CYTOPHOTOMETRIC DETERMINATION OF INTRASPECIFIC VARIATION OF DNA PER CELL IN *Pinus banksiana*

By

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ABSTRACT

Intraspecific DNA variation among 16 provenances of *Pinus banksiana* was demonstrated cytophotometrically with chicken erythrocyte nuclei used as an internal standard. The measured factor of increase was 1.18. The use of a single source of pine nuclei as an internal standard was also evaluated and the pine nuclei cannot be recommended for internal reference without careful testing, since extreme variation in DNA amounts was observed. The use of chicken erythrocyte nuclei as an internal standard is recommended.

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INTRODUCTION

Deoxyribonucleic acid (DNA) constancy per cell within a species was one of the basic concepts ascribed to identify DNA as the genetic material (Mirsky and Ris, 1949). However, the organization of DNA into genes has been elucidated and the constancy concept has not held true for all species and the amount of DNA per cell was found not totally related to unique genetic messages (Hall, 1974).

Sparrow, et al. (1972) reviewed and summarized reports of cellular DNA variation in both plant and animal species. Rees and Jones (1972) noted an increase in the amount of DNA per cell with increasing complexity of more advanced organisms, but the increase was not rigidly correlated with evolutionary advancement. Intraspecific DNA variation in coniferous species has been reported (Table 1).

Stable changes in cellular DNA content have been induced within one generation in Linum usitatissimum, variety Stormont Cirrus (Durrant, 1962) and Nicotiana rustica (Hill, 1965) by varying the nutrient content of the growth medium. Evans (1968) measured the induced variation in flax to be 16%.

If DNA amount is truly important in determining where a certain species or population within that species

may be successfully cultivated, then we should try to understand the modes of DNA variation. Such knowledge may help in predicting which species are suitable for introduction into new regions, the regions where new species may be cultivated and the optimal siting for breeding programs. If achieving the desired phenotype requires a change in the DNA amount rather than a genotype change, then breeding by conventional methods is unlikely to succeed (Bennett, 1976).

This study is concerned with measuring DNA contents among provenances of Pinus banksiana using an internal standard. The validity of chicken blood and pine nuclei as internal standards is also evaluated.

MATERIALS AND METHODS

DNA values from 16 provenances (seed sources) of Pinus banksiana were measured. Seed source number, geographic location, and DNA values are listed in Table 2.

Preparation of test material: Seeds from 16 test sources and from one local source (5150) were germinated for 9 days at room temperature, in a covered chamber on moistened Pearlite. The seedlings were harvested and

fixed in Carnoy's 6:3:1 (95% ETOH: chloroform: glacial acetic acid). Fixation was for one hour at room temperature. The seedlings were rinsed in 70% ethyl alcohol (ETOH) and stored in the refrigerator (in 70% ETOH) for subsequent processing.

Blood smears from a single chicken were made on microscope slides, air dried and stored in the freezer.

Hydrolysis curve preparation: The first test run was performed to establish a proper hydrolysis time for both chicken erythrocyte and pine nuclei.

One-half of each of five chicken blood slides was cleared with distilled water and a Kimwipe, and all were fixed in the same manner as the test material. Five vials of the 5150 pine were removed from the refrigerator and with the chicken blood slides were hydrated through five minute changes in 70%, 50%, 30% ETOH and distilled water. Both were then placed in 5N HCl (412.5 ml conc. HCl plus 587.5 ml H_2O) equilibriated to 25° C in a water bath. One slide and one vial were removed at the end of each ten minute period and hydrolysis was stopped by rinsing in distilled water at 4° C. The vials of 5150 pine were labeled and stored in an ice bath until all samples were collected. The chicken blood slides were dehydrated through an alcohol series (30%, 50%, 70%, 95% - five minutes each) and aired dried. Five

root tips were excised and squashed on the prescribed slide in distilled water, first by maceration with a flattened end of a glass rod, then by pressure applied to a coverslip over the material. The slide was then placed on dry ice until frosted and the coverslip was flipped off with a razor blade (Conger and Fairchild, 1953). The slide was rinsed in absolute alcohol and air dried. All slides were stained with Feulgen stain (Berlyn and Niksche, 1976), pH 1.8 for two hours at room temperature in a light tight drawer. Staining was followed by three, ten-minute rinses in bleach (10 ml 1N HCl and 10 ml 10% potassium meta-bisulfite in 180 ml H_2O) and dehydration through an alcohol series.

