CONTENTS

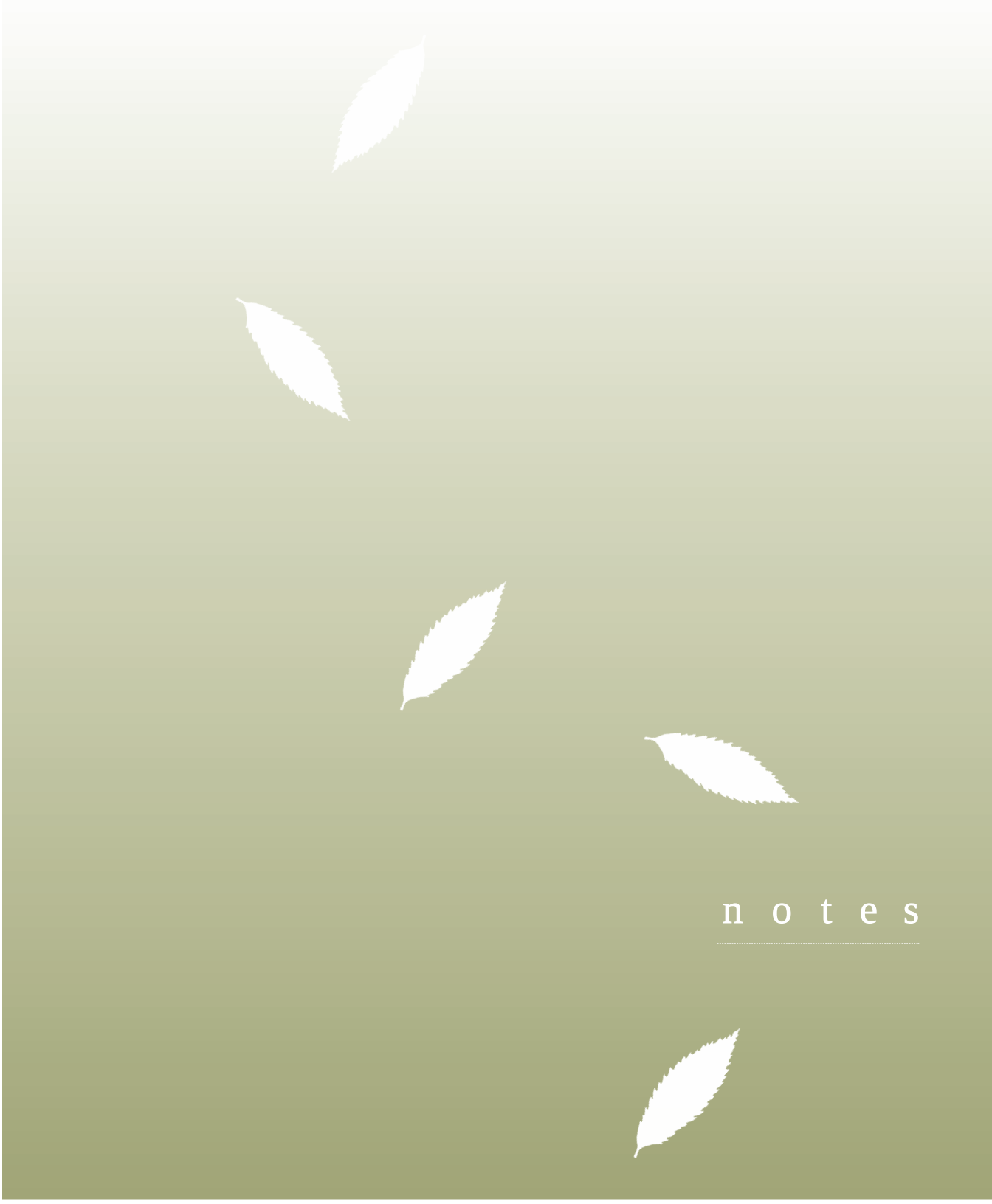
NOTES

MEMORIES

SCIENCE AND NATURAL HISTORY

CASTANEA GUIDE

Blank Page 2


notes

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FROM THE EDITOR

It has been nearly one hundred years since chestnut blight, *Cryphonectria parasitica*, was first identified in the Bronx Zoological Park. The blight has converted the American chestnut from the towering and dominant species of the East, to a sickly tree whose numbers are in continual decline. Over the years, individuals have labored hard and are finally very close to a resolution of the tree's plight.

Despite our armory of scientific tools to aid the American chestnut, and our progress, there is natural mystery at every turn. We still have not determined whether there are two or three or more genes that together confer blight resistance. We do not fully understand why Chinese trees are orchard-like and American tall and straight. Yet, we have enough information to move forward toward our goal.

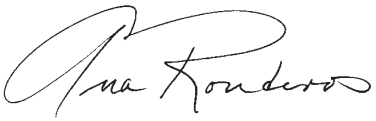
In the pages of this issue of *The Journal* a web of scientists unfolds, each outfitted with different tools and frameworks, working to bring back the American chestnut. From Meadowview, TACF scientists provide an update on breeding work. In the report that follows, a team of scientists provides our breeding program a solid seal of approval and offers guidelines to keep our efforts on the right track despite limits in our knowledge. Nobel laureate Norman Borlaug provides other suggestions in a letter to TACF's executive director.

While TACF researchers focus on breeding, scientists elsewhere look at techniques that can support and augment TACF's efforts. Christopher Holliday and Scott Merkle investigate the possibility of cryostorage of chestnut embryos, potentially useful to ensure the distribution of highly blight resistant material and to preserve germplasm collected from the shrinking genetic pool of American chestnuts surviving in the wild. Doug Boucher, on the other hand, looks at the wild and presents the projection matrix model, a method for orderly prediction of growth and regeneration of forest trees under varying conditions.

In the middle of this issue, you will find an interlude from science in excerpts from Ellsworth Barnard's *In A Wild Place* where he emphasizes nature's ability to survive, a stance developed from his observations of the American chestnut before and after the blight. Wood engravings by Charles H. Joslin from that same book capture the beauty of the American chestnut. Harold Bower suggests that American chestnuts actually encouraged

the growth of White oaks in an ironic twist of nature. His observation reminds us that despite our science, we still sometimes have no idea why nature (or the American chestnut) does what it does.

Lastly, please check out our new section "Castanea Guide" that provides useful information for many of our new and old supporters who may not have a background in breeding or science.



MEADOWVIEW NOTES 1999–2000

by Fred V. Hebard, Paul H. Sisco, and Peter A. Wood

In 1999 Meadowview was blessed with adequate to abundant rains until September, when drought set in and persisted until March 2000. Fortunately, we received enough rain during most of the growing season — there was drought 50 miles to the east, north and west of us.

In general, the growth of trees was good, although this year it became very clear that trees grow much better on hilly areas of the Glenn C. Price Research Farm than on flatter areas, even those with a slight elevation (none of the ground at the Price farm is swampy). Nevertheless, we expect it will be adequate for our purposes. We are continuing to investigate reasons for the poorer growth.

Current holdings are indicated in Table 1, and Table 2 indicates changes from last year. We now have well over 14,000 trees in the ground covering about 45 acres. This is becoming a big operation!

1999 Harvest

This was a good harvest. We are back up in the 5,000 to 6,000 nut range we achieved when we first acquired a bucket truck in 1996. Totals do not exceed that range because we had fewer American chestnut mother trees available than in previous years. That will be the case next year, but numbers should increase in 2001 as more Price Farm American chestnuts come into production. Pollen contamination was low, confined to only a few crosses.

We made a strenuous effort to finish up all desired crosses from trees screened for blight resistance prior to 1998. In general, we succeeded. The year 2000 will be the last one when our most advanced cross will be straight third backcrosses (BC_3s). In 2001, we will begin intercrossing BC_3s produced back in 1995, to make BC_3-F_2s.

Blight Resistance

We have stated over the years that we should be able to backcross the blight resistance of Chinese chestnut into American chestnut. The basis for this statement is that we recovered highly blight-resistant progeny from intercrosses of F_1s and from two BC_1s. We also were able to recover straight backcross BC_1s and BC_2s that had levels of blight resistance as

The list of volunteers who helped with bagging and pollination this year is long and has been published in *The Bark*. Thank you very much! We wouldn't get it done without your help.

If you are interested in helping pollinate next year, plan on any time in June after the 11th. (Call 540 944-4631 around June 1st.) Elder Hostelers will be helping with inoculations next year. If you are interested, call 617 426-8055 or write 75 Federal St., Boston MA 02110.

good as, or better, than that of F_1s, with levels of resistance between that of Chinese and American chestnuts.

In 1999, we extended the experimental basis for our claim. First, we tested the blight resistance of some F_3s whose F_2 parents had been highly blight resistant. Two of three F_3 families we tested had only highly blight-resistant progeny (Table 4). This suggests that their F_2 parents were homozygous for blight resistance, and that they were highly blight resistant. Our first general release of blight-resistant, American-type chestnut trees will be in the form of BC_3-F_3s. Based on the results with the straight F_3s, we expect that most of BC_3-F_3s will be highly blight resistant.

In the same experiment as the F_3s, we also tested for blight resistance some progenies from open pollination of 'Clapper' BC_2 trees, and recovered some highly resistant progeny (Table 4). Because some of these trees were probably Clapper BC_2-F_2s, this gives us encouragement that our next step of intercrossing BC_3 will also produce progeny with high levels of blight resistance, comparable to that of the Chinese parent.

The Number of Genes Controlling Blight Resistance

Although not necessary for breeding purposes, it would be very helpful for us to know how many genes control blight resistance and how they act. If it is two, then we would only need to grow about 35 progeny from each backcross to have a high probability of recovering both genes, whereas if it is three, 75 are needed. For intercross generations, the number of progeny needed escalates even more steeply with an increase in the number of resistance genes.

In 1993, the data suggested that two or three incompletely dominant genes control blight resistance. Seven years later, we still cannot pin the number down. However, a fair amount of evidence is suggesting at least three rather than two genes.

The results of screening second backcross trees for blight resistance (Table 5) suggests that three genes control blight resistance. We selected trees as having adequate levels of blight resistance at a frequency of about 1 in 8, which fits a three-gene model. Another piece of evidence comes from the results of screening the progeny from open pollination of 'Clapper' BC_2s mentioned above (Table 4). We recovered 6 highly blight-resistant trees out of 284, which fits the 63:1 ratio of susceptible

to resistant expected in F_2 if three incompletely dominant genes control blight resistance.

