is to use a reciprocal seedling transplant approach to assess the ECM community composition in pure stands of American, Chinese, and backcross chestnuts and colonization patterns of seedlings of these three lineages planted in each stand.

Methodology

This proposed work will take place at The American Chestnut Foundation's Meadowview Research Farm (MRF) in Virginia. Staff at MRF will collect seeds from these different lines of chestnut breeds, aiming for at least 300 of each lineage. One hundred seeds of each of the three chestnut types will be out-planted and seedlings will be used as "bait" (an approach used in other studies by the PI: Walker et al 2005; Walker et al. 2008, Caruso et al. 2021) in a randomized block design within pure stands of American, Chinese, and backcross chestnuts at the MRF in a 3 x 3 factorial design. Seeds will be planted after cold stratification in spring 2025. Additionally, an extra cohort of each lineage will be planted for early harvest shortly after emergence to determine initial size parameters (Number of leaves, stem length, basal diameter, and root and stem biomass). When seedlings are harvested at the end of the growing season, we will use these initial parameters to estimate seedling growth.

Once seeds germinate in spring 2025, we will tally the number of germinants in each 3x3 combination and will base future sampling on a balanced design from these germinants. The PI and undergraduate research students from UNCA will begin monthly (June, July, and August) trips to MRF in summer 2025 to assess growth, survival, and physiological condition of seedlings. Because of the differences in crown architecture of the different overstory chestnut types, we will assess the seedling light environment with hemispherical canopy photographs (Diaz 2023). These measurements could be used as a covariate in comparisons among stands. Photosynthetic capacity will be measured on 3-5 randomly selected seedlings of each type from

each stand using steady-state light response curves using two portable photosynthesis systems (Li-6400, LiCor Biosciences, Lincoln, NE). We will randomly select twenty seedlings of each type in each stand for morphological measurements. Each month we will measure basal diameter, total stem length, and total number of leaves. If a seedling dies, it will be replaced with a randomly chosen seedling of similar initial basal diameter. These parameters will be used to assess growth in the different seedling x stand combinations. In addition to seedling surveys, we will collect any ECM fruiting bodies from the three stands and take them back to the lab at UNCA for identification, DNA barcoding (see below) and curation in the UNC Asheville Fungarium. It is known that sampling only fruiting bodies for fungal community composition has limitations (Baptista et al. 2015), and these collections are meant to add DNA barcodes to our internal database (and Genbank) to assist with identification of ECM root tip fungi.

Half of the seedlings will be harvested in mid-August 2025, with care will be taken to ensure all fine roots are collected. The remainder will be harvested in August 2026 (with supplemental funding from the Undergraduate Research Program at UNCA). Leaves will be excised and total leaf number will be counted and total leaf area will be measured with a leaf area meter (LI-3100C, LiCor Biosciences, Lincoln, NE). Roots will be excised from stems for processing for mycorrhizal colonization. Stems, leaves, and roots (after ECM processing – see below) will be dried at 60^{o}C for 48 hours and weighed to get biomass for each organ.

Root ECM colonization will be examined by first manually washing soil from the seedlings root systems. Roots will then be examined under a dissecting microscope and a total of 10 cm of root length will be sub-sampled from the top, middle, and bottom of the secondary root system (sensu Walker et al. 1999). All root tips in that 10 cm root length will be counted and observed for mycorrhizal colonization and colonization frequence will be calculated as the percentage of total root tips that have been colonized by ECM. ECM root tips will be excised and identified using morphotyping and DNA barcoding, except root tips colonized by *Cenoccocum geophilum* Fr., which is usually very abundant and easily identified visually by its stiff black rhizomorph. We will extract, amplify, and sequence DNA from unique morphotypes to determine operational taxonomic units (OUT's).

DNA extractions from ECM root tips will be done with Qiagen DNeasy Plant Kits (Qiagen, Germantown, MD). We will amplify the internally transcribed spacer region (ITS) using primers ITS1-F and ITS4 (White et al. 1990; Gardes and Bruns 1993), a commonly used locus for DNA barcoding. We will use a protocol developed by Russell (2023) to sequence DNA amplicons using UNC Asheville's Minion Sequencer (Oxford Nanopore Technologies, Oxford, UK). Sequences will be aligned using Geneious v. 10 and aligned sequences will be compared to the NCBI Genbank sequence library with the nucleotide BLAST multiple alignment tool to determine OTU's.

We will use pooled OTU data from all seedlings of each type to estimate ECM richness and diversity within each monotypic stand. We will compare ECM community similarity among the

three monotypic chestnut stands by calculating the Coefficient of Community (CC) with this equation from Baird et al. (2014).

Coefficient of community (CC) = 2c/(a+b)

Where a = total number of species present in the first community, b = total number of species present in the second community, and c = number of species present in both communities

We will assess any differences in ECM community among the different seedling lineages within each monotypic stand using the same CC calculation. Richness and diversity indices will be compared among the monotypic stands using analysis of variance (ANOVA). We will assess whether there were differences in colonization rate, growth parameters, or physiological characteristics among seedlings in the different monotypic stands with two way ANOVA with stand and seedling types as treatment variables. Growth and physiological parameters will be related to colonization rate with regression analysis.

