Comparative Analysis of Phyllosphere Microbiota in Backcross, Transgenic, and Wild Type American Chestnuts

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Co-PI: Paula Jackson, Ph.D. (Kennesaw State University, Kennesaw, GA 30144)
Collaborator: Katherine Dobson (Kennesaw State University, Kennesaw, GA 30144)

Summary

The composition and diversity of the microorganisms that inhabit the surface of leaves (i.e., phyllosphere microbiome) is governed by host plant genetics and leaf morphology. The importance of the phyllosphere microbiome on regulating plant growth, pathogen susceptibility, and even ecosystem-scale processes necessitates a holistic understanding of the phyllosphere microbiome, especially for species of conservation concern. The objective of our study is to perform a metagenetic survey of backcross, transgenic, and wildtype American chestnuts to test whether they exhibit comparable phyllosphere microbiomes (i.e., similar ecological functioning), and whether leaf morphology and genetics are better predictors of the phyllosphere microbiome than location.

Project Duration: 2 years (January 2024 - December 2025)

Amount requested:	*Year 1:*	*$35,310*
	Year 2:	*$14,682*
	Total:	*$49,992*

Short and Long-Term Goals

Short term goals:

1. To identify and describe the microbial (fungal and bacterial) community of leaves (phyllosphere) from wild type, transgenic, and backcross American chestnuts.
2. To determine differences in the bacterial communities of the above-mentioned Chestnut varieties.
3. To test for a relationship between phyllosphere microbiome and location, genetics, and leaf traits.

Long term goals:

1. To establish if differences in the microbiome phyllosphere (if present) are associated with differences in plant health (e.g., growth, pathogen resistance).
2. To use the intentional inoculation of beneficial bacteria as an additional mechanism towards the conservation and restoration of the American chestnut.

Background/Introduction

Biological relationships such as predator-prey interactions and food web dynamics have been extensively studied and serve as classic examples of interactions shaping population dynamics and ecosystem processes. However, much less research has been conducted on how the microbial community can influence these biological relationships and the organisms within them (Baldrian, 2017). No organism is without microbial influence (Cavicchioli et al., 2019); Human disease is impacted by gut bacteria (Shreiner et al., 2015), orchids are dependent on fungi for seed germination (Yeh et al., 2019), and growth-promoting rhizobacteria in plant roots mediate stress (Bhat et al, 2023; Lugtenberg and Kamilova, 2009). These examples are proponents that no organism exists in singularity and that these macro and micro relationships have important consequences. Though microbiome research has gained popularity in recent years, there is still ample work to be done in ecosystem and trophic level microbiome interactions (Zhu et al., 2023; Weems et al., 2021). Such knowledge is especially valuable to assess and improve conservation and species reintroduction efforts.

The interface between plants and the environment is not sterile but instead harbors an astounding diversity of microorganisms, including bacteria, fungi, and algae. Seeds, roots, flowers, and leaves all possess their own microbiome that affect metabolic processes including nutrient acquisition, stress tolerance, plant growth, and defense against pathogens (Compant et al., 2019; Hassani et al., 2018). For example, grapevines showed increased resistance to drought stress when inoculated with certain rhizospheric bacteria (Rolli et al., 2015) and inoculated *Arabidopsis thaliana* leaves showed resistance to a fungal pathogen (Ritpitakphong et al., 2016). However, previous research in the plant microbiome has been focused within the roots, or rhizosphere (Müller et al., 2016), and less is known about the leaf microbiome, or phyllosphere, and how the microbiota affects its host organism (Kembel et al., 2014). A recent review summarized that phyllosphere microorganisms can influence individual plants by altering plant pathogen resistance and productivity, and even impact ecosystem scale processes such as nitrogen and carbon cycling (Sohrabi et al., 2023). The authors of that review highlight the need for further research and concluded that "*Comprehensive inventories of phyllosphere microbiotas across plant lineages, plant developmental stages, and geographic regions will likely remain a high priority in the coming decade*".

