Proposal

Project Title: Vegetative compatibility groups of *Cryphonectria parasitica* in American chestnut, Chinese chestnut, and backcross hybrids across eleven field sites in the southeastern United States.

Project Summary: Blight disease (*Cryphonectria parasitica*) sampling of 11 American chestnut (*Castanea dentata*) reintroduction test plantings (ca. 2009-2011) established by the Southern Research Station and partners covering NC, TN and VA was conducted in 2014. Over 130 *C. parasitica* isolates were obtained during the first year. Trees from parents (American and Chinese) and the BC_3F_3 generation were ranked according to canker severity from 2014 to 2017. Over 400 cankers were verified using morphological and molecular identification. We will determine vegetative compatibility (VC) groups across the planting sites, and determine if VC groups affected tree disease distributions from the 2014 isolates since all hybrids and parents were sampled during that period.

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Project Duration: 11/01/2018-10/31/2019

Total Amount Requested: ~~$10,000~~ $6,074 approved, SBowers

Goals:

Short-Term Goal:

1. Define isolate vegetative compatibility (VC) groups of *C. parasitica* of stored isolates from chestnut genotypes from parental species and backcross generation sources [American chestnut, BC_1F_3 , BC_2F_3, BC_3F_2, BC_3F_3, Chinese chestnut] across 11 reintroduction test plantings.

Long-Term Goals:

1. Data from short-term goal 1 can be used to determine if disease rankings can be correlated with dominant VC groups within and across individual sites.
2. Compare our results to previously published results on dominant VC groups of American chestnut and chestnut in Europe and Asia.

Project Narrative:

Justification

Forest reintroduction trials were established from 2009 to 2011 to examine how American chestnuts bred for blight disease resistance would perform in forest environments of the Southern Region of USDA National Forest System (Clark et al. 2014, 2016). This project represents a long-term, highly advanced research project using the best nursery seedling technology available (Clark et al. 2012), complex statistical designs, and yearly measurements on survival, growth, and competitive ability (Clark et al. 2016). Over time, *C. parasitica* has naturally infected planted seedlings, thereby providing an opportunity to study natural blight disease in populations with known pedigree and age. Chestnut blight evolves quickly, and dozens of strains are known to exist in eastern North America with various levels of pathogenicity (MacDonald and Double, 1978; Double and MacDonald, 2014). However, virtually no research has been conducted to determine how these various blight strains may effect expression of blight resistance in traditionally bred material.

Vegetative compatibility (VC) or incompatibility of *Cryphonectria parasitica* strains has been studied in forest stands containing native chestnut and oak (*Quercus*) for many years in the eastern United States and Europe (Anagnostakis 1978, McDonald and Double 1978, Nash and Stambaugh 1982, Akilli et al 2009, Double and McDonald 2014). Vegetative compatibility of fungi refers to the ability of hyphae to connect, and anastomosis occurs followed by the exchange of cellular contents including cytoplasm and nuclear materials (Van Alfen et al, 1975). In the most recent study by Double and McDonald (2014) 52 vegetative compatibility groups from Great Smoky Mountains National Park were determined when compared against 64 European (EU) tester strains (Cortsei and Milgroom 1998). In European/Asian continents VC groups are in much smaller numbers, with 5 groups being identified across 11 provinces in Turkey (Akilli et al 2009).

In North America, *C. parasitica* populations quickly evolved leading to relatively high levels of vegetative incompatibility (*vic*) or individual genetic fungal diversity (Milgroom and Cortesi, 2015). Multiple loci (six) are reported to be involved in *C. parasitica* strain compatibility (Dutech et al, 2012). Short et al (2015), determined that there were 39 unique *vic* genotypes of *C. parasitica* from 5 locations in eastern United States. These results were based on 116 isolates obtained across those sites. Furthermore, genetic variability were compared using PCR methodology to traditional tester pair-wise coinoculated assays using the 64 EU strains. Based on the past study (Short et al, 2015), cultural methods using the EU testers were believed to overestimate genetic diversity. Therefore, a duel approach to determining genetic diversity is proposed using pair-wise cultural methods of field isolates compared with 64 EU tester strains. Secondly using multilocus (6 loci) PCR assays for defining vegetative incompatibility gene profiles of the same field isolates.

