

SCIENCE AND NATURAL HISTORY

Using DNA markers to distinguish among chestnut species and hybrids

by Tom L. Kubisiak



Dr. Tom Kubisiak of the U.S. Forest Service's Southern Institute of Forest Genetics and Dr. Robert Bernatzky of the University of Massachusetts have been using molecular genetic markers to pinpoint hot spots of American chestnut genetic diversity. Using samples collected on a roughly 135-mile grid covering the entire range of the tree, the two researchers are analyzing chestnut DNA to assess overall levels of diversity and to map out the sources of the greatest genetic variation. This paper grew out of an analysis of a sample from a Maine chestnut conducted as part of that study.

Identification of American chestnut trees in the wild for inclusion in breeding programs is currently done using morphological traits. Distinguishing traits include leafshape, stipule size, presence or absence of leaf and stem trichomes, and stem color. Application of these traits is reasonably clear if the trees are pure American chestnut, but identification of hybrids is very difficult. Hybrids that are primarily American chestnut may look like American chestnut. Such individuals can be frequently found in densely forested areas because of extensive plantings of species and hybrids in public and private woodlots. Since the breeding programs are designed to include as much native diversity of American chestnut as possible, positive genetic identification would be very helpful.

Along these lines, I recently received a bag of chestnut leaves and a letter from Mr. Robert P. BaRoss of Cape Elizabeth, Maine. In this letter, Mr. BaRoss explained that the chestnut leaves he sent were taken from a 70-foot chestnut tree on the Ram Island Farm in Cape Elizabeth. Mr. BaRoss stated that Maine members of The American Chestnut Foundation (TACF) were interested in using this tree in their breeding program, but the leaves and bark of the tree did not look typical of American chestnut. Therefore, there was some suspicion as to the tree's ancestry. Maine members hoped that DNA marker techniques would be able to determine whether this tree was a "pure" American chestnut that should be included in their breeding program. I promptly replied to Mr. BaRoss that it should be possible to determine whether the tree is of hybrid ori-

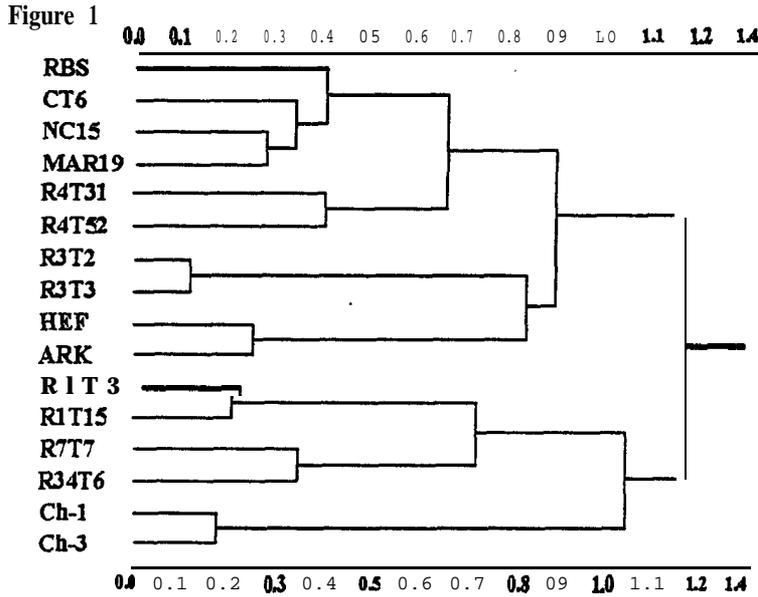


Figure 1. Unweighted pair group mean cluster dendrogram constructed from a distance matrix based on 97 random amplified polymorphic DNA markers collected on 16 chestnut samples. The samples included: one unknown chestnut tree from Cape Elizabeth, Maine (RBS); three American chestnut trees (CT6, NC15, and MAR19); two Chinese chestnut x American chestnut F1 hybrids (R4T31 and R4T52); two European chestnut trees (R3T2 and R3T3); two chinquapin trees (HEF and ARK); two Chinese chestnut trees (RIT3 and RIT15); two Japanese chestnut trees (R7T7 and R34T6); and two Henry chestnut trees (Ch-1 and Ch-3).

gen using DNA techniques, and that I would be happy to genotype the tree in light of its importance to the Maine program. The following is a description of the results of this study.