Several studies on trees have demonstrated that phyllosphere microbial community compositions are species specific. For example, Redfort et al. (2010) provide support that interspecific variability in the phyllosphere microbiome was greater than intraspecific variability, even across continental geospatial scales, and Laforest-Lapointe et al. (2016) found host species identity to be the strongest driver of variation in phyllosphere bacterial community. Kelly et al. (2021) report that American chestnuts (*Castanea dentata*) exhibited the most distinct rhizosphere microbiome, both functionally and taxonomically, among the tested sympatric species (red oak *Quercus rubra* and black cherry *Prunus serotina*) but did not test the phyllosphere. Brown et al. (2022) found evidence that soil fungal communities became more dissimilar over time between planted red oaks and American chestnuts, and even between

American chestnuts that differed in their blight occurrence. However, no studies known to the authors of this proposal have investigated the phyllosphere microbiome of American chestnuts.

Leaf characteristics (e.g., leaf economic spectrum, cuticle properties, stomatal density) have been identified as a likely driver of bacterial community composition (Redford et al., 2010). Recent research on the mechanisms of how plants regulate the phyllosphere microbiome points towards genetic control, with mutant plants hosting significantly altered microbial communities compared to wild types individuals (Reisberg et al., 2013, Bodenhausen et al., 2014, Chen et al., 2020; Pfeilmeier et al., 2021). The associated dysbiosis, or imbalance of the organism's microbiota, has even been experimentally linked to leaf tissue damage (Chen et al., 2020). Given their genetic differences, it is reasonable to expect that backcross, transgenic, and wildtype American chestnuts exhibit distinct phyllosphere microbiotas. Differences in the composition and diversity of the leaf microbiome would have several implications for American chestnut conservation, spanning from organismal to ecosystem scale. At the organismal scale, an altered phyllosphere microbiota can have implications on plant physiological processes (e.g., gas exchange), disease resistance, and potential to adapt to a changing environment (refer to Hassani et al., 2018 and Sohrabi et al., 2023 for a review). At the ecosystems scale, transgenic and backcrossed American chestnuts that harbor different bacterial and fungal species than the wild type would alter the species' ecological functioning in its range and potentially impact ecosystem processes including carbon and nutrient cycling (Sohrabi et al., 2023).

The main objective of the proposed research is to **compare the phyllosphere microbiome of backcross and transgenic American chestnuts to wild type American chestnuts**, thus directly addressing critical research area three (i.e., comparative studies in ecological functioning) as identified in the 2023 RFP. Within this framework, we propose a comprehensive metagenomic study utilizing both 16S (microbial) and ITS (fungal) sequencing and integrating the results with genetic and leaf-morphological data. We hypothesize that

1. transgenic, backcross, and wild type American chestnuts differ significantly in their phyllosphere microbiota; and that
2. leaf morphology and genetic makeup of American chestnut trees are better predictors of phyllosphere microbial species diversity and community composition than location.

Our results fill a critical knowledge gap and provide valuable data to inform and optimize conservation efforts for this important keystone species.

Methodology

Sample Collection - To cover a broad geographic area, we will reach out to chestnut conservation partners across the United States about their willingness to participate in our study. Partners who agree to participate will be sent sampling packages comprising a return box with prepaid overnight return shipping labels, sterile sample bags, sterile gloves, alcohol wipes, ice packs, and step-by-step sampling instructions. Sampling encompasses collecting 100 g of healthy leaf material per tree according to provided instructions. Because there can be significant intra-individual variation in phyllosphere community structure (Laforest-Lapointe et al. 2016), each sample will comprise of multiple leaves from different positions within the canopy of each plant.