Project Goals

The overall goal of the proposed project is to advance American chestnut restoration by more closely examining disease strains that affect resistance to *C. parasitica*. We build upon existing research and methodology to meet current goals of this proposal. Results will be used to improve understanding of resistance and disease interactions of traditionally bred American chestnut seedlings.

Proposed Impact of Research

The proposed research is crucial to improving our limited understanding of field performance and blight resistance of traditionally bred material in 'real-world' forest settings. Documenting blight strains on trees with known pedigree and age will help refine blight-resistance testing of trees planted in reforestation test plantings. Results will provide recommendations for restoration plantings when blight resistant material becomes available and will assist breeding efforts, particularly those of TACF.

Study site and experimental material

Eleven plantings were established in three national forests (Nantahala, Cherokee, and George Washington & Jefferson), and nine are still being followed (Clark et al. 2014 and 2016): three plantings established in 2009, one in 2010, and five in 2011. Two plantings, one established in 2010 and one established in 2011, were removed from further study due to low survival from Phytophthora root rot disease. In the nine remaining plantings, 3075 trees were planted from four backcross breeding generations and two parental species [American chestnut, BC_1F_3 , BC_2F_3, BC_3F_2, BC_3F_3, Chinese chestnut]. All material was obtained through collaboration with TACF, from their orchards in Meadowview, VA or from open pollinated wild (American) or urban (Chinese) trees. Trees were planted using an incomplete block design, and family identity has been maintained throughout the study (Clark et al. 2016).

Previous Methodology

Blight data were recorded in late growing season of each year (August-October) after planting. For the first four years, we recorded blight data as presence/absence of symptoms consistent with blight disease. In years 5-9 after planting (years 2013-2017, depending on year of planting), a blight ranking was assigned to each tree as follows: 1=no visual evidence of blight disease symptoms; 2=cankers were in early phases of development or were exhibiting signs of resistance, including a lack of orange stromata and usually accompanied by slight swelling at the site of infection; 3=cankers had abundant orange stromata with a portion of the tree killed above the point of infection; and 4=trees were assumed to have died from *C. parasitica* infection. The highest canker ranking was used for trees with multiple cankers. Although we did not measure canker lengths, this qualitative ranking system closely resembled that of the ranking system used following stem inoculations (Hebard, 2005). If the tree died-back completely from *C. parasitica* infection and produced a new sprout, a yearly resistance ranking value of 3 was recorded for the first and second year of the new sprout, but a value of 1 was assigned thereafter until new pathogen infection occurred. If the tree died before infection occurred, yearly resistance ranking was recorded as missing data for the year of mortality and each year thereafter.

To confirm the cankers were result of *C. parasitica* infections, two bark samples (5 x 5 mm) were removed along margins on boarders of questionable cankers, which primarily consisted of bark wounds with no visible stromata or probable cankers that appeared to be in the early stages of development. Generally, cankers accompanied by abundant stromata were not sampled. The bark samples were placed into microtiter plates so that they could later be identified as to specific cankers and locations on a tree. All samples were stored at 4°C in the laboratory until processed using isolation procedures (Baird, 1991). The bark samples were surface disinfected in 0.525% w/v sodium hypoclorite solution for 10 min, rinsed in sterile distilled water and cultured onto glucose-yeast extract medium (GYE) in 10 X 1.5 cm Petri plates for 10-d at room temperature exposed to fluorescent light and diffused sunlight. Colonies were then transferred to potato dextrose agar (PDA, Difco Lawrence, KS). Colony morphology was verified after 10-d incubation at 20°C under fluorencent light. When isolate morphologies were uncertain, molecular sequencing was conducted using ITS primers and sequencing methods as described previously (Baird et al. 2014, Short et al 2015).

In 2014, a blight sample was collected from every American chestnut, Chinese chestnut, and BC_3F_3 with a suspected blight canker. Blight samples were collected, assayed as described above, and isolates were stored in 15% glycerol at -80°C. Will ~130 isolates of *C. parasitica* collected.

