on loblolly, but the results at these young ages are inconclusive.

Discussion

Results of the search for putative hybrid pines reveal an optimistic picture of growth when these trees are compared with associated shortleaf pines. The progeny of the putative hybrids may offer a possible alternative to loblolly pine, whose long range adaptability to poor sites in the Piedmont is open to question. As shown by the results, certain lines of these putative hybrids produce progeny with growth rates and survival equivalent to those of young loblolly pines. Because the results of the progeny tests apply only to young trees, all conclusions are tentative. As the trees mature, more meaningful results should become available.

Summary

Putative hybrid pines were found on the Clemson Experimental Forest in the South Carolina Piedrnont. Measured traits indicate that those trees are probably hybrids from *Pinus echinata* X *Pinus taeda* crosses. In natural stands performance of the putative hybrids, when compared with that of associated shortleaf pines, is superior in terms of volume and form. Open-pollinated progeny, three to seven years of age, of the putative hybrids were generally intermediate in height growth between loblolly and shortleaf pine when planted on eroded heavy-textured soils on which littleleaf develops. However, in a few cases, individual progenies had growth rates equivalent to those of young loblolly pines.

Key words: Putative hybrids, Rust resistance, Littleleaf sites.

Zusammenfassung

Im Versuchswald S. C. Piedmont, USA, wurden in Form und Wachstum abweichende Kiefern beobachtet. Aufgrund von Merkmalsuntersuchungen wird vermutet, daß diese höchstwahrscheinlich Hybriden aus Kreuzungen zwischen *Pinus taeda* und *P. echinata* sind. Frei abgeblühte Nachkommenschaften sind intermediär, das heißt es sind nur wenige Individuen vorhanden, die *Pinus taeda* ähnlich sind.

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Mikrospectrophotometric Determination of DNA per Cell and Polyploidy in Fraxinus americana L.

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Introduction

The natural range of white ash *(Fraxinus arnericana* L.) extends from Minnesota to Nova Scotia south to Florida and west to Texas (FOWELLS, 1965). A diploid (2N = 46), tetraploid (2N = 92), and hexaploid (2N = 138) euploid series has been described for the species as well as distribution and evolutionary considerations (WRIGHT, 1944).

Various morphological criteria have been used to ascertain ploidy levels of white ash. WRIGHT (1945) found stomata size reliable for polyploid differentiation of nursery grown trees. Stomata size of field-grown individuals, however, was only found reliable for separation of diploids and polyploid~.SANTAMOUR (1962) examined nursery grown stock and reported that bud and leaf scar morphology could discriminate diploids from tetraploids. Hexaploids were similar to tetraploids in these character differing only by leaf and twig pubescence. MILLER (1955), however, found the pubescent character too variable to distinguish polyploid individuals.

Several investigators have employed cytophotometry as a tool to study polyploidy in plants. SOUTHERN (1967) attempted taxonomic clarification for species of *Tulipa*. GRANT (1969) determined that species of *Betula* with lower ploidy levels (2N = 28, 42, 56, 70) did have corresponding DNA values; whereas, the highest ploidy level (2N = 84) had a DNA value corresponding to approximately 63 somatic chromosomes.

Determination of chromosome numbers for polyploid species which possess large numbers of very small chromosomes is a difficult and time consuming task. The problem becomes compounded when a large number of individuals need to be determined; therefore, Feulgen cytophotometry was used in this study to correlate nuclear DNA content with chromosome number to explore the pattern of ploidy levels of white ash throughout its natural range and thereby aid in further understanding the genetic variation in the species.

Material and Methods

White ash seed provenances from three open-pollinated families (single tree collections) were used *(Table* 1).

DNA Feulgen measurements were made from radicle apex cells of three embryos per slide and contiguous with

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Table 1. — Provenance collection number, location, tree number and DNA quantity per root tip of *Fraxinus americana* L.

Identification and seed source numbers	Latitude °N	Longitude °W	Single tree collection number	DNA in Picograms $\bar{x}$	s	$s_{\bar{x}}$
			04	2.85	0.09	0.06
1. NC-6786	44.7	68.9	07	3.08	0.20	0.09
			09	3.10	0.17	0.08
			01	2.75	0.10	0.06
2. NC-6803	44.4	73.7	04	3.46	0.13	0.07
			07	3.02	0.06	0.05
			01	3.12	0.09	0.06
3. NC-6793	42.7	76.7	04	3.30	0.14	0.07
			06	3.21	0.20	0.09
			02	3.25	0.15	0.08
4. NC-6769	42.2	83.7	04	3.40	0.27	0.10
			06	3.92	0.31	0.11
			01	3.26	0.21	0.09
5. NC-6723	45.2	89.0	03	3.31	0.16	0.08
			09	2.93	0.06	0.05
			02	3.32	0.18	0.08
6. NC-6794	41.2	73.0	05	3.56	0.18	0.08
			10	2.82	0.16	0.08
			01	3.21	0.15	0.08
7. NC-6732	40.8	81.9	03	3.49	0.33	0.11
			07	3.38	0.33	0.11
			02	3.68	0.30	0.11
8. NC-6778	39.0	79.5	07	3.16	0.05	0.04
			10	3.90	0.36	0.12
			01	8.01	0.60	0.15**
9. NC-6798	39.4	84.1	04	3.41	0.17	0.08
			05	3.09	0.12	0.07
			01	7.08	0.57	0.11&
10. NC-6795	38.4	85.7	07	9.64	0.60	0.15**
			09	9.90	1.41	0.24**
			01	2.83	0.15	0.08
11. NC-6771	39.1	88.4	06	3.27	0.14	0.08
			10	3.02	0.12	0.07
			06	9.56	0.53	0.15**
12. NC-6722	37.6	88.3	08	2.81	0.18	0.08
			10	3.45	0.15	0.08
			01	2.98	0.08	0.06
13. NC-6721	37.7	89.2	03	2.90	0.06	0.06
			12	8.24	0.80	0.18**
			03	8.27	0.80	0.18**
14. NC-6792	37.2	87.4	05	8.60	0.46	0.14**
			10	3.33	0.11	0.07
			03	7.79	0.36	0.12**
15. NC-6797	35.7	78.7	04	7.84	0.28	0.11**
			07	8.20	0.27	0.10**
			07	7.99	0.33	0.11**
16. NC-6783	35.3	82.6	08	8.03	0.46	0.14**
			10	3.56	0.21	0.09
			03	3.20	0.07	0.05
17. NC-6728	35.2	85.9	06	3.08	0.07	0.05
			10	3.10	0.05	0.04
			02	3.52	0.16	0.08
18. NC-6535	36.4	92.8	06	2.73	0.11	0.07
			08	3.33	0.10	0.06
			02	3.27	0.14	0.07
19. NC-6740	33.4	88.8	04	5.91	0.50	0.14*
			06	3.01	0.04	0.04
			05	3.24	0.15	0.08
20. NC-6737	31.0	88.8	07	2.97	0.17	0.08
			10	3.48	0.13	0.07
			01	5.27	0.15	0.08*
21. NC-6738	30.5	91.0	03	5.61	0.20	0.09*
			10	5.60	0.27	0.10*
			04	6.28	0.26	0.10*
22. NC-6768	30.3	94.4	05	6.42	0.26	0.10*
			06	6.63	0.24	0.10*