Metagenomics - Once received, samples will either be frozen or immediately processed. Sample processing will follow the same protocols as used by PI Bretfeld and lab members for USDA-funded research on the tomato phyllosphere: For each replicate, an equal amount of leaf material will be clipped and aseptically transferred to round-bottom centrifuge tubes containing 30 mLs of autoclaved 1X Phosphate Buffer Saline (PBS) containing 0.1% Tween 20 using sterilized tweezers and scissors. Tubes will be sonicated for 3 minutes in a Bransonic 2800 ultrasonic bath (Branson Ultrasonics, Brookfield, CT) and vortexed at maximum intensity for 1 minute. Leaf material will be removed from the suspension which then is centrifuged at 15,000 g for 10 minutes. The resulting supernatant will be removed, and the suspension continuously recentrifuged until at most 0.250 mLs of pellet suspension remains. DNA will be extracted from pellets using the DNA Powersoil Kit (Qiagen, Hilden, Germany). Processed samples will be shipped to LC Science (LC Science, Houston, Texas, USA) for PCR amplification, subsequent ITS and 16S rRNA sequencing, and basic community analysis. Additional statistical analyses will be performed in R (R Core Team, 2023).

Leaf Traits - For each tree, a subset of the leaf material will be used to assess stomatal density and size, and leaf mass area. Leaf mass area will be derived from leaf area, dry weight, and leaf thickness. Protocols will follow Cornelissen et al. (2003) and Wright et al. (2002). Briefly, leaf area will be measured using a Leaf Area Meter (LiCor LI-3100C) and dry weights will be determined after leaves were oven dried at 80℃ for 48 hours. Stomatal density and size will be determined via leaf impressions outlined by Xu and Zhou (2008) with minor modifications. Areas between leaf veins will be painted with clear nail polish and allowed to dry. A piece of tape will be applied to the dried polish, removed, and placed on a microscope slide. Stomatal density and stomatal aperture will be measured using a compound microscope.

Project/Budget Timeline

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<tr><td colspan="5">reach out to conservation partners</td><td></td><td colspan="2">sampling</td><td colspan="3"></td><td colspan="8">data analysis and thesis/manuscript writing</td><td></td><td colspan="4">manuscript submission</td></tr>
<tr><td colspan="2"></td><td colspan="4">prepare and send sampling packages</td><td></td><td colspan="5">sample processing</td><td colspan="7"></td><td>ESA</td><td colspan="4"></td></tr>
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Activities | *Spending*

Intellectual Merit/Broader Impacts

Our proposed study will fill a critical knowledge gap on the composition of the phyllosphere microbiome of American chestnuts. Most importantly, our study will elucidate whether disease resistant transgenic or backcross varieties exhibit similar or distinct phyllosphere microbiomes, and thus have the potential to fulfill the same ecological functioning, as the wild type - a research priority identified by the American Chestnut Foundation. Our results will lay the foundation to potential areas of cross-inoculation studies between American chestnut varieties and the potential of such treatments for increasing disease resistance, stress tolerance, and ability to adapt to changing environments. Findings from our study will not only be shared with conservation partners but also presented at a national conference (e.g., Ecological Society of America) and submitted for publication in a peer-reviewed journal.

Funding from this proposed study offers excellent opportunities for involvement of undergraduate and graduate students at KSU, a majority-minority institution. Our study integrates cutting-edge metagenomic science with real conservation needs, fitting well into KSU's biology graduate program in Integrative Biology, and will lay the foundation for the MS thesis of collaborator Dobson. Involvement of undergraduate students will be supported from KSU through research credits and lab fees, and students will present their findings at the Undergraduate Research Conference at KSU in Fall 2024.

Lastly, our project provides an excellent opportunity to integrate citizen science. By soliciting participation in sample collection from conservation partners and growers, we will be able to cover a much broader geographic range that includes growers from across the southeast, thus making this study a truly collaborative science and conservation effort.

Budget Justification

Contractual ($19,440)

- ITS and 16S samples ($90 x 240 samples = $21,600 - 10% discount = $19,440)

Charges for metagenomic sequencing and data analysis at LC Sciences (https://lcsciences.com/). Prices are $45 per ITS and $45 per 16S sequencing, with a 10% discount for bulk analyses (>50 samples).

