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ORIGINAL PAPER

Role of genomics in the potential restoration of the American chestnut

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Abstract The development of genomic tools will enhance traditional tree breeding technologies leading to more certain and timely recovery of the American chestnut, a keystone heritage tree of the eastern United States. Major efforts are being made in gene discovery, genetic marker development, construction of a BAC-based physical map, and DNA transformation technology. A strategy of map-based cloning, association genetics, and genetic engineering, combined with traditional and marker-assisted backcross breeding is proposed for the long-term genetic restoration of this iconic tree species.

Keywords Genomics · Chestnut · Restoration

Introduction and historical context

The American chestnut (*Castanea dentata* [Marsh.] Borkh.) was once one of the most important tree species in America (U.S. Census Bureau 1908; Davis 2006) but virtually ceased to exist as an economically and ecologically relevant forest tree by the mid 1900s, having fallen victim to the chestnut blight, (*Cryphonectria parasitica* [Murr.] Barr), an introduced fungal pathogen. The blight killed some four billion trees, one of the greatest ecological disasters in American history. Decades of tree breeding efforts and research on chestnut and the fungal pathogen engender hope that the tree species will be restored. Breeding is now at the third generation of backcrossing, with genotypes expected to be 15 out of 16 American germplasm. This paper briefly reviews the status of the American chestnut and discusses how genomic science may complement ongoing efforts and accelerate the reintroduction of the species in American forests. Chestnut may become a model for application of genomic technology to other threatened tree species, particularly as increased stresses come to our forests through climate change and introduced pests/diseases.

The role of chestnut in America's forest ecosystems has been shaped by glaciation and settlement. Chestnut probably survived the Wisconsin glaciation in small Southern Appalachian refugia and migrated north along the mountain chain as the climate started warming about 10,000 years ago, reaching the current northern limit of its natural range (Fig. 1), within the last few thousand years (Russell 1987; Russell and Davis 2001; Anagnostakis 2001). Likely uncommon in precolonial times, the American chestnut expanded rapidly following disturbance caused by settlers, no doubt a result of the species ability to sprout prolifically from cut or burned stumps, quickly establishing dominance on cleared sites (Paillet 2000). Today, chestnut survives as rare, large "escapes" or as numerous small understory sprouts in the heart of its range (Stephenson et al. 1991).

The American chestnut possessed a remarkable array of desirable traits. It grew very rapidly, often to a great size, with outstanding form and wood quality. The wood was very resistant to rot and therefore was used extensively in construction as lumber and roofing, poles, masts, and railroad ties. Tannins were extracted from bark and wood chips, and the chips were subsequently pulped for the production of paper. The tree grew well on dry uplands, a trait that today would make it a valuable biofuel species in