Outcomes

This study will provide a better understanding of the ECM communities that develop in monotypic stands of American, Chinese, and backcross chestnuts. This study will yield data that can facilitate the successful deployment of trees improved through genomic selection and other tree improvement strategies for ***establishment and restoration*** of blight-resistant chestnuts in its former range. It will also help to understand the ability of seedlings to connect with existing ECM networks in these monotypic stands. It will also relate ECM colonization and community composition to aspects of successful seedling establishment, specifically photosynthetic capabilities and growth and survival. This understanding addresses TACF Priority Research Area 2. *Studies which compare ecological functionality, local adaptation, and/or stress tolerance of backcross or other hybrid American chestnuts to wild type American chestnuts*. Additionally, this work will support undergraduate research students . This real-world, hands-on research experience and collaboration with TACF MRF staff will help in their professional development and will help train the next generation of scientists working on the successful reintroduction of chestnut to its former ecological range.

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H. Timeline (Start and completion dates for each goal)

Seedlings will be outplanted in fall 2024. Some will be destructively harvested to get initial size parameters so that relative growth rate can be calculated at harvest.

Activity	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct
Student training												
Seedling Work												
Seedling monitoring												
Seedling Physiology												
Harvest and processing												
Molecular Work												
DNA extractions												
PCR Amplification												
Sequencing												

The molecular work will continue beyond the funding period.

Students will analyze data, prepare manuscripts and presentations during the spring 2026 Semester. They will present at UNCA's Spring Undergraduate Research Symposium as well as the Annual Meeting of the Association of Southeastern Biologists. They will prepare a manuscript for UNCA's peer-reviewed Journal of Undergraduate Research. Manuscripts will be prepared for publication in external peer-reviewed journals also.

I. Results Reporting

Data and metadata will be stored in files on PI Horton's work computer and University Google Drive. Students will prepare manuscripts to submit for publication and will present their finding at regional or national meetings and, if possible, at the annual meeting of The American Chestnut Foundation. Students will also present their work at UNC Asheville's Undergraduate Research Symposium the semester they graduate. Any fungal fruiting bodies collected will be curated in the UNC Asheville Fungarium and morphological data will be uploaded to the UNCA Asheville Fungarium Mycoportal (an international database of Fungaria) database. DNA barcode sequences of fruiting bodies and ECM root tips will be submitted to Genbank and those for fruiting bodies in the UNCA Fungarium will be linked to their Mycoportal entries.

J. Budget and Justification

Expense	**Amount Requested**
Undergraduate Student Wage	**$6000**
Fringe benefits (7.65%)	**$460**
Travel	**$360**
Field Supplies	**$500**
Molecular Supplies	**$2680**
Total	**$10,000**

Budget Justification

Student Wage

Funds are requested to support two undergraduate students. These students will 1) work over the summer to monitor and measure leaf-level physiology over the summer, 2) harvest and process seedlings in late summer and fall and 3) extract DNA, amplify, and sequence DNA from mycorrhizal root tips and fruiting bodies collected on site. Funds are requested for FICA for these students at the calculated rate of 7.65% of wages.

Travel

We request funds to support four trips to the TACF Meadowview Research Farm in Virginia. These trips will allow monthly physiology measurements during the summer and harvest in late summer. Using University vehicles the costs are $50 per day plus fuel (estimate $90/trip).

Field and Molecular Supplies

We request funds for field supplies (consumables for the physiology equipment, wax bags for collecting fungal fruiting bodies, and Ziploc bags for collecting harvested seedlings. We also request funds for the DNA extraction, amplification, and sequencing using protocols developed for fungal DNA barcoding using UNCA's Oxford Nanotech Minion DNA sequencer. Funds will be used to by reagents, flow cells, and laboratory consumables (e.g. microfuge and PCR tubes, micropipette tips, etc.)

K. Curriculum Vitae

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Education

2000 - 2003	Postdoctoral Fellowship, Plant Physiological Ecology, Virginia Tech
1996 – 2000	Ph.D. Forestry, Northern Arizona University
1993 - 1996	MS Biology, Appalachian State University
1986 – 1990	BS Biology, University of North Carolina

Professional Experience

2020 – present	Professor and Chair, Biology Department, UNC Asheville
2016 – present	Professor, Biology Department, UNC Asheville
2009 – 2016	Associate Professor, Biology Department, UNC Asheville
2003 – 2009	Assistant Professor, Biology Department, UNC Asheville
2000 - 2003	Postdoctoral Fellow, Virginia Tech
1997 - 2000	EPA-STAR Research Fellow, Northern Arizona University

Professional Societies/ Service:

Ecological Society of America, Southern Appalachian Botanical Society, The American Chestnut Foundation

Recent Publications (2015 – present * denotes undergraduate author)

Cleary* MS, **JL Horton**, and CC Fiilgueiras. (in review). Physiological comparison of pure American and hybrid American-Chines chestuts. *Castanea*

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Current and Pending Support

1. JL Horton (this proposal). $10,000. Investigating the growth, performance, and mycorrhizal communities in seedlings of American, Chinese, and hybrid chestnuts planted in stands of different chestnut types.
2. Filgueiras, CC and JL Horton (2022-2024). $10,000. Herbivory and Stress Responses of Outplanted Wild-Type and Backcross Hybrid American Chestnuts in Western North Carolina. The American Chestnut Foundation. Ends 9-30-2024.

L. COI

There are no known conflicts of interest for the PI conducting this project.