Supplies ($15,870)

- DNA extraction kits ($454 x 5 = $2,270)
- shipping supplies (240 samples = 120 packages, $80 per package; $9,600)
- general lab supplies (reagents, slides, containers, ppt, etc., $4,000)

General lab supplies and reagents to prepare buffer solutions for separation of microorganisms from leaf surfaces, DNA extraction kits (DNeasy PowerSoil Pro Kit (50)), and shipping supplies for sample collection from conservation partners across the country (shipping packages include pre-paid overnight postage to return samples to KSU and all necessary sampling supplies).

Student Support ($11,682)

- MSIB graduate student support ($1650 per month x 6 months + $1782 fringe; $11,682)

Support for one semester for one graduate student in the Masters of Integrative Biology program (MSIB; including fringe) at KSU. Remaining semesters will be funded by KSU.

Other ($3,000)

- Travel support ($1500)
- Publication cost ($1500)

Support to cover conference related expenses for students and PI and to cover publication expenses.

Total $49,992

COI

The authors declare no COI.

Literature

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Bodenhausen, N., Bortfeld-Miller, M., Ackermann, M., & Vorholt, J. A. (2014). A synthetic community approach reveals plant genotypes affecting the phyllosphere microbiota. *PLoS genetics*, *10*(4), e1004283.

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Chen, T., Nomura, K., Wang, X., Sohrabi, R., Xu, J., Yao, L., ... & He, S. Y. (2020). A plant genetic network for preventing dysbiosis in the phyllosphere. *Nature*, *580*(7805), 653-657.

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Lugtenberg, Ben, and Faina Kamilova. "Plant-growth-promoting rhizobacteria." Annual review of microbiology 63 (2009): 541-556.

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Weems, Ebony I., et al. "Centering microbes in the emerging role of integrative biology in understanding environmental change." Integrative and Comparative Biology 61.6 (2021): 2145-2153.

Yeh, Chuan-Ming, et al. "New insights into the symbiotic relationship between orchids and fungi." Applied Sciences 9.3 (2019): 585.

Zhu, Yong-Guan, et al. "Ecosystem Microbiome Science." mLife 2.1 (2023): 2-10.

Mario Bretfeld

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Professional Preparation

Carl-von-Ossietzky University (Oldenburg, Germany)	Landscape Ecology	Diploma (2010)
University of Northern Colorado (Greeley, CO, USA)	Biological Education	Ph.D. (2014)
Smithsonian Tropical Research Inst. (Rep. of Panama)	Plant Ecophysiology	(2015-2017)
University of Wyoming (Laramie, WY, USA)	Plant Ecophysiology	(2017-2019)

Appointments

Kennesaw State University (Kennesaw, GA)	Assistant Professor	(2019- current)
University of Wyoming (Laramie, WY)	Research Assist. Scientist	2017-2019

Five Most Recent Publications

Knowles, J. F., Bjarke, N. R., Badger, A. M., Berkelhammer, M., Biederman, J. A., Blanken, P. D., **Bretfeld, M.**, Burns, S.P., Ewers, B.E., Frank, J.M., Hicke, J.A., Lestak, L., Livneh, B., Reed, D.E., Scott., R.L., Molotch, N. P. 2023. Bark beetle impacts on forest evapotranspiration and its partitioning. *Science of the Total Environment*, DOI 10.1016/j.scitotenv.2023.163260.

Cheng, Y., Leung, R. L., Huang, M., Koven, C., Detto, M., Knox, R., Bisht, G., **Bretfeld, M.**, Fisher, R. A. 2022. Modeling the Joint Effects of Vegetation Characteristics and Soil Properties on Ecosystem Dynamics in a Panama Tropical Forest. *Journal of Advances in Modeling Earth Systems.* DOI 10.1029/2021MS002603.

Bretfeld, M., Speckman, H.N., Beverly, D.P., Ewers, B.E. 2021. Bayesian predictions of bark beetle attack and mortality of three conifer species during epidemic and endemic population stages. *Frontiers in Forests and Global Climate Change*. DOI 10.3389/ffgc.2021.679104.

Howell, A., **Bretfeld, M.**, Belmont, E. 2021. Impacts of bark beetle-induced tree mortality on pyrogenic carbon production and heat output in wildfires for fire modeling and global carbon accounting. *Science of the Total Environment*. DOI 10.1016/j.scitotenv.2020.144149.

