A Tree Improvement Program for Eastern Cottonwood in the Southeastern United States

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ABSTRACT

A southwide project was initiated in 1995 to develop eastern cottonwood (*Populus deltoides* Bartr. ex Marsh. var. *deltoides*) clones capable of producing 5-10 dry tons/acre/year in short rotation woody crop culture. The Southeast was subdivided into six subregions: Southeast Atlantic (SA = NC to eastern GA), East Central (EC = TN and northern AL), East Gulf (EG = south AL, west GA, northwest FL, and east MS), Lower Mississippi River Valley (LM = Memphis, TN to New Orleans, LA), West Central (WC = west AR and east OK), and West Gulf (WG = west LA and east TX). Open-pollinated seeds have been collected from several mother trees in each of 72 natural stands in the three eastern subregions (SA, EC, and EG), and cuttings have been collected from 208 clones of prior genetics programs for the three western subregions (LM, WC, and WG). DNA analyses of leaf samples from the mother trees in the 72 natural stands and from part of the 208 clones were conducted at LSU for evaluation of geographic patterns in RAPD and AFLP markers. The open-pollinated seeds were germinated at the University of Florida and vegetatively propagated to produce rooted seedling cuttings for four field trials located in eastern NC, northwest FL, southwest AL, and southeast MO.

Results are presented for phenotypic variation in wood specific gravity of trees and clones from the six subregions (collected and analyzed by Mississippi State University). Summaries are given for variation in rooting ability of seedlings (graduate project at University of Florida) and geographic patterns of RAPD and AFLP markers (graduate project at LSU). Geographic trends in first-year results for height growth and *Melampsora* leaf rust resistance at the four field sites are described.

INTRODUCTION

Short-rotation woody crops (SRWC) can be used for rapid production of biomass energy and wood fiber. Growth rates of 5-10 dry tons per acre per year are required in SRWC to help meet the nation's annual energy consumption and to economically justify this alternative source of fiber for the pulp and paper industry. Indirect benefits are reduction in dependence on foreign oil, mitigation of atmospheric carbon dioxide buildup, and supply of homogeneous raw material from accessible sites near mills.

Poplars (Populus sp.) have long been used throughout the world for SRWC (Land *et al.* 1996, Land and Singh 1997). They are suitable for paper and wood manufacture and as a feedstock for ethanol production (Dinus *et al.* 2001). Hybrid poplar in the Northwest is already

producing 5-10 dry tons per acre per year. The Southeast has high potential for SRWC, since the region has the second largest excess/idle agricultural acreage of all regions in the United States and also has a long, moist growing season. Eastern cottonwood (*Populus deltoides* Bartr. ex Marsh. var. *deltoides*) represents the best choice for SRWC in the region, because of its rapid growth and ease of vegetative propagation. Some local selection has been done (Land *et al.* 1996), but no southeast-wide collection, testing, and breeding of cottonwood was done before 1995 (when the current project was initiated).

The goal of the project is to develop genetically suitable eastern cottonwood clones for SRWC in the southeastern United States. Objectives are to identify/produce clones with increased growth rate, increased wood specific gravity, and *Melampsora* spp. leaf-rust resistance for each of six geographic subdivisions (subregions) of the Southeast. Mississippi State University (MSU) is coordinating the project, and Louisiana State University (LSU) and the University of Florida (UFL) are subcontractors. Funding is provided by (1) the Department of Energy (through the Bioenergy Feedstock Development Program at Oak Ridge, TN), (2) the U. S. Forest Service (through the Southern Research Station at Asheville, NC), and (3) three timber companies (Boise Cascade Corporation, International Paper Company, and Westvaco Corporation).