* tetraploid; ** hexaploid; & putative pentaploid.

chicken erythrocytes (BERLYN and MIKSCHE, 1976) for a total of 66 slides.

Tissue and Slide Preparation. Seed was removed from the samaras and placed in distilled water for five days at room temperature. The imbibed seeds and previously prepared air-dried chicken blood smear-slides were simultaneously fixed with Carnoy's No. 2, ethyl alcohol, chloroform, glacial acetic acid (6 : 3 : 1), fixative (BERLYN and MIKSCHE, 1976) for 2 hours with the application of intermittent vacuum during the first 15 minutes of fixation.

Hydration was accomplished by 10 minutes in each of 50% and 30% ethyl alcohol rinses, followed by 2 hours in distilled water during which time the embryos were excised from the seeds. Squash preparations were then made on cleared areas (areas from which erythrocytes were removed) of chicken blood smear slides using the following methods: (1) a 3 to 4 mm segment of radicle apex was excised and placed on a cleared area; (2) the segment was mashed and spread within the cleared area with a blunt glass rod; (3) material was squashed with a coverslip; (4) steps 1 through 3 were repeated for two more embryos on two more areas of the same slide; (5) and finally, the cover slips were removed according to the method of CONGER and FAIRCHILD (1953). The slides were then simultaneously hydrolyzed and stained.

Hydrolysis and Feulgen staining were accomplished by the following steps: (1) slides were placed in 5N HCl at 21° C for 45 minutes *(Fig. 1):* (2) rinsed in cold distilled water (4° C) twice for 2 minutes; (3) transferred to 2.9 pH adjusted (GHOSH, 1969; MIKSCHE, 1971; JACQMARD and MIKSCHE, 1971) Schiff's reagent and placed in dark for 2 hours; (4) bleached twice in 10 minute baths of potassium metabisulfite solution (BERLYN and MIKSCHE, 1976, p. 274); (5) rinsed twice in distilled water for 10 minutes each; (6) dehydrated to absolute ethyl alcohol via 30% — 50% — 70% — 95% — 100% steps; (7) and allowed to air dry.

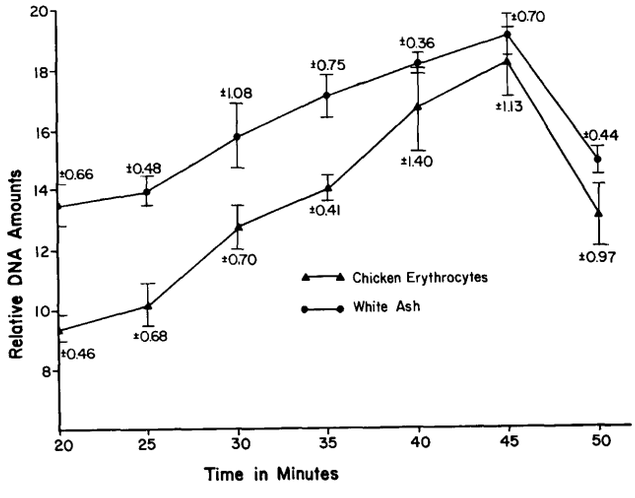

Fig. 1. — Hydrolysis Curves. A 45 minute hydrolysis in 5N HCl at 21° C was established for both white ash and chicken erythrocyte nuclei. Each point was derived from the mean of 20 white ash and 6 chicken erythrocyte nuclei by two-wavelength cytophotometry using wavelengths of 545 nm and 500 nm.

Non-specific light loss was minimized for cytophotometry by mounting the preparations in matching refractive index oil (n_D 1.556, R. P. CARGILLE Laboratories, Inc., Cedar Grove, New Jersey).

Two-wavelength Cytophotometry: Two wavelength cytophotometry was accomplished by instrumentation described by MIKSCHE (1971).

Measurements were taken using a 95X objective with immersion oil and two-wavelengths of 545 nm and 500 nm were determined from spectral adsorption curves *(Fig. 2)* (ORNSTEIN, 1952; PATAU, 1952). Tables were used to compute amounts of relative Feulgen absorption (MENDELSOHN, 1958).

