External Science Review

Blight Resistance Breeding and Research Programs of

The American Chestnut Foundation

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Summary of findings

- The review panel was impressed with the work, and in-depth self-analysis and planning, presented by TACF.
- The panel was pleased with TACF efforts to use leading edge science and technology—specifically the use of genomic selection and genetic engineering tools—to improve the efficiency and rate of improvement for blight resistance, while maintaining local adaptation of germplasm in the face of climate change.
- The panel complimented TACF in planning to broaden the resistance base for breeding beyond the primary Graves and Clapper sources and urged them to consider substantially expanding the number of sources and genotypes through innovative breeding methods (e.g., use of pollen vs. seeds, early testing methods, and use of genomics to increase introgression/backcrossing (BC) efficiency).
- The panel supported the use of genetic engineering (GE) tools and current focus on the OxO gene but believed that too much reliance is being placed on a single insertion event and its deregulation. A single event may impair fitness when made homozygous due to the high likelihood of expression of deleterious rare recessive alleles when made homozygous in further breeding, its possible silencing/attenuation over years and generations, and that it may not be durable in the face of pathogen evolution in the long term. The panel urged the development of alternative forms of OxO transgenes with distinct promoters and coding regions to avoid gene silencing when they are combined, and development of alternative genes that, among others, might include those for resistance to *Phytophthora*.
- The panel believed that TACF should complete its evaluation and progeny tests of the best backcross materials in a focused and rigorous manner, but given that blight resistance levels and tree form appear to be short of hopes for this stage of the program, consider reducing investment in these materials in the future in favor of investments in the infusion and rapid breeding with new sources of genetic diversity, use of both the current OxO and novel forms of OxO and other transgenes, and selective combination of the best BC and OxO materials in a manner that maximizes effective population size (as TACF has proposed).
- In addition to developing resistance, we urge expanded studies on seedling culture, chestnut silviculture, establishment of high-quality field trials, and release of the best seed lots for restoration.
- The review process would have been strengthened by more detailed and integrated summaries of progress and work illustrating the complementary efforts by TACF Chapters and cooperators (breeding, testing, cooperative activities, and the small grants program), awards and impacts of their small grants programs, and database/informatics systems.

Introduction

A notable strength of The American Chestnut Foundation is its willingness to periodically invite outside scientists to review its research strategy and processes. Previous reviews, in 1999 and 2006, contributed significantly to guiding the program. With recent developments in genomic technologies and resources, and exciting progress in biotechnology, TACF recognized the need for a new review. A panel of five senior scientists with backgrounds in tree improvement and forest genetics research relevant to TACF's organizational goals, met for two days in Abingdon, Virginia, hosted by TACF staff. They attended presentations and field trips in the nearby Meadowview breeding and testing properties. Subsequently, the panel convened to discuss their observations and to develop a preliminary list of recommendations. These recommendations were then discussed in detail with TACF staff.

Visiting Review Panel Participants

Dr. Sally Aitken, University of British Columbia Faculty of Forestry, Professor and Associate Dean - Research and Innovation

Dr. John Davis, University of Florida, Associate Dean for Research, Associate Director of Florida Agricultural Experiment Station

Dr. Richard Sniezko, Center Geneticist, USDA Forest Service, Dorena Genetic Resource Center

Dr. Steven H. Strauss, Distinguished Professor, Oregon State University, College of Forestry

Dr. Nicholas Wheeler, Review Committee Chair

TACF Participants

Mr. Stephen Barilovits (Electronics Engineer, Systems Design), Chair-Elect of Science & Technology Committee, member of TACF Board of Directors

Ms. Laura Barth, TACF Horticulture and Pathology Specialist

Ms. Sara Fitzsimmons, TACF Director of Restoration

Dr. Brian McCarthy (Ohio University), Chair of Science & Technology Committee, Chair-Elect of TACF Board of Directors

Dr. William Powell (SUNY-ESF), member of Science & Technology Committee, Director, American Chestnut Foundation Research and Restoration Project

Dr. Kim Steiner (Penn State University), TACF Senior Science Advisor, Past-Chair of TACF Board of Directors

Ms. Lisa Thomson, TACF President and CEO

Dr. Jared Westbrook, TACF Director of Science

The review process

The review process was well defined by TACF and most of the relevant review materials provided well in advance of the actual review. Past reviews and current summaries of the status of the science program were helpful in providing a working framework for our own review. We thank Executive Coordinator Ms. Cherin Marmon-Saxe for clear and timely communications and logistical arrangements.

Most of the review panel came to this meeting with only passing knowledge of the program history and scope of activities. The brief exposure, regardless of intensity, gave us relatively little time to fully grasp the essence of the program. Future reviews may take an extra day to more fully develop the story to be told. A flow-chart type illustration of the genetic resources currently used in the program and their origins, and at least near-term breeding plans, would help the committee "see the forest for the trees." This might also be useful for education and outreach (e.g., a large poster or interactive multimedia presentation).   More details on the Chapter programs (strengths as well as challenges), and the work and impacts of the small external grant programs, would help the committee to better understand how all TACF, not just the science and Meadowview core, operated and interacted. Conveying a clearer idea of what end products they wish to put out would be good to both reviewers and their publics: most resistance programs in trees, and certainly for restoration will not wait to have 100% of the progeny genetically resistant, and will try to get trees on the landscape soon (e.g. white pine blister rust (multiple species), Port-Orford-cedar, *Acacia koa*). Getting trees into the field— even if only a certain percentage will survive—will allow some land managers to start using the species and give feedback to the program. What level of resistance (survival) has been promised to the cooperators and is it time to revise the message in a positive manner?

Evaluation

The review panel was impressed with the scope, scale and quality of the overall program. Notable features include:

Model
TACF, with its unique organizational structure, has positioned itself to be one of the world's model programs for the rescue and restoration of a forest tree species facing functional extinction. The organization's support network, fueled by the passion and donated time and resources of thousands of contributors, has contributed significantly to the long-term survival of the program. The program has benefitted greatly due to the continuity of purpose and institutional memory of its employees. The lack of such traits has contributed to the demise of many forest tree improvement programs around the globe in the past (Wheeler et al. 2015).

We share your concerns regarding the maintenance of volunteer support (fiscal, in-kind) as membership ages and restoration programs are delayed.

Staff
The existing staff, from top to bottom, appear to be excellent. Our commendations begin with the leadership provided by the principals (President and CEO – Lisa Thomson; Chair of Science and Technology Committee – Brian McCarthy; Board Member – Stephan Barilovits; TACF Senior Science Advisor – Kim Steiner). As with all those associated with this program, we commend these individuals for their passion and excellence in leadership. We are particularly happy to see the long-term continuity of research guidance provided by Dr. Steiner. We were impressed with the apparently seamless addition of science lead Dr. Jared Westbrook, supported of late by horticulturist Ms. Laura Barth. We believe their skills sets are strong and appropriate and will be instrumental in moving the program into the next phase of research and development. Dr. Bill Powell's contributions on genetic engineering (OXO and new genes) and rapid breeding (early flowering and resistance testing) have provided key innovations in support of the program. Sara Fitzsimmons' long-term contributions and institutional memory are irreplaceable – a real key to eventual restoration success. The field technicians are knowledgeable and deeply immersed in the program. They are critically important to program success and continuity.