MATERIALS AND METHODS

The Southeast was subdivided into six subregions: Southeast Atlantic (SA = NC to eastern GA), East Central (EC = TN and northern AL), East Gulf (EG = south AL, west GA, northwest FL, and east MS), Lower Mississippi River Valley (LM = Memphis, TN to New Orleans, LA), West Central (WC = west AR and east OK), and West Gulf (WG = west LA and east TX). Two-hundred-and-eight clones of known origin from genetics studies by the U. S. Forest Service at Stoneville, MS, and Oklahoma State University at Idabel, OK, were selected to represent the three western subregions (LM, WC, and WG). Each of these three subregions was subdivided into four "Areas" (different rivers or different segments of rivers), and each "Area" was represented by at least ten clones. Since there were no tested clones of known origin from the three eastern subregions (SA, EC, and EG), 24 natural stands of eastern cottonwood were located in each of these three subregions. To do this, each subregion was subdivided into two halves (usually two different major river systems), and each half was represented by six stands on "bottomland" sites (river floodplains) and six stands on "upland" sites (uplands, terraces, or minor streams in the uplands). Thus, a total of 72 natural stands were located in the three eastern subregions.

Half-inch (12mm) diameter wood cores were extracted from pith to bark at breast height from 206 of the 208 clones and from 314 trees in the 72 stands. Since the ages of ramets were known for the 206 clones, cores from trees greater than ten years old were divided halfway from pith to bark. The half coming from nearest the pith was designated "juvenile", and the half from nearest the bark was designated "mature". One-hundred-and-seven of the clones had ramets sufficiently old (12 to 20 years old) to allow this subdivision. Specific gravities were measured as the ratio of unextracted dry weight to green volume on the "juvenile" and "mature" cores from the 107 clones and on the whole cores from the remaining 99 clones and 314 trees. Proc GLM and Scheffe's method of mean separation tests (SAS 1999) were used to test differences between "juvenile" and "mature" specific gravities and to test differences among subregions.

Open-pollinated seeds and green leaves were collected from 222 mother trees in 69 of the 72 natural stands in the three eastern subregions. Cuttings were collected from the 208 clones that represented the three western subregions, and green leaves were taken from 57 of these clones that were present at LSU. DNA analyses of the leaf samples were conducted at LSU.

Amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) markers were employed to determine if the 57 clones could be "fingerprinted" by DNA markers and to study population structure and estimate genetic variation in the 202 trees from the three eastern subregions. The cuttings were planted in clone preservation nurseries and breeding orchards at MSU, LSU, and UFL. The open-pollinated seeds were germinated at UFL and vegetatively propagated to produce rooted seedling cuttings for four field trials.

A study was conducted at UFL to investigate genetic variation in greenwood propagation success of eastern cottonwood. Greenwood cuttings from 5-7 seedling clones from each of seven of the open-pollinated families were propagated under mist. Stock plants were 2.5 years old and had been normalized under greenhouse conditions for more than a year. Cuttings were harvested and stuck in January 2000. The top-most cutting from each stem was discarded. It was cut directly above the auxiliary bud at leaf plastichron index 0 (LPI index leaf 40mm) (Larson and Isebrands, 1970). Subsequent cuts were made directly above the third lateral bud down from the previous cut, thereby producing two-node cuttings with some variation in length. A half leaf was retained on each cutting. Cuttings were dipped in water, stuck in a 2:1:1 (Pro-Mix®: Fafered®: perlite) rooting medium, and arranged into 4 randomized blocks in 7x14 Traymaster® cells. After 18 days, a total of 328 cuttings were assessed for survival, root initiation, number of roots, root dry weight, callusing, and shoot flush. Leaf area was measured for each cutting. Effects were tested by Proc ANOVA (SAS 1999).

Four sites were selected for field trials: (1) an agricultural field on an upland site in northwest Florida (FL), (2) an agricultural field on a Mississippi River alluvial plain site in southeast Missouri (MO), (3) a recently-harvested hardwood plantation site on the Tombigbee River floodplain in southwest Alabama (AL), and (4) a recently-harvested hardwood plantation site on a terrace of the Roanoke River in northeastern North Carolina (NC). The FL and MO sites were planted in June-July 1999 and have drip irrigation. The AL site was planted in December 1999, and the NC site was planted in March 2000. These latter two sites have no irrigation and are one-half year younger than the first two sites. Only the seedling clones from the three eastern subregions and a "check" group of 12 clones from the LM and WG subregions are used in these field trials. There were insufficient rooted cuttings to plant a balanced experimental design across the four sites. Each site has a "main study" with three replications for clones having three cuttings, a "supplemental study" with two replications for clones only having two cuttings available, and "borders" for clones with one (or extra) cuttings. Clones may be represented at one to four sites. Measurements of height and *Melampsora*
leaf-rust infection were taken in September-November 2000. The leaf-rust infection severity was scored as follows:

1 = no rust (no yellow urediospores on any leaves),
2 = little rust (isolated spores on several leaves, but no "crinkled" leaves),
3 = medium rust (many spores, some coalesced necrotic spots, often has "crinkled" leaves, and some defoliation in interior of crown),
4 = heavy rust (most of crown defoliated, with only young leaves at branch tips).

Analyses of the unbalanced data were conducted with Proc GLM (SAS 1999). A completely random design (CRD) was assumed at each site. Means were calculated by the LSMEANS option and tested by the Scheffe method. Scale effects and heterogeneity of variances for the different sites were removed by expressing each tree's value (for height or leaf-rust score) as a

"performance level". Performance level was calculated as the individual value minus the site mean, with the difference being divided by the standard deviation for that trait at that site. A completely random model was used for a Proc GLM analysis of variance of first-year height to provide conservative estimates of variance components for main effects and site-by-main-effect interactions for (1) subregion effects, (2) stands within subregions, (3) families within stands, and (4) clones within families.

RESULTS AND DISCUSSION

Specific Gravity

The overall mean specific gravity for all 520 trees was 0.36. All but three of these trees had specific gravities between 0.28 and 0.44, indicating a lot of individual tree variation. "Juvenile" and "mature" wood specific gravities were not significantly different, but "whole-core" specific gravity was greater for the three western subregions than for the three eastern subregions (Table 1). The lack of a difference between the "juvenile" and "mature" portions of the tree is not surprising, as Dinus *et al.* (2001) reviewed literature for other poplar species that reported gradual declines in specific gravity through the tenth annual ring and then a gradual rise across subsequent rings. Thus, values at two to four years of age are similar to those at ages beyond ten years of age. But, this is the first report of a geographic pattern of increase in specific gravity from east to west in the southern population of cottonwood. One must not get too excited about

Table 1. Phenotypic means and mean separation tests for eastern cottonwood specific gravities from (1) "juvenile" and "mature" wood and (2) six subregions of the southeastern United States.

Class	Number of Trees	Mean Specific Gravity	Scheffe's Test [a]
"Juvenile" wood	107	0.363	A
"Mature" wood	107	0.369	A
Subregion = SA	104	0.339	b
EC	126	0.348	b
EG	84	0.351	b
WC	52	0.374	a
LM	113	0.376	a
WG	41	0.381	a
Overall (for "whole-core" values across subregions)	520	0.358	

[a] Similar letters indicate no significant difference ($\alpha = 0.05$). Upper case and lower case letters are used to designate different comparisons.

this phenotypic pattern, however. The trees were measured on different sites under different environmental conditions. Dinus *et al.* (2001) noted that specific gravity is a composite of several properties, including (1) cell-wall thickness and (2) types, numbers, and sizes of wood cells. The geographic pattern may simply represent an environmental effect on one or more of

these properties, rather than a genetic difference. Clones from all subregions must be grown together on the same site before the cause of the pattern can be verified.

DNA Analyses

Using 14 primers, a total of 101 polymorphic RAPD markers were amplified from leaves of 57 clones from the LM, WC, and WG subregions. Six AFLP primer pairs resulted in a total of 457 polymorphic markers. The three most polymorphic RAPD primers or any of the six AFLP primer combinations generated enough polymorphisms to uniquely identify all clones. This indicates that extensive genetic diversity exists among the clones, and it demonstrates the efficiency of these markers for DNA fingerprinting.