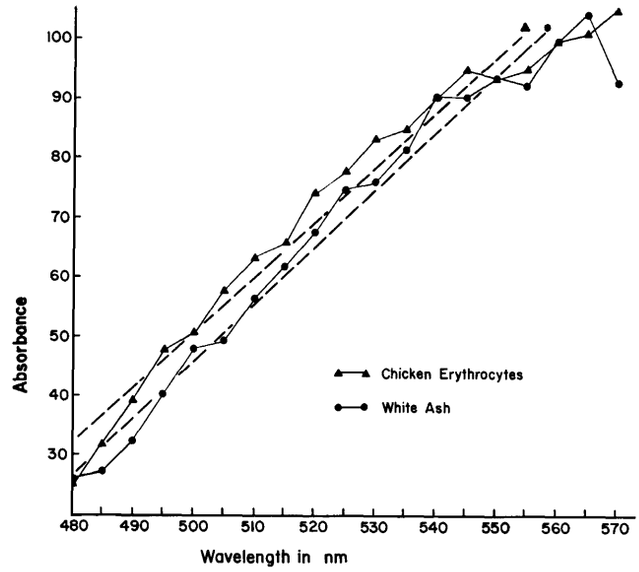

Fig. 2. — Spectral absorption curves for both white ash and chicken erythrocyte nuclei were determined by setting the 560 nm optical density absorbancy for each curve equal to 100 for purposes of comparison (GARCIA and IORIO, 1966; RASH, 1974). Each point represents the mean of 5 white ash and 3 chicken erythrocyte nuclei. Since no significant differences in absorption maxima or general curve shapes were detected, the same wavelengths were chosen for both white ash and chicken erythrocyte nuclei. $A\lambda_1$ = 500 nm and $a\lambda_2$ = 545 nm (ORNSTEIN, 1952; PATAU, 1952) were selected from regression lines in which y = ax + b. The equation for the white ash plotting was y = (0.97) (x) + (—439.08) and he chicken erythrocyte plotting was y = (0.93) (x) + (—414.24).

Three tree preparations were empirically selected on the basis of previously determined chromophore values to establish population profiles of the three ploidy levels. For each tree, relative DNA amounts of 75 nuclei were measured. The intent, by sampling a large cross section of each nuclear population, was to determine if the nuclei were parametric in distribution and to determine sample size. A normal distribution was observed in the three ploidy populations and, therefore parametric statistical analysis could be applied *(Fig. 3).*

The sample size used was determined by measuring 75 randomly assigned nuclei from each tree. The standard error of the mean leveled off at 25 measurements and the "t" test between 25 and 75 measurements yielded values of 0.13 (diploid), —0.12 (etraploid), and 0.11 (hexaploid), each with 98 degrees of freedom and were not significant at 5% (SNEDECOR, 1956). Therefore, 25 nucleu were measured from each of the remaining tree preparations.

Requirement of an Internal Standard and Conversion of Relative DNA to Absolute Amounts: Chicken erythrocyte nuclei, which served as an internal reference value, were smeared on microscope slides. (BERLYN and CECICH, 1976; BERLYN and MIKSCHE, 1976; DHILLON, BERLYN and MIKSCHE, 1977; and DOERSCHUG, MIKSCHE and STERN, 1976). Prior to fixation, the erythrocyte nuclear dry mass was determined

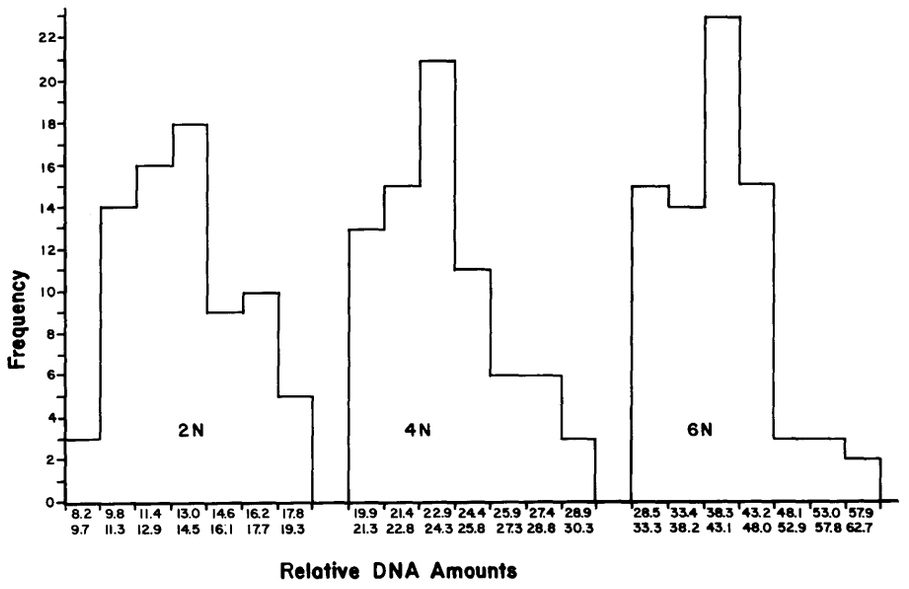

Fig. 3. — Nuclear DNA population profiles of three white ash trees representing different ploidy levels.

from 150 polarizing interference microscope readings by using the formula given by BERLYN and MIKSCHE (1976). The procedure was repeated after treating the cells with DNase, the difference in dry weights representing the amount of DNA per nucleus. The ratio of nuclear dry mass to Feulgen absorption in erythrocyte nuclei was used to calculate the absolute amounts of DNA in white ash nuclei. The NDA content of erythrocyte nuclei as determined by interferometry (2.62×10^{-12} g) is close to published values determined chemically (SHAPIRO, 1970).