Key documents
We find the current Strategic Plan and the 3- BUR documents to be excellent for defining goals, strategies, missions and visions. There should be no confusion as to where this program seeks to go, and the path that will take TACF there, using appropriate adaptive management approaches to scientific methods, tools and genetic materials.

Progressive science
We applaud TACF's adoption of cutting-edge methods and tools which we believe will 1) yield increased precision in the breeding, testing and selection of superior individuals and 2) dramatically improve the chances of developing multiple sources of resistance to both *Cryphonectria* and *Phytophthora*. Notable are the adoption of quantitative genetic methods, use of genomic tools and resources to enhance selection and track pedigrees, and the well– thought-out acceptance of biotechnology (i.e., genetic engineering) as a resistance breeding tool.   The key in the latter may continue to be discussions and communications with state Chapters and land managers interested in planting chestnut.

Phenotyping

We applaud the team's recognition that phenotyping is key to long-term progress. It should receive increased attention and resources to improve methodologies and increase the heritability of phenotypic scores. This cannot be emphasized too much – phenotyping is key to success. Having germplasm demonstrations on silviculturally appropriate field sites (and resistance that is expected to persist) is strongly encouraged. Added benefits would accrue from good field trials that relate to short-term testing technologies that confirm their utility. The oldest field trials are not TACF's, but rather were installed by the USFS, so strengthening that relationship would be good. Based on conversations between Dr. Sniezko and USFS colleagues after the review, there appears to be some useful genetic resistance from at least one of the oldest (9-year-old) USFS trials. TACF does have younger field trials, but it was unclear whether these were on optimum sites.

Climate change

We agree with the team's recognition that climate change will have a significant impact on population development and deployment for restoration and must be planned for pro-actively in their conservation, breeding and restoration work.

Cooperative studies

We strongly support TACF's continued efforts to develop cooperative studies, through grant applications or other means of leveraging resources. Notable here are efforts to better understand existing genetic diversity in surviving chestnut populations and the relationship of genetics to environments (ecological and provenance phenotyping, and landscape genomics). The American Chestnut Cooperators' Foundation (ACCF) is reported to have seed orchards and is distributing seed (pure American chestnut) with some level of resistance. Cooperation with ACCF should be considered if feasible and may help public perceptions of TACF and ACCF joint efforts.

Competitive grants program – Though few details were provided, the committee believed that the TACF competitive grants program, as a means for seeding future research and leveraging resources, is likely to be useful and worthy of continued support. For example, it could aid in illuminating several modest enquires like differential reproductive success of pollen polymixes and cloning methods.

Database

The importance of the project database, and its continued development and maintenance, can't be overemphasized. While the committee received only a brief overview of the system in place, the TACF staff appeared to appreciate it's importance. We recommend that TACF ensure

that there is qualified personnel, with backup expertise, in place for continuity and curation. It is also important that the system be connected to, and include, Chapter activities.   A written overview of the full system would help TACF to coordinate and monitor performance, and would have helped the review committee in it's evaluation.

Technical Feedback

To provide review comments that are directed to strategic questions most important to TACF, below we specifically comment on the stated program objectives.   We present the objectives verbatim (bold), then summarize the committee's responses to each of them.

In Appendices I and II we consider specific queries that were provided to us shortly before and during our review, providing tentative observations, as the committee did not consider them in-depth or necessarily reach consensus about them.

Objective 1. Finish selection in Clapper and Graves BC3F2 seed orchards at Meadowview Research Farms

Objectives 1 and 3 constitute the essence of the original backcross breeding plan to introduce and interbreed resistance into native American chestnut. As such they have been the focus of TACF efforts until now. These efforts are worthy of attention, offering the best available information on functional resistance. The work should be concluded as expediently as possible, unless results from other approaches suggests shifting course will lead to more rapid progress soon. These efforts should include field testing on appropriate chestnut sites to confirm efficacy of resistance and to inform development of silvicultural prescriptions.

Phenotyping

TACF staff may wish to take a fresh look at past methods of inoculation and scoring. We encourage collaboration with the PA Chapter to evaluate the relationship between short-term inoculation protocols and older field trials of non-inoculated stock. One recent European chestnut study suggests inoculation studies before the age of 4 may mask what might otherwise be usable levels of resistance, leading to unnecessary early culling (Pazitny et al. 2018). Obviously, early testing is desirable to move the program faster but if it is significantly inaccurate, it will be counter-productive. The European results should be scrutinized to evaluate the importance and benefit vs. risk of this tradeoff and contact with the publishing scientists made to clarify their strengths and limitations.

TACF response: Our methods of phenotyping for blight tolerance have been and continue to be a subject of debate. On one side of the debate, there are some that argue we should wait fifteen years to assess long-term blight tolerance of individual trees. This group also argues that blight tolerance is best assessed with natural infection of stems with *Cryphonectria parasitica* as artificial inoculation circumvents physical barriers to blight infection on the stem. At the other extreme, we have artificially inoculated seedlings in their first year of growth with a highly pathogenic strain of *C. parasitica*. So far we have performed these small stem assays (SSAs) primarily for backward selection – meaning we inoculate seedling progeny to estimate the relative genetic resistance of their open-pollinated mother trees. We are also testing if we can use small stem assays as a forward selection method to eliminate the most susceptible trees prior to planting in orchards. Before we adopt SSAs for forward selection, we are testing whether the inoculated seedlings survive after planting in the field and whether seedling canker severity and survival is correlated with these same traits assessed in later stages of growth.

With the addition of genomic selection to predict progeny blight tolerance on large numbers of parent trees in our seed orchards, we found correlation between late-developing blight tolerance phenotypes of parent trees and the predicted or observed average short-term canker severity on their progeny. Specifically, we found that a selection index based on five traits indicative of long-term blight tolerance of parent trees (age 8 to 16 years) is negatively correlated (r = -0.65) with average canker severity (predicted or observed) on their two to three year old progeny six months after inoculation with *C. parasitica* (Westbrook et al. 2019a).

We have also found that blight tolerance of BC_3F_2 parents as assessed with small stem assays on progeny is correlated with blight tolerance of the same parents as assessed from the average canker severity on different subsets of progeny inoculated at age three in orchards. Specifically, among American chestnut BC_3F_3 families, within family survival rates of first year seedlings inoculated with a highly pathogenic strain of *C. parasitica* is negatively genetically correlated ($r_{genetic}$ = - 0.75 ± 0.30) with family average canker severity on three year old seedlings six months after inoculation with weakly and highly pathogenic strains of *C. parasitica* (Saielli & Levine 2019).

These studies demonstrate that blight tolerance of parents and progeny are correlated and family rankings are similar whether progeny are inoculated in their first year or at age three. In practice, we select trees in our Meadowview seed orchards based on a selection index composed of the long-term blight phenotypes of parent trees and the average canker severity of progeny, either observed from progeny tests or predicted using genomic selection. (Westbrook et al. 2019a).

TACF cited their preferential use of a highly virulent strain for controlled inoculation; the panel was concerned that this might eliminate genotypes and resistance genes that are useful for

providing a broader resistance base, thus a mixed strategy might be considered (e.g., two stage or multiple source inoculations).

