

MOLECULAR MARKERS LINKED TO RESISTANCE TO CRYPHONECTRIA PARASITICA IN CHESTNUT

T.L. Kubisiak

Plant Research Geneticist

USDA Forest Service

Southern Research Station

Southern Insititute of Forest Genetics

Saucier, MS

In recent issues of *The Journal* several articles have appeared which describe The American Chestnut Foundation's breeding program for backcrossing the blight resistance of Chinese chestnut into American chestnut (Hebard 1994a). The breeding program involves repeated crossing of selected *C. dentata* x *C. mollissima* hybrids and



progeny to *C. dentata* (American chestnut), hence reconstituting the *C. dentata* genome with the addition of genes that condition resistance from *C. mollissima* (Chinese chestnut).

Recent issues of *The Journal* have also discussed the application of molecular markers in the backcross breeding program and advantages that such markers offer (Mulcahy and Bernatzky 1993; Ellingboe 1994; Hebard 1994a; Hebard 1994b). Ellingboe

their (1994) pointed out two things that The American Chestnut Foundation hoped to gain by applying molecular markers in the backcross breeding program. First, molecular markers and a corresponding genetic linkage map would help ACF identify the markers that bracket

genes (or gene families) that condition blight resistance. Second, markers and such a map would give ACF the information to select against unwanted Chinese chestnut DNA in advanced backcross generations, greatly reducing the number of backcrosses required to reach a relatively "pure" American chestnut.

In the following paragraphs I describe how I came to work on the chestnut blight problem. I touch on the underlying theory behind recombinational linkage mapping, mention some current results in work with chestnut, and how these results compare to prior knowledge regarding the suspected pattern of inheritance of blight resistance. Finally, I look ahead and suggest where our efforts might best be focused next.

THEORY OF RECOMBINATIONAL LINKING

I met Fred Hebard of The American Chestnut Foundation in January 1995 when he visited the Southern Institute of Forest Genetics in Saucier, Miss., to discuss strategies for a joint molecular-marker mapping project in chestnut between the USDA Forest Service and The American Chestnut Foundation. Fred's study particularly interested me at the time because I had just finished my Ph.D. at Louisiana State University; my dissertation had focused on molecular-marker mapping in the southern pines. Since this visit we have spent numerous hours in the laboratory, on the computer, and on the phone analyzing and discussing the results of our efforts.

To understand how a recombinational linkage map is constructed, it is first necessary to recognize that genes are arranged on chromosomes. Chromosomes are microscopic structures that can be observed in actively dividing cells. Chestnut species have 24 chromosomes (Jaynes 1962) which are arranged in pairs during meiosis. Meiosis is a special form of cell division leading to the formation of pollen in male flowers and eggs in female flowers. During meiosis, chromosome pairs separate, and at fertilization, when the pollen grain and egg unite, the "normal" or diploid number of 24 chromosomes is restored. For example, in normal first hybrids between Chinese and American chestnut there will be one Chinese chromosome and one American chromosome in each of the 12 pairs of chromosomes after fertilization (see Figure 1).