A total of 736 chicken erythrocyte nuclei were measured by two-wavelength cytophotometry. These values were pooled and a mean of 9.47 determined for the relative DNA amount per nucleus equivalent to the pre-determined dry mass value. Pooling these nuclear DNA values was presumed valid since the slide mean values depict a normal distribution and the slide to slide coefficient of variation was 11%. This variation was of the same order of the 10% uncertainty seemingly inherent in comparative cytophotometry (RASCH, 1974; GARCIA and IORIO, 1966).

The estimates of DNA white ash amounts determined by the comparative ratio of relative DNA to chicken erythrocyte nuclear dry mass is a reliable estimate because both cell types were simultaneously processed, had uniform hydrolysis curves and were measured under identical instrumentation. However, when using this method of DNA estimation, the caution extended by RASCH (1974) is also applicable here and should be considered.

Chromosome Count Determination: Chromosome counts were made after cytophotometric determinations for DNA amounts were completed, to insure that measured DNA amounts corresponded to levels of ploidy.

To initiate germination, seeds were soaked for 3 days at room temperature in distilled water after which time the

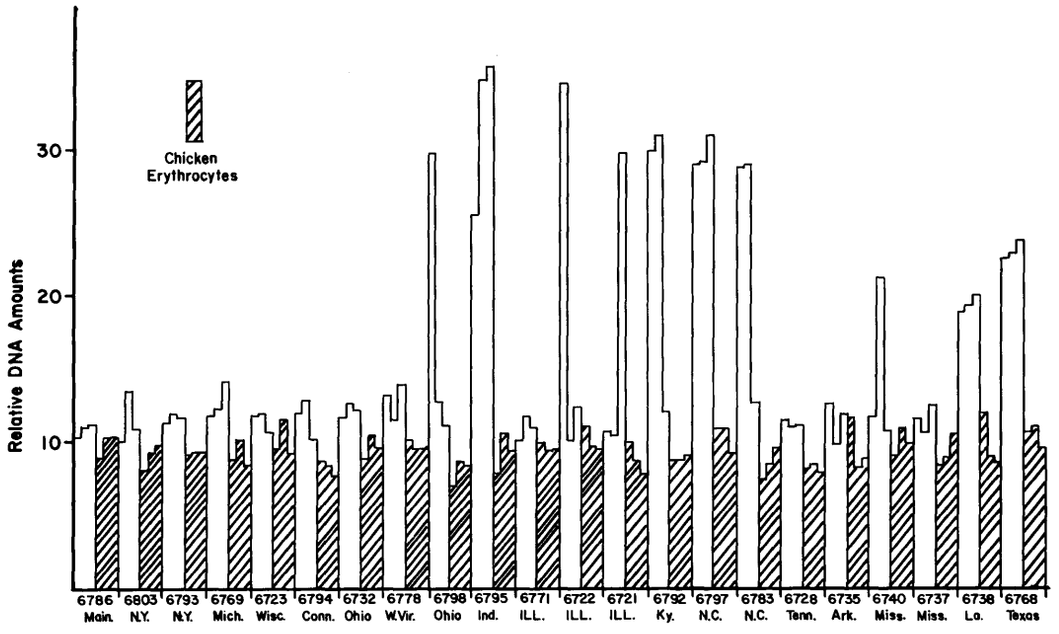

Fig. 4. — Relative nuclear DNA amounts for individual trees and chicken erythrocytes.

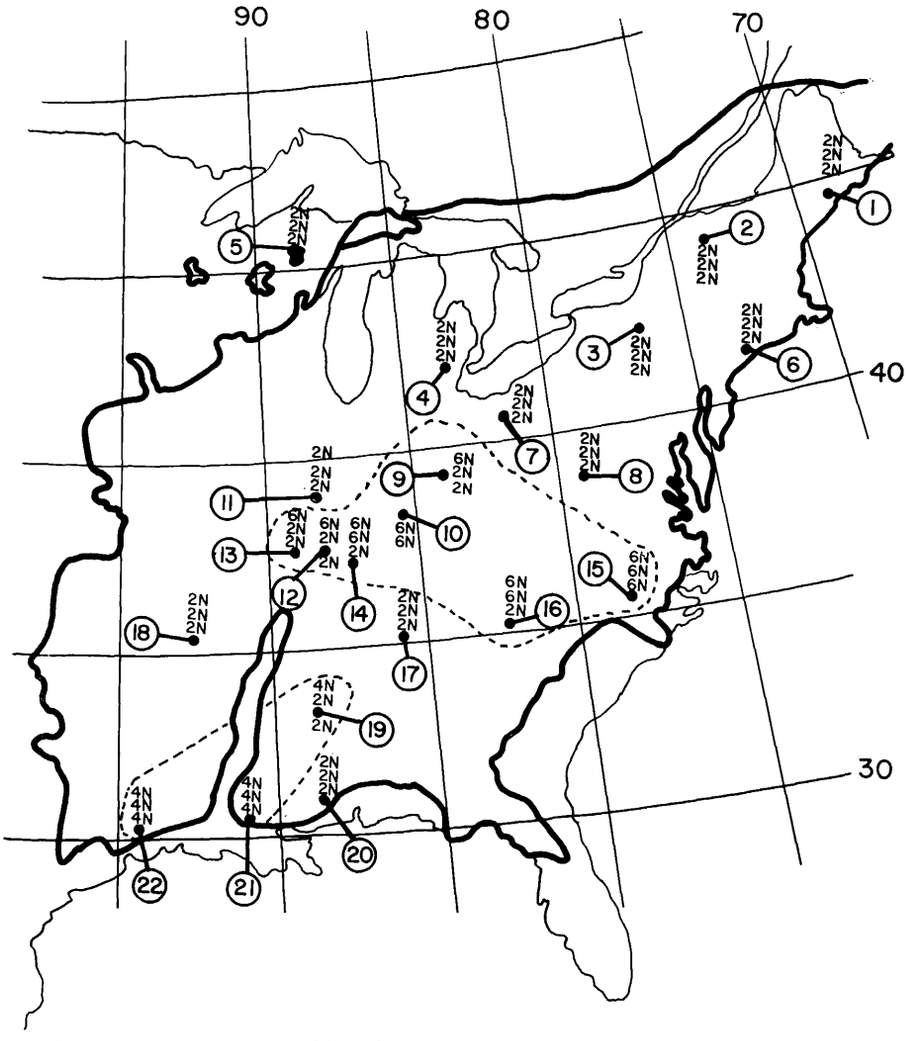

Fig. 5. — Natural range of white ash, location of provenances included in the study (circled numbers) correspond to numbered provenances and further details listed in *Table 1* and cytophotometrically determined ploidy levels found at each location. The putative pentaploid, which has not been shown, was from provenance number 10.