TACF response: We seek to balance expedience and accuracy in making our selections. Our standard method for selection within seed orchards is to inoculate trees at age two with a weakly pathogenic strain (SG2,3) of *C. parasitica*. We remove 60% – 80% of trees that demonstrate significant canker expansion 6 months to 1 year after inoculation. We have performed addition culling in Meadowview seed orchards based on progeny testing, genomic selection, and long-term blight phenotypes as described previously. Some chapters also perform a second inoculation at age four with a more pathogenic strain of *C. parasitica* to cull additional individuals from seed orchards. We have found strong correlation in family rankings for blight tolerance when we inoculate with a weakly pathogenic v. strongly pathogenic strains of *C. parasitica* (Steiner et al. 2017; Westbrook & Jarrett 2018). We have also found correlation between parent blight phenotypes and progeny canker severity. With the addition of genomic selection and the corroboration between various blight tolerance assessment methods, we have become more confident that our selection methods are identifying the most blight tolerant trees.

Phenotyping studies at the Meadowview site provided the committee with cause for concern on two fronts. The first is the somewhat complex nature of parentage likely found in the open-pollinated progeny of BC3F2 parents, and how the results of progeny tests should be interpreted. TACF staff reported a poor correlation between BLUP-generated breeding values and progeny test phenotype results, and suggested there may be strong deviations from random mating that caused errors in estimation of breeding values. We recommend time and resources be spent on characterizing mating patterns and levels of inbreeding to better understand how the use of open-pollinated progenies may have affected progeny phenotypes. This problem appears to be a function of haphazard placement of heterogenous research materials (e.g., nearby plantings of Chinese source near some parents and clusters of siblings). Mapping BLUP values spatially and considering the composition of paternal parents nearby to mother trees, may help to interpret and possibly adjust BLUP values.

TACF response: We are in the process of removing contaminating chestnut pollen sources that are adjacent to open-pollinated mother trees. We have removed the Chinese chestnut and American chestnut trees in the 'Clapper' BC3F2 seed orchard on the Duncan farm. We are also in the process of removing an old BC3F3 progeny test that is adjacent to the 'Graves' BC3F2 seed orchard. Finally, we are removing Chinese chestnuts on the Price farm that are adjacent to the BC3F1 parents of the BC3F2 trees in seed orchards.

Through genotyping-by-sequencing of BC3F2 trees and reference panels of Chinese chestnut and American chestnut, we have estimated the proportion of BC3F2 genomes inherited from

Chinese chestnut v. American chestnut. Through these analyses we have identified a number of 'pseudo-F1s' in our seed orchards. These trees inherited 50% or more of their genome from Chinese chestnut and are presumably the progeny of BC3F1 trees that were open-pollinated by Chinese chestnuts on TACF's Price Farm. We plan to monitor these pseudo-F1 trees over the next few years to determine if they are male sterile. If they are not male sterile, we will eventually remove the trees from the seed orchard after we have collected seed from these trees to advance new backcross lines from novel Chinese chestnut sources of resistance (see response to Objective 2 for further explanation).

The BC3F3s in our progeny tests were also generated through open pollination among the BC3F2 trees. We found that a BC3F2 mother with an inferior blight tolerance phenotype (e.g., dead main stem) had progeny with the least severe cankering among all families tested so far. This tree was planted near a stand of Chinese chestnuts on the Duncan farm, which we have since removed. For rare cases where the mother tree with inferior blight phenotype has progeny with high blight tolerance, we will perform controlled pollinations with BC3F2 pollen on the mother tree in question. We will evaluate the progeny blight tolerance again to ensure that the apparently high blight tolerance of the mother tree is not an artifact of pollination by Chinese chestnut trees.

We appreciate the reviewer panel's suggestion to genotype the BC3F3 trees as well to determine levels of inbreeding and percent Chinese chestnut ancestry in this generation. We plan to genotype these trees after we have finished culling susceptible trees from seed orchards and have planted BC3F3 progeny in demonstration plantings to assess their level of blight tolerance.

The second issue posed by the Meadowview site is the recognition that the site is non-optimal for growing chestnut. The soils appear to be too moist and poorly drained, thus trees are under stress and not likely to fully express their genetic potential for growth, form, and resisting disease. The site may suffice for genetic conservation, plant propagation and short-term inoculation purposes, but serious consideration should be given to moving long-term seed orchard, demonstration plantings[1] and progeny trials to more optimal sites.

TACF response: We agree that Meadowview is not an optimal site for growing American chestnuts due to the poor drainage in some areas and occasional limestone outcrops, which hinder chestnut growth, survival, and possibly blight tolerance. TACF has begun looking for better land to plant the next generation of backcross and transgenic seed orchards.

It would be desirable to quantitatively define the selection target – often called an ideotype. Is the goal to produce (1) trees that are canker-free in the field, (2) trees that live with cankers, (3)

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[1] Alternatively, demonstration plantings at this site may be used to contrast results at more optimal sites.

seedlings that might have some partial resistance but eventually die (but might have some value in breeding in different components of resistance), or (4) seedlings (trees) with fewer cankers, or all of the above?

TACF response: We propose the following selection ideotypes to define success in the breeding program:

- Main stem survives indefinitely with blight infection.
- Blight cankers superficial and extensively callused with minimal exposed wood.
- Trees grow as single-stemmed trees at rates and maximum heights more similar to American chestnut than Chinese chestnut.
- Leaf, twig, and nut characteristics similar if not indistinguishable from American chestnut.
- Trees compete and reproduce under competition in the eastern hardwood forests. Seedling recruitment is observed near parent trees.
- Populations adequately represent the diversity and adaptive capacity remaining in the post-blight *C. dentata* population.
- A subset of the population has tolerance to both *Cryphonectria parasitica* and *Phytophthora cinnamomi*.

We have observed a negative correlation between percent of the genome inherited from American chestnut and blight tolerance in backcross populations (Westbrook et al. 2019a). This result suggests that alleles for blight tolerance are segregating at more genomic loci than previously assumed and that phenotypic selection has not been sufficiently accurate to select for all resistance alleles in all backcross lines and generations. Generating hybrid chestnut trees that are indistinguishable from American chestnut yet also have high levels blight tolerance is proving to be difficult with backcross breeding. We remain optimistic that we will be able to meet these selection criteria by outcrossing transgenic American chestnut with the OxO gene to pure American chestnuts and backcross trees (see response to Objective 4). We are also advancing new backcross lines from additional Chinese chestnut sources of blight tolerance through fewer backcross generations (BC1 and BC2) to find an adequate balance between blight tolerance and American chestnut growth characteristics (see response to Objective 2).

Depending on the ideotype, does any particular phenotypic screen give all the necessary information? What inoculum sources (e.g. strains, etc) would identify particular ideotypes as well as illuminate underlying types of resistance? For example, in blister rust resistance work, MGR type resistance can be detected in inoculations of very young seedlings and fairly quickly (<1 year from inoculation); while in looking for partial resistance older (2 year) seedlings are used and evaluated for up to 5 years after inoculation. For Port-Orford-cedar, the assessment period was extended (on inoculated 1-year old seedlings) from ~1 year (the previous 'standard'

until the 2005 test year) to up to 3 years to pick up the quantitative resistance and to identify the best seedlings in each 'good' family (and to clearly delineate which families were MGR vs. quantitative resistance).