Chinese Chestnut Grandparent

American Chestnut Grandparent

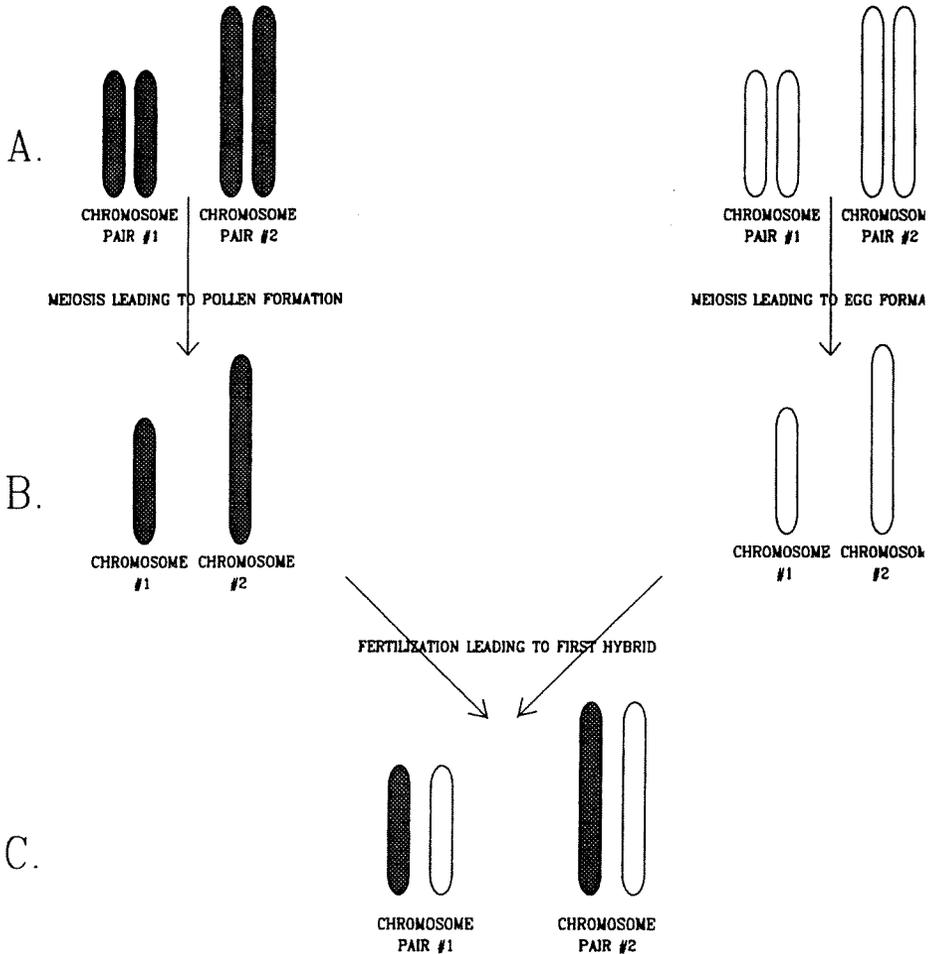


Figure 1. Two-chromosome example of the process leading to the formation of an interspecific F1 hybrid. A Chinese and American chestnut grandparental chromosomes. Chinese chromosomes are cross-hatched and white. B. Meiosis in each of the respective grandparents and the formation of a single pollen cell and egg cell and the fusion of egg and pollen to form an F1 hybrid.

Usually, every gene on a chromosome will also be found on its counterpart, or homologous chromosome, at the same location, although frequently a gene will differ slightly between the homologous chromosomes. Those genes that are slightly different but are located at the same place on homologous chromosomes are called alleles. Allelic differences between or among various individuals are often referred to as polymorphisms. In American chestnut, likely there are genes that match those conditioning blight resistance in Chinese chestnut. The difference is they are alleles for susceptibility to blight, instead of alleles for resistance. This difference between alleles of the same gene is crucial to plant breeding.

The idea that chromosomes behave as units for segregation of alleles on chromosome pairs during meiosis led to the expectation that all genes located on the same chromosome should be transmitted as a unit, and therefore show linkage (Sturtevant 1913). Likewise, genes on separate chromosomes are never linked.

However, linkage between two separate genes on the same chromosome is never complete, because each pair of chromosomes usually exchanges segments of DNA during meiosis. The occurrence of such an exchange event, called a cross-over, results in recombination between genes located on the same chromosome pair. For instance, one would expect that most chromosomes in pollen or eggs formed by a first hybrid between Chinese and American chestnut would be part Chinese and part American (see Figure 2), unlike the chromosomes in the first hybrid, which are either pure Chinese or pure American. The alleles on the chromosomes in the pollen or eggs of the first hybrid would have recombined during meiosis.

The frequency of crossing over between any two genes serves as a measure of the genetic distance between them. This has been discussed in a recent issue of *The Journal* (Hebard, 1994b), although that discussion only briefly described the process by which the genetic distances between separate genes on a chromosome are transformed so they can be related in a linear fashion. For instance, for three linked genes the distances from the middle gene to each flanking gene should add up to the distance between the flanking genes. The percent recombination values are mathematically transformed by an appropriate mapping function (Haldane 1919; Kosambi 1994) into a