embryos were dissected and placed on filter paper moistened with 50 μM aqueous zeatin in petri dishes (GENDEL, S., personal communication) to procure uniform germination. After 10, 16-hr photoperiods, the seedlings were transferred to 0.25% Actidione solution (SHARMA and SHARMA, 1972; CUMMINGS, BREWER and RUSCH, 1965) for 4 hours at 25° C. Feulgen squash preparations of the root tips were made as previously described and restained with 45% aceto-orcein (occasionally warming from a slide mounting hot plate) for 5 minutes. Cover slip mounts were made with immersion oil (refractive index 1.515).

Photomicrography was accomplished with a Wild M-12 microscope using 50X, 100X objectives and EKtapan film.

Results and Discussion

Two-wavelength cytophotometrically determined mean relative DNA absorption values of white ash and companion chicken erythrocyte nuclei are presented in *Figure 4*. Each of these mean values was determined from 25 white ash and 10 chicken erythrocyte nuclei for direct comparison. Slide to slide variation of white ash DNA amounts was found to be independent of the slide to slide variation of chicken erythrocyte DNA amounts.

Relation between Ploidy Levels and DNA Quantity: With the exception of one tree, observed DNA values fell within one of three ploidy levels. Forty-six diploid trees with mean DNA values ranging from 2.73 to 3.92 pg, 7 tetraploid trees with mean DNA values ranging from 5.27 to 6.63 pg, and 12 hexaploid trees with mean DNA ranging from 7.79 to 9.90 pg represents the euploid series of the natural range of white ash *(Table 1, Fig. 5)*. A correlation analysis between mean DNA values and 2N, 4N, and 6N, ploidy levels, respectively, yielded a highly significant r value of +0.98 *(Fig. 6)*. The correlation in *Figure 6* was determined from the mean DNA values of each ploidy group. The results support both the previously reported euploid series (WRIGHT, 1944) and the use of cytophotometry as a means of differentiating ploidy levels for the white ash complex.

The one tree excluded from the above analysis had a DNA value of 7.08 pg and was therefore approximately halfway between the tetraploid and hexaploid DNA levels: a putative pentaploid or possible aneuploid. Since uniform techniques were maintained, the reason the one tree did not conform to one of the three DNA levels measured for the other 65 trees would not be expected to be of a technical nature. Unfortunately, sufficient putative pentaploid material was not available for chromosome preparations.

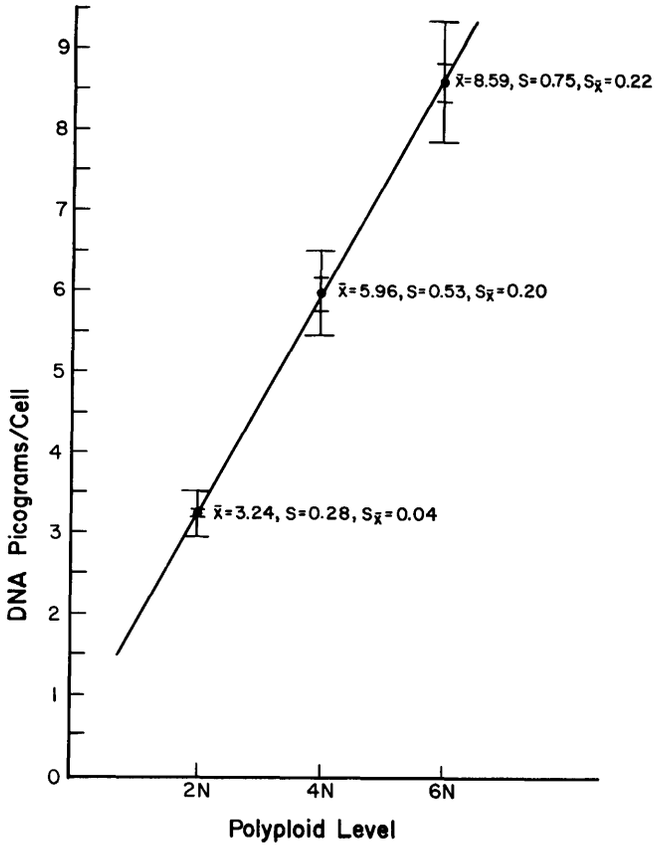

Fig. 6. — Relationship between ploidy levels and mean nuclear DNA values of 65 white ash trees (r = +0.93).

Collection 6778-10 with a mean nuclear DNA value of 3.90 ± 0.12 pg had a diploid complement of 46 chromosomes within a maximum interpretation error of 6%. Collection 6768-05 with a mean nuclear DNA value of 6.42 ± 0.10 pg had a tetraploid complement of 92 chromosomes within a maximum interpretation error of 1%. Collection 6795-09 with a mean nuclear DNA value of 9.90 ± 0.24 pg had a hexaploid complement of 138 chromosomes within a maximum interpretation error of 10% *(Fig. 7 a, b, c).*

Aneuploidy Considerations: Though the regression analysis between mean DNA amounts and the previously reported euploid series of 2N, 4N and 6N demonstrated a highly significant correlation, the average tetraploid DNA value of 5.96 pg was 7.5% less than twice the average diploid DNA value of 3.22 pg and the average hexaploid DNA value of 8.51 pg was 11.9% less than three times the average diploid DNA value. In all probability, a combination of the inherent variation attributable to cytophotometry and the samll number of measured tetraploid and hexaploid trees were responsible for these lower than expected readings.