Yet this evidence is inconclusive. Further data are needed. The most important data will come from test crosses of some of our plants. In a test cross, the genotype of a parent tree is examined by looking at its progeny. For instance, a test cross could be used to confirm the three-gene hypothesis from Table 5. Some of the trees classified as adequate might not have had a full complement of resistance genes, especially if four genes control resistance. In this case, the test cross progeny of some "adequate" trees would have lower levels of blight resistance than that of other "adequate" trees.

One such test cross will come as we start in earnest this year screening BC_3s for blight resistance; each set of BC_3s derived from a BC_2 constitutes a test cross for that BC_2. In the backcross method of plant breeding, each backcross generation is a test cross for the previous generation.

Molecular mapping of crosses has been another means we have used to investigate the number of genes controlling blight resistance. In last year's "Meadowview Notes," we presented a fairly detailed summary of our mapping results up to that time. The preliminary data indicate that different Chinese trees may contain different genes for resistance, while they may have a few resistance genes in common. A team of three geneticists and plant breeders who reviewed our scientific programs last August suggested that we use 15 different Chinese sources of resistance to expand our base of resistance genes, in case the blight manages to overcome any one set of them.

It is clear that the molecular mapping of blight resistance genes would be more informative if we increased the precision of our measurement of blight resistance, map larger groups of trees, and add more genetic markers to the maps. It would also help if we mapped each resistance gene by itself. Last year we planted an orchard to do that. We expect to have the results from that planting in three years, although one planned extension of the experiment will take several more years.

Increasing the Precision of Blight Resistance Measurements

We are almost finished installing an irrigation system at the Price farm. By irrigating the trees, we will be able to get more uniform growth and

A Quick Guide to Chestnut Breeding Terminology

American x Chinese	= F_1
F_1 x F_1	= F_2
F_2 x F_2	= F_3
F_1 x American	= BC_1
(also known as BC_1F_1)	
BC_1 x BC_1	= BC_1-F_2
BC_1-F_2 x BC_1-F_2	= BC_1-F_3
BC_1 x American	= BC_2
BC_2 x BC_2	= BC_2-F_2
BC_2-F_2 x BC_2-F_2	= BC_2-F_3
BC_2 x American	= BC_3
BC_3 x BC_3	= BC_3-F_2
BC_3-F_2 x BC_3-F_2	= BC_3-F_3

BC (often written as B) indicates the offspring of a backcross, the breeding of a pure American chestnut with a tree that is a genetic mixture of blight resistant and pure American stock.

F indicates the offspring of an intercross, the breeding of two genetically "pure" trees or two trees of the same generation that are already a genetic mixture of blight resistant and pure American stock. Lowered numbers indicate the number of times a breeding procedure has occurred in a tree's lineage.

thus a more uniform response when inoculated with the chestnut blight fungus to screen for resistance. At the Wagner Research Farm, we have been irrigating trees when they are screened for blight resistance, but we have not been able to irrigate them in the years prior to screening. So there is more variation in tree size and vigor from those prior years than would occur with irrigation.

To increase the precision of blight resistance measurements, we also plan to increase the number of cankers initiated on trees destined for molecular mapping, from 4 to 15. Such a high number of cankers on breeding trees is not advisable — fifteen would kill them. In addition, an increased number of cankers will require more frequent measurements of canker size (requiring more of our limited labor resources). Therefore, the increased number of cankers will only be initiated on trees destined for molecular mapping.

Chinese Chestnut Tree Form

As many of you know, most Chinese chestnut trees have a more rounded form than American chestnut trees. Americans tend to grow with a single trunk whereas Chinese chestnut trees tend to have multiple branches. We select for the American form in our crosses.

One factor that influences branching in chestnut is the timing of bud break in the spring. If buds break before a late spring frost that kills them, then suppressed buds begin to expand, leading to multiple branches. At Meadowview, Chinese chestnut trees generally begin to break bud prior to May 1, whereas American chestnut generally begins after May 1. Hard frosts often occur around May 1, which does not harm the tight buds on American chestnut trees, but kills the growing shoots on Chinese chestnut trees. This early growth trait is dominant in crosses of Chinese and American chestnut.

The early budding of Chinese chestnut trees combined with killing frosts is not the only factor leading to their branchiness. For many years we have investigated the morphological basis of this difference. Data collected last year suggest that three to four buds at the branch tips of Chinese chestnut trees begin growing at bud break in the spring, whereas only the terminal bud grows in American chestnut.

This spring the weather turned cool in April and there was very little suppression of bud break in many trees, including American chestnut.

However, when the weather warmed up, the broken buds were suppressed from expanding further in American chestnut. The pattern of expression varied depending upon the time of initial bud break relative to cool period. Perhaps the cool weather depressed metabolism in the terminal bud so that it was unable to suppress the subjacent buds.

The involvement of cool, but not freezing weather at the time of bud break in the lack of suppression of buds below the terminal may be interacting with early bud break to lead to the branchy form of Chinese chestnut trees in years without late spring frosts. We will have to look again next year.

Cytoplasmic Male Sterility

As reported previously (Shi and Hebard, *The Journal*, 11:38-47, 1997), male sterility occurs in progeny when a Chinese chestnut male is crossed to an American chestnut female, but not when the Chinese chestnut is used as female. With the help of Tom Kubisiak of the Southern Institute of Forest Genetics, we mapped one gene controlling male sterility coming from the Chinese chestnut cultivar, 'Nanking.' With the help of our Price Scholar Michelle Phipps and graduate student Timothy McKechnie, we took additional data on a large Clapper BC_2 family. Again with Tom Kubisiak's help, we found that two genes appear to control male sterility in the large Clapper family.

Male sterility, the failure of a plant to produce pollen, is not something that we would want to have in our trees. However, it can be very useful. First, just like hairy leaves, large stipules, and early leaf emergence, it is clearly a trait coming from the Chinese parent, so it is a marker we can select against in our efforts to eliminate as much of the Chinese genome as possible. Secondly, it can help control the pollen parent in crosses. We could create an isolation block of male-sterile F_1 trees surrounded by pure American chestnut trees and allow open pollination. The seed harvested from those F_1s would be guaranteed to be BC_1 seed.

Science Audit

An important and exciting scientific event of 1999 was our Science Audit by a review team of three geneticists and plant breeders. An abbreviated summary of their report is included in this issue of *The Journal*.

TABLE 1

Type and number of chestnut trees and planted nuts at the TACF Meadowview Research Farms in May 2000, with the number of sources of blight resistance and the number of American chestnut lines in the breeding stock.

	Number of		
	Nuts or Trees	Sources of Resistance	American Lines*
Type of Tree			
American	1614		91
Chinese	751	37	
Chinese x American: F_1	630	22	66
American x (Chinese x American): BC_1	1347	11	45
American x [American x (Chinese x American)]: BC_2	2723	9	65
American x {American x [American x (Chinese x American)]}: BC_3	4801	2	103
(Chinese x American) x (Chinese x American): F_2	310	3	4
[Ch x Am) x (Ch x Am)] x [Ch x Am) x (Ch x Am)]:F_3	10	1	2
[Amer x (Chin x Amer)] x [Amer x (Chin x Amer)]: BC_1-F_2	462	2	2
{Am x [Am x (Ch x Am)]} x {Am x [Am x (Ch x Am)]}:BC_2-F_2	655	3	7
Chinese x (Chinese x American): Chinese BC_1	142		
Chinese x [American x (Chinese x American)]	41		
Japanese	3	2	
American x Japanese: F_1	1	1	1
(American x Japanese) x American: BC_1	73	1	1
(American x Japanese) x Japanese: Japanese BC_1	5		
Castanea seguinii	48	1	
Chinese x Castanea pumila: F_1	2		
Large, Surviving American x American: F_1	452	10	12
(Large, Surviving American x American) x American: BC_1	189	2	7
Large, Surviving American x Large, Surviving American: I_1	93	4	4
Large, Surviving x American: $F_2 = F_1 x F_1$, same LS parent	192	2	2
Irradiated American x American: F_1	44	1	1
Other	40		
Total	**14,628**		

* The number of lines varied depending on the source of resistance. We will have to make additional crosses in some lines to achieve the desired number of 75 progeny per generation within a line. In keeping with past practice, the number of lines for each source of resistance are added separately; thus, progeny from two sources of resistance with the same American parents would be counted as two lines rather than one line(this only occurs rarely).

TABLE 2

Changes between 1999 and 2000 in the number of chestnut trees and planted nuts of different types at TACF Meadowview Research Farms, including changes in the number of sources of blight resistance and the number of American chestnut lines in the breeding stock.

	Increase or Decrease* in Number of		
	Nuts or Trees	Sources of Resistance	American Lines
Type of Tree			
American	226		9
Chinese	258	4	
Chinese x American: F_1	173	4	11
American x (Chinese x American): BC_1	337	0	4
American x [American x (Chinese x American)]: BC_2	-30	1	5
American x {American x [American x (Chinese x American)]}: BC_3	1528	0	30
(Chinese x American) x (Chinese x American): F_2	0	0	0
[Ch x Am) x (Ch x Am)] x [Ch x Am) x (Ch x Am)]:F_3	1	0	0
[Amer x (Chin x Amer)] x [Amer x (Chin x Amer)]: BC_1-F_2	-2	0	0
{Am x [Am x (Ch x Am)]} x {Am x [Am x (Ch x Am)]}:BC_2-F_2	27	1	3
Chinese x (Chinese x American): Chinese BC_1	0		
Chinese x [American x (Chinese x American)]	0		
Japanese	0	0	
American x Japanese: F_1	0	0	0
(American x Japanese) x American: BC_1	40	0	0
(American x Japanese) x Japanese: Japanese BC_1	0		
Castanea seguinii	0	0	
Chinese x Castanea pumila: F_1	-7		
Large, Surviving American x American: F_1	110	0	1
(Large, Surviving American x American) x American: BC_1	-9	0	0
Large, Surviving American x Large, Surviving American: I_1	0	0	0
Large, Surviving x American: $F_1 = F_1 x F_1$, same LS parent	-6	0	0
Irradiated American x American: F_1	44	1	1
Other	6		
Total	**2696**		

* The decrease in BC_2 trees reflects roguing of trees with inadequate levels of blight resistance, combined with low levels of production of BC_2 trees intended to be planted in Meadowview. The increases in BC_1 and BC_3 trees and in (Am x Jpn) x Am BC_1 and in Large Surviving American F1 trees reflects further breeding. The increase in irradiated American x American chestnut trees reflects correction of an incorrect classification from 1999.

TABLE 3

The American Chestnut Foundation 1999 nut harvest from controlled pollinations and selected open pollinations.