Leaf or dormant bud tissue from a total of 16 trees was collected and shipped to the USDA Forest Service, Southern Institute of Forest Genetics, in Saucier, Mississippi. The samples included: one unknown chestnut tree from Cape Elizabeth, Maine; three American chestnut trees (one from Connecticut, one from North Carolina, and one from West Virginia); two Chinese chestnut trees (RIT15 'Mahogany' PI#70315 and R7T3 'Nanking' from the South and Spring Lots, respectively, of the Sleeping Giant Chestnut Plantation in Hamden, Connecticut); two Japanese chest-



nut trees (**R7T7** and **R34T6** from the South and West Lots, respectively, of the Sleeping Giant Chestnut Plantation in **Hamden**, Connecticut); two European chestnut trees (**R3T2** and **R3T3** from the Humphrey Hill Lot of Lockwood Farms in **Hamden**, Connecticut); two Henry chestnut trees (both from Guang Xi, China); two chinkapin trees (one from Mississippi and one from Arkansas); and two Chinese chesnut x American chesnut **F₁** hybrid trees (**R4T31** and **R4T52** from the Spring Lot of the Sleeping Giant Chestnut Plantation in **Hamden**, Connecticut).

DNA was isolated from approximately two grams of leaf tissue or 0.25 grams of dormant bud tissue using a modification of the CTAB-based procedure outlined in Wagner et al. (1987). The polymerase chain reaction (PCR) technique was used to generate random amplified polymorphic DNA (**RAPD**) markers. **Oligonucleotide 10-mer** primers used in PCR were obtained from either Operon Technologies (Alameda, CA), or J. Hobbs (Univ. of British Columbia, Vancouver, B.C., Canada). **RAPD** amplification was based on the protocol reported by Williams et al. (1990). The completed reactions were electrophoresed in 2% agarose gels, stained with ethidium bromide, and photographed under *W* light using Polaroid 667 film.

To identify informative **RAPD** markers, 24 primers were screened against DNA extracted from the 16 different chestnut samples. Those **RAPD** markers showing differences among the 16 samples were scored as potentially informative. Markers were subjectively chosen based on the intensity of amplification (only intensely amplified bands were scored) and the absence of co-migrating DNAs. **RAPD** fragments were identified by the manufacturer primer code corresponding to the primer responsible for their amplification, followed by a four-digit number indicating the approximate fragment size in base pairs. (A list of these loci is available upon request from the author.)

Chestnut samples were placed into groups or clusters using the unweighted pair-group mean method (UPGMA) available under the CLUSTER procedure in the statistical analysis software SAS (SAS Institute Inc., Cary, NC). A distance or dissimilarity matrix was constructed based on the **RAPD** fragment data. Chestnut samples were scored for the presence or absence of a band at each of the **RAPD** markers. The distance matrix was constructed by tallying the total number of marker differences found between pair-wise comparisons of samples. In other words, I sim-



ply counted up the number of markers at which two trees **differed**. For the 16 chestnut samples, a total of 136 pair-wise comparisons were made.

A total of 97 **RAPD** markers were identified and scored on the chestnut samples. UPGMA analysis suggested two primary groups; one including all the eastern or Asian chesnut species and another including the western or European and North American chestnut species (Figure 1). Within the eastern or Asian chesnut grouping, each species formed its own distinct cluster, with Henry chestnut being further separated **from** Chinese chesnut and Japanese chesnut. within the western or European and North American grouping, each species formed its own distinct grouping. European chesnut and chinkapin were slightly more separated from American chestnut than were the Chinese chestnut x American chestnut **F₁** hybrids. Although the Chinese chestnut x American chestnut **F₁** hybrids clustered closely with the American chesnut trees, they did form their own distinct cluster. The unknown tree **from** Cape Elizabeth clustered very closely with the three American chestnut trees.

Using **the RAPD** marker data, it was possible to distinguish among the six chesnut species and Chinese chesnut x American chestnut **F₁** hybrids included in this **study**. The American chesnut trees appear to be quite different than all of the other chestnut species. The American chestnut grouping differed from the European chesnut grouping by an average of 26 markers, the chinkapin grouping by an average of 27 markers, and the Chinese chesnut, Japanese chesnut, and Henry chesnut groupings by an average of 40 markers each. Based on this analysis, the unknown tree from Cape Elizabeth appears to be of American chestnut ancestry as it clusters very closely with the other American chesmuts. It does not appear to be an **F₁** hybrid with any of the **other** chesnut species as it did not form its own distinct cluster, as did the Chinese chesnut x American chestnut **F₁** hybrids (Figure 1).

This **study** provides support to the hypothesis that **RAPD** markers can be used to quickly determine the genetic identity of putative American chestnut trees in the forest for inclusion in breeding programs. **RAPD** markers will be useful for quickly identifying "pure" species and first generation hybrids, but may not be useful for identifying more advanced generation hybrids unless large numbers of markers-are assayed to provide the **desired** resolution.

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