The Feulgen absorption was measured for five nuclei of both chicken blood and 5150 pine on each slide by the two-wavelength method of cytophotometry (Ornstein, 1952: Patau, 1952). The average readings were plotted and the proper hydrolysis time for the experimental and standard materials was determined to be 30 minutes in 5N HCl at 25° C (Figure 1).

Stain tests: Lillie (1951) and Berlyn and Miksche (1976) stain preparations were tested for differences and for proper stain formula preparation. The stain was 99% pure basic fuchsin (lot 63019, Harleco,

Gibbstown, N.J.).

The formula for Lillie's stain is: 5g basic fuchsin stirred in one liter of distilled water for two hours. The mixture is then filtered into a brown bottle and 100 ml of 1N HCl, five drops of concentrated HCl, and 10g of potassium meta-bisulfite are stirred in. The mixture is then allowed to stand for 24 hours in the dark prior to use. The mixture for the Berlyn and Miksche stain is: 2g basic fuchsin plus 3.8g potassium meta-bisulfite in 200 ml of .15N HCl (12.375 ml conc. HCl plus 987.625 ml H_2O), stir for two hours in darkness, add 1g activated charcoal, stir five minutes, filter into a dark bottle and store in the refrigerator.

The pH of both stains was adjusted to 1.8 with 1N NaOH and two chicken blood slides which had been fixed and hydrolzed for 30 minutes (as described above) were stained in each. A chicken blood slide which had been stored in 70% ETOH in the refrigerator for one week was also processed and stained in the Berlyn and Miksche stain to determine whether alcohol storage affected stain uptake.

Thirty nuclei were read from each slide and the results were analzed for differences.

Preparation of slides for cytophotometry: Fifty chicken blood slides were removed from the freezer and

areas were cleared on both ends, leaving a strip of chicken blood in the middle of the slide. These slides were placed in Carnoy's (6:3:1) for one hour at room temperature, then rinsed with 70% ETOH. The test and 5150 sources were removed from the refrigerator and rinsed in 70% ETOH. Both chicken blood slides and seedlings were hydrated to water and hydrolyzed for 30 minutes in 5N HCl at 25° C. Hydrolysis was stopped with 4° C water and seedlings were stored in an ice bath while the chicken blood slides were dehydrated through an alcohol series and air dried. Five root tips from a test provenance and five root tips from the 5150 pine were squashed on opposite ends of a chicken blood slide (as described under hydrolysis curve preparation). Three slides (replications) were made up for each provenance to give a total of 48 slides. All slides were stained in Feulgen stain (pH 1.8), prepared according to Berlyn and Miksche (1976). After staining the slides were bleached in three, ten-minute rinses of bleach solution, dehydrated through an alcohol series and air dried.

The slides were placed in a slide box and a cover slip was applied with refractive index oil (n_D 1.556) just prior to reading.

Spectral curve preparation: A spectral curve was

generated for determination of the maximum chromophore absorption for the stained material. The spectral curves were determined as described in Berlyn and Miksche (1976). The absorbance of a plug of five different nuclei of chicken blood and 5150 pine was measured at wavelengths from 470 to 600 nm at 10 nm intervals. The averages of the readings were used to determine the spectral curve described by quadratic regression (Figure 2).

Determination of the method for reading the material: On one slide three 5150 pine nuclei were measured ten times by both the one-wavelength, two-area method (Garcia and Iorio, 1966) and the two-wavelength, one-area method (Ornstein, 1952; Patau, 1952). The one-wavelength, two-area method displayed a lower coefficient of variation, and was therefore applied.