TACF response: We have not been able to distinguish major gene resistance from quantitative resistance in our chestnut blight tolerance evaluations. The positive correlation between blight tolerance and the proportion of backcross genomes inherited from Chinese chestnut suggests that blight tolerance is a polygenic trait rather than primarily conferred by major effect alleles.

Cloning

Previous reviews have called for the use of cloning techniques to enhance the program, while also noting that the species is rather recalcitrant to such efforts, particularly through rooted cuttings. Thus, to date cloning has been used to a very limited degree. However, as outlined below we believe the benefits of having cloning tools are too great to ignore. We suggest more effort to develop high-throughput cloning capability, centered around micropropagation and/or grafting, either in-house or through contract to a local horticultural center. It would be helpful to inquire as to the use of cloning in other chestnut species programs around the world.

TACF response: Cloning a diverse collection of American chestnut has thus far proved to be elusive and difficult. It took more than a decade to work out methods for cloning with somatic embryogenesis in chestnut (Merkle et al. 1991, Polin et al. 2006). The Canadian Chestnut Council has worked for nearly decade to induce rooting from chestnut scion material that has been grafted onto etiolated seedlings (Galic et al. 2012). Lovat & Donnelly (2019) have recently worked out protocols to clone a diverse collection of American chestnuts with axillary shoot culture; however, the concentration of hormones and culture temperature must be optimized to clone individual genotypes. In general, while some individual genotypes have been cloned with some methods, the success in regenerating plants has been low and not all genotypes could be cloned with specific methods. To our knowledge, success with rooting other members of the Fagaceae has been similarly poor or uneven, despite considerable horticultural interest in cloning oaks, for example.

TACF is interested supporting additional research to further refine cloning methods for American chestnut. Given the high risk and potentially low success rates, we would prefer that these cloning methods be worked out by qualified collaborators, rather than by TACF staff. TACF could potentially provide "seed funding" through its small grants program or other funding sources support research in cloning methodologies.

Grafting is our current method for cloning diverse collections of American chestnut for future use in breeding. Trees often get blight in the graft unions and, unlike seedlings, grafted trees do not resprout. To mitigate the risk of failure from graft incompatability prior to use in breeding,

we plan to grow a portion of grafted trees under high light conditions to stimulate catkin (pollen) production. We will then apply this pollen to transgenic or wild-type American chestnuts to conserve the genetics of parent tree in the seedling progeny. Among field-grown grafted trees, we will also collect seeds from these trees as soon as they flower so we can plant seedling progeny in the event that the grafted parent trees die.

The potential uses of cloning in breeding, and for achieving other program goals, include:

- Pseudo-cloning – Establish progeny, from seed, on optimal test sites. Coppice main stem after year one. Save 3-4 coppice shoots that arise in year two and prune the rest. Apply different treatments to surviving shoots such as different inoculation strains, controls, and treatments to promote early flowering capacity. This potential method will need to be tested to ensure that treatments on one shoot does not affect phenotypes on another.

TACF response: We will try coppicing future progeny tests and inoculating the different stems with different strains of *C. parasitica*.

- Progeny testing – Clonal propagation of genotypes provides superior estimates of heritability and breeding values based on replicated trials, either on a single site, or on multiple sites across a range of environments. Some replications may be placed in forest settings without inoculation, or in settings free of blight to evaluate growth and form.

TACF response: We have planted progeny tests composed of BC3F3 families from Meadowview on 30 + forest and old-field sites across the eastern U.S. Each site typically has 20 – 30 families. Sites share common families, although no site contains all families planted among all of the sites. We do not plan to artificially inoculate any of these tests and instead will assess blight incidence and severity from natural inoculum. The majority of these tests were planted in 2011 through 2014 and many of the trees have not yet been infected with chestnut blight. As trees get infected with blight in the next five years, we will compare the average blight incidence, severity, and mortality within families to canker severity of the same families that have been artificially inoculated in Meadowview orchard progeny tests and small stem assays.

GWAS (genome-wide association studies) or provenance trials – Use of replication could improve phenotyping capacity and improve the accuracy and precision of genetic estimates.

- Seed orchards – Consider locating orchards on blight-free sites (e.g., Pacific Northwest, use of hypovirulence, chemical control) where disease pressure is minimal, and trees can be grown to optimize seed production. Multiple ramets of each clone can speed up breeding progress and production of material for restoration.

See response in Objective 4 about growing American chestnuts west of the Mississippi for germplasm conservation

<u>Demonstration plots</u>

The panel supports frequent use of demonstration plots at Meadowview and Chapter locations for program marketing purposes. Row plantings of materials of different ancestry and resistance will better illustrate genetic improvement than data to stakeholders and the public. Such trials should be maintained as long as they provide an accurate reflection of breeding progress. Cloning of highly resistant trees of good form (and suitable comparators), planted in pairs or row plots, would help to establish convincing demonstrations. If demonstration plots are replicated within sites, some replicates may be inoculated while others are not. If a demonstration plot does not appropriately represent the known results of genetic testing, the plot should be removed.

TACF response: TACF is currently applying for funding with the U.S. Forest Service Landscape Scale Restoration program to plant a network of restoration trials in the eastern U.S. These trials would be composed of progeny from our most blight tolerant BC3F2 selections from Meadowview, Pennsylvania, and North Carolina seed orchards. We are planning on planting these restoration trials in eight states within the historical range of *C. dentata* range. Each planting would consist of 250 to 500 trees from 10 to 25 BC3F3 families. We are planning to have two plantings in each state. Only sites that are suitable for growing American chestnut will be selected (i.e., well drained soil with a pH 5-6). One major objective of these plantings is to assess the long-term blight tolerance of BC3F3 trees after we complete selection in our BC3F2 seed orchards. A second objective is to assess the regional adaptability of backcross material when we plant it in states close to v. in different climactic zones from the origin of the *C. dentata* parent trees.

<u>Clarifying the relative levels of resistance in BC3F2 and BC3F3 generations</u>

The committee was undecided on whether there was a reduction of resistance between these generations. Is this uncertainty due to phenotyping difficulties, or is there a dilution of resistance factors in the more advanced interbred generations? Is resistance sue to more than three major genes? Thus, it is unclear to us if there is value in F4, F5 or higher crosses. We suggest concentrating on high quality, convincing studies, supported at least in part by clonal trials of the best materials, before incorporation of other sources of resistance into these materials (or otherwise going too far down this BC pathway).

TACF response: We found that the true BC3F2 selections, on average had blight tolerance that was significantly less than pseudo-F1s, but greater than American chestnut in our Meadowview seed orchards (Westbrook et al. 2019a). A negative correlation between proportion of

backcross trees' genomes inherited from American chestnut genome and blight tolerance suggests that Chinese chestnut alleles for blight tolerance segregate at more than three loci. Polygenic inheritance coupled with low accuracy phenotypic selection for blight tolerance may explain why there has been a dilution of resistance alleles across backcross generations. After finishing selection our Clapper and Graves BC3F2 seed orchards, we plan to combine BC3F3 progeny from Clapper and Graves in a next generation seed orchard. We will use this seed orchard to determine how much blight tolerance can be enhanced with additional selection in the BC3F3 generation. We are not planning to pursue recurrent selection for all of the chapter breeding programs as this would be an onerous task and it is not clear at this time how much recurrent selection will enhance blight tolerance.