Nut Type	Female Parent	Pollen Parent	Pollinated			Unpollinated Checks			Number of American Chestnut Lines*
			nuts	bags	burs	nuts	bags	burs	
BC_1	Miller 72-211 F_1	American	15	23	47	0	3	6	1
BC_1	American	Nanking F_1	50	51	149	0	4	10	4
BC_1	Mahogany F_1	American	148	180	276	0	21	35	3
BC_1	Nanking F_1	American	365	194	469	11	19	38	4
BC_2	S.LotR4T23 BC_1	American	6	18	41	0	1	5	1
BC_2	Mahogany BC_1	American	103	99	181	0	9	17	2
BC_2	Nanking BC_1	American	527	327	936	3	27	52	3
BC_2	OTR1T7 BC_1	American	4	55	134	0	6	9	1
BC_2-F_2	Clapper BC_2	Clapper BC_2	122	190	550	0	12	45	1
BC_2-F_2	Clapper BC_2	op	2399	open pollinated				6	
BC_2-F_2	Graves BC_2	Graves BC_2	102	146	312	1	7	23	2
BC_3	American	Clapper BC_2	719	785	1758	2	71	177	36
BC_3	American	Graves BC_2	317	316	683	3	29	55	12
BC_3	Clapper BC_2	American	750	601	1918	0	47	148	12
BC_3	Graves BC_2	American	1124	1120	2748	1	73	210	21
Chin I_1	Chinese	Chinese	533	496	723	8	37	67	8
F_1	Other Chinese	American	109	91	158	0	8	16	6
F_1	Nanking Chinese	American	159	165	346	0	17	28	4
LS F_1	American	Corrigan	41	30	53	0	2	6	2
LS F_1	American	NC Champ	206	94	200	9	6	16	4
Unclassified			4	73	184				
Total Controlled Pollinations			**5404**	**5054**	**11866**	**38**	**399**	**963**	

*The number of American lines for this table is restricted to the number of American chestnut trees that were direct parents, not grand parents, of progeny.

TABLE 4

Number of chestnut trees in various blight resistance classes for three F3 families and a composite of families from open-pollination of Clapper BC_2 trees. Results from control trees also are presented.

	Resistance Class*							
Family	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**5**
Controls								
'Meiling' Chinese	2	1	1	0	0	0	0	0
Seedling Chinese	0	1	5	0	0	0	0	0
'Kuling' Chinese x American	0	0	2	1	1	0	0	0
Seedling American	0	0	0	0	0	0	1	5
F_3 A	1	1	0	0	0	0	0	
F_3 B	1	2	1	0	0	0	0	
F_3C	1	2	0	0	1	0	0	
open-pollinated 'Clapper' BC_2	1	5	43	13	75	28	82	37

* Class 1 is the highest level of resistance, where there is little or no canker expansion. Class five is the lowest level of resistance.

TABLE 5

Number of second backcross chestnut trees derived from the 'Clapper' and 'Graves' first backcross trees that were selected or not selected as having adequate levels of blight resistance between the years 1994 and 1998, and the probability of fit of these data to the hypothesis that blight susceptibility:resistance were segregating with a ratio of 7:1, acceptance of which suggests that three incompletely dominant genes might be controlling blight resistance.

	Clapper			Graves		
Year	**Selected**	**Not Selected**	**p***	**Selected**	**Not Selected**	**p***
1994	12	107	0.43			
1995	11	50	0.19	10	71	0.97
1996	14	123	0.42	6	53	0.59
1997	13	88	0.91	7	60	0.61
1998	15	64	0.08	18	146	0.56
1998	6	56	0.50	21	92	0.05
1998				1	31	0.11
Totals	**71**	**488**	**0.89**	**63**	**453**	**0.84**

*Probability values (p) greater than 0.05 imply acceptance of a hypothesis, while values less than 0.05 imply rejection of the hypothesis. Ratios of 3:1, which suggest that two incompletely dominant genes might be controlling blight resistance, and 15:1, which suggest that four genes might be controlling blight resistance, did not fit these data ($p<0.000001$).

THE TACF BREEDING PROGRAM REVIEW, CONDENSED

August 12–15, 1999

by Dr. Shawn A. Mehlenbacher, Professor of Horticulture, Oregon State University, Dr. Ronald L. Phillips, Regents' Professor of Plant Genetics, University of Minnesota, and Dr. J.P. van Buijtenen, Professor Emeritus of Forestry, Texas A&M University

(Editor's Note: This document has been significantly reduced in length. Should you desire a full-length copy, please print from the web site address http://www.acf.org/review.htm. TACF Science Staff responses to this report are available at http://www.acf.org/response.htm. You may also write TACF, P.O. Box 4044, Bennington, VT 05201 for a printed copy of the full Breeding Program Review and responses.)

INTRODUCTION

The vision of The American Chestnut Foundation to restore the American chestnut to its native habitat in the United States is being accomplished through the breeding program and various other activities….

The review team [comprised of the three authors, each with expertise in different areas of tree breeding and/or genetics] was impressed by the progress made to date in the breeding program. [TACF] scientists are focused on the goals as set forth originally by Dr. Charles R. Burnham and advanced through the many efforts of The American Chestnut Foundation. These efforts represent an exceptional example of how volunteers with a highly focused mission can accomplish a goal of broad interest to the American people but one for which federal and state funds are extremely limited. We commend the staff scientists for their dedication and sincere interest in achieving the goal. The overall goal of the Foundation is quite ambitious; that is, to restore the species in the United States. The review team is pleased that the Foundation recognizes the long-term effort required in the breeding program and that members are providing sound advice and support….

The focus of this review is principally on the breeding program and an examination of the underlying science. While the review team has several suggestions, we recognize that there is a restricted budget and that keeping focused on the primary objectives is quite important….

Defining and revisiting objectives periodically is an essential part of a vibrant research program. In this regard, the objectives should be more explicitly stated relative to the breeding program per se, as opposed to the goal of restoring the chestnut to its native range and perhaps to future goals of improving timber quality, nut production, and other characteristics….

Parents

Choosing Parents. The backcross method being used requires identification of sources of blight resistance (donor parents) and susceptible American chestnuts (recurrent parents).

Sources of Resistance. Most of the advanced selections in the TACF breeding program now incorporate resistance from one of three sources: Clapper, Mahogany / Graves, and Nanking. Additional sources would be highly desirable. We encourage the TACF scientists to assemble a collection of potentially resistant parents (25-30) and to evaluate [them], and to use the best 15 or so as parents. The review team is concerned about the Clapper defect…. This problem may become even more serious in the future.... The possible existence of pathogenic variation in the chestnut blight fungus is an additional reason for the use of additional sources of resistance….

American Parents. The number of American parents used in each backcross has been limited in past years. Their inherent disease susceptibility leads to early death, although mud packs can prolong their lives somewhat. Efforts are now being made to broaden the genetic base on the American side, and we commend these efforts…. By using cooperators in Pennsylvania, Tennessee, and other states, diversity in the American chestnut can be incorporated…. The continued use of large surviving American trees in breeding is encouraged, as some of these trees do indeed appear to transmit some resistance.

Seed Stratification. Procedures for seed stratification appear to be adequate. Refrigerated storage facilities have been adequate in past years, but additional space may be needed in the future.

Crossing

Pollination. The large number of outcrosses (pollen contamination) in past years is a concern. We encourage the staff to further investigate different materials (bagging materials, bag sizes, etc.) and procedures (time

of bagging, pollen collection and storage, etc.) to reduce the incidence of outcrossing. Removal of adjacent trees and some pruning would be expected to result in increased flowering and set on selected trees….

Research on Pollen-Stigma Incompatibility. At this time, we feel that this research problem is of low priority….

Breeding Strategy. The backcross method appears to be working, as advanced selections from the TACF breeding program combine desirable growth habit with a moderate level of resistance. A higher level of resistance is expected in the BC_3F_2 generation. We support continued use of this method (see Figure) and the development of additional BC_3F_2 selections. These BC_3F_2 selections could be clonally propagated (by grafting or stooling) and used as parents of a seed orchard. BC_3F_2 selections could be released as clones for limited use (homeowners, small farmers), but the vast quantities of trees needed for reforestation would be from seed orchards. These same BC_3F_2 selections (or their BC_3F_1 parents) could be used as parents for additional generations of backcrossing. Additional backcrosses may be needed to achieve adaptation to local conditions, and State chapters could play a very important role…. [A]dvancing [additional sources or resistance] to the BC_3F_2 generation by staff at Meadowview should receive a higher priority than advancing the Clapper, Mahogany / Graves, and Nanking sources to the BC_6….

FIGURE

Chinese x American = F_1
F_1 x American = BC_1F_1 *(Note: Clapper and Graves are BC1F1 selections)*
BC_1F_1 x American = BC_2F_1
BC_2F_1 x American = BC_3F_1
BC_3F_1 x BC_3F_1 = BC_3F_2 *(Controlled pollinations)*

Then with the population of BC_3F_2:
- clonally propagate the best selections
  - ➔ recurrent selection (based on disease resistance, growth rate, form, wood quality)
  - ➔ further backcrossing (if needed)
- Establish seed orchards ➔ seed or seedlings for distribution
- Progeny tests (or further evaluation of parent clones)
- Remove inferior clones from the seed orchard.

Argument Against Going to BC_6. At the BC_3, it appears that the desired combination has been obtained of blight resistance and American type trees. Many examples can be cited in fruit and nut crops where the objectives were met long before the BC_6. If the BC_3 is the last generation, then locally adapted American parents should be used to produce this generation.... Remember that for forest establishment, a heterogeneous population of heterozygous trees should be the objective. It seems that the use of unevaluated American parents in the backcross generations is equally (if not more) likely to introduce undesirable traits than the relatively few Chinese genes that would remain....

Arguments for Advanced Backcrossing. The importance of the advanced backcross program depends on many factors that are largely unknown at this time and revolves around how deleterious the 6-7% Chinese genes remaining in the released germplasm will be to the ultimate survival and uses of the material.

Although theory dictates that the recovery of American genes in the BC_3 will be 93-94%, this is an average figure.... The actual percent American in any one selected line may be rather different.... [T]he remaining Chinese genes could lead to problems in the future.

A trade-off exists between utilizing breeding time and resources for advanced backcrossing versus starting over with additional sources of resistance. The review team favors the development of useful materials with other sources of disease resistance since we know that susceptibility to the blight can devastate the species and that the pathogen will undoubtedly undergo change over the years and could well overcome any specific resistance genes bred into the American germplasm.... We would recommend that further backcrossing, if performed, be done with the aid of molecular markers to maximize the recovery of the recurrent parent and perhaps reduce the number of backcross generations needed... [and] that BC_3 trees be used based on BC_3F_3 progeny tests that will be available. The State chapters of The American Chestnut Foundation may play a role here since many will be crossing the released germplasm to locally adapted American trees....