The techniques for reading Feulgen absorption with the one-wavelength, two-area method are as follows:

1) set monochromator at setting determined from the spectral curve (the peak of the curve).
2) locate the cell to be measured and place in the center of the photocathode diaphragm.
3) close the diaphragm until it contains only the nucleus and a narrow surrounding clear

area, record this diaphragm size (A_i).

4) move to an adjacent clear area and zero the photometer transmittance.

5) return cell to diaphragm and record transmittance (T_i).

6) close diaphragm to contain largest possible area of nucleus with no clear areas within diaphragm.

7) move to clear area and zero transmittance.

8) return nuclear plug to diaphragm and record transmittance (T_1) and absorbance (O.D.).

To calculate extinction for this method, the following equation is used:

$$\text{Feulgen extinction units } (E_1A_1) = \frac{(O.D.)(1-T_i)\ A_i^2}{(1-T_1)}$$

Reading of test slides: The instrument used consisted of a Leitz monochromator (for wavelength selection), a Leitz Ortholux microscope with a microphotometer head, and a Photovolt multiplier photometer.

Reading the test slides consisted of 25 readings each of chicken blood, 5150 pine, and the test material. The readings were done under oil using a 54X objective.

DNA dry mass determination: The DNA content of chicken erythrocyte nuclei was determined by dry mass measurements on an interference microscope. Fifty nuclei

were located on a slide and their areas were determined by measuring the major and minor axes with the micrometer in one of the eyepieces. The extinction was measured for each nucleus by rotating the analyzer from its zero setting until the bright nucleus is as dark as the background was at the image analyzer zero setting (Berlyn and Miksche, 1976).

The optical path difference (Δm) is determined by the formula:

$$[(\text{degrees extinction} \times 2)/360] \times 5462 \times 10^{-8}\text{cm}$$

where 5462×10^{-8}cm is the wavelength of the monochromatic light which illuminates the cell.

The dry mass of the nucleus is determined by the equation:

$$M = (\Delta m \times A)/100\alpha$$

where M=dry mass of a living or fixed cell mounted in water.

Δm = optical path difference of the nucleus in medium m.

A = area of nucleus.

α = material constant (0.0018 for proteins) (Berlyn and Miksche, 1976)

Following this initial measurement the blood smear slide was incubated in 0.5N perchloric acid (PCA)(13.6 ml 60% PCA in 236.4 ml H_2O) at 70° C for 60 minutes to hydrolyze

the DNA. Each nucleus was then relocated and measured as before. The difference in dry mass between the first and the second readings is the DNA content for that cell. The value determined for chicken blood was 2.8 picograms per nucleus.

RESULTS

A student-t distribution test was run on 60 observations each of chicken blood nuclei stained with Feulgen stain mixed according to Lillie (1951) pH 1.8 and according to Berlyn and Miksche (1976) pH 1.8. A t-value calculated at the 5% level of signficance (118 degrees of freedom) showed significant difference between the means for the two methods (Lillie stain-$\bar{X}$ = 17.5; Berlyn and Miksche stain-$\bar{X}$ = 19.8 Feulgen absorption units). Less residual stain was present in Berlyn and Miksche stain mixture and this stain preparation displayed a significantly higher mean extinction for stained nuclei than the Lillie method, so this stain mixture was chosen for use.

The chicken blood slide which had been stored in 70% ETOH showed no signficant difference in stain uptake from the unstored slide.

In choosing the technique for reading the material three 5150 pine nuclei were read ten consecutive times by both the one-wavelength, two area and two-wavelength,

one-wavelength methods. No significant variation was found between the coefficients of variation for the means of the nuclei (n=30) for the two methods (F_{test}= 1.12, $F_{.95}$= 1.84). However, the coefficient of variation was considerably lower for the repetitive readings of the single nucleus with the one-wavelength method [2-λ, CV = (5.7%, 5.8%, 5.1%): 1-λ, CV = (1.9%, 1.9%, 1.7%)].