Project timeline: (Funding will be requested in 2018 and 2019)

Table 1. Project timeline for each goal.

Goals	Start Time	End Time
Short Term	August 2014	October 2019

Goal 1		
Short Term Goal 2	August 2014	October 2019
Long-Term Goal 1	October 2019	October 2021

How Results Will Be Measured

Short-term Goal 1: A blight ranking (previously described) will be assigned to each tree in 2018 and 2019 based on blight disease symptoms. Survival and growth (height, ground-line diameter, and diameter at breast height) will be also be measured.

Using the stored isolates from collections made in 2014, VC groups using EU tester strains of *C. parasitica* will be assaying using traditional cultural methods as per Double and MacDonald (2014). Molecular methodology will done at same time comparing allelic differences among them using primers for the six known diallelic vic genetic loci developed by Short et al (2015). In the paper, primer sequences are provided for vic1a, vic2, vic3a, vicd, vic6 and vic7. Furthermore, the diallelic vic locus amplicons are listed in GenBank library for comparisons using *vic* allele-specific amplicons of known sizes compared to EU tester strains (Cortesi and Milgroom, 1998).

Depending upon results from the VC studies it may be necessary to revisit specific planted trees to observe and attempt to reisolate the *C. parasitica* using bark sampling methods (previously described). This may be necessary especially if VC groupings show unique or uncertain results. Often isolates will sector in culture making impossible to verify barrages or compatibility. New samples from cankers will be cultured using standard practices to identify them as *C. parasitica*. Isolates will continue to be processed, identities confirmed through October 2019 (year 1 of study) and segregated based on colony morphology, sporulation potential, and compatibility.

Long-term Goals 1 and 2: We will conduct various statistical analyses to evaluate the relationships between VC groups from isolates collected in 2014 to the blight disease rankings and mortality in subsequent growing seasons (2014-2019). Various abiotic and biotic factors (e.g., seedling growth, planting location, competition, canopy cover) and seedling type (breeding effect, and genetic family) can be incorporated into these analyses to help refine and better quantify blight resistance of each genotype at each site. We will compare our results to those of previous reports within and outside the United States.

Monitoring of Progress

Progress will be monitored by the PI and co-PI to ensure that laboratory work conducted by undergraduate, graduate students, post-docs, is being conducted efficiently and correctly. Weekly reports from workers will be requested to ensure quality control. The PI will seek advice from Dr. William MacDonald and Mr. Mark Double, West Virginia University concerning methodologies, data collection and result interpretations as needed to ensure quality of the research. A statistician in the Animal Science Department at the University of Tennessee has been involved in the American chestnut plantings since establishment and will assist with statistical analyses, and will be co-author on reports and future manuscripts.

How Results Will Be Reported

Results from this project will be reported at the annual meeting of the USDA regional project on chestnuts (NE-1333), at the annual meeting of the American Phytopathological Society, and to The American Chestnut Foundation. Peer-reviewed publications will be expected to come from this research and potential outlets include:

Baird, R., Clark, S.L. Schlarbaum, S.E., Saxton A.M. 2019. Evaluation of genetic diversity of *Cryphonectria parasitica* populations within reforestation sites of backcross bred American chestnut hybrids in southeastern United States.

Clark, S.L., Baird, R., Schlarbaum, S.E., Saxton A.M. 2020. Temporal comparisons of disease patterns and epidemiology of *Cryphonectria parasitica* to growth and isolate genetic diversity data of field planted backcross hybrids of American chestnut.

Any publications resulting from the project will list TACF as a source of support and a reprint (PDF) will be sent to TACF. We will prepare a final report, and we will publish a research summary for a general audience to be published in *Chestnut*, the magazine of TACF. Currently, a publication has been submitted for a professional journal based on our first eight years of disease resistance testing.