Selection

Seedling Growth. An average of 80% survival from direct planting of stratified seeds in the orchard would be adequate. Growing seedlings in the

greenhouse and then transplanting the seedlings to the field is an alternative that the staff should consider. Greenhouse facilities and transplanting will be necessary if marker-assisted selection is adopted. The planting of seedlings in the field in such a way that collected data lends itself to statistical analysis is commended. The inclusion of resistant Chinese checks is part of the field plot layout…. The precocity shown by many selections was striking, allowing a breeding cycle of only six years. This is truly remarkable for a timber crop species…. It has been repeatedly shown that rapid seedling growth is a key to early fruiting (precocity) in fruit and nut crops.

Selection for Blight Resistance. The currently-used methods of evaluating levels of resistance are the result of years of effort on this subject and we are confident that genetic resistance is being identified through these tests. Investigation of alternative methods that would allow selection at an earlier age, either in the field or greenhouse, or using detached shoots, is encouraged.

Gall Wasp and Other Pests. At this time, TACF staff should keep abreast of the current location of the gall wasp in the southeastern states. By encouraging plantings by cooperators in areas where the gall wasp is present, sources of resistance to this pest might be identified.

Evaluation

Evaluation of Growth Habit. In seedling blocks, a range in growth habit is evident. The vigorous, upright growth of the best BC_3F_1 selections is striking. A wide range in precocity is also evident, and there may be an undesirable relationship between desirable growth habit and lack of precocity. Experience over the next few years will reveal if there is need for concern.

Evaluation of Morphological Traits. The leaf hairs, green twigs, and large stipules of the Chinese species mapped to linkage group C in the Mahogany F_2 population, and these loci were independent of the three identified resistance loci. Thus, in this population, it appears that there would be no disadvantages to selecting against these Chinese traits. Since so many loci are located on linkage group C, there would be no selection pressure against Chinese alleles at loci on other linkage groups. This may not be the case in other populations.

Clonal Propagation of Selections. Clonal propagation by grafting can be done routinely in chestnut…. Grafted trees of advanced selections

could also be used to establish trials to determine their region of adaptation. Also, grafting would allow the establishment of seed orchards from selected parent clones. Stooling is an alternative method to grafting and has been used widely in France. At this time, tissue culture does not appear to be a cost-effective means of clonally propagating chestnut selections. Clonal propagation would also allow more efficient use of prime chestnut land by the breeding program. Effective breeding strategy requires rapid advance through the generations, and with limited land and resources, this means a need to eliminate seedlings that do not meet the stated objectives of the program…. Wise use of land will become even more critical in future years, as more resistance sources are used and population sizes increase….

Molecular Markers. From a genetics standpoint, the goal of the chestnut breeding program is to recover from an interspecific hybrid the chromosome segments carrying genes of the American chestnut except for those that confer blight resistance. That is currently being accomplished based on using the American chestnut as the recurrent parent in sequential crosses and selection for disease resistance and readily visible American morphological traits. Since several of the traits readily distinguishing American and Chinese types are now known to be located on the same chromosome, selection on the basis of morphological traits alone may be problematic.

Molecular markers are "neutral markers" in the sense that they have no effect on the phenotype of the plant. Hundreds and even thousands can be monitored in a single cross. The DNA fragments used as molecular markers distinguish American from Chinese…. Scoring segregating progenies (mapping populations) for the markers and traits of interest allows associations to be made between the markers and the genes controlling the trait….

Such molecular markers could be immediately used in The American Chestnut Foundation breeding program for detecting pollen contamination and documenting pedigrees…. Molecular markers are also used to fingerprint germplasm for future identification, patent applications, and protection of patented materials.

[W]e do not recommend a "marker assisted" breeding approach at this time (except for disease resistance). However, we do favor the use of markers in the ways mentioned above. To accomplish this task, the review team recommends that a proposal be developed that would lead to useful molecular markers for the chestnut. This proposal can be discussed with

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OPEN CHESTNUT BURRS

[varioius funding agencies]… Although we do not recommend a molecular marker lab at this time for The American Chestnut Foundation, equipment and facilities should be provided to foster and facilitate the work….

RELEASE

Seed Orchard Design. The design proposed by Hebard consists of 8 orchard blocks of approximately 5 acres. Each block is divided into 16 sub-plots consisting of one BC_3-F_2 family. Within each subplot the 160 full-sib seedlings are arranged in 5 rows of 32 trees each. Spacing is one foot within rows and 7 foot between rows. After testing for blight resistance only one tree will be left per subplot. This will result in a final stocking of about 30 trees per acre. The total size of the orchard will be approximately 4 acres. Production at age 10 is estimated at 1500 lbs. per acre or 6000 lbs. for the whole orchard. This is equivalent to about 480,000 nuts. The blocks are laid out in a staggered pattern to minimize crossing between related individuals.

An alternate layout could be achieved by laying out the subplots in rows and shifting the crosses two positions to the right each time a new row is started. This would give adequate spacing between related indi-

viduals and would not require staggering the blocks thus leading to a more efficient utilization of the available land….

Expansion of Orchards. The 4-acre orchard is probably adequate while the orchard is being progeny tested, since the early releases will be primarily for the purpose of evaluation. Once some or all of the selections have proven resistant, the orchard could be expanded quickly by grafting the resistant parents. Not enough information is available to estimate the size of the expansion needed. If the need for seed is substantial, serious consideration should be given to contracting out the work….

Progeny Testing. The selections made in the BC_3-F_2 orchard need to be tested for resistance, for the absence of Chinese characteristics, and for growth. If suitable markers are available, homozygosity at the putative resistance loci could be determined on the selections themselves. This might best be done on all the candidates that made the short list. The results would be a major consideration in making the final selections. It would also be desirable to make controlled crosses on the selections and test them for resistance by inoculation. Finally the seeds given to the cooperators should be used to evaluate performance in the field. This should be done according to a valid statistical design.…

Germplasm Agreement. [T]he panel believes that it would be useful to have two different agreements. One would cover materials provided to cooperators for research purposes; the other would be for contractors, who agree to mass produce propagules for the marketplace.

It would also be prudent to seek patents for appropriate materials such as clones of selections in the BC_3-F_2 generation and beyond.

Equipment

Basic equipment is available, but somewhat old. It might be wise to replace one of the trucks in the near future. A smaller tractor will be needed to be able to work in the narrow rows in the BC_3-F_2 orchard. A -80° C freezer will be needed for storage of pollen and samples for DNA extraction. Facilities for seed stratification are limiting and an additional refrigerator will be needed. Better office space is needed and plans are being made to make some improvements on the house at the Wagner farm and turn it into office and laboratory space. A handheld computer would be very handy for recording of measurements, storing maps, and storing information on germplasm, tests, and pedigrees.

RECOMMENDATIONS:

1. **We endorse** the backcross strategy as appropriate for the stated objective of combining blight resistance with American timber-type growth habit.

2. **We recommend** the use of additional sources of resistance and the development of BC_3F_2 selections from each of these sources.

3. **We recommend** expansion of the number of American parents used in breeding.

4. **We recommend** incorporation of clonal propagation (by grafting or stooling) as a routine procedure in the breeding program.

5. **We recommend** investigation of methods to increase seed yields from controlled pollinations and seed orchards while minimizing contamination.

6. **We recommend** more rapid elimination of undesirable seedlings from plots. The clonal propagation of the best seedlings will allow wiser use of the available land.

7. **We recommend** improved weed control and use of herbicides other than Roundup.

8. **We recommend** the extensive use of stakes in plots and labels on trees.

9. **We recommend** additional research on methods to determine levels of blight resistance, particularly methods applicable to young trees.

10. **We recommend** that seed orchards be established using clonally propagated clones, and that such orchards be established on well-drained sites where chestnuts will thrive.

11. **We recommend** that as superior selections are identified and seed (or seedlings) from seed orchards become available, that trials be established to determine adaptation of this chestnut germplasm.

12. **We recommend** that the current TACF Germplasm Agreement be replaced with two separate types of agreements. The first type would be a memorandum of understanding or material transfer agreement. This type of agreement would allow cooperators to use TACF selections in breeding and for evaluation purposes. A second type of agreement should be developed to cover the propagation and marketing of new cultivars developed by TACF.

13. **We recommend** the establishment of an attractive, well-manicured, and well-labeled collection of parents and advanced TACF selections at the entrance to the Wagner Farm as part of TACF's efforts to educate the public about its activities.

14. **We recommend** the purchase of a freezer (-80°C) for storage of leaf and pollen samples.

15. **We encourage** the use of DNA markers, primarily through grants or contracts with outside agencies, for the detection of pollen contaminants, mapping resistance loci, recovery of the recurrent parent genome, and possible patent protection of advanced selections.

(Editor's Note: Dr. Norman E. Borlaug, 1970 Nobel laureate for crop breeding research and Honorary Board Director of The American Chestnut Foundation, provides commentary on the TACF Breeding Program Review in a letter to executive director Marshal Case.)

COURTESY OF CHARLES H. JOSLIN AND THE MASSACHUSETTS AUDUBON SOCIETY

CHESTNUT BLOSSOMS

January 25th, 2000

Mr. Marshal Case
The American Chestnut Foundation
PO Box 4044
Bennington, VT 05201-4044

Dear Mr. Case:

….I have been fascinated by the progress that has been made in the incorporation of blight resistance into the American Chestnut. All who have collaborated in this adventure, are to be congratulated for the progress that has been achieved.

I can fully appreciate the importance of this project, for I was a young forester working at the Northeastern Forest Experiment Station in 1936 at the Hopkins Experimental Forest at Williamstown, Massachusetts when the last of the big chestnuts were being killed by the blight. I was too young and inexperienced at that time, to really understand its implications. But as I continued my studies, and especially when I studied forest pathology and later plant pathology, I came to realize what a disaster I had witnessed in those early years.

After shifting from my forestry career to genetics and plant pathology, I have spent most of my career breeding of wheat, for high grain yield, broad ecological adaptation and resistance to diseases-especially against the rust fungi (three species of Puccinia). Having worked in innumerable countries around the world, I have come to appreciate the great genetic variation in pathogens that attack our crops and forest trees. For that rea-

son, I am especially fascinated by the work you are doing.

I would like to make a few comments on the TACF Breeding Program Review that was done in August 12 to 15, 1999. I believe this is an excellent review and I want to congratulate the Committee who made this study and published the report. I do want to add a couple of comments, however, which I hope might be helpful.