For analysis of variance and testing of several cytophotometric techniques, the Feulgen absorption units of the test material were converted in four ways:

1) to picograms using the mean of the 25 chicken blood readings within the slide in the formula (F.A.U. = Feulgen absorption units):

$$\frac{\text{pg (test)}}{\text{F.A.U.(test)}} = \frac{\text{2.8 pg/chicken blood nucleus}}{\text{F.A.U.(cb)}}$$

2) to picograms using the mean of the chicken blood readings from all slides (n = 1200) using the above formula;

3) the ratio was calculated between the test material and the mean of the 5150 pine readings on that slide;

4) the ratio was calculated of the test material to the mean of all 5150 pine readings (n = 1200).

The 5150 pine readings were also converted as in 1) and 2) above for comparison purposes.

For conversion 2) above, the mean for all chicken blood nuclei readings (n = 1200) was 25.422 Feulgen absorption units. This average has a coefficient of variation of 7.9%, s = 2.015, and $S_{\bar{x}}$ = .058. For conversion 4) the mean for 5150 pine was 323.771 Feulgen absorption units, coefficient of variation = 12.5%, s = 40.226, $S_{\bar{x}}$ = 1.173. The normal distributions of the two populations have been illustrated with probability curves (Figure 3).

A two-way analysis of variance (Snedecor, 1956; Steel and Torrey, 1960) was run using the six groups of converted figures and is summarized in Table 3.

There were significant differences at the 5% level among provenances with each of the four conversion methods and no significant difference between replications except when comparing ratios of the test material to the 5150 pine within each slide.

The converted 5150 pine values showed significant differences among the 16 groupings of 3 slides as well as among the 3 slides in each group.

A one-way analysis of variance (Snedecor, 1956) was performed on the conversions of test material which showed no signficant difference among replications. The data were evaluated as 75 readings per provenance rather

than three groups of 25 each to eliminate the extra error involved in separating the data into three replications. The summary of these tests is contained in Table 4.

A Student-Newman-Keuls (SNK) test was applied to data from the one-way analysis of variance to illustrate those means which contributed to the significant difference. These tests are summarized in Table 5.

Linear regressions were run between DNA amount and latitude, longitude, and altitude. No correlation was found for any of the parameters (lat., $r = 0.312$; long., $r = 0.123$; alt., $r = 0.278$; $r_{0.95} = 0.482$).

The validity of the Feulgen absorption readings for anaphase and telophase figures (2C) used for DNA determination in this study was tested by reading metaphase figures (4C) for five provenances. A chi-square test was applied to a 2:1 ratio between these readings. A calculated χ^2 of 0.0311 demonstrates an existence of a 2:1 ratio at the 5% level ($\chi^2_{.95} = 7.26$).

DISCUSSION

Differential stain uptake by the pine nuclei presented an immediate problem. Light and dark nuclei were evident within the pine material on any one slide. Hall (1974) encountered a similar problem when working with Pinus banksiana and attributed the variation partly

to the fact that the root tips were stained whole (bulk stained). However squash preparations were utilized in this study and the problem persisted. Hall abandoned the use of root tip material to use the more uniformly staining embryonic and gametophytic tissue. However, since the populations were easily discernible, and the darker readings gave good correlation with the DNA mounts determined biochemically (Miksche, 1968), dark readings were used in this study.

To test for normality among the lightly stained cell population the distribution of 20 readings from one slide was plotted on probability paper (Figure 4). The maximum deviation from the normal distribution line was 35%. The acceptance limit at the 95% level is 20% (Kung 1973), therefore the hypothesis of normal distribution was rejected. It can be assumed that these cells do not represent a true population, but only groups of intermediately stained nuclei.

Garcia and Iorio (1966) compared the one-wavelength and two-wavelength methods with readings on human leucocytes. They found a variation in the means of only 6% for cells having quite different nuclear configurations. They also found the grand mean to be 5% lower using the one-wavelength method, but the coefficients of variation fell within the same range. In this test (performed on pine material) the one-wavelength method gave a 6% lower

mean than the two-wavelength method (this poses no problem since all readings are relative). The coefficient of variation of readings between unclei was not significantly different; however, the coefficient of variation between repetitive readings on a single nucleus was considerably lower for the one-wavelength method than for the two-wavelength method. The one-wavelength method also offers one practical advantage: both measurements can be made at the level of maximal absorbance. (Also the extinction is computed from the intranuclear transmittance so that the noise of the apparatus is brought to a minimum (Garcia and Iorio, 1966).