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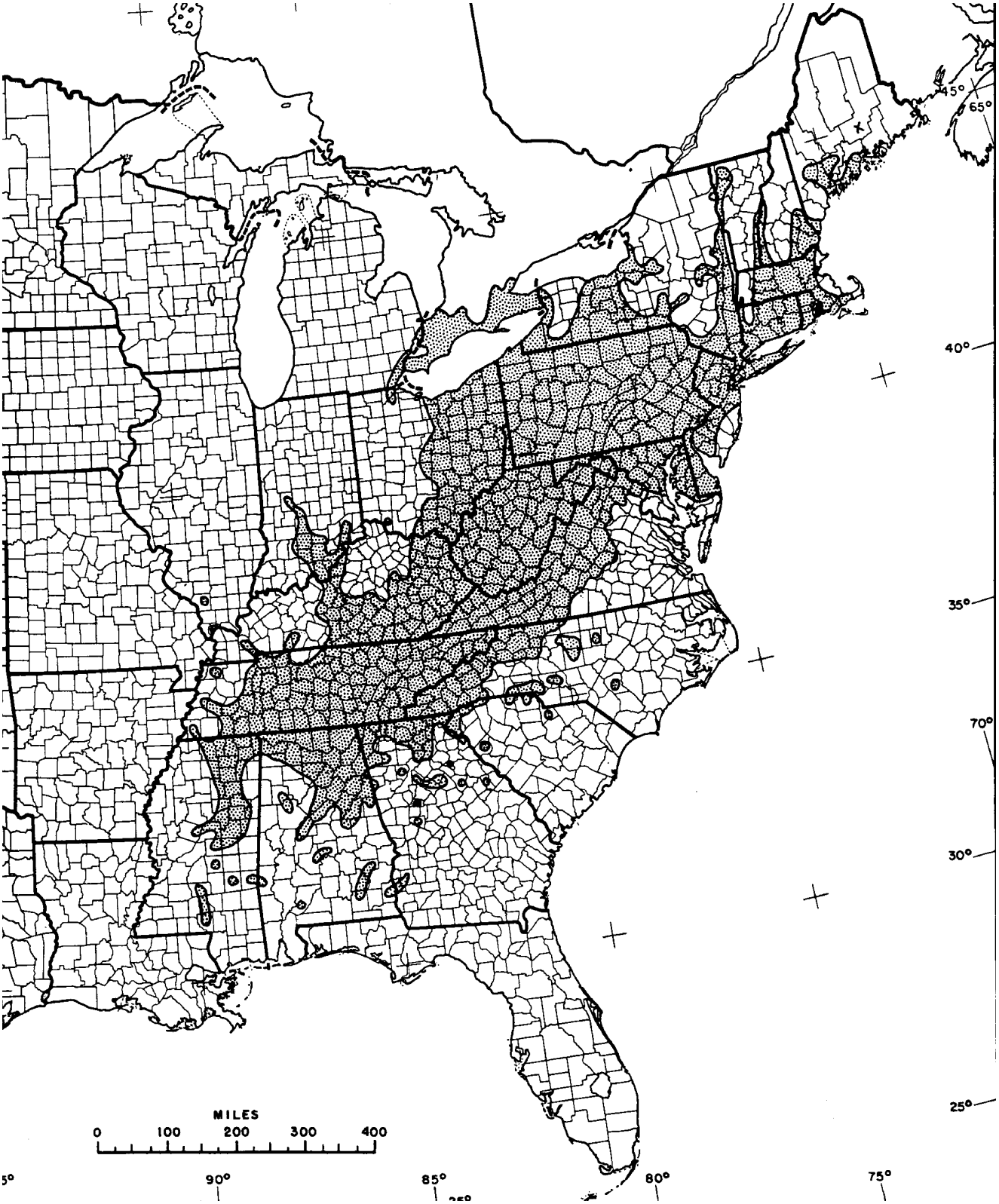

Fig. 1 Natural range of the American chestnut circa 1900 (Saucier 1973)

these regions (Jacobs 2005). Historically, its seeds provided food and revenue for rural communities, and a wide range of animals were dependent on the mast, including black bear, turkey, deer, raccoon, and the passenger pigeon (Hill 1994). Chestnut has been described as "the perfect tree" (Freinkel 2007). Within a few decades, the chestnut blight altered these ecosystem interactions completely.

Chestnut blight typically enters a tree through wounds, infecting and killing cambial tissues and ultimately girdling the tree (Anderson 1914). Low levels of resistance to the blight have been reported in native populations of American chestnut (http://ipm.ppws.vt.edu/griffin/accf.html), but moderate to high levels of resistance appear not to exist.

Current approaches to developing disease-resistant American chestnuts

Three approaches to developing blight-resistant American chestnut are being pursued: breeding for resistance, biological control of the blight, and genetic transformation.

Breeding for resistance Both hybrid breeding (introgression of major effect resistance alleles) and within species breeding for quantitatively inherited resistance (additive) are being pursued. Attempts to introduce resistance factors into American chestnut via interspecific crosses with Asian chestnut species began in the 1930s, but failed to recover trees that physically resembled the native American chestnut (Jaynes 1994). In the early 1980s, backcross breeding of interspecific hybrids to American chestnut was initiated by the Connecticut Agricultural Experiment Station (CAES, http://www.ct.gov/caes), and The American Chestnut Foundation (TACF) (http://www.acf.org). Good progress has been made by both institutions in introgressing resistance from Chinese chestnut (*Castanea mollissima* Blume) and Japanese chestnut (*Castanea crenata* Siebold & Zuccarini) into a modest array of genetic backgrounds. TACF is already in their sixth generation of backcross and intercross matings (Hebard 2006). The first tests of what are hoped to be blight-resistant, American-type trees are now being planted, and operational releases of verified resistant material is 7–12 years in the future.

Griffin et al. (2006) report modest success in continuing efforts to breed among surviving American chestnuts that possess putative quantitatively inherited blight resistance.