Objective 2. Advance 10 Chinese chestnut sources of resistance to BC2 in Meadowview breeding program

The review committee was unanimous in support of this objective and suggested it be given high priority. However, we believe the stated objective of advancing 10 Chinese sources of resistance may be far too conservative when the program effective population size, and the possibility of introgressing new, distinct, and more numerous resistance genes, is considered. We recommend expanding the number of sources of new resistance by an order of magnitude or more, via pollen as well as seed. Furthermore, new sources should not be restricted to Chinese chestnut. Consider all Asian sources of resistance, as well as European sweet chestnuts and as many Large Surviving American (LSA) sources that can be obtained.

TACF response: We agree with this recommendation, with a caveat acknowledging the operational difficulties. During the 2018 breeding season at Meadowview, TACF staff performed 25 controlled pollinations and harvested 1,350 seeds from these crosses to advance eight Chinese chestnut sources of blight tolerance to BC2F1 and BC2F2 generations. Also, the staff harvested 525 seeds from pseudo-F1 trees in Meadowview seed orchards. The pseudo-F1s were presumably pollinated by neighboring BC3F2 trees, hence their progeny would be pseudo-BC1s. The Chinese chestnut grandparents of the pseudo-BC1s are unknown, but could be the Chinese chestnut progeny of intercrosses among 19 different Chinese chestnut parents that are currently planted adjacent to the BC3F1 selections on TACF's Price Farm. We are also using large surviving American chestnuts (i.e., trees whose main stem survived the blight pandemic) as American chestnut parents in these crosses in hopes that they will also contribute to the blight tolerance of the backcross progeny. We are taking these new backcross lines through one or two rather than three or four backcross generations to avoid diluting out genes for blight tolerance in each backcross generation. We will plant the progeny in a "mixed-source" breeding orchard and perform staged inoculations with increasingly virulent strains of *C. parasitica.* We will perform final selections to maximize the proportion of the genome inherited from American chestnuts via genotyping the selection candidates. We will generate large segregating populations of BC1F2 and BC2F2 trees through controlled and open-pollinations among the

selections in these orchards. These BCxF2 progeny will be planted in a separate seed orchard where further selection for blight tolerance will be performed. Seeds from the intercrosses among BC1F2 and BC2F2 trees will be deployed for restoration trials (Westbrook 2018).

We plan to expand on this program by advancing additional Chinese chestnut and Japanese chestnut sources of blight tolerance at Meadowview to BC1 or BC2. We are also planning to generate BC1F2s and BC2F1s by intercrossing or backcrossing BC1F1 trees that descended from 10+ sources of *C. mollissima* resistance that are currently reproductive in the House Rock orchard in PA. TACF's chapters that do not have unique sources of blight tolerance (other than Clapper and Graves) may choose to participate in this program by pollinating wild-type trees in their region with pollen from selected backcross trees from Meadowview and PA.

Overall, we plan to expand this multi-source breeding effort to 20 Asian sources of blight tolerance and approximately 100 *C. dentata* backcross lines. We do not plan to expand this program to 100 + Asian sources due to the large effort relative to the uncertainty of gain. While TACF has enough reproductive F1, BC1, and BC2 trees available now to advance 20 additional sources, we do not have enough unique Asian chestnut sources of resistance from these generations to advance 100 additional sources. Advancing additional sources (beyond 20) would require two or three additional generations to make F1 crosses with Asian chestnut parents and then perform one or two generations of backcrossing to *C. dentata* prior to intercrossing the selections. We are planning to expand the effective population size of transgenic trees to > 500 (Westbrook et al. 2019b). Thus with the existing Clapper and Graves backcross programs, plus the planned transgenic breeding program, our breeding programs together should adequately represent diversity and adaptive capacity within *C. dentata*.

Accelerated breeding methods such as those recommended by Powell (induced early flowering, juvenile blight testing) and others (Meilan 1997; Wheeler et al. 1982; Wheeler and Bramlett 1990) should be considered to infuse these new sources as quickly as possible.

TACF response: Accelerated breeding methods are useful for generating pollen from first year seedlings, but we need large trees to generate large numbers of seeds. Hence accelerated breeding methods have limited applicability to the backcross program. We will need to generate large numbers of progeny (50 – 200) per cross to perform selection within backcross populations segregating for blight tolerance alleles. We need large reproductively mature trees to generate large numbers of seed. Furthermore, phenotypic selection accuracy for blight tolerance is limited given that blight phenotypes have low to moderate heritability ($h^2 < 0.5$, Westbrook et al. 2019a). Thus we plan to progeny test backcross selection candidates that pass the first round of phenotypic selection.

We strongly recommend using controlled crosses rather than open pollinated material for progeny testing, to the extent possible. This need not be bi-parental crosses, and in fact, it may be preferable to use polymixes to increase diversity (Lambeth et al. 2001; Wheeler et al. 2006).

Concerns about differential reproductive success or exclusion could be studied relatively easily and quickly with genetic markers (Apsit et al. 1988; Nakamura and Wheeler 1992a, b) and could be accomplished without great cost or delay with low cost genetic markers (e.g., by an academic collaboration, perhaps with a TACF seed grant).

TACF response: We plan to progeny test backcross selection candidates through controlled pollinations with polymixes. We also plan to genotype progeny of intercrosses among these selections to ensure that particular parents are not over-represented among the progeny.

To the extent possible, Chapter programs could also benefit from similar expansions in their genetic base as opportunities arise (though we understand, and were pleased to learn, that some of this work is already underway).

Objective 3. Finish selection in Clapper and Graves seed orchards in TACF's Chapter breeding programs

The review committee recommends some continued work to develop BC populations in the Chapters, but suggests that the work here be limited relative to the other major objectives, with the following qualifications:

- Select and guide Chapters – Emphasize a small number of Chapters that have unique characteristics and/or environments, such as zones of ecological adaptation, extent of other diseases (especially *Phytophthora*), and the unique quality of the American chestnut parents employed. Emphasize Chapters or regions where the extent of progress, investment, and phenotype quality are highest.

TACF response: The southern chapters (GA, NC/SC, AL, TN, VA, and KY) are planting or plan to plant orchards composed of backcross trees selected for resistance to *Phytophthora cinnamomi* for eventual breeding with blight tolerant trees (Westbrook et al. 2019c). All chapters will participate in germplasm conservation of American chestnuts in their regions. These American chestnuts will be used for transgenic outcrossing and to create new backcross lines from additional Asian sources of blight tolerance. Preliminary chapter targets for numbers of trees to conserve are in Westbrook (2018). We may revise these targets after we obtain results from a range-wide study of genetic diversity and adaptation in American chestnut.