The need for standardization of cytophotometric readings is obvious when reviewing the results of previous experiments. Feulgen absorption can vary greatly between stain batches and processing techniques. "The use of an internal standard has the advantage of permitting presentation of the data in actual mass units and obviates the use of arbitrary units which are wavelength dependent. The use of actual mass units of DNA per cell makes not only the data between slides and staining batches directly comparable, but also makes the values obtained by different investigators subject to direct comparison (Dhillon, Berlyn and Miksche, 1977)."

Chicken erythrocytes are becoming a common internal

standard and are recommended by Rasch (1974) and Berlyn and Cecich (1976), because they are nucleated and the DNA is non-replicated (2C) at maturity.

Chicken erythrocytes were used as an internal standard and the squash from a local source (5150) of Pinus banksiana was placed on each slide to determine its usefulness as an internal standard.

The chicken erythrocyte nuclei demonstrated good internal standard performance whether used on a per slide basis or on a total mean basis. No signficant variation was found between the replications for either conversion method. When using 5150 pine as an internal standard on the basis of total mean, no signficant difference was found between the replications. However, when used on a per slide basis, there was significant variation.

When values for 5150 pine were analyzed with a two-way analysis of variance, significant differences were present among slides whether converted on the per slide or total chicken blood mean basis. Although the differences were reduced if converted with the total chicken blood mean (factor of increase with per slide conversion = 1.21; with total mean conversion = 1.07), which indicates unreliable variation.

If unstained experimental material is squashed on the chicken blood slides and the erythrocytes and experimental material are stained simultaneously, they

can be considered as paired comparisons. Therefore after conversion to absolute amounts, slide to slide variation can be omitted. (Berlyn and Cecich, 1976; Berlyn and Miksche, 1976).

Since the pine and chicken nuclei were stained together for this experiment, the DNA values which were converted using the mean extinction of the standard within each slide is the best estimation of the DNA value for the test material on that slide. Using the internal standard in this way is the only available method for eliminating slide to slide variation.

In a two-way analysis of variance on the test material converted to absolute DNA values on a slide to slide basis, there were highly significant differences among the provenances, and no significant differences among the replications in mean DNA values (see Table 3). The factor of increase among the provenances was 1.18.

The variability within the 5150 provenance was also measured to test the usefulness of a pine source as an internal standard. When grouped into 16 "provenances" (same 3-slide replicate groups as test material) and converted to absolute values on a per slide basis, there was significant variation among the "provenances" and among replications. The factor of increase among the "provenances" was 1.21. This variation is probably due to the fact that 5150 is a multiple tree collection of

seed and DNA content within the collection area may be extremely variable. The fact that the measured variation is so great indicates that the seed source is not suitable as a standard for slide to slide comparison. It also points out the need for comprehensive tests on any material prior to use as a standard. Chicken erythrocytes are highly recommended as the internal standard for use in comparative cytophotometric studies from the results of this study, Rasch(1974), Berlyn and Cecich, (1976), and the recommendation of Berlyn and Miksche (1976).

The results of this study indicate that the overall mean of the 5150 pine could possibly be used as an internal standard for converting relative absorption values to absolute DNA values. This is due to the fact that the mean value encompasses the spectrum of readings within the normal distribution of 5150 pine, whereas the per slide measurement of 5150 pine are not representative of the provenance. However, this type of conversion would not correct for slide to slide variation and for proper conversions a less variable standard should be used and should be applied on the slide to slide basis.

Significant differences were found among the means of the provenances when converted to absolute values in any of the four ways listed in the Results section (chicken blood standard-per slide and total mean; 5150

pine standard - per slide and total mean). On those which showed no significant