Based on my long experience in back-crossing to control various diseases of wheat, while at the same time trying to improve grain yield and agronomic characters, I have come to realize that by growing a large population of F_1 plants of the second back-cross (BC_2), and selecting vigorously in the progeny for the morphologic phenotype of the recurrent commercial parent with seedlings with resistance to the disease you are breeding for, you can skew the selection more rapidly back towards the morphologic phenotype of the recurrent parent than if it is done at random, in which case you will probably need to use BC_3 or BC_4.

If this procedure is followed, and if a large number of F_1 seeds of the second back-cross are used, I think that you can greatly save on the land required for "out-plantings" and at the same time make more rapid progress in obtaining good blight resistance in forest phenotypes similar to those of the native American Chestnut. If this procedure is followed in the BC_2, rather than carried on to the BC_3 and BC_4, a large percentage of those seedlings in the last back-cross (BC_2) will be within the acceptable morphologic traits of the native American Chestnut.

I would urge that you inoculate aggressively all segregating populations with *Endothia parasitica* inoculum taken from infected sprouts taken from as many different parts of the range of the American Chestnut as possible.

If your breeding program is using more than one species as a source of resistance to the blight organism, I suggest you attempt to make a few crosses between the different F_1 single crosses.

The second point which I would like to make, once you have a few outstanding seedlings identified in the second back-cross (BC_2) with good resistance to Endothia in acceptable morphologic makeup, I would suggest that some vegetative cuttings or clones be tested for their scope of resistance in the several areas of the original home of the Chestnut blight pathogens, namely China or Korea.

I feel quite confident if a concerted effort is made that satisfactory arrangements can be made for such testing in the People's Republic of

China, where you are likely to have good collaboration. I am sure that through the International Union of Forest Research Organizations (IUFRO), such arrangements can be made.

If you encounter unmanageable problems in making arrangements for such testing, perhaps I can serve as an intermediary, since I have been working in cereal production and disease problems in the People's Republic of China for more than 25 years.

With best wishes for continued success on this very worthy program — the TACF Breeding Program for incorporating chestnut blight resistance into the American Chestnut — I remain,

Sincerely,

Norman E. Borlaug

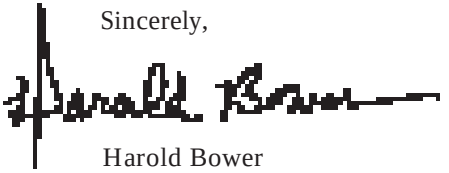
(Editor's Note: A response to William Lord's article, "Chestnuts and Wildlife —Then and Hereafter," in the winter 1999-2000 issue of The Journal. *In that same issue, John J. Morgan and Sara H. Schweitzer write on the chestnut—wild turkey connection, "The Importance of the American Chestnut to the Eastern Wild Turkey.")*

February 25, 2000

Dear Friends,

It is interesting to read William Lord's writings about chestnuts and wildlife. It brings to mind the connection between oak and chestnut silviculture and turkey ecology. At the time that the Europeans were coming to America to occupy these lands, there were large flocks of turkeys, some containing hundreds and others containing thousands of birds. These birds are gallinaceous, meaning they are chicken-like and scratch the ground while foraging. The new maple seedlings would be ripped out of the ground, while the oak seedlings often remained intact (because of a stronger root?). "Ah!, you say, the turkeys would eat the acorns before they could grow." Some would, but if there were chestnut seeds available, turkeys often overlooked the acorns. So, the ground was scarified, with the litter layer torn apart and the acorns had good root-soil contact immediately. (Incidentally, the maple samaras were some of the earliest mast in the year available to the birds.) So, some of us believe that turkeys were more responsible (than fire) for establishing oaks (especially white oaks) in the forest on the mesic sites as long as the chestnuts were available (food level #1). The sooner you have chestnuts for field planting, the sooner the oaks will benefit. Thank you!

Sincerely,



Harold Bower
Forester and TACF member
Mount Vernon, OH


memories

EXCERPTS FROM

IN A WILD PLACE: A NATURAL HISTORY OF HIGH LEDGES

by Ellsworth Barnard

In northwestern Massachusetts lies a little known Wildlife Sanctuary, High Ledges. Ellsworth Barnard, a retired University of Massachusetts (Amherst) English professor, grew up on this property, observed its natural changes throughout the years, and ensured that others will be able to enjoy it as well by donating it to the Massachusetts Audubon Society. In his book, ***In a Wild Place****, he celebrates his years of intimate knowledge of this site in gracefully written discussions on birds, trees, flowering and non-flowering plants, and ecological niches of the land. Most importantly to TACF members, he dedicates a chapter to the American chestnut, partially reprinted here.*

Wood engravings by Charles H. Joslin, a science illustrator and well-known wood engraving artist, elegantly illustrate the beauty of High Ledges in ***In A Wild Place****.*

(***In A Wild Place: A Natural History of High Ledges***, 1998, reprinted with permission of author Ellsworth Barnard, illustrator Charles H. Joslin, and publisher the Massachusetts Audubon Society. To receive a full copy of *In A Wild Place*, contact Kristin Eldridge at the Massachusetts Audubon Society, 781-259-9506 ext. 7255.)

Chapter 1: The View from the Ledges

There is a spot along the crest of the ridge that, in the town of Shelburne, forms the steep eastern slope of the Deerfield River valley, where a rock fall in some distant past has left an overhanging cliff known locally as the High Ledges. Traditionally, this was a favorite destination for hikers….

Visitors to the site were more than rewarded for the effort. Indeed, though "breathtaking" is a trite term, many first-time visitors have found it accurate. Facing a little south of west, one looks down almost a thousand feet to the village with its toylike houses peeping out from among the sugar maples that in summer shade the streets and in autumn flame

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A VIEW FROM THE LEDGES

with color. Beyond, the hills of Buckland rise sharply to the still higher summits of the town of Ashfield….

A mile or two north of the village, the river makes an abrupt turn to the west; and from High Ledges one can look straight up its v-shaped valley, with a series of ridges running down to the river on either side, each one rising beyond the other toward the level line topping the Hoosac range—a line broken, beyond, by the massive upward surge of Mount Greylock toward a perfect dome whose summit is the highest point in Massachusetts.

This is the panorama when the air is clear and the face of nature is unshadowed. But the scene is ever-changing from season to season, from day to day, from hour to hour….

So, here is one small, precious piece of earth that will remain unspoiled, where refugees from the restless rush of the human world may find in the presence of nature a moment of peace.

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MAPLE TREE

Chapter 5: The Fall and Rise of the American Chestnut

Though in my childhood innocence I had no notion of the many facets of the world of trees, and found only an unreflective pleasure in their companionship as I wandered about the farm, there is one species that stands out in memory more vividly than all the rest…. This is the American chestnut.

Among the brightest memories of my early childhood is that of some blue and gold October morning when I would set out with one or more of my older brothers to "go chestnutting." Chestnut trees were common around the farm, and in early July they would be covered with thick clusters of cream-colored, yarnlike blossoms, to be followed by small, green, spiny burrs, which would grow during the next three months to spheres a little smaller than tennis balls. Then, in early October, they would split into four segments, which, opening as if on hinges, would reveal a

pale, gleaming, velvety lining that offered a dramatic contrast to the harsh exterior; and inside, cushioned in a row, there would be three glossy, reddish brown nuts, whose color would later darken to that to which they lent a name….

Tapering from a broad base to a pointed tip, they were about half the size of those sold in markets, which are imported mostly from Italy (where in season one finds the hillsides here and there softly quilted with creamy blossoms). Beneath a hard but thin covering was a crunchy nut with a distinctive flavor that a child's adventurous taste found agreeable. But only when roasted (or, less romantically, boiled) would it acquire the melting sweetness that is unique.

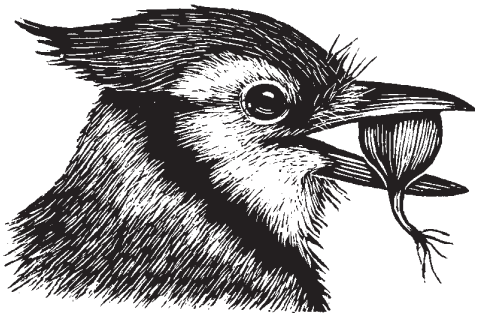
A few years later, my way to school was regularly "across lots" through a neighbor's pasture where there was a scattering of huge old chestnut trees, and under their spreading branches the ground in autumn would be so thickly strewn with fallen nuts that, on my way home, it would only take a few minutes to fill my lunch box to overflowing….

There was, to be sure, a downside to these joys. The following summer, the dry, spiny burrs, sometimes half-hidden beneath dead leaves, would lie in wait for bare unwary feet, and the spines would all too readily transfer themselves to the flesh pressed against them. And, though so slender as to be barely visible, they pierced deep and produced a pain when pressed upon that made one willing, for relief, to endure the sharper but temporary pain of extraction by means of a firmly held needle.

A delectable but not essential addition to the human diet, the nuts were a staple food for many kinds of wildlife. Perhaps no other form of plant life contributed so much to so many of the species of mammals, from mice to squirrels to deer and bear, that inhabited the eastern forests, as well as larger birds such as jays, crows, ruffed grouse, and wild turkeys.

But to the human population, the role of the nuts as a source of food was minor compared to that of wood, whose qualities gave it a unique value. Though it was relatively soft when green, when seasoned it took on an ironlike hardness. An old-time woodsman was quoted as saying, "Nothing dulls a crosscut saw as fast as a dead chestnut." It also, though straight grained, possessed unusual tensile strength, which made it less likely than many other woods to break or splinter under stress. Add to this its almost unique resistance to decay and to insect attacks, and the reason is clear for its multitude of uses: for railroad ties and utility poles,

ILLUSTRATIONS COURTESY OF CHARLES H. JOSLIN AND THE MASSACHUSETTS AUDUBON SOCIETY



BLUE JAY AND CHESTNUT

for the sills and joists and rafters and siding of frame buildings, and planks for the floors of barns and bridges and other structures subject to heavy use. It was also a standard wood for kitchen furniture, since, though plain grained and not richly colored, it was not unattractive and would withstand much wear and tear. Before the advent of barbed wire, it supplied the rails for fences that were easier to erect than stone walls…. And as firewood, dry chestnut burned with a quick hot flame that made it ideal "sugar wood" to fuel evaporators used in making maple syrup. Finally, the bark was a main source of tannin, used in tanning hides.

WHITE-FOOTED MOUSE

The American chestnut was also set apart from many other trees by its size. A mature tree might measure four feet in diameter at the base and eighty feet or more in height. I remember that at the family sawmill a twelve-foot chestnut log sawed out 360 board feet of lumber.

This account makes clear the immensity of the tragedy when, early in the twentieth century, the American chestnut was swiftly and, it seemed, permanently destroyed by the chestnut blight….