- Leverage Meadowview results – Use results from Meadowview BC studies and Chapter plantings to select programs to emphasize. This should help spread and efficiently use resources but should be done carefully so as not to disengage or create animosity amongst the Chapters. For instance, host volunteers from the Chapters to help with increased breeding loads at Meadowview or other future orchard sites, using Chapter genetic resources.

TACF response: Considering the finding that the Meadowview Clapper and Graves BC3F2 seed orchards are expected to generate progeny with low to intermediate blight tolerance (Steiner et al. 2017; Westbrook et al. 2019a), we recommend that chapters scale back on planting Clapper and Graves BC3F2 seed orchards from nine blocks of 20 backcross lines per chapter to a minimum of three blocks (Westbrook 2018). The suggestion of organizing foundation-wide volunteer events at Meadowview is interesting, and we shall consider the possibilities.

- Integrate GE – Emphasize work to integrate GE trees into the BC populations rapidly in selected Chapters if the strength, stability, and deregulation status of the transgenic OxO trees are favorable and there is support in the Chapters for transgenic work.

TACF response: TACF staff have administered a questionnaire to assess to what extent specific chapters would like to be involved in a transgenic breeding program. Chapter involvement with the transgenic breeding program may vary from collecting scions from naturally occurring American chestnuts to maintaining germplasm conservation orchards, performing pollinations with transgenic pollen, and planting orchards composed of transgenic progeny.

Objective 4. Diversify GE populations and combine blight and PRR resistance

We strongly support expanded efforts to increase diversity in the transgenic materials. Consideration needs to be given both to the number of transgenic events and genotypes that are transformed, and to the range of genotypes that will be pollinated with transgenic trees. The biggest constraint to effective population size will be the number of transgenic trees used. We think this should be increased both by developing OxO transformants in several more American chestnut genotypes, and potentially by developing transgenics with resistance from genes other than OxO. The objectives outlined in 4b-4g are aggressive and desirable.

Additionally:

- Climate change considerations – When expanding the number of transgenic genotypes and the non-transgenic trees that will be pollinated with transgenics, select good genotypes from across the range of the species, including both leading and lagging edges of the current range. This will capture the range of adaptive diversity within natural populations.

See our response below to the suggestion for a landscape genomics study.

- Consider *ex situ* plantings of collections in western refugia, free of disease at this time, to ensure availability for crossing in years to come.

TACF response: A permit is required to import American chestnuts from the eastern United States into Oregon, Washington, and California. Care would need to be taken to disinfest seeds of blight propagules prior to shipping west of the Mississippi river. If we can find reliable

collaborators that are willing to maintain pure American chestnut plantings on the west coast, then these blight-free plantings could be useful for germplasm conservation and breeding. Pending U.S. regulatory approval, SUNY-ESF and TACF may be able to ship pollen generated from plants grown in blight-free growth chambers to the Pacific Northwest for pollinating healthy American chestnut trees in the Pacific Northwest to increase the diversity in the transgenic breeding program.

- Linkage drag – We suggest careful consideration of linkage drag that may result in expression of recessive deleterious alleles when individual transgenic events are made homozygous in seed orchards. Diversifying transgenic events will ameliorate this issue, although we recognize there are some potential concerns around gene silencing. In natural populations, if gene silencing occurs in some genotypes, natural selection will quickly remove or reduce those in the population. Population simulation modelling could be used to evaluate this risk.

TACF response: In a paper in review (Westbrook et al. 2019b), we have simulated transgenic outcross pedigrees and marker-assisted introgression of the OxO transgene. The abstract of the paper summarizes our plan for diversifying the transgenic population of American chestnut:

Summary

- Breeding transgenic blight tolerant American chestnuts with susceptible wild-type trees is potentially an efficient method to rescue the genetic diversity and adaptive capacity of the American chestnut population for large-scale restoration.
- To develop a breeding plan to diversify a transgenic blight tolerant population, we simulated pedigrees to estimate inbreeding coefficients and effective population size in scenarios involving outcrossing 1 to 4 transgenic founders to a maximum of 1500 wild-type trees over 1 to 5 generations. We also simulated marker-assisted introgression scenarios to minimize the extent of the transgenic founder genome, especially on the transgene carrier chromosome.
- Simulations suggest that the effective population size may be increased to > 500 and? the average inbreeding coefficient reduced to < 0.01 by outcrossing a single transgenic founder over five generations to 2, 25, 50, 150, and 450 (677 total) wild-type parents. Three generations of marker-assisted introgression with 50 to 100 progeny per cross is predicted to decrease the length of founder genome to 7% to 13% of the transgene carrier chromosome length as compared to 42% with event selection only. Transgenic outcross progeny may be intercrossed to generate a population of trees that is homozygous for blight tolerance.
- A diversified population of transgenic blight tolerant American chestnut is estimated to be available for use in large-scale forest restoration 20 to 35 years after federal approval to distribute the trees. In contrast, trees from earlier generations would be available almost immediately after federal approval for personal or horticultural plantings. Methods to accelerate outcrossing are discussed.

- Gene diversification – The committee urges that additional blight resistant GE events are produced to protect against unexpected silencing, pleiotropy, and drag of deleterious alleles when homozygous. These can be alternate forms of OxO overexpression but lacking contiguous regions of sequence identity.

TACF response: We have included an excerpt from Westbrook et al. 2019b on our plans for creating new transgenic founders:

"We currently plan to create up to three additional transgenic founder lines through Agrobacterium-mediated insertion of OxO into three additional American chestnut trees' genomes. Creating additional transgenic founder lines alleviates the founder bottleneck on effective population size once transgenic founders are outcrossed to wild-type trees. Having additional founders also mitigates the risk that deleterious mutations in linkage disequilibrium with OxO in a single founder tree's genome will have negative effects on fitness among progeny that inherit OxO in a homozygous state. However, outcrossing with multiple transgenic founders also carries the risk that the OxO transgene could be silenced in progeny that inherit multiple copies of OxO at different locations in the genome. To mitigate the risk of gene silencing, we intend to express OxO with different promoters in different transgenic founders. In the 'Darling 58' founder, OxO is expressed with the constitutive CaMV 35s promoter. Additional transgenic lines of American chestnut will be developed in which OxO is expressed from a wound-inducible promoter (win3.12) and different constitutive promoter (UBQ11). Expressing the OxO transgene with different promoters reduces the risk of silencing due to methylation of a specific promoter region, though we acknowledge that post-transcriptional gene silencing would not be affected by specific promoters. In a forest setting, blight infection will eventually kill trees that have the OxO gene silenced; therefore, natural selection could maintain transgenic blight tolerance even if silencing occurs."

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- It may be desirable in the future to only create T2 crosses among T1 individuals from different genetic backgrounds.

TACF response: Once SUNY-ESF has created multiple transgenic founder lines with OxO inserted at different locations in different *C. dentata* genetic backgrounds, we plan to intercross these trees over two generations to compare OxO expression among progeny that inherit 1, 2, 3, or 4 copies of OxO at two genetic loci. The purpose of this experiment will be to test if OxO expression is reduced or silenced among progeny that inherit multiple copies of OxO.

- Gene insertion event stability – We urge continued studies of OXO and new transgenes' expression levels and potential silencing as they are moved with additional crosses, as trees age and among different environmental stresses. If this turns out to be infrequent

or sporadic, natural selection should effectively cleanse the population of most of the silenced (blight susceptible) trees.