On the other hand, a curious observation (factor) was manifested with regard to the hexaploid measurements which may indicate yet another reason for the deviation from the ploidy multiplication factor. When the mean DNA values for each of the N, 4N and 6N groups were plotted on normal probability graphs, a test for normality revealed that both the diploid and tetraploid values conformed to normal distributions at the 5% level *(Figures 8 and 9).* The hexaploid values, however, displayed a deviation from normality at the 5% level (Kung, 1973) *(Fig. 10).*

Comparing estimated values obtained for the hexaploid levels on probability plots; 3 of them were separated from the remaining 9 by a 10% margin. When these two hexaploid groups were plotted separately on normal probability graphs, each group displayed a normal distribution *(Figures 11 and 12).* The average value for the group of 9 mean DNA values then became 8.11 ± 0.08 pg with a 16% difference from three times the average diploid value, while the average value for the group of 3 became 9.70 ± 0.10 pg; only a 0.4% difference from expected. Since these 3 mean values came from trees confined to two geographically close provenances and were not subject to bias when they were measured, it is tempting to give serious consideration to Cimino's (1974) speculation that some loss of chromosomal material is responsible for the lower average found in 9 of the 12 hexaploid trees measured.

Aneuploidy occurs in polyploid systems (Khush, 1973) and is probably a factor in the population adaptation processes of white ash. Verfication of such chromosomal phenomena could be accomplisled by examining meiotic chromosome

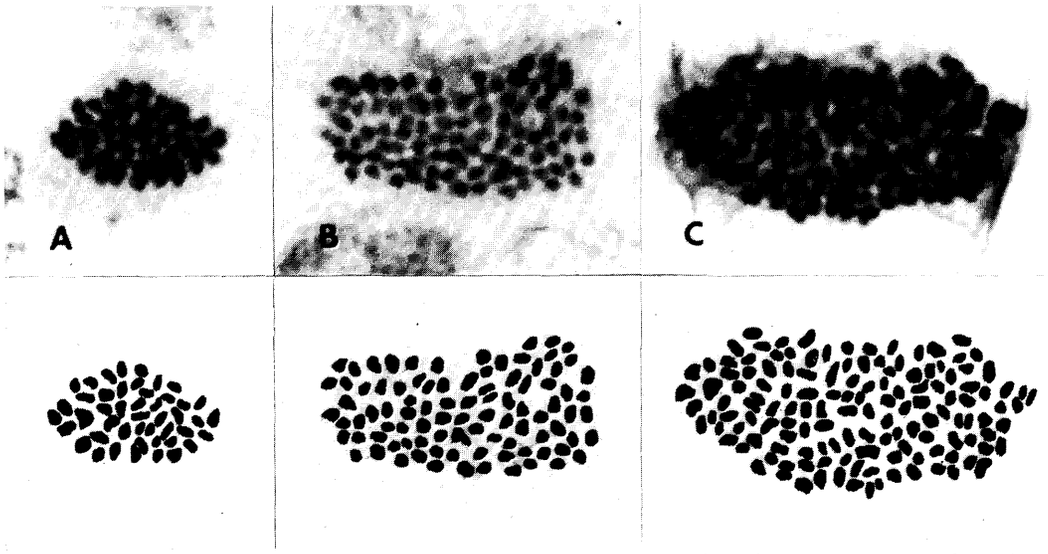

Fig. 7 A, B, C. — Photomicrographs and corresponding drawings of white ash chromosome complements of diploid (46), tetraploid (92) and hexaploid (138) levels. X1685.

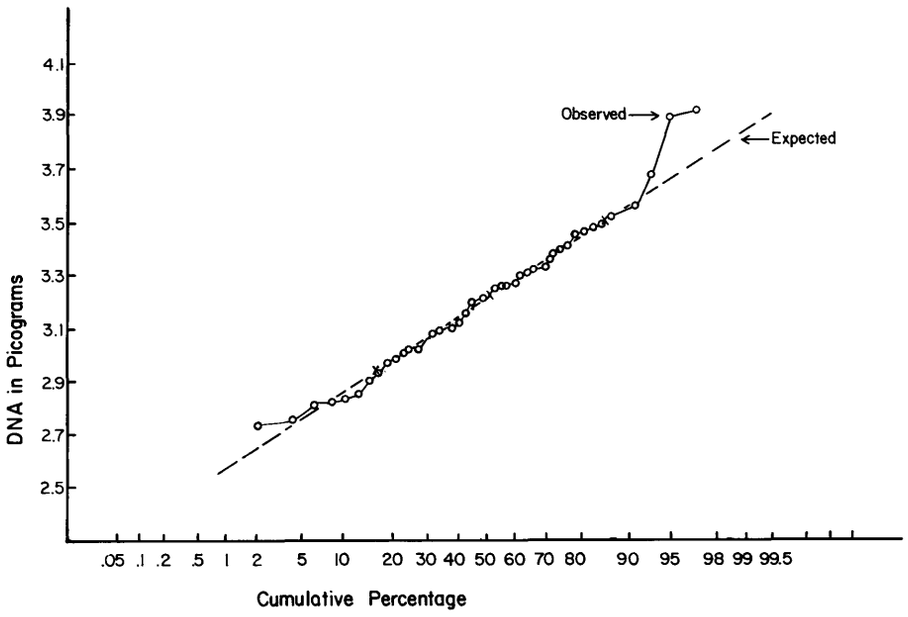

Fig. 8. — Normal probability plot of the 46 diploid white ash mean nuclear DNA values.