The agent of the blight is a certain fungus whose scientific name for many years was *Endothia parasitica* but recently, through what seems to the layperson the impenetrable mystery of scientific nomenclature, has been burdened with a name as formidable as the disease itself, *Cryphonectria parasitica*. The tiny spores are spread by wind, rain, and

perhaps small birds and mammals. Entering the bark through any small wound, they quickly send out a network of fine fibers called the mycelium that attacks the cambium layer (between the bark and the wood, where growth takes place) and in effect girdles the tree.

Often the first symptom of the disease is the appearance on the bark of minute orange dots, the size of small pinheads. Then, as the tree dies, a sort of brown scurf spreads over the bark, which dries and breaks open, revealing the lifeless wood beneath. All this may take place within a single year….

[M]y personal observations during the seventy years since the blight invaded western Massachusetts suggest that nature itself, unaided, has been mounting a successful response. Although, as I have said, the sprouts that sprang from the roots of blighted trees seemed destined at first to share their parents' fate, a few persisted for a longer time and achieved a larger size before succumbing, to be replaced by others that were still more stubbornly resistant. And this process has continued….

And though, in the normal course of things, I shall not live to see the outcome of this particular act in nature's endless drama, I am confident that it will have, from our limited human viewpoint, a happy ending.

RED SQUIRREL

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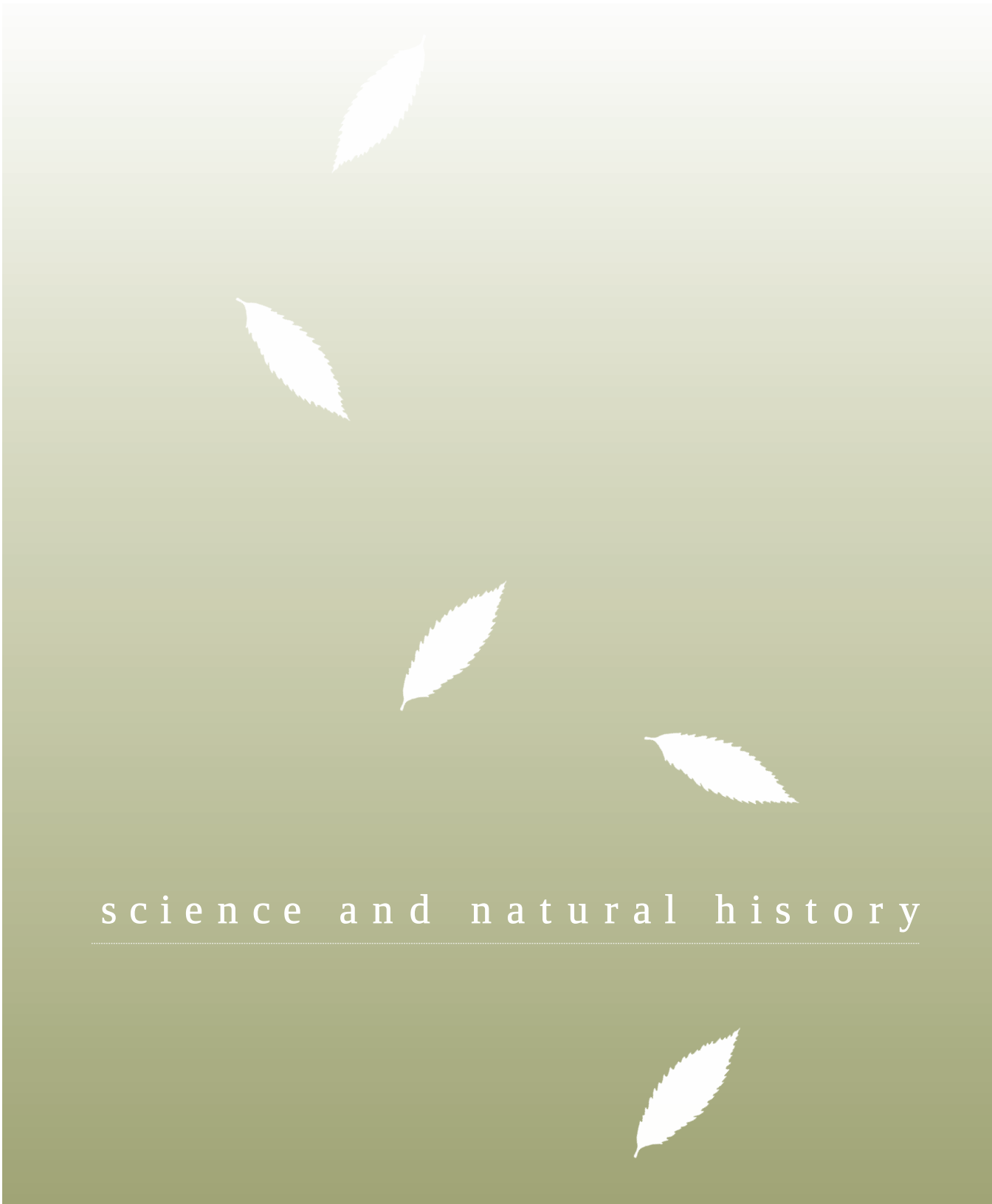
science and natural history

POPULATION DYNAMICS OF AMERICAN CHESTNUT SPROUTS: THE PROJECTION MATRIX APPROACH

by Douglas H. Boucher, Department of Biology,
Hood College, MD

INTRODUCTION

There are now grounds for optimism that approaches such as The American Chestnut Foundation's backcross breeding program will lead to at least partially blight-resistant American chestnuts in coming years. However, the scaling-up of such efforts from single individuals to the literally billions of trees involved in forest restoration will be a daunting task. In order to implement resistance on a forest scale, one of the things we will need to know is what conditions and how much improvement in growth and survival, will be necessary for chestnut populations again to become self-sustaining.

A striking example of the possibility of restoration has been provided by the until recently blight-free population at West Salem, Wisconsin, established from a few trees planted outside the natural range of the species in the 1880s (Paillet and Rutter 1989, Cochran 1990). Over several decades, the offspring of these trees outreproduced and outgrew the oaks and hickories that had dominated the forest in which they were planted, and took over the canopy over a substantial area. Furthermore, chestnut seedlings were found to be colonizing forests as much as 1 km away.

But the other side of the coin is shown by sites where chestnut is failing to reproduce and sometimes even declining rapidly. A chestnut sprout population studied near Mountain Lake, Virginia, showed a precipitous drop with 62% of the stems dying in just 5 years between 1982-83 and 1988 (Parker et al. 1993). Furthermore, Ribbens and Paillet (1996) have shown that even in planted groves where the blight does not yet occur, rates of seedling production can be inadequate to sustain the population.

These positive and negative examples make ecological studies of chestnut sprout populations very timely. A new way to do these ecological studies is to use the technique of projection matrix modeling, which allows us to ask "what-if" questions about possible chestnut restoration strategies (Caswell 1989, Cochran and Ellner 1992, Davelos and Jarosz 1998). This method allows us to answer the questions:

- If we are going to plant relatively resistant individuals in the wild, at what age or size should they be planted?
- If we plant trees that are only partially blight resistant, will this be sufficient? How much resistance – how much of an increase in the trees' survival and growth rates — is necessary?
- How much does the effect of better survival and growth depend on the environment — e.g. on such variables as deer browsing, squirrel predation, light levels, or climate?

This paper summarizes some early results of applying the projection matrix method to chestnut populations in two regions of the U.S.

MATERIALS AND METHODS

Study Areas

From 1995 through 1998 I annually tagged and measured chestnut stems at Gannett Hill, in the Bristol Hills of upstate New York, and since 1996, I have done the same at several sites near Frederick, Maryland. All the sites are comparable in that they are oak-hickory forests on slopes and ridges, but light conditions and herbivory vary among them quite substantially. The population at the Knapp farm in Germantown, MD, was tagged in the summer of 1996, three others in the summer of 1997, and three more (not discussed in this paper) in 1998.

The Projection Matrix

The projection matrix is simply a square table of numbers which give the probabilities that an individual of a particular size will survive, grow, and / or produce new individuals (offspring) in the coming year. These probabilities vary by the tree's size (e.g. for chestnut sprouts of different heights) so the population is divided into several "stage" categories; e.g., seedlings, saplings, small trees, medium trees, and large trees. Each number in a projection matrix (the "transition probabilities") tells how likely it is that an individual in a given stage this year (year t) will survive and grow to be in a particular stage (which may be the same stage or a different one) next year (year t+1). Thus, for example, the number corresponding to the seedling-small sapling transition probability tells what proportion of this year's seedlings (0-1 m high) will still be alive next year and that, by then, will have grown to small sapling size (1-2 m high). Since

the seedling stage is the first one and the small sapling stage is the second one, this probability (0.072 in Table 1) appears in the first column and second row of the matrix.

Together, the numbers in the projection matrix contain all the information necessary to measure how fast the population is growing or declining, and to predict how many individuals of each stage class will be present in future years. It tells us the same things that life tables, used by both actuaries and animal ecologists, express in a different form.

Field Methods

In order to construct a projection matrix, we need to collect the data that will allow us to estimate all these transition probabilities. In general, this involves marking a sizable population of individuals of all sizes and following their fate (survival, growth and reproduction) for at least one

TABLE 1

Average projection matrix for the chestnut sprout population at Gannett Hill, Ontario County Park, New York from 1995 to 1998. Stages are defined by the heights of the largest stem in each chestnut clump, in meters (0 to 1 m, 1 to 2 m, etc.). This matrix was calculated by averaging the matrices for 1995-96, 1996-97 and 1997-98. Blank entries are equal to zero.

Height category in year t+1	Height category in year t						
	0-1 m	1-2 m	2-3 m	3-4 m	4-5 m	5-8 m	8 m +
0-1 m	0.908	0.116	0.023	0.028	0.052	0.022	
1-2 m	0.072	0.817	0.114			0.030	
2-3 m	0.009	0.067	0.783	0.028			
3-4 m			0.068	0.767	0.075	0.030	
4-5 m				0.162	0.750	0.068	
5-8 m				0.014	0.099	0.765	0.133
8 m +					0.024	0.085	0.867

[AR0] In the process, observations are noted such as: groups of stems growing in clumps, which are thought to correspond to single root systems (Paillet 1993); the appearance of blight cankers; other blight symptoms; and signs of deer or antler rubbing.

year. In our studies, each stem's diameter at breast height (DBH), diameter at the base (10 cm above the ground), and height is measured. The grouping of stems into clumps, which are thought to correspond to single root systems (Paillet 1993) is noted, as are the appearance of blight cankers and other blight symptoms on each stem and any signs of deer browsing or antler-rubbing. In the spring of each year (April-May) we survey the area to look for newly germinated seedlings (which are rare). These are tagged, measured and their location noted.