Solander, K.C., Newman, B.D., Carioca de Araujo, A., Barnard, H.R., Berry, Z. C., Bonal, D., **Bretfeld, M.**, Burban, B., Candido, L.A., Célleri, R., Chambers, J.Q., Christoffersen, B.O., Detto, M., Dorigo, W.A., Ewers, B.E., Ferreira, S.J.F., Knohl, A., Leung, L.R., McDowell, N.G., Miller, G.R., Monteiro, M.T.F, Moore, G.W., Negron-Juarez, R., Saleska, S.R., Stiegler, C., Tomasella, J., Xu, C. 2020. The pantropical response of soil moisture to El Niño. *Hydrology and Earth System Sciences*. DOI 10.5194/hess-24-2303-2020.

Grants

Total awarded: > $270,000

2023	Bretfeld. M. (PI). Testing the efficacy of hydroponic propagation of American chestnuts (*Castanea dentata*). Kennesaw State University College of Science and Mathematics Mentor Protégé Award. – *$4,000*
2022	Bretfeld, M. (PI), Dice, C., Blackwell, M. Offsetting the Quality-Quantity Tradeoff of Hydroponically-Grown Tomatoes via Foliar Inoculation. USDA Specialty Crop Block Grant. – *$80,000*
2022	Brooks, M. (Graduate student mentee), Bretfeld, M. Optimizing conservation efforts for Georgia's endangered Royal Catchfly (*Silene regia*). Georgia Botanical Society - Marie Mellinger Field Botany Research Award. – *$2,000*
2020	Bretfeld, M., Grant, "LI-COR Environmental Education Fund (LEEF)", LI-COR Biosciences, Private – *$33,081.65*
2020	Bretfeld. M. (PI), Gurupatham, S. Measuring the variation in thermo-biological properties for organic vs conventional tomatoes. Kennesaw State University College of Science and Mathematics Mentor Protégé Award. – *$2800*
2018	Bretfeld, M. (PI), Frank, J., Parsekian, A.D., Ewers, B.E. RAPID: Forest Ecosystem Response to Interacting Bark Beetle and Fire Disturbance are set up by Regeneration in the First Year. NSF Ecosystems RAPID. – *$164,997*

Synergistic Activities

- Mentored and trained over 30 undergraduate and graduate research assistants in plant physiological data collection and analysis.
- Member of the Ecological Society of America's National Vegetation Classification Panel, facilitating and supporting the development, implementation, and use of a standardized vegetation classification for the United States to aid in research, management, and conservation.
- Member of the Georgia Plant Conservation Alliance and Georgia Native Plant society, safeguarding *Silene regia* and developing protocols to optimize conservation efforts.
- Integrated over 50 undergraduate students in plant physiological and plant conservation research as part of course-based undergraduate research (CURE) classes at KSU, including Plant Physiology and Plant Ecology.

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Professional Preparation

University of California, Los Angeles, USA	PhD in Biology	1996
Universidad Simón Bolívar, Sartenejas, Venezuela.	B.S. in Biology *(Licenciada en Biología)*	1986

Appointments

2022-Present	Department Chair, Department of Ecology, Evolution and Organismal Biology, Kennesaw State University, GA.
2021 - 2022	Interim Chair, Department of Ecology, Evolution and Organismal Biology, Kennesaw State University, GA.
2020 - 2022	Interim Associate Dean for Research, College of Science and Mathematics. Kennesaw State University, GA.
2016 - 2017	Professor and Interim Chair, Department of Ecology, Evolution and Organismal Biology, Kennesaw State University, GA.
2010- Present	Professor, Department of Ecology, Evolution, and Organismal Biology Kennesaw State University, GA.
2005 – 2010	Associate Professor Department of Biology and Physics Kennesaw State University, GA.
1999 – 2004	Assistant Professor Department of Biology and Physics Kennesaw State University, GA.
1996 – 1999	Instructor Department of Biology University of California, Los Angeles, CA.
1996	Instructor Department of Biology Santa Monica College, Los Angeles, CA.
1989 – 1993	Teaching Assistant/Teaching Fellow Department of Biology University of California, Los Angeles, CA