TACF response: We acknowledge that expression of OxO may be attenuated or silenced in different *C. dentata* genetic backgrounds, different generations, or under environmental stress conditions. In 2019, we applied for APHIS permits to outcross T1 progeny of the 'Darling58' transgenic founder to additional wild-type *C. dentata* parents. We will measure OxO expression among the progeny from these crosses to determine if expression varies among progeny of different *C. dentata* parents. We plan to conduct future studies where we will compare the expression of OxO among progeny of different *C. dentata* outcross generations including comparing OxO expression in hemizygous versus homozygous trees. We also plan to measure the expression of OxO in common families planted in different environments.

- Landscape genomics – The landscape genomic studies proposed may be helpful for choosing native chestnut sources for diversification/outcrossing. In addition, because of the use of cutting edge molecular genotyping technology and interest from academic collaborators, significant external grant funding may be obtained to support the work. A genomic approach may be useful and cost-effective in evaluating the extent to which extant populations are locally adapted to climate and may indicate how many seed zones are needed once resistant material is available for restoration. This approach might substitute for provenance testing for climate adaptation (an approach that could be expensive and possibly ineffective as non-resistant material from different locations might succumb to blight before reliable adaptive patterns are expressed in those trials that are planted within the current range of chestnut and blight). However, the use of ecographic information (climate of origin, e.g., from ClimateNA) and climate change modeling will also be helpful for choosing diverse and adapted sources for breeding and future restoration efforts. The extent to which genomic studies, or provenance trials on non-blight locations, would add to ecographic variables to predict adaptive patterns is unclear, and might be investigated in pilot studies (e.g., a subset of locations, perhaps funded by a pilot grant) before major investments in either approach were made.

TACF response: Jason Holliday (VA Tech) and Jared Westbrook (TACF) received a grant from the USDA NIFA program in 2019 to sequence the genomes of 500 American chestnut trees from across the historical species range to study diversity and climate adaptation in remnant American chestnut populations. We have included an excerpt from the proposal that addresses how we will use the genomic data to develop targets for germplasm conservation:

"Results from Objective II (delineation population structure) and Objective III (identification of loci underlying local adaptation) will be used to develop targets for number of American chestnut trees to propagate from each management unit and adaptive unit, respectively. Targets for the number of wild-type trees to propagate within each management unit will be proportional to allelic diversity among putatively adaptive loci and to the predicted future

spatial extent of suitable climate associated with each adaptive unit. Our rationale for conserving larger numbers of trees within more adaptively diverse sub-populations is that these sub-populations are expected to respond more strongly to natural selection. To incorporate future climate into our sampling scheme, numerical ranges in climate variables that define adaptive units within *C. dentata* will be identified by clipping historical climate data based on the geographic extent of each adaptive unit in ArcGIS. The total geographical extent of suitable habitat for *C. dentata* will be predicted for the year 2080 with Maximum Entropy (MaxEnt) modeling, which combines grids of climate variables with habitat occupancy data to predict suitable conditions for a species. The inputs for MaxEnt prediction of suitable habitat will be spatial variation in density of American chestnut stems estimated from Forest Inventory and Analysis data; historical climate data (1895 – present) for the current range of American chestnut (www.prism.oregonstate.edu); soil survey data (websoilsurvey.sc.egov.usda.gov), elevation from the U.S. Geological Survey 3D Elevation Program (nationalmap.gov/3DEP); and Intergovernmental Panel on Climate Change predictions of climate in 2080 using two representation concentration pathways (RCP 4.5 and RCP 8.0) for greenhouse gases (http://sedac.ipcc-data.org/ddc/). The geographical area associated with each adaptive unit will be estimated for the year 2080 by partitioning climate variation within the predicted suitable habitat for *C. dentata* in 2080 range by ranges in the historical climate data associated each adaptive unit."

- GE caveats -- While OxO transgenic trees offer great hope, they should be embraced with caution as they have not yet begun to be used in field trials of a size and duration that is comparable to those in forestry or agricultural biotechnology breeding programs. They are also dependent on a single gene insertion event that is the best of only a few dozen that have been produced. Thus, the committee considers USDA deregulation and its equivalent at EPA/FDA not a commitment to commercial use, as is common in agriculture, but as a license to do essential breeding research. If they are deployed at scale and start to be used in restoration, care must be taken to insure the first efforts are successful (high quality sites, management, and outreach). Early failures in this contentious area could put the future of the program at risk. This message needs to be conveyed to cooperators to avoid overpromising/underdelivering.

Appendix I - Comments on Additional Questions Posed by Jared Westbrook

Question 1a. Do the reviewers see merit in continuing planting and selection in TACF's backcross program (especially in the volunteer-led Chapter programs) when intermediate resistance to chestnut blight is expected after selection is complete in BCxF2 seed orchards?

We assume that the question is asking if TACF should move to the BC3xF3 generation and beyond (F4, F5). Simulations that were presented to the committee suggested that average levels of resistance should continue to increase, but actual results to date have not reached full expectations[2]. The real prospect of investing years more of breeding and testing with little return exists. As discussed above in response to the Objectives, the committee believes TACF should explore all options. For the mainline program we suggest intercrossing Graves and Clapper as soon as possible, using controlled crosses, adding variable virulent strains for inoculations, and relying on genomic / genetic marker / accelerated flowering selection methods to accelerate the process. You may wish to follow the original plan in selected Chapter programs and disbanding others. We also proposed investing in additional sources of resistance.

If grant funds could be obtained by a collaborator, a genomic approach could be very useful in determining the number of loci or genomic regions involved in resistance from Chinese chestnut, providing a potential explanation for why resistance is lower than expected in the BC3F2 materials, and informing the decision of whether to continue with more generations of backcrossing. It could also be useful for selecting BC3F2 or BC2F2 individuals that have higher than average American chestnut ancestry as ancestry should be quite variable after two or three generations of backcrossing. The role of inbreeding, and the extent of inbreeding depression, on blight resistance and growth in these BC materials also warrants study.

Question 1b. What are pros and cons for completing selection at BCxF2 in the backcross program if transgenic American chestnut trees with potentially high levels of blight resistance are approved for use in breeding and restoration by the U.S. federal government?

The obvious "con" is that transgenics may not be deregulated, or that the cost for each added event will not be affordable or expeditious. That is potentially a major concern. We are not sure what level of investment is needed for "completing the selection at the BCxF2 level" but if it can be done in 2 years or less, then by all means do it and move onto the other approaches discussed earlier in this report, as funds and timing permit. Crossing BC trees with transgenics should help build greater resistance to blight.

Question 2. Do the reviewers see merit in raising grant funds and philanthropic investments for genomic selection (GS) in Chapter breeding programs? Alternatively, do you recommend mostly phenotypic selection and limited progeny testing in Chapter breeding programs?