A total of 492 AFLP markers and 104 RAPD markers were used in analyses of the leaf samples from 202 trees in the EC, EG, and SA subregions. Each subregion was subdivided into four populations ("upland" and "bottomland" within each of two "halves" or rivers) to investigate population structure and estimate genetic variation. The within-population genetic diversity was estimated to be 0.2543 from AFLP data and 0.2619 from RAPD data. The coefficient of genetic differentiation among populations (F_{ST}) was estimated to be 0.0663 and 0.0536 for AFLP and RAPD data respectively (P<0.001). Genetic distance matrices were calculated based on Nei's standard genetic distance (Nei 1972, Nei 2000). The correlation between AFLP and RAPD data matrices as measured by Pearson product moment correlation was 0.425 (P= 0.027). Dendrograms were constructed using the unweighted pair-group method (UPGMA) and Neighbor-joining (NJ) based on Nei's genetic distance matrices. Populations from the EG subregion grouped together by both methods when AFLP data were used. The UPGMA tree from RAPD data suggested that populations from the EC and EG subregions were genetically similar, whereas the NJ method only grouped populations from the EC subregion. Variances associated with the population parameters from AFLP analysis were significantly lower than those from RAPD analysis, suggesting that AFLP markers are more reliable than RAPD markers for population studies. Figure 1 provides an UPGMA dendrogram of a combined data set of AFLP and RAPD markers.

Greenwood Propagation

Measurements of the greenwood cuttings at 18 days after being stuck revealed that the number of roots per ramet ranged from zero to 73. However, the only significant source of variation for number of roots was "among clones within families". Leaf area was correlated with root number ($r = 0.49$, $P > .0001$) and dry weight ($r=0.66$, $P > .0001$). Family means for number of roots per cutting ranged from five to 20. Although significant variation between families for root number was not observed, the significant variation among clones within families and the small numbers of families (7) and clones within families (5-7) may have prevented the detection of family differences. This conclusion would support earlier findings of Ying and Bagley (1977), who found significant differences in rooting among open-pollinated families of eastern cottonwood when they used a greater number of families.