Project Budget and Justification

Wages and Salaries: *Undergraduate student support and training*: Undergraduate student from the Biochemistry unit of Baird's department will be hired ($4,000/yr)= 4,000 + fringe (0.41%) at $16 **=$4,016**. The student will be involved in laboratory fungal culture work and molecular identifications. **Travel:** will be required to collect data at research sites. The PI will travel to one chestnut meeting and travel to support the field bark sampling at the 11 American chestnut planting sites total trips = **$2,000.** The sites are located from Blacksburg, Va to Hayesville, NC. **Supplies** will be needed for field sampling of chestnut bark tissues from each site including flagging, sampling tools, and sample carriers. In the laboratory, growth media preparation for culturing and compatibility studies require isolation media in Petri plates and long-term storage of isolates; additional costs include molecular supplies for DNA extract and allelic mapping to 64 EU tester strains for VC studies. Total supply costs are = **$3,984.** The overall total is = **$10,000**.

Budget: Year One (2018/2019)

Expense	Amount
Undergraduate Student Salary	$4,000
Fringe benefits .41%	$16
Travel	$2,000
Supplies	$3,984
Overhead / F&A Not allowed	$0
TOTAL	*$10,000*

Conflict of Interest or Commitment Statement- Dr. Baird was a former student of Dr. William McDonald and Mark Double from West Virginia University from 1978-1980. They could be possible reviewers of this proposed study due to their involvement with TACF. Dr. Stacy Clark has been collaborating with TACF since 2007, has spoken to several of their local chapters, science board, and one national meeting. She recently formed an agreement with them, the 'Biennial Plan of Work' to strengthen communication and collaboration between TACF and the Southern Research Station (SRS). This Plan was signed by Lisa Thomson, President of TACF, and Rob Doudrick, Station Director of the SRS.

REFERENCES

Akilli S., Y.Z. Katircioglu, and S. Maden. 2009. Vegetative compatibility types of Cryphonectria parasitica, causal agent of chestnut blight, in the Black Sea region of Turkey. For. Path. 39:390-396.

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Clark, S.L., Schlarbaum, S.E., Saxton, A.M., Hebard, F.V., 2012. Nursery performance of American and Chinese chestnuts and backcross generations in commercial tree nurseries. Forestry 85, 589-600. doi:10.1093/forestry/cps068

Clark, S.L. Schlarbaum, S.E., Saxton, A.M., Hebard, F. 2014. The First Research Plantings of Third-Generation, Third-Backcross American Chestnut (*Castanea dentata*) in the Southeastern United States. In: Double, M.L., and MacDonald, W.L. (eds.), Proceedings of the Fifth International Chestnut Symposium, ISHS. Acta Horticulturae 1019: 39-44.

Clark, S.L. Schlarbaum, S.E., Saxton, A.M., and Hebard. F.V. 2016. Establishment of American chestnuts (*Castanea dentata*) bred for blight (*Cryphonectria parasitica*) resistance: influence of breeding and nursery grading. New Forests 47(2):243-270. doi 10.1007/s11056-015-9512-6.

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Short D.P., M. Double, D.L. Nuss, C.M. Strauder, W. MacDonald, and M.T. Kasson. 2015. Multilocus PCR Assays Elucidate Vegetative Incompatibility Gene Profiles of *Cryphonectria parasitica* in the United States. Appl Environ Microb 81:5736-5742.

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Professional Preparation:

B.S. West Virginia University. 1978 Forestry Management
M. S. West Virginia University. 1980 Plant Pathology/Forest Pathology
Ph.D. University of Tennessee. 1984 Botany/Mycology

Appointments:

1999 to present	Research Plant-Forest Pathologist/Mycologist/Botanist/ Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University.
1994 to 1999	Plant Pathologist, University of Georgia, Tifton, GA.
1991 to 1994	Extension and Research Plant Pathologist, Purdue University, Vincennes, IN.
1989 to 1991	Research Postdoctorate (Two year position), Coastal Plain Experiment Station, Tifton, GA.

EMPLOYMENT:

1999-Current

Research Plant Pathologist/Mycologist (80% research-20% teaching), Mississippi State University, Starkville, MS. Duties include research on forest and agricultural mycological and pathological investigations involving exotic pests' determinations, reforestation efforts and understanding of changing microbes (eg. mycorrhizal fungi and soil pathogens) associated with changing climates. Teaching includes courses in mycology, clinical plant pathology, and a team taught Pest Management course (undergraduate). During the last 18 years an agricultural-forest heath-mycology-ecology program was initiated working in various states throughout the southeastern region of the United States. See below for more details.