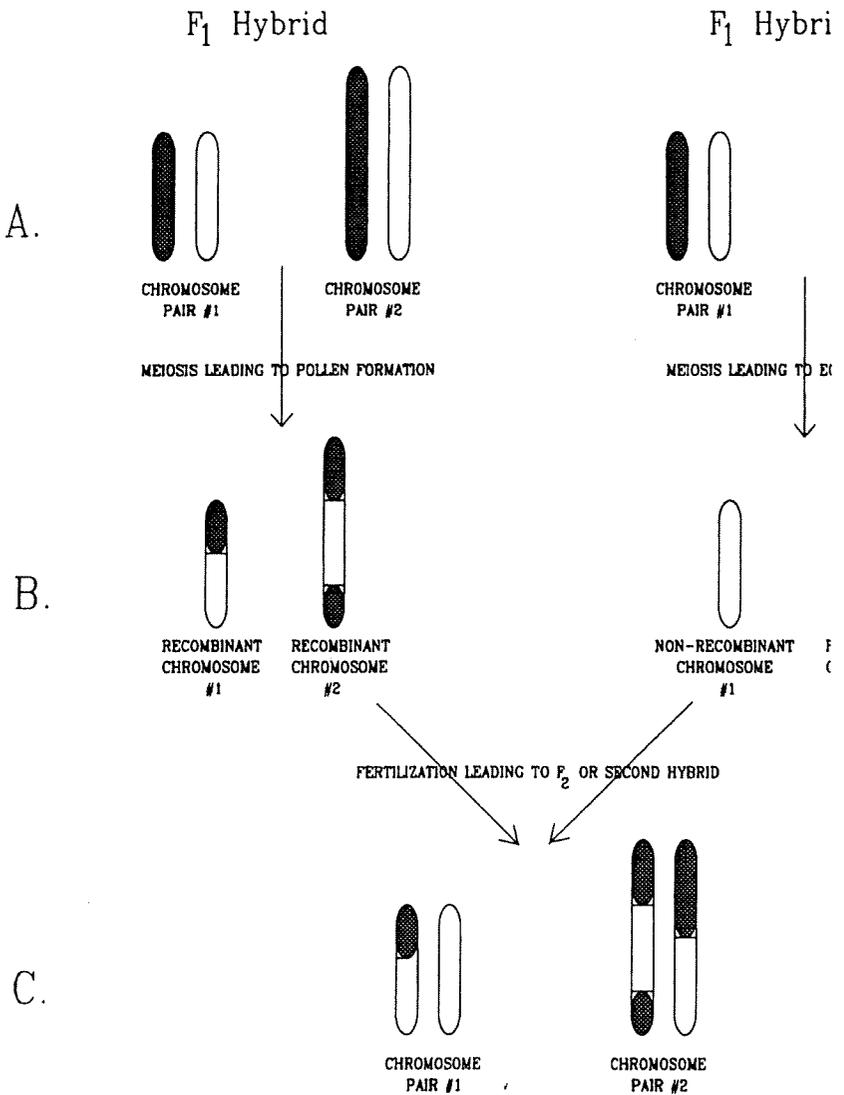


Figure 2. Two-chromosome example of the process leading to the formation of an F_2 or second hybrid of F_1 hybrid showing Chinese and American chestnut derived chromosomes. B. Meiosis in each hybrids and the formation of a single pollen cell and egg cell. Note that cross-over has resulted in wh of Chinese and American DNA within each chromosome pair. C. Fertilization and the fusion of egg an second hybrid.

measure of genetic distance known as a centiMorgan (cM). The cM distances between genes are linearly related and can be used to construct a genetic map of each chromosome.

CURRENT RECOMBINATIONAL MAPPING EFFORTS

Using various molecular markers, I constructed a genetic linkage map for the Chinese x American hybrid chestnut genome using an F₂ population developed by Hebard. The map is based on segregation data for a maximum of 102 offspring. In our F₂ population, 241 polymorphisms were scored; 45 were found to deviate significantly from their expected Mendelian inheritance ratio based on chi-square analyses ($p \leq 0.01$). The 45 distorted polymorphisms were excluded from any further analyses as I could not confidently conclude that each represented a single genetic locus, or location on a chromosome. Additionally, the use of distorted polymorphisms can often lead to the declaration of false linkages. The remaining 196 loci (genetic locations) were employed for linkage mapping and in the search for genomic regions conditioning a blight-resistant response in the tree. Six isozyme, 14 RFLP, and 176 RAPD loci were entered into the computer program JoinMap (version 1.1)(Stam 1992).