The committee believes that it would be wise to first show proof of concept in the mainline programs and pencil out the cost/benefits before making decisions for Chapters. For Chapter programs that relied on the same sources of resistance, it is also essential to determine if one training population is predictive for other populations—which we regard as unlikely unless the materials are identical. The

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[2] See Stacy Clark's field results. Functional resistance may be greater than suggested by trials at Meadowview. More actual field results on good chestnut sites are needed before making definitive conclusions.

combination of genomic selection, use of less virulent strains for inoculation (so a wider variety of resistance genes can be recognized and selected for), and hopefully continued reduction in genotyping and bioinformatics costs, may make this a viable option in the future. No matter whether genomic selection is used or not, reliance on progeny testing will not go away for many years, since it is necessary to provide training populations and advanced generation crosses with multiple sources of resistance. Consideration should be given to testing with and without inoculation.

Aside from GS, there is a very strong role to be played by genomics in selecting among BC2 and BC3 trees for the highest proportion of American chestnut ancestry, as well as to reduce the size of regions flanking QTL for blight resistance if sufficient numbers of individuals can be genotyped for mapping these loci or genomic regions. Genomic approaches can also provide a good picture of the extent of local adaptation to guide the number of breeding populations needed. Given the need for access to laboratory facilities and bioinformatics expertise, perhaps the best path towards bringing a genomic component into the TACF science program is through collaborations with academic partners who can seek funding from federal grant programs. The partnership TACF has developed with Dr. Jason Holliday is an excellent example of this.

Question 3. *Do the reviewers see added value in pursuing regulatory approval for transgenic trees with resistance to Phytophthora cinnamomi? Consider that backcross trees have been demonstrated to have intermediate to high levels of resistance to P. cinnamomi.*

If natural resistance to *Phytophthora* is only found in rare germplasm, it will make breeding for resistance to it and chestnut blight, plus selection for adaptation and growth/form, extremely complex (a secondary BC program). If GE approaches to *Phytophthora* resistance are effective, the resistance genes can be combined into single constructs or co-introduced, then inserted and crossed into diverse base germplasm. Having both GE and natural forms would also be highly desirable for durability.

Question 4. *To what extent do the reviewers recommend breeding transgenic trees with backcross trees and to what extent do you recommend keeping the backcross and transgenic programs separate? In other words, what proportion of transgenic breeding lines do you recommend creating via outcrossing transgenic trees to backcross trees v. crossing transgenic trees to wild-type American chestnuts? What are the social implications of keeping these programs separate v. combining them?*

Assuming transgenics are permitted, the committee believes that it will be necessary to follow both paths, simply because transgenics will not be permitted everywhere in the restoration and forest product market landscape, at least not for many years. It might be wise to mix the lines in proportion to the areas in which mixing will be permitted, as this should provide the most durable resistance.

Question 5. *Do the reviewers recommend considering local adaptation and climate change in our plans for propagating wild-type trees for outcrossing and diversifying transgenic populations?*

The committee unanimously agreed, as discussed above. When materials are ready for restoration, encourage "assisted gene flow," climate-based seed transfer to match genotypes with new rather than last-century local climates (Aitken and Whitlock 2013; Aitken and Bemmels 2016). If results are available

from ecographic or landscape genomics studies they can inform seed transfer; otherwise climate models such as the US Forest Service's Seedlot Selection Tool can be used (https://www.fs.usda.gov/ccrc/tools/seedlot-selection-tool). While the development of resistant material far outweighs climate adaptation concerns for American chestnut, if resistant genotypes are planted in environments they are maladapted to restoration will fail, and most widespread tree species show considerable local adaptation to climate.

Do the reviewers agree with our approach of propagating trees from subpopulations in proportion to diversity at genetic loci that potentially underlie local adaptation and in proportion to projected range area for each locally adapted subpopulation?

In early stages of restoration, it is not essential to maximize diversity on each site but to match genotypes to sites to which they should be able to grow well (which may be limited in early plantings). However, some level of diversity is highly desirable (e.g., a minimum of 5 genotypes for specific locations). Over time diversity should grow as new resistant materials are developed and planted, and trees outcross in the wild.

Alternatively, we could propagate germplasm to represent the range of climate and soil conditions over the historical range of American chestnut without considering climate change and local adaptation.

As discussed above, in addition to historic range and adaptation considerations, we believe it is wise to consider climate change, including likely changes to current pests and pathogen ranges, in the light of available ecological and genotypic information.

Appendix II - Comments on Queries from Ms. Sara Fitzsimmons

Question 1. How much emphasis should be placed on conserving wild trees in situ vs. ex situ?

We do not advise spending resources on *in situ* conservation efforts except for large American survivors, but if volunteers and cooperators want to do this, don't discourage them. Focus some effort on *ex situ* conservation for lagging edge populations given climate change (Aitken et al. 2008). Get pollen via high light intensity floral induction, or transplant trees from new basal sprouts from the field and graft or micropropagate, if wild trees do not live long enough to provide pollen or outbred seeds.

In addition, *ex situ* plantings that could be used as common gardens for GWAS or provenance trials in blight-free western plantations, using clonally replicated propagules, could be helpful. Plagiotropic growth habit can be overcome by coppicing after year one if rooted cuttings are employed.

Question 2. At what point should materials be released for "restoration"?
We urge that the early demonstration trials have high levels of resistance (perhaps selectively using clones for quality assurance) be established on high quality and publicly accessible sites. We support using a mix of materials for restoration on a broader scale, but temper expectations as some or many trees will die and others won't look great. Place signs near demonstration plantings in a way that captures and amplifies the educational component of TACF and its many programs.

Question 3. How much "diversity" needs to be represented in a single "restoration planting". If a planting is 300 trees, how many backgrounds/lines/pedigrees should be established within that planting?
The committee believes that high diversity is not essential in early plantings, but not fewer than about 5 genotypes that are expected to be adapted to the area should be used. Iterative establishment of 5 or more new adapted genotypes every few years in a given area, and as new materials come on line, using both the best BC and transgenic sources if available, is a reasonable silvicultural scheme. Diversity of trees, and landscape-level diversity, will also increase over time with naturally outcrossing and regeneration. Of course, the minimum number of unrelated genotypes in the breeding program should be much higher than the minimum number in a single restoration planting.

Question 4. Should TACF continue to have material transfer agreements of some kind with partners who plant materials? I welcome any comments regarding the stringency/outline of these types of agreements.
Presuming there are no significant patent, market, or regulatory constraints (as the committee believes that the TACF desires and will work to put in place), we recommend a low-obstacle instrument for distribution and tracking. This might be a simple, one-page material transfer agreement that requires a commitment to annual or biannual reporting of planting size and location (at least to county level as many will not wish to disclose specific locations), and tree health and form. The MTA requirement would also establish paper and email contact information so TACF or others could query about status, and to provide new information to those planting about the materials as research proceeds.

Question 5. Should TACF plan on overseeing restoration activities to ensure 1) blight-resistant materials are diversified to an appropriate level for species restoration and 2) those materials are then planted in enough locations and in the most suitable areas for the species to resume ecosystem services. What is the minimum number of locations or areas which need to be established.

If TACF is producing materials, then it can control, to a point, when, with what materials, and where restoration plantings are established. This goal is unlikely to be feasible in all places; however, TACF could produce and publish maps/locations that prioritize high quality sites for American chestnut rather than marginal sites, suggest them most appropriate genetic materials, and update users (see comments on question 4) as new performance, genetic, and ecological information becomes available.

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