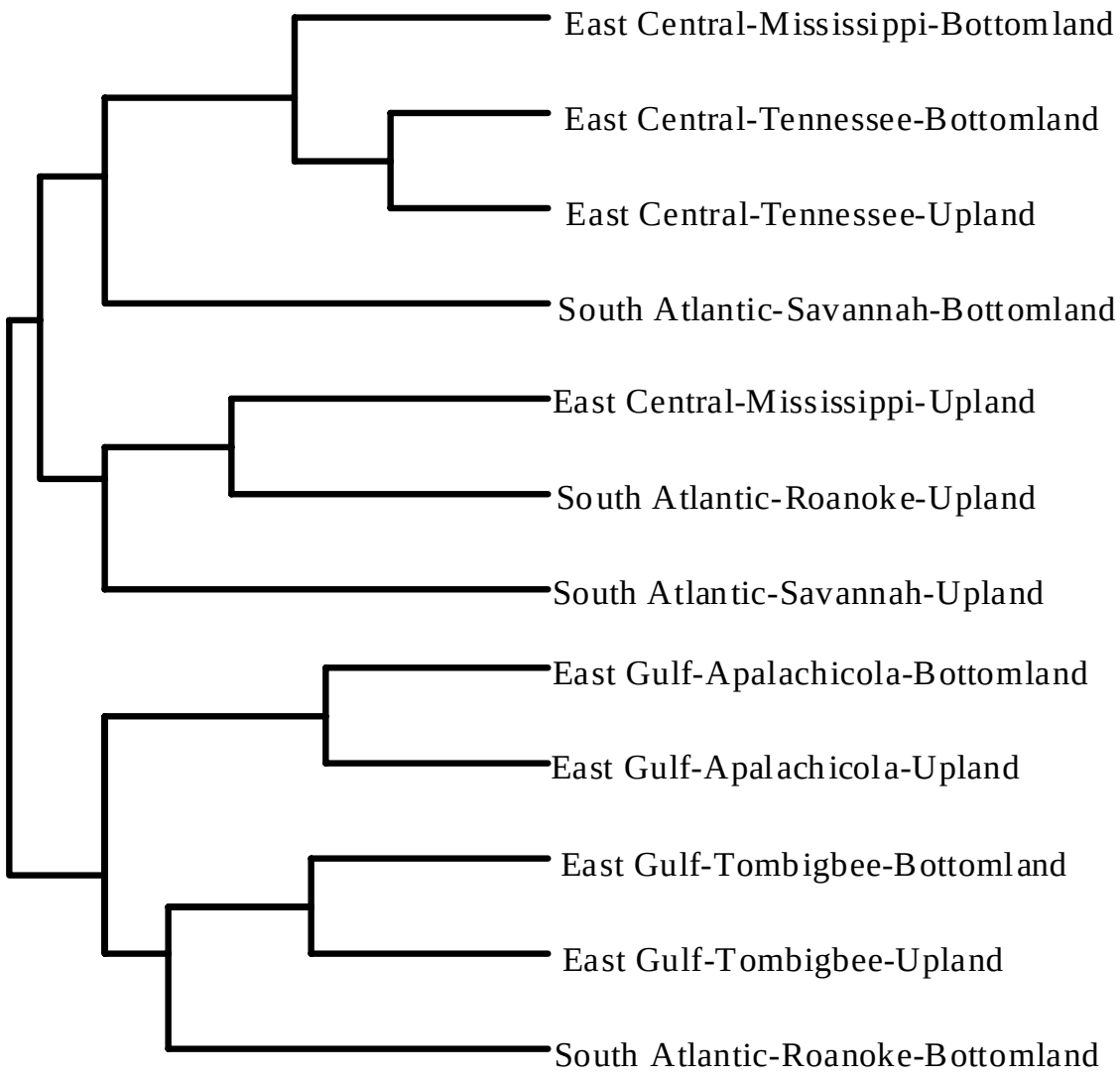

Figure 1. UPGMA dendrogram for 12 eastern cottonwood populations based on 492 AFLP and 107 RAPD markers using Nei's standard genetic distance.

Leaf Rust and First-Year Height

Very little *Melampsora* leaf-rust was observed at the AL and NC test sites during the fall of 2000 (Table 2). This was a very dry year, and the lack of moisture at these two sites may have been responsible. In the MSU nursery and orchard, where the same clones were represented in an irrigated situation (nursery) and a non-irrigated situation (orchard) at the same site, there was much more leaf rust in the nursery than in the orchard. The FL and MO sites had drip irrigation and had more rust than the AL and NC sites. At these two irrigated sites there appears to be more rust in the more northerly and interior EC subregion. A similar trend of greater leaf infection on seedlings from more northerly stand origins (central TN) was observed by Friend (1981) in a two-year-old cottonwood nursery at the MSU campus in east central Mississippi.

Table 2. Means and mean separation tests for *Melampsora* leaf rust infection and first-year heights of cottonwood clones from three southeastern subregions and a check lot at four test sites.

Subregion [a] (or Check)	Test Site [b] AL	FL	MO	NC	Subregion Average
	- Height (ft) -				
SA	5.3 A	10.4 A	12.9 C	6.1 B	8.8
EC	4.9 AB	10.2 A	12.8 C	6.6 A	8.9
EG	5.1 A	10.8 A	13.9 B	6.6 A	9.1
CK	4.4 B	10.8 A	15.3 A	6.7 A	8.2
Site Average	5.0	10.5	13.3	6.4	
	- - - - - - Leaf Rust Score (1 = none, … 4 = heavy infection) - - - - - -				
SA	1.8 b	2.5 a	3.4 a	2.1 a	2.5
EC	1.9 b	2.9 b	3.6 b	2.1 a	2.7
EG	1.8 b	2.8 b	3.2 a	2.0 a	2.4
CK	1.5 a	2.5 a	3.2 a	2.0 a	2.1
Site Average	1.8	2.7	3.4	2.0	

[a]CK = Genetic check of 12 tested clones from the Lower MS River Valley and East Gulf (clones 110116, 110531, 110804, 111101, 111733, 112127, 112620, 112830, 3324, S7C1, ST-121, & ST-71)

[b]Mean separation tests were performed by Scheffe's test on Least Squares adjusted subregion means of performance-level values by SAS Proc GLM. The alpha level used for significance was 0.05.