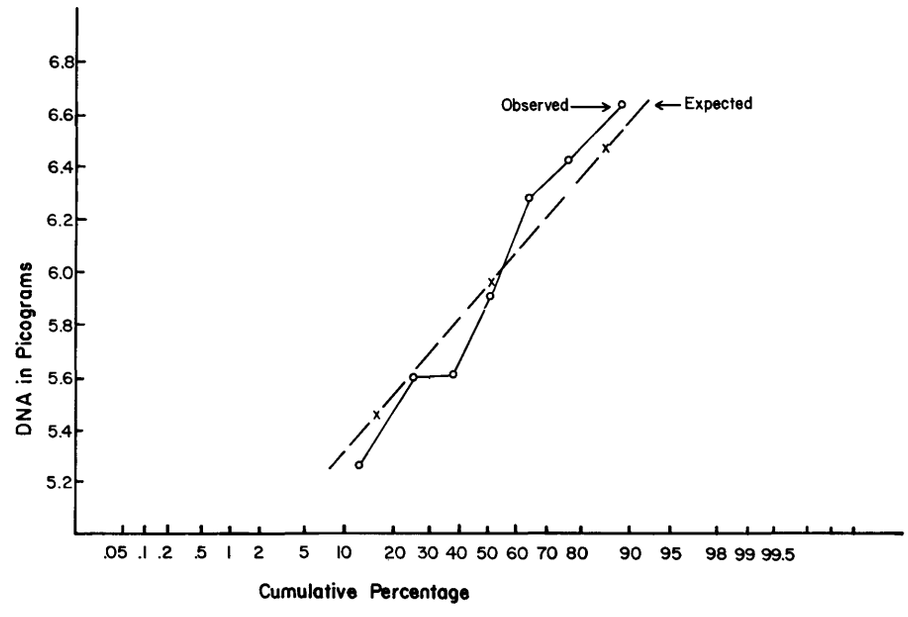

Fig. 9. Normal probability plot of the 7 tetraploid white ash mean nuclear DNA values.

configurations of the putative aneuploid and known hexaploids and by performing a cytophotometric study of a more intense nature than attemped in this study.

Ploidy Distribution: The 46 diploid: 7 tetraploid: 12 hexaploid ratio obtained cytophotometrically in this study was strikingly similar to the 42 diploid: 8 tetraploid: 13 hexaploid ratio observed by WRIGHT (1944). Whether or not these ratios depict the frequency distribution of the polyploidy series for the range of white ash population needs further verification since neither WRIGHT's nor this study was constructed for resolving this question. Only the Wayne County, Ohio provenance was in common to both investigations.

Diploid trees were found throughout the natural range. Hexaploids were found between 40° and 35° north latitude and tetraploids were found south of 35° north latitude *(Figure 5).* The only intraprovenance mixture of ploidy was either diploid and hexaploid or diploid and tetraploid. WRIGHT (1944) found all three ploidy levels in two inermediate states Pennsylvania and Maryland as well as tetraploidy in one southern state, Alabama. The reason for this difference in polyploid status is not readily apparent. It may simply be due to the small number of trees sampled per provenance in this study or inaccurate chromosome number estimates from stomata size by WRIGHT (1944). Regardless of this discrepancy, the results of this investigation

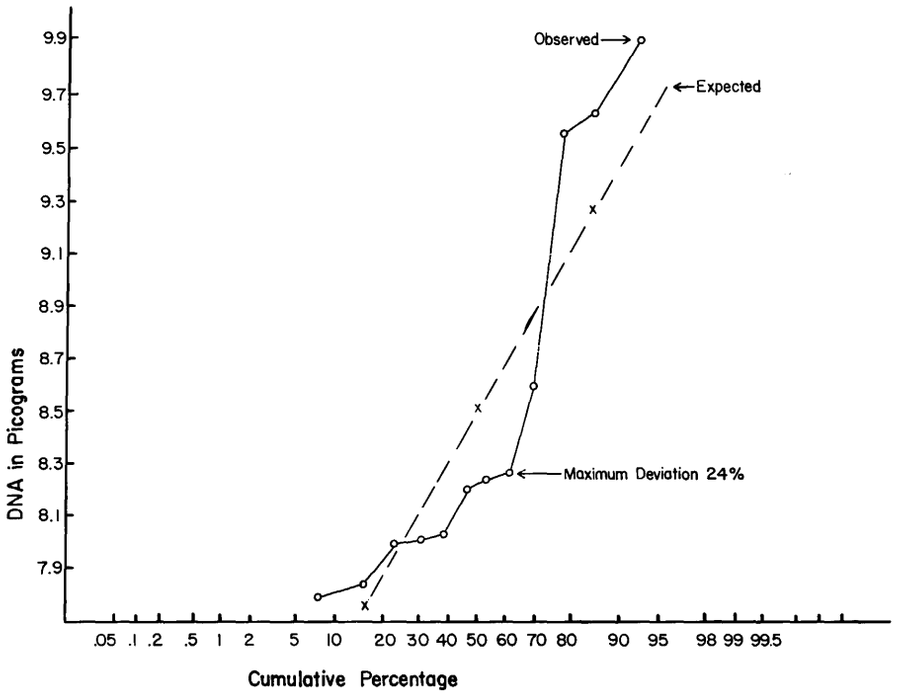

Fig. 10. — Normal probability plot of the 12 hexaploid white ash nuclear DNA values and the 24% significant deviation from the expected (KUNG, 1973).