We try to mark hundreds of stems over as broad a range of sizes as possible at each site. The marking and measuring is generally done in midsummer, so that we can note which individuals are flowering at the same time. In the fall of each year, we census all individuals in each population for the presence of fruits. Numbers of burrs per stem are counted using binoculars and are then combined with the seedling counts from the following spring to estimate fertilities.

Once the data are collected and the matrix is calculated, we can use it to estimate the population's growth rate (λ, "lambda"). If the population is stable, $\lambda = 1.0$ exactly; increasing populations have $\lambda > 1.0$ and decreasing populations have $\lambda < 1.0$. By changing different figures in the matrix, we can see how they would affect λ, and in particular whether they could make a declining population into a stable or increasing one. Other calculations with the matrix allow us to estimate how old individuals are (Cochran and Ellner 1992) without needing to do tree-ring analysis — which in the case of the chestnut can be both inaccurate due to repeated sprouting and dangerous due to its providing infection points for the blight.

Results

A Stable Chestnut Population in New York

At the New York site the matrices for the three years 1995-1998 were quite similar, so I used the average matrix for the whole period (Table 1). Analysis of this matrix showed:

- The population is nearly stable at this site ($\lambda = .9939$) indicating a decrease of the population by only about 0.6% each year.
- However, an average of 9.2% of the individuals each year show "degrowth" (reduction in height). Most of these have severe blight symptoms, and about two-thirds of them also show signs of heavy deer browsing.

- As a result of this "degrowth", the size distribution of the population is shifting towards lower heights. Simulation using the average projection matrix predicts that, over a 50 year period, the number of trees over 2 m in height will decline by 54.6%.

Thus this population appears to be roughly in equilibrium with the chestnut blight. However, over time the population will have fewer and fewer tree-size individuals and more and more small sprouts.

Growing and Declining Populations in Maryland

The trees at the Knapp site in Maryland are on average considerably larger than the New York population, and fruit production at this site is also substantially greater. In 1996-97 this population was growing rapidly — by about 12% per year, according to its value of λ (1.118). This may not seem particularly rapid, but at this rate the population would double in just 8 years and would increase ten-fold in just 25 years.

The Knapp site and the three other sites in Maryland which were tagged in 1997 vary considerably in light level, making it possible to compare in 1997-98 how population growth rates (measured by λ) and mortality rates were affected by light. The highest population growth rate in 97-98, and the only λ above 1.0, was at the intermediate-light-level Knapp site (λ = 1.043). Populations were stable or declining at sites with both high light levels (Right Hand Fork Road, λ = .869) and low light levels (Gambrill, λ = 1.000; Coleman, λ =0.915).

Analysis of variance of tree mortality rates at the Maryland sites, using the light level at the site and the 1997 size of the tree as independent variables, showed a significant effect of light level ($F=6.79$, $P = .019$) and a weakly significant effect of tree size ($F=3.15$, $P = .094$). Mortality was highest at the two sites with the highest and lowest light levels (Right Hand Fork Road and Coleman, respectively).

Discussion

These results show how population matrix modeling can provide ecological information that will be useful for chestnut restoration. For many years, the principal role of the American chestnut in ecology has been as a lesson to students of the potential devastation that can come through

the introduction of exotic species and as the creator of a natural experiment in succession through its disappearance. Foresters' interest in the species gradually declined as it no longer reached tree size, while the overwhelming impact of the blight made its population dynamics seen atypical among native plants. And so, although excellent ecological studies have been done in recent years (e.g. Brewer 1995, Davelos and Jarosz 1998, Griffin 1989, 1992, Griffin et al. 1991, Paillet 1993, 1996, Paillet and Rutter 1991, Parker et al. 1993, Ribbens and Paillet 1996, Russell 1987, Stephenson et al. 1991), most of the effort to restore the American chestnut has come from disciplines other than ecology.

Now, nearly a century after the blight's introduction, we hope to be reaching a point where we will be able to partially reverse its devastating effects. In this context, ecologists using the techniques of matrix population modeling can play an important role. We will not be the ones who discover "the cure," but we hope to offer some help to those who do.

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AMERICAN CHESTNUT LEAVES

COURTESY OF CHARLES H. JOSLIN AND THE MASSACHUSETTS AUDUBON SOCIETY

PRESERVATION OF AMERICAN CHESTNUT GERMPLASM BY CRYOSTORAGE OF EMBRYOGENIC CULTURES

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INTRODUCTION

During the past decade, research in our lab has focused on in vitro clonal propagation of a number of hardwood forest trees via somatic embryogenesis, a tissue culture process that produces clonal populations of structures resembling seed embryos. These somatic embryos can be germinated to produce seedling-like plants (somatic seedlings). Somatic embryogenesis is a very powerful tool, having the potential not only to generate thousands of clonal plants, but also to provide a route for producing genetically engineered plants. A decade ago, our lab began research aimed at regenerating American chestnut via this process, with the long-term goal of introducing fungal resistance genes into the cultures and regenerating transgenic, blight-resistant trees from them. We were able to initiate embryogenic American chestnut cultures and to use microprojectile bombardment to introduce foreign DNA into the cells (Merkle et al. 1990, Carraway et al. 1994, Carraway and Merkle 1997), but we failed to regenerate transgenic trees. Dr. Charles Maynard's lab at the State University of New York at Syracuse, has also adopted this strategy and has made significant progress towards producing transgenic chestnut trees with genes that may confer blight resistance (Maynard et al. 1998, Xing et al. 1999).

Although we have suspended for now our transgenic research with embryogenic American chestnut cultures, we continue to try to improve the frequency of somatic seedling production from them, which has been very low in our hands. One source of this difficulty we discovered was that, compared to embryogenic cultures of most of the other species with which we work, American chestnut cultures rapidly lose their ability to produce somatic embryos capable of germinating into somatic seedlings. For example, while embryogenic yellow-poplar cultures maintained by serial culture for up to 5 years can still produce plants, our American chest-

nut cultures seem to completely lose this ability within 2 years. Thus, long term maintenance of the cultures by serial transfer to fresh medium is not a suitable way to keep productive cultures of this tree. This problem is compounded by the fact that it takes several years of growth in the field to evaluate the performance of trees (of any species) derived from somatic seedlings. Thus, by the time trees derived from the embryogenic cultures have been tested in the field, the cultures from which they were derived have long since lost their capacity for embryo and plantlet production. Other problems associated with long term-maintenance of cultures by serial transfer include increased risk of contamination, the potential for unwanted new genetic variation to arise in the cultures (somaclonal variation) and, of course, the labor and supply costs of transferring the cultures to fresh medium every 3-4 weeks.

Because of these problems associated with long-term maintenance of cultures, we began testing protocols for long-term storage of embryogenic cultures of all the species with which we work. The most promising long-term storage approach is cryopreservation (or cryostorage) in which cultures are frozen in liquid nitrogen (LN_2), which has a temperature of -196° C. At this temperature, living cells virtually halt metabolism and therefore can be held indefinitely without transfer or its associated problems. Fortunately, embryogenic cultures make excellent material for cryopreservation, and embryogenic cultures of several forest trees have been stored this way. While most of these reports have involved conifers, such as white spruce (Kartha et al. 1988), hardwood embryogenic cultures should also make excellent candidates for cryopreservation. Here we report the successful cryostorage and recovery of American chestnut embryogenic cultures.

Materials and Methods

The two embryogenic culture lines (designated A and B) used in the experiment were derived from seeds collected from two different American chestnut trees in 1996 and 1997 and cultured following protocols we have described previously (Merkle et al. 1991, Carraway and Merkle 1997). Immature nuts used to start these cultures were generously provided by personnel at The American Chestnut Foundation's Research Farm at Meadowview, Virginia. Embryogenic cultures were maintained on semi-solid woody plant medium (WPM; Lloyd and McCown 1980) supple-

mented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g/l sucrose and 1 g/l casein hydrolysate, and gelled with 3g/l Gel-rite gellan gum.

For cryostorage of American chestnut cultures, we adapted a cryostorage protocol originally developed for radiata pine by Hargreaves et al. (1999). Previously, we had successfully tested variations of the same protocol with embryogenic cultures of yellow-poplar and sweetgum. The two most successful treatments from experiments with these hardwood trees were selected for testing with American chestnut.

The cryopreservation experiment had three phases: preconditioning, freezing and recovery. For preconditioning, approximately 1 g of embryogenic culture material was inoculated into 25 ml Erlenmeyer flasks containing 2.5 ml of liquid osmotic pretreatment medium, which was the same as the maintenance medium described above, but supplemented with 0.4 M sorbitol. The negative osmotic potential of the sorbitol-supplemented medium draws water out of the cell, preventing cell bursting due to ice crystal formation upon freezing (Kartha 1985). Flasks were agitated on a gyratory shaker at 100 rpm for 24 hrs and then chilled to 4° C. Treatment with dimethylsulfoxide (DMSO) was used to prevent cell dehydration and freeze injury (Kartha 1985). Since DMSO may be toxic to plant cells at higher concentrations, two concentrations were tested. DMSO was added to the same sorbitol-supplemented medium described above to make media with a final DMSO concentration of either 10% or 20%. These media were chilled to 4° C, filter-sterilized and added (2.5 ml per gram of tissue) to the prechilled cultures just prior to freezing, giving final DMSO concentrations of either 5% or 10%.

For freezing, 1 ml aliquots of the embryogenic suspensions were pipetted into prechilled (4° C) 2 ml cryovials (Nalgene) and placed directly into a prechilled (4° C) "Mr. Frosty" freezing apparatus (Nalgene). The freezing apparatus, which is filled with isopropanol, is designed to lower the temperature in the cryovials at a controlled rate of -1° C per minute when placed into an ultra-cold freezer (-80° C). After 1.5 hours, the cryovials were removed from the "Mr. Frosty" and loaded into a cryostorage box (Nalgene) which was lowered into a tank filled with LN_2 for 24 hours.

For recovery, the cryostorage box was removed from LN_2 and placed directly on ice. Cryovials were removed from the box and thawed for two minutes in a 40° C water bath. In order to separate cell clusters from the

toxic DMSO, suspensions were poured through a single layer of Nybolt (30 µm pore size nylon mesh) overlaid on a disk of filter paper (Whatman No. 1). Once filtered, the nylon mesh with the cells was placed onto the same semisolid maintenance medium described above and transferred to fresh medium after 1hr., 24hrs., and 7 days. After the final 7 day period, culture material was removed from the mesh and plated directly onto maintenance medium.