Five Recent Publications

Andrade, J.L. and **P.C. Jackson**. 2023. Insights on Rooting Depths for Woody Species of Barro Colorado Nature Monument. *In press*. A Century of Plant and Ecosystem Research at Barro Colorado. In The First 100 Years of Research on Barro Colorado Island: Plant and Ecosystem Science, ed. H. C. Muller-Landau and S. J. Wright: Smithsonian Institution Scholarly Press.

Paula C. Jackson, José Luis Andrade, Casandra Reyes-García, Olivia Hernandez Gonzalez, Thomas McElroy, Roberth Us-Santamaria, Jose Luis Sima, and Juan Manuel Dupuy. 2018. Physiological Responses of Species to Microclimate Help explain Population Dynamics along Succession in a Tropical Dry Forest of Yucatan, Mexico. *Forests*. 9(7) 411.

Lucía Sanaphre-Villanueva, Juan Manuel Dupuy, José Luis Andrade, Casandra Reyes-García, **Paula C. Jackson**, and Horacio Paz. 2017. Patterns of Plant Functional Variation and Specialization Along Secondary Succession and Topography in a Tropical Dry Forest. Environmental Research Letters. 12(2017):1-9.

Frisch-Kreps Jennifer, **Paula C. Jackson**, and Meg Murray. 2017. Transforming undergraduate biology learning with inquiry-based instruction. *Journal of Computing in Higher Education*. Doi: 10.1007/s12528-017-9155-z

Lucía Sanaphre-Villanueva, Juan Manuel Dupuy, José Luis Andrade, Casandra Reyes-García, Horacio Paz, and **Paula C. Jackson**. 2016. Functional Diversity of Small and Large Trees along Secondary Succession in a Tropical Dry Forest. *Forests*: 7, 163; doi:10.3390/f7080163. http://www.mdpi.com/journal/forests

Externally Funded Federal Grants as PI:

Jackson P.C. (PI) Meg Murray and Jennifer Frisch (CoPIs) WIKIed Biology**.** Transforming Undergraduate Education in Science, Technology, Engineering, and Mathematics (TUES) National Science Foundation (NSF; $199,544) Grant. 2010 -2015

Jackson, P.C (PI) RUI Differences in Source Water Use of Woody Vegetation of Dzibilchaltún in the Yucatan Peninsula, Mexico National Science Foundation (NSF $ 300,000. 2005-2009

Externally funded State Grants as PI

Jackson, P, (PI) Bell, M, Reese, S, and Callahan, K (CoPIs) University System of Georgia USG STEM IV Initiative Grants Year 1, Year 2, Year 3 (USG $75,000 ea. year. 2020, 2021,2022)

Funded Federal Grants as CoPI:

Jennifer K. Frisch (PI) Neporcha Cone, Brendan Callahan, Paula C. Jackson (CoPIs). TelBIO: Teaching English Learners Biology. National Science Foundation Noyce Scholarship Grant. (NSF $1,198,224**.** 2014 -2017

Funded Federal Grants as Faculty Associate:

Marcus C. Davis, Scott J. Nowak, John C. Salerno, Martin L. Hudson.MRI: Acquisition of a Confocal Microscope for Multidisciplinary Research. National Science Foundation. (NSF $ 334,632**,** 2012 -2015)

Synergistic Activities

- Mentored over 70 undergraduate and graduate students.
- Organized the Undergraduate Institute in STEM on Mentoring Undergraduates and Building an Inclusive Community of Researchers. Registered 11 to 15 faculty each year (2020, 2021, and 2022. Funded by the USG STEM IV Initiative).
- Distinguished Professor Award, College of Science and Mathematics, Kennesaw State University. 2014).
- Distinguished Teaching Award, College of Science and Mathematics, Kennesaw State University (2010).