Biological Control Blight was also introduced to Europe, from Asia. In 1965, a strain of the blight fungus, unable to kill the European chestnut (*Castanea sativa* Mill.), was reported in France (Grente 1965). Subsequent inoculation of cankers with this "hypovirulent" strain gradually resulted in remission of the disease throughout Europe (Anagnostakis 2001). Hypovirulence is caused by a fungal virus. Attempts to replicate this success with American chestnut have been only marginally successful (MacDonald and Fulbright 1991; Griffin 2000; Anagnostakis 2001, MacDonald and Double 2006). Spread of the hypovirulence in forest settings is constrained by a genetic system that restricts fusion of hyphae among diverse strains of the fungus (Anagnostakis 1977). Apparently, the fungus in America is highly diverse, genetically, and not uniformly affected by the fungal viruses. Still, hypovirulence remains a viable component of an integrated disease control program that includes genetic resistance (Freinkel 2007; http://ipm.ppws.vt.edu/griffin/accf.html).

Genetic Transformation The use of transgenes has been proposed for restoration of species threatened by introduced pests and pathogens (Merkle et al. 2006). *Agrobacterium*-mediated transformation of embryogenic lines and plant regeneration from somatic embryos have been achieved for American chestnut (Carraway et al. 1994; Andrade et al. 2005). A gene encoding an antifungal enzyme, oxalate oxidase (OXO), also known as wheatgermin, has been recently introduced into chestnut (Polin et al. 2006; Welch et al. 2007). Oxalate is an inhibitor of the hypersensitive response. OXO is expressed in plants to degrade the oxalic acid produced by fungal pathogens, and at the same time, OXO produces hydrogen peroxide, which serves as a fungicide and as a signal for the plant defense response. Transfer of a wheat OXO has generally improved resistance to fungal pathogens in dicots (Lane 2002). Other antifungal genes that have been used successfully in plants, might increase resistance in chestnut, such as genes encoding chitinases (Dana et al. 2006) or antifungal peptides (Huang et al. 2002; Castro and Fontes 2005). If resistance genes from chestnut were isolated, it would be possible to combine, in one plant, multiple resistance genes to create a more general and stable type of resistance. To date, no genes with antifungal properties have been identified in *Castanea* species. In hazelnut (*Corylus avellana*; family Betulaceae) resistance to eastern filbert blight is controlled by a single locus (Mehlenbacher et al. 2004) and therefore might be isolated before any genes from *Castanea* are available.

The potential role of genomics in American chestnut restoration

Genomics is being applied to virtually every major crop plant and to several tree species to identify genes that can accelerate improvement (Morgante and Salamini 2003; Neale 2007). Genomic analysis of chestnut will (1) identify

many of the genes of the organism and their locations, (2) provide for complex trait dissection of blight resistance and other important growth, form and adaptability traits (Kubisiak et al. 1997; Neale and Savolainen 2004; Verhoeven et al. 2006; Neale and Ingvarsson 2008), (3) provide tools for association of genes and traits (Rafalski 2002; Neale and Savolainen 2004), (4) identify and clone specific resistance factors, for genetic engineering or marker-aided selection (Salvi and Tuberosa 2007), and (5) speed backcross breeding and provide pedigree identity capability (Hospital et al. 1992; Dekkers and Hospital 2002; Lecape et al. 2007; see Fig. 2). Beyond providing insights into the chestnut genome, genomics will provide better understanding of the blight fungus, and the interaction with its chestnut host and viral parasite (Allen et al. 2003). Full genome sequencing of *Cryphonectria parasitica* has been completed by the Joint Genome Institute of the Department of Energy (http://genome.jgi-psf.org/euk_home.html) which may lead to new strategies for biological control.

Genomic tool development

Three genomic tools currently under development (http://www.fagaceae.org) should advance the progress toward restoration of American chestnut. These are, (1) large-scale gene discovery through high throughput Roche/454 sequencing of expressed genes (ESTs) in American and Chinese chestnut (Margulies et al. 2005), (2) development of large numbers of genetic markers from this sequence data identifying markers showing polymorphisms within and between species, and (3) the construction of a high resolution bacterial artificial chromosome (BAC)-based physical map. Genomic work has focused on Chinese chestnut because a major goal is the identification of the resistance factors in this species. New mapping populations in Chinese chestnut, American chestnut, and hybrids are also being created to permit high resolution mapping and comparative mapping studies.