The rooted cuttings grew to a height of five to six feet after one full growing season at the AL and NC sites, and they were twice as tall after 1.5 growing seasons under drip irrigation at the FL and MO sites (Table 2). Separate analyses of variance and mean separation tests for each site revealed significant height differences among subregions / checks at the MO, AL, and NC sites, but not at the FL site. The genetic check of 12 previously selected clones from the LM and WG subregions had the tallest trees at the MO site and the shortest trees at the AL site. These "check" cuttings were rooted from hardwood cuttings that were stuck a month later than the greenwood cuttings from the seedlings. Thus, their poor performance at AL may indicate that they have not yet had enough time to overcome their late start. Their superior performance at MO, where they have had an additional half growing season for growth, may reflect the previous selection for improved growth that these clones had undergone. The means for the other three subregions are based on unselected clones, many of which are inferior in growth. There may still be individual clones from these three subregions that are superior to the performance of the check clones. When the check is excluded from consideration, only the MO and NC sites exhibit significant differences among the three eastern subregions. Surprisingly, the "local" subregion at each of these more northerly sites has grown the least in height (SA at the NC site and EC at the MO site). These very early results suggest that the EG subregion may provide greater height growth than local sources when grown in the northern part of the southeastern United States.

A combined analysis of variance across the four sites for first-year height (with the check clones excluded) indicated that most of the clonal variation is associated with (1) variation among clones within mother-tree families and (2) variation among mother-tree families within stands (Table 3). Since height differences among the sites resulted in heterogeneity of variances

and scale effects, the heights were "transformed" into performance levels to remove these differences before the combined analysis was conducted. These performance levels can subsequently be used in selection of superior clones, since clone means across sites will not be biased by incomplete representation at all sites in the unbalanced study. "Site-by-Subregion", "Site-by-Stand-within-Subregion", and "Site-by-Clone-within-Family" interactions were all significant, even at this young age and even after removal of the scale effects. The error component of variance, which represents environmental variation among ramets within clones, accounts for two-thirds of the sum of all the variance components. Much of this within-clone variation resulted from non-uniform rooted cuttings that were planted because of a shortage of material. First-year heights are still greatly affected by the size of the material that was planted, and these effects may decline with age. However, future trials must use more uniform planting material. In summary, there is evidence from these early height results that the greatest gains from clonal selection of eastern cottonwood will come from family and within-family selection, and different clones may have to be selected for different sites. Because of the large error variance and the need to also test rooting ability, second-stage trials will follow these initial "screening" trials and will be established with uniform, non-rooted cuttings of the top 25% of the clones in the present trials.

Table 3. Combined analysis of variance across four test sites for seed origin and clone effects on first-year height (Performance Levels) of rooted cuttings from cottonwood seedlings collected in three southeastern subregions (SA, EC, and EG). Analysis of unbalanced data was conducted by SAS Proc GLM, and all effects were considered random.

		Type III		**Variance Component**	
Source of Variance	d.f.	M.S.	Pr > F	Estimate	% of Tot.
Test Sites [=T]	3	0.433	.9594^{ns}	- 0.00939	0.0
Clones [=C]	973				
Among subregions [=R]	2	9.375	.2450^{ns}	0.00633	0.7
Stands w/i R [=S(R)]	61	2.755	.1063^{ns}	0.01939	2.0
Families w/i S(R) [=F(SR)]	90	1.829	.0002**	0.05341	5.6
Clones w/i F(SR)[=C(FSR)]	820	1.035	< .0001**	0.07472	7.8
Sites x Clones [=TxS]	1240				
TxR	6	4.556	.0003**	0.02398	2.5
TxS(R)	172	1.115	.0279*	0.02830	2.9
TxF(SR)	201	0.844	.2628^{ns}	0.01156	1.2
TxC(FSR)	861	0.805	.0002**	0.08683	9.1
Error	1784	0.653		0.65315	68.2

SUMMARY AND CONCLUSIONS

Eastern cottonwood is an important species for SRWC because of its rapid early growth and ease of vegetative propagation. An early requirement of tree improvement for this species in the southeastern United States is the collection and establishment of tests of genetic material from throughout the region. Such a project was initiated in 1995 and now involves three universities, two federal agencies, and three timber companies.