Of the 196 loci analyzed, 185 were mapped. In total, 2 isozyme loci, 14 RFLP, and 169 RAPD loci mapped to 12 linkage groups spanning a total genetic distance of 660.9 cM. Using the partial genetic linkage data and the method-of-moments estimator (Hulbert et al. 1988), I obtained several estimates of genome size. My current estimates suggest a genome size ranging from approximately 780 cM to 900 cM. Based on these results, the map spans at least 73% of the Chinese x American hybrid genome.

Once I had the map, the next step was to search for genes that condition a blight-resistant response. Many of the statistical tests employed for mapping traits such as blight resistance are based on specific underlying assumptions (Ott 1991). A major assumption for many of these statistical models requires that the phenotypic trait data be normally distributed. Indeed, the resistance data collected for the central trees used in this experiment, American and Chinese chestnuts and their first hybrid, were approximately normally distributed, with similar variances.

In order to determine whether any of the markers linked to genes conditioning resistance in the chestnut standard analysis of variance was carried out using marker groups as class variables (Young et al. 1993). An association marker and a blight-resistant response was considered probability of observing an F-value as extreme or more than the observed value was less than or equal to 0.001. This level was chosen to ensure that a minimum number of markers was declared throughout the experiment (Lander and Lincoln et al. 1992). Using single-marker analyses, four unlinked regions were found to be conditioning blight resistance.

To confirm the results of the single-marker analyses, we also analyzed using the program MAPMAKER/QTL (Lincoln et al. 1992). Upon further investigation with ER/QTL and multiple locus models, only two of the markers were found to be significantly associated with resistance to blight. Unfortunately, I cannot yet publish the map, but I can give the approximate location of the genes conditioning a blight response.

TABLE 1.

Markers bracketing regions conditioning a blight resistant response. Primers beginning with C were obtained from J.E. Carlson (University of British Columbia, Vancouver, British Columbia, Canada), primer X03 was obtained from Operon Technologies Inc. (Alameda, California, USA).

Primer	Sequence (5' to 3')	Molecular Weight of Marker Band (bp)
C258	CAGGATACCA	450
X03	TGGCGCAGTG	1050
C153	GAGTCACGAG	1900
C202	GAGCACTTAC	1025

The first region was on linkage group B; most likely it is located between RAPD markers amplified by the primers C258 and X03 (see Table 1 for primer information). The second region was located on linkage group G between RAPD markers amplified by primers C202 and C153. These two marker-intervals were responsible for explaining as much as 56.8% of the phenotypic variation observed.

RESULTS IN HISTORICAL CONTEXT

These results are extremely promising in light of the long-standing hypothesis that blight resistance is conferred by two incompletely dominant genes (Clapper 1952). If blight resistance were controlled by only two genes, then it should be a relatively straightforward task to introduce these genetic regions into American chestnut using the backcross method. Of course, mapping results suggest that the situation is somewhat more complex, with over 40% of the resistance response unexplained.

WORK AHEAD

In spite of these promising results, a tremendous amount of research still needs to be conducted. Integrating the morphological markers discussed in a recent issue of *The Journal* (Hebard 1994b) into the molecular map must still be completed.

It would also be helpful to confirm the influence of the regions conditioning a blight-resistant response in both the first and second backcross generations. If the influence of these regions is confirmed, the breeding program will serve to introduce these regions into many different selections of American chestnut. One unique approach might be to identify shrubs and sprouts of American chestnut still persisting in the wild throughout the natural range, bring them into flower by exposing them to full sunlight, hybridize these to select hybrid individuals, and repeatedly backcross to restore native genotypes.

In addition, molecular studies of other pedigrees involving two or more species should be undertaken as these may help determine whether genes for blight resistance from different sources (such as Japanese chestnut) map to the same or different locations in the genome. Such information would help identify other possible sources of blight resistance, avoid the use of redundant sources of resistance,

and greatly increase breeding efficiency in terms of the number of offspring needed for selection efforts.

Hopefully the development and re-introduction into the wild of blight-resistant, primarily American, chestnut trees will restore the chestnut throughout much of the eastern United States.

To conclude, I want to inform the readers of *The Journal* that I would be happy to supply as much information as possible about the markers used in this study.

ACKNOWLEDGEMENTS

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