Current research includes studies of microbial (mycological) communities within the Southern Appalachian Mountains as they relate to forest tree heath and now climate change impacts. My overall goal is to obtain baseline data of microbial communities which are indicators of forest health with the diverse geographical region. Since 2014 I have been working with Forest Service personnel and Steve Jeffers, Clemson University, doing holistic studies on outplanted resistant backcrosses of American chestnut where the work was initiated in 2009. The research continues with the team to study the survival of resistant backcrosses at 10 locations in southeastern United States.

Related American Chestnut Research Publications:

Baird, R. E. 1991. Mycobiota of bark associated with seven strains of *Cryphonectria parasitica* on two hardwood tree species. Mycotaxon 40:23-33. West Virginia MS

Baird, R. E. 1991. Growth and sporulation of hypovirulent and virulent strains of *Cryphonectria parasitica* on dead *Quercus rubra* and *Acer rubrum*. Mycologia 83:158-162. West Virginia MS
Thesis (American Chestnut Study)-Major Professor

D. McNeill, III (M.S.)-Determination of hypovirulent and virulent isolate occurrence and their vegetative compatibility of *Cryphonectria parasitica* collected from the Great Smoky Mountains National Park (2005-2008).

Reazin, C.†, Clark, S., Jumpponen, A., Baird, R†., 2017. Hybridization of American and Chinese chestnuts alters seedling recruitment of fungal symbionts and other guilds from shared nursery soil. Fungal Diversity (Submitted). †=equally contributed to paper.

Clark, S.L., S.E. Schlarbaum, A.M. Saxton, and R.Baird. 2017. Eight-year blight (*Cryphonectria parasitica*) resistance of backcross-generation American chestnuts (*Castanea dentata*) planted in the southeastern United States. Forest Ecology and Management (Submitted).

Other General Publications

Baird, R., C. A. Wood-Jones, J. Varco, C. Watson, W. Starrett, G. Taylor, and K. Johnson. 2014. Rhododendron decline in Great Smoky Mountains and surrounding areas: Intensive site study of biotic and abiotic parameters associated with the decline. Southeastern Naturalist 13:1-25.

Bily, D. S. †, Diehl, S. V. Cook, M., Wallace, L. E., Sims, L., Watson, C., R.E. Baird†. 2018. Temporal and locational variations of Phytophthora spp. community in a forested urban water drainage and stream runoff system. Southeastern Naturalist 17:176-201. †=equally contributed to paper.

Veach, A. M†, Stokes, C.E, Knoepp, J., Jumpponen, A., Baird, R†, 2017. Fungal guilds and community composition vary across environmental gradients in the southern Appalachian Mountains. Microbial Ecology 761:156-168. †=equally contributed to paper.

Wadl, P. A., S. Boggess, R. Trigiano, T. Rinehart, R. Baird and B. Scheffler. 2018. Transcriptome profiling of *Cornus florida* in response to *Erysiphe pulchra* infection. J. Amer. Phytopathologica Society. (Submitted).

Grants:

Comprehensive field and laboratory study of *Cryphonectria parasitica* and *Phytophthora cinnamon* epidemiology using resistance lines of American chestnut (Chestnut Foundation). Sponsor: USDA-Forest Service 06/30/2014-06/30/2016 $50,000

Continuation of Short and Long Term Studies of Fungi as Symbiotic, Saprophytic and Pathogens in Reforestation and Established Forest Ecosystems of Southern Appalachian Mountains. Highlands Biological Station Foundation 05/01/2016-12/31/2016 $3,600 Grant-In-Aide.

Continuation of Short and Long Term Studies of Fungi as Symbiotic, Saprophytic and Pathogens in Reforestation and Established Forest Ecosystems of Southern Appalachian Mountains. Highlands Biological Station Foundation 06/01/2017-12/31/2017 $3,600 Grant-In-Aide.

Impacts of wildfire on recovery of forest microbial populations. Friends of GRSM 5/1/18-12/30/18 $2,000