About 10,000 expressed genes (EST) have been sequenced and annotated (http://www.fagaceae.org) with a SNP frequency of about one in 600 bases of consensus sequence. Hundreds of polymorphic simple sequence repeat (SSR) sequences have been detected in the EST sequences. SSRs are particularly useful in comparative genomics studies with other *Castanea* or *Fagaceae* species (Barreneche et al. 2004; Sisco et al. 2005). A major objective is to obtain an integrated high-resolution genetic and physical map, which would be a major step toward cloning of blight resistance genes. Identification of markers very close to resistance factors may allow identification of candidate genes for resistance. Cloning of resistance genes would facilitate development of desirable combinations of resistance genes in the best genotypes either by breeding or by genetic engineering.

Cytogenetics

Substantial segregation distortion and an inability to resolve two linkage groups was observed in the comparison of the American/Chinese hybrid map with that of European chestnut (Sisco et al. 2005), suggesting chromosome rearrangements within the genus. Recently, Islam-Faridi et al. (2008) provided cytogenetic evidence of a translocation, as a quadrivalent in pollen mother cells of an F_1 hybrid (*C. mollissima x C. dentata*). If rearrangements are associated with chromosomes carrying resistance factors, as the genetic data suggest, introgression of resistance in backcross breeding may be hindered by segregation distortion

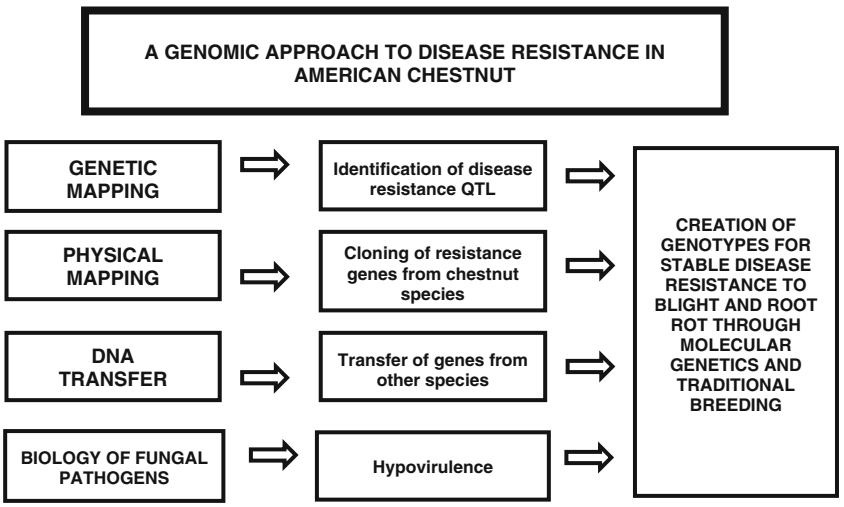

Fig. 2 A genomic approach to enhance disease resistance breeding in American chestnut

and reduced recombination (Rieseberg et al. (1995). Comparative mapping that elucidates chromosomal rearrangements will enhance the effectiveness of backcross breeding (introgression) in efforts to disseminate resistance factors in breeding populations.

Marker-enhanced breeding

Genomics will have the largest and most immediate impact on recovery of the American chestnut through development of DNA markers that will guide backcross breeding (both foreground and background selection) and early culling in recurrent lines. Highly informative markers reduce overall time to introgress resistant loci into production populations (Hillel et al. 1990; Hospital et al. 1992, 2002; Hospital and Charcosset 1997). One or more generations of backcrossing may be eliminated through use of markers to select against donor DNA in progeny. As nearly complete American chestnut resistant lines are produced, markers could virtually replace the need for disease inoculation protocols in recurrent selection programs designed to spread the resistance factors. Markers will also be invaluable in pyramiding genes for resistance (Friedt and Ordon 2007; White et al. 2007).

Using markers for fingerprinting and pedigree confirmation can save breeding program years of effort. New markers in chestnut have already proven useful. Markers would be particularly useful for determination of the breakpoints of the chromosomal rearrangements to maximize the efficiency of selection for resistance in spite of rearrangements. If specific resistant lines are commercialized and protected, markers will be required to maintain pedigree fidelity and provide legal identification.

Trait dissection using genomics

Blight resistance in chestnut appears to be an oligogenic trait. QTL mapping experiments (Casasoli et al. 2001, 2006; Kubisiak et al. 1997) conducted with interspecific crosses between American and Chinese chestnuts suggest at least three resistance loci on separate linkage groups. These loci account for up to 42% of the phenotypic variation in blight resistance as measured by canker size in inoculated interspecific chestnuts. The large number of genetic markers and polymorphisms now available, in combination with large backcross breeding populations, suggests the possibility of disease gene identification through association genetics, as a complement to map-based cloning. Association genetics is a direct method of identifying genes, by correlation of specific SNP polymorphisms in known genes with specific phenotypes (Tenaillon et al. 2001). Association genetics requires large populations, good phenotyping capability, SNP single nucleotide polymorphism) markers in most genes, and linkage disequilibrium appropriate for the marker density (Rafalski 2002). Because of their life history characteristics, most tree species are well suited for association genetics (Neale and Savolainen 2004; Gonzalez-Martinez et al. 2007). Association genetics could significantly speed up the identification of genes controlling blight resistance and other economic and adaptive traits.

Beyond initial recovery: an expanded role for association genetics

Recovery of American chestnut will be a slow process as resistance factors are introgressed or engineered into the broad array of genetic backgrounds necessary for adaptation across the former natural range of the species. To date, selection of American chestnut trees for inclusion in the breeding program has been dictated largely by availability of stump sprouts with pollen or seed catkins. Selection of parents based on desirable wood or nut traits has not occurred. It is suggested here that a clonally replicated provenance trial designed to simultaneously serve as an association genetics population could enhance selection of existing resistant breeding lines, through use of markers associated with desirable traits, and also provide genotypes for future infusion into recurrent populations. To be successful, these genetic trials would have to be established, following appropriate quarantine protocols, in locations not currently influenced by the chestnut blight (e.g., west coast of the US), so they may develop unhindered. The establishment of a population in the near term could benefit the chestnut recovery program for years to come.

Map-based cloning and association genetics will play key roles in identification of disease resistance genes in chestnut

A common strategy for the identification of resistance genes in a plant species is map-based cloning (Tanksley et al. 1995; Bent 1996, McHale et al. 2006). In this approach, colocalization of resistance and genetic markers to regions of known sequence leads to the isolation, identification, and characterization of plant disease resistance genes. To do this, high-resolution genetic mapping, local physical mapping, and local genome sequencing are required. When candidate genes are identified, their functional resistance is verified by DNA transformation or genetic complementation. Disease resistance genes have been cloned from many plants, including *Arabidopsis*, other dicots, and several cereals. Most plant genes for

disease resistance (R genes) belong to related families of nucleotide-binding site/leucine-rich repeat genes, (NBS-LRR). Plant genomes sequenced so far, have 150 (*Arabidopsis*) and 400 (rice and poplar) NBS-LRR homologs (McHale et al. 2006; Kohler et al. 2008). If the genes underlying the blight resistance QTLs in Chinese chestnut could be identified, isolated, and characterized in this way, it would be possible to pyramid several different resistant factors in selected American chestnut genotypes either by transgenic methods or by marker-assisted breeding, adding speed and confidence to traditional breeding approaches.

Map-based cloning is routine in *Arabidopsis* due to its small genome size (<150 Mb), full genome sequence, and highly saturated genetic maps (Jander et al. 2002). The prospect of cloning of blight disease resistance QTLs in chestnut appears reasonable once high resolution genetic maps and contiguous physical maps exist for the regions of interest. The overall ratio of base pairs (genome size of 800 Gb) to recombination map distance for chestnut is about 800 kb/cM (Casasoli et al. 2006), compared to *Arabidopsis* with a general ratio of 250 kb/cM.

Current plans include the localization of 500 to 1,000 SNP and SSR markers on genetic maps and to identify additional markers closely linked to resistance. The large number of ESTs and SNPs in these ESTS should aid greatly in establishing contigs and a minimum tiling path for targeted sequencing based on the integrated genetic and physical maps. Fine scale genetic mapping of QTLs may be possible using association genetics in the large backcross inbred populations made by TACF.

An additional challenge

Public acceptance of transgenic crops has been slow due to fears of new technology and the potential for release of artificial genes into the environment. It is expected that there will be similar opposition to a genetically modified chestnut (Strauss and Bradshaw 2004). However, chestnut is a special case, where an ecological disaster has already occurred and the genetic technology could aid in the restoration of a spectacular forest tree. Chestnut may become the first case of the application of genetic technology for ecological restoration and lead to similar applications for the protection or conservation of many threatened forest tree species, in these particularly difficult times (Merkle et al. 2006).

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