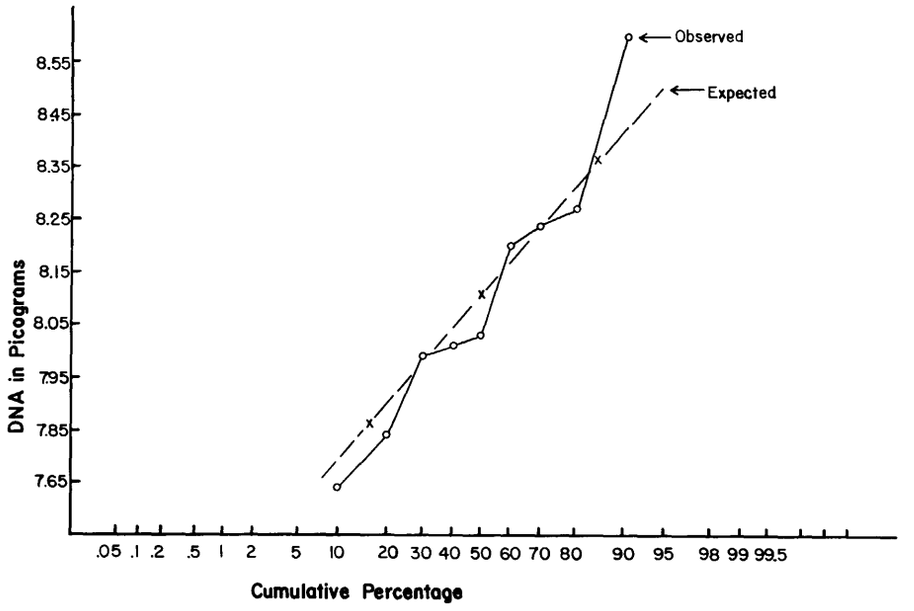

Fig. 11. — Normal probability plot of the 9 separated hexaploid white ash mean nuclear DNA values.

indicate that south of 40° north latitude, polyploidy for white ash is extensive and this finding is in agreement with WRIGHT (1944).

From past evidence, the frequency of polyploidy would be expected to increase in a northerly direction with polyploids having a selective advantage in fluctuating habitats (STEBBINS, 1960, 1971; GRANT, 1971). This is to say that polyploids were believed to be pioneering individuals found spreading into new habitats due to some selective advantage over diploid individuals. This does not seem to be the case for the white ash complex. In neither this investigation, WRIGHT's (1944) nor SANTAMOUR's (1962), has polyploidy been found north of approximately 40° north latitude. It is possible that white ash polyploid individuals do exist in the northern states but sampling to date may not have been extensive enough to verify their existence. However, evidence indicates that, if at all present, the frequency of white ash polyploidy in its northern range is very low. Assuming that past glacial periods temporarily eliminated tree growth in what is now the northern range of white ash, it would appear that the pioneer individuals posses a diploid chromosome complement.

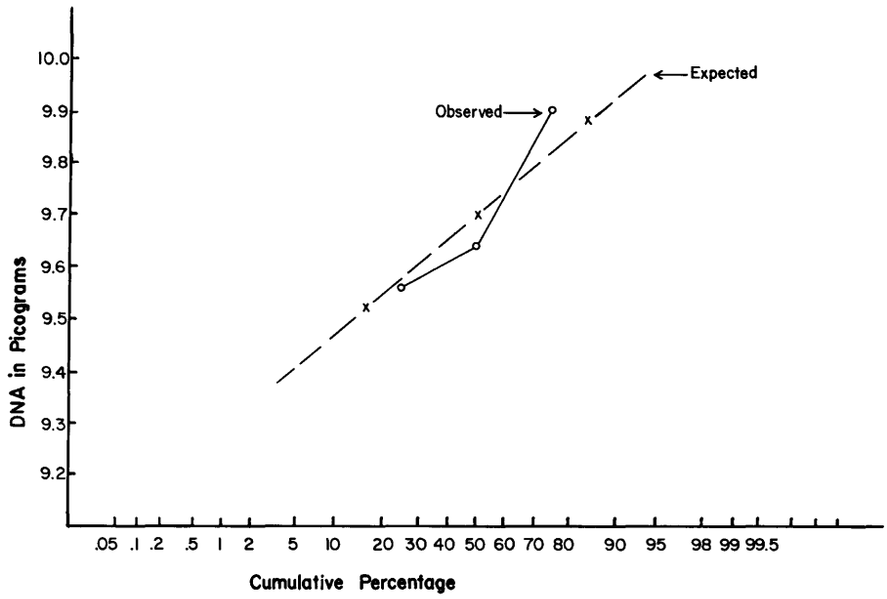

Fig. 12. — Normal probability plot of the 3 separated hexaploid white ash mean nuclear DNA values.

Acknowledgement

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Part of M.S. Thesis submitted by V. G. Schaefer, University of Wisconsin — Stevens Point.

Abstract

The DNA quantity per root tip cell of 66 *Fraxinus americana* L. trees from 22 geographic locations was determined by Feulgen microspectrophotometry. A direct correlation was found between observed DNA amounts of 3.22, 5.96 and 8.51 pg for the respective diploid (2N = 46), tetraploid (2N = 92), and hexaploid (2N = 138) chromosome levels. A deviation of observed ploidy DNA values from a direct multiplication factor was observed. South of 40° north latitude both diploid and polyploid individuals were represented, while north of 40° north latitude only diploid individuals were found. These may be pioneer individuals of the white ash complex.

Key words: DNA, Microspectrophotometry, Polyploidy.

Zusammenfassung

Bei 66 Einzelbäumen von *Fraxinus americana* L. aus 22 Herkünften wurde der DNA-Gehalt mikrospektrophotometrisch (Feulgen) bestimmt. Dabei ergab sich eine Beziehung zum jeweils gefundenen Chromosomensatz. Dieser betrug südlich 40° nördl. Breite diploid 46, tetraploid 92 und hexaploid 138. Nördlich des 40. Breitengrades wurden nur noch diploide Individuen gefunden.

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