The southeastern region was subdivided into six subregions. Two-hundred-and-eight clones from previous genetics trials were selected to represent the three "western" subregions, and 72 natural stands were located for seed, wood, and leaf collections in the three "eastern" subregions. Specific gravities have been determined from the wood-core collections, DNA markers have been identified from the leaf samples, and rooted cuttings have been produced from seedlings derived from the seed collections. Four field trials in AL, FL, MO, and NC have been established with the rooted cuttings, and first-year results for height growth and *Melampsora* spp. leaf-rust infection are reported here.

Extensive variation among clones within families and/or among families (or mother trees) within stands has been detected for specific gravity, AFLP and RAPD markers, rooting ability of greenwood cuttings, and first-year height. The same may also be true for leaf-rust infection, but it was not analyzed in this paper. In addition, possible evidence of geographic patterns of variation among subregions have been detected for all these traits. Phenotypic values for whole-core specific gravity increased from east to west across the six subregions, groupings from RAPD and AFLP markers (based on genetic similarities) were detected among the three eastern subregions, leaf-rust susceptibility increased from southern to northern origins (from EG to SA and EC), and height growth increased from northern to southern origins (when planted at the more northern sites). Site-by-subregion, site-by-stand-within-subregion, and site-by-clone-within-family interactions were significant for first-year heights, indicating that different clones may be required for different sites. Two-thirds of the variation in the heights was associated with differences among ramets within clones, however. This may be due to non-uniformity in the rooted cuttings that were planted. A second set of trials will be established with non-rooted cuttings from the best 25% of the clones (for growth rate and leaf-rust resistance) to improve uniformity (precision of test) and to evaluate rooting ability.

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LITERATURE CITED

Dinus, R.J., P. Payne, M.M. Sewell, V.L. Chiang, and G.A. Tuskan. 2001. Genetic modification of short rotation poplar wood: Properties for ethanol fuel and fiber productions. Crit. Revs. Plant Sci. 20(1):51-69.

Friend, M.M. 1981. Genetic variation in juvenile traits of eastern cottonwood from the southern United States [MSc thesis]. Mississippi State, MS: Mississippi State University. 117 p. Available from: Dept. of Forestry, Mississippi State, MS.

Land, S.B. Jr., A.W. Ezell, S.H. Schoenholtz, G.A. Tuskan, T.J. Tschaplinski, M.Stine, H.D. Bradshaw, R.C. Kellison, and J. Portwood. 1996. Intensive Culture of Cottonwood and Hybrid Poplars. In: Growing Trees in a Greener World: Industrial Forestry in the 21st Century. 35th LSU Forestry Symposium. M. C. Carter (ed.). School of Forestry, Wildlife & Fisheries, LSU Agricultural Center, Louisiana Agricultural Experiment Station, Baton Rouge, LA. pp. 167-189.

Land, S.B. Jr., and N.B. Singh. 1997. *Populus* tree improvement in northern India. Proc. 24th So. Forest Tree Impr. Conf. 1997:224-33. Available from: NTIS, Springfield, VA; PB 97-186217.

Larson, P.R. and J.G. Isebrands. 1970. The plastochron index as applied to developmental studies of cottonwood. Can. J. For. Res. 1:1-11.

Nei M. 1972. Genetic distance between populations. Am. Nat. 106:283-292.

Nei M., and S. Kumar. 2000. Molecular evolution and phylogenetics. Oxford University Press, New York, NY 10016.

SAS Institute Inc. 1999. SAS/STAT® User's Guide, Version 8. Cary, NC: SAS Institute Inc. 3884 p.

Ying, Ch. and W.T. Bagley. 1977. Variation in rooting capability of *Populus deltoids*. J. Paper No. 5254. J. Ser., Nebaska Agr. Exp. Sta.