Results and Discussion

Evaluation of recovery and regrowth of cryostored material was based on visual assessment and fresh weight gain. The cell clusters appeared to be brown to black immediately after thawing (Figure 1). Within 2-3 weeks, however, clear to pale yellow globular-stage embryos arose from the darker tissue (Figures 2 and 3). After 4 to 6 weeks, heart, torpedo and cotyledon stage embryos formed (Figure 4) and were later transferred to medium lacking 2,4-D to mature further.

Fresh weight data were collected at 20, 32, 45, and 61 days post-thaw, and transfers were made to fresh medium on the same day the fresh weight data were collected. Both treatments yielded 100% recovery for both genotypes. However, the data for both genotypes suggested that cryostored cultures treated with 5% DMSO recovered more rapidly (Figure 5). The fresh weight gain data also indicated a significant effect on recovery from cryostorage due to genotype, with line A recovering more quickly than line B, regardless of the DMSO concentration used.

Embryogenic American chestnut cultures appear to be excellent material for long-term storage via cryopreservation. Cultures of both genotypes we tested fully recovered following cryostorage and proliferated as well, or better than they had prior to freezing. In addition, although a qualitative judgement, we believe the embryos derived from the cryostored cultures appeared to be better-formed and more vigorous than embryos from non-frozen cultures. Although embryos derived from the cryostored cultures are still in plantlet regeneration tests, we believe they will germinate and grow at least as well as non-frozen embryos.

Cryostorage may prove to be an invaluable tool to TACF's breeding program. For example, seeds resulting from TACF's most promising crosses could be used to initiate embryogenic cultures, each of which would then be divided. Some material from each line could be cryopre-

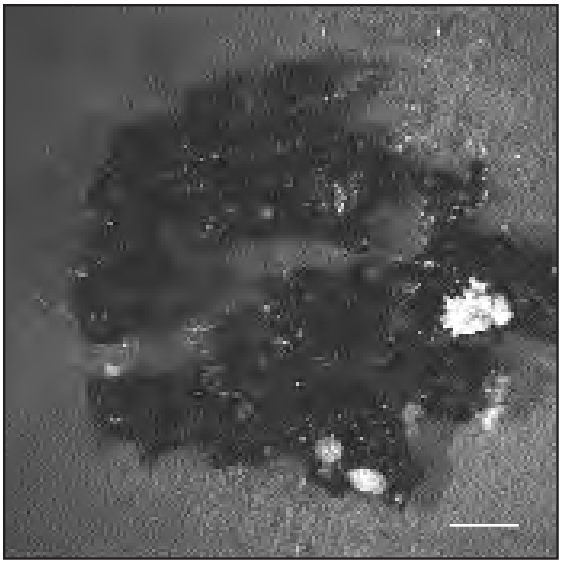

Figure 1. American chestnut embryogenic material approximately 1 week following removal from liquid nitrogen. Bar = 2 mm.

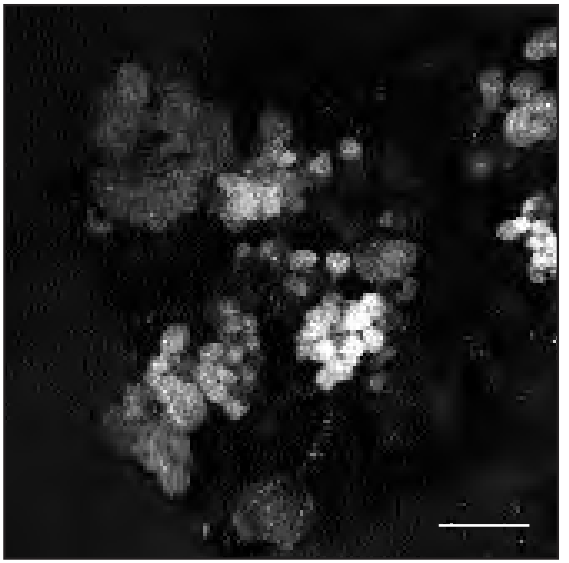

Figure 2. American chestnut embryogenic material approximately 2 weeks following removal from liquid nitrogen, showing some regrowth. Bar = 2 mm.

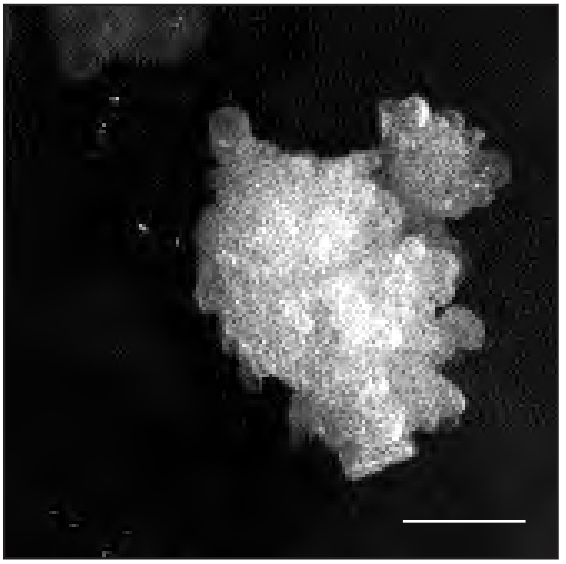

Figure 3. American chestnut embryogenic material approximately 3 weeks following removal from liquid nitrogen, showing some regrowth. Bar = 1 mm.

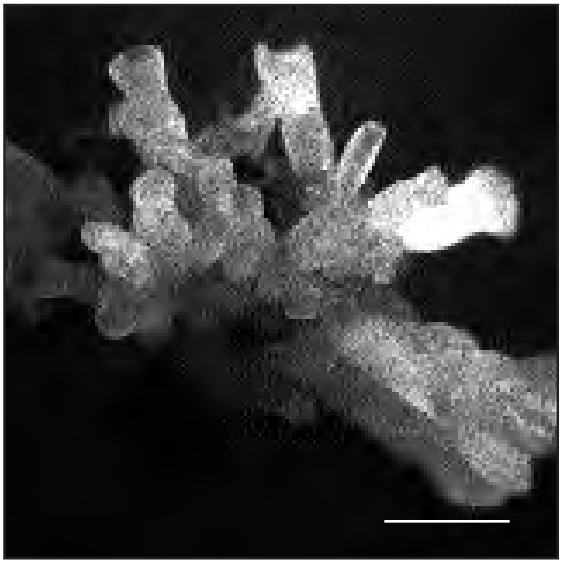

Figure 4. Cluster of developing somatic embryos at various stages derived from cryostored American chestnut culture. Bar = 1 mm.

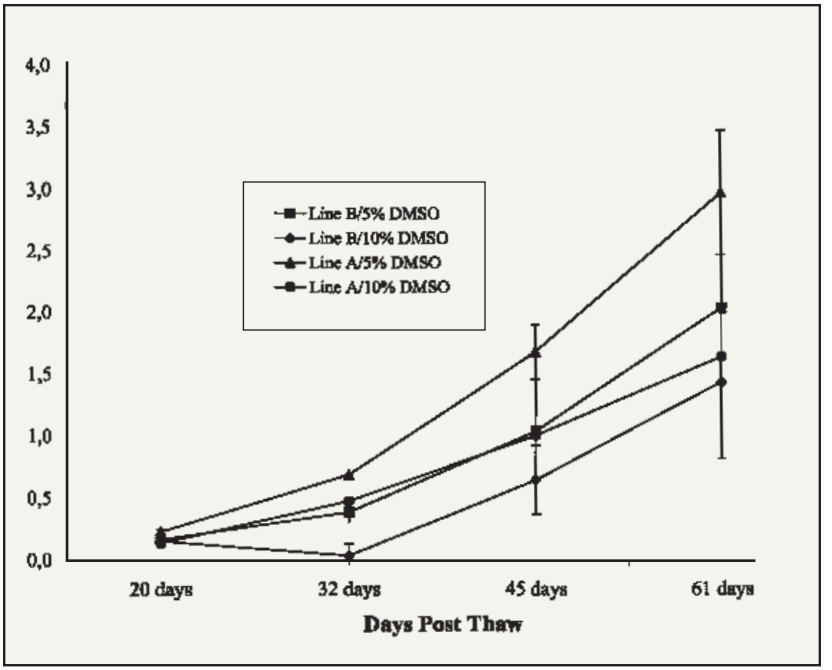

Figure 5. Regrowth of two American chestnut embryogenic culture lines (A and B) cryoprotected with either 5% or 10% DMSO, following cryostorage.

served while somatic seedlings from the cultures are tested for field performance for however many years are necessary. Once field evaluations are completed, those clones that perform well could be thawed and somatic seedlings produced from them to supply TACF members and cooperators with the best material.

Finally, cryopreservation of embryogenic American chestnut cultures may help meet the critical need for conservation of American chestnut germplasm, as indicated in a publication by Huang et al. (1998). These authors noted relatively high levels of genetic diversity in the southernmost populations of American chestnut in Alabama, and recommended that conservation efforts should consider such populations as a focal point for capturing much of the species' genetic variation. However, Huang et al. (1998) also noted that these relict populations are rapidly being lost and that resprouting stumps appear to be declining. Embryogenic cultures initiated from seeds collected from these populations could be cryostored to preserve germplasm that might otherwise be lost.

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Castenea Guide

An Insider's Guide to the American Chestnut and TACF Science

THE PATH TO MOST RESISTANCE

(Based on three genes for resistance)

PARENTS

100% Chinese chestnut *(Blight resistant/orchard type)* **X** **100% American chestnut** *(Blight susceptible/timber type)*

Resistant American Characteristics & Average % American Parentage		Degree of Resistance		
		Low to No	Intermediate	Fully Resistance
50%	(F_1) First Generation Hybrid		100%	
75%	Backcross: American x F_1 = BC_1	87.5%	12.5%	0%
87.5%	Backcross: American x BC_1 = BC_2	87.5%	12.5%	0%
93.75%	Backcross: American x BC_2 = BC_3	87.5%	12.5%	0%
93.75%	Intercross: BC_3 x BC_3 = BC_3F_2	34.4%	64.0%	1.6%
93.75%	Intercross: BC_3F_2 x BC_3F_2 = BC_3F_3	0%	0%	100%?*

Each generation of trees is inoculated with blight fungus and selected for resistance and American characteristics. Each backcross generation requires a minimum of 5 years to complete. The F_1 generation can be completed in 3 years. For an explanation of terms please refer to the Quick Guide to Terminology on page 9.

* This precentage of highly resistance trees is a hope of The American Chestnut Foundation. It is based upon certain scientific assumptions and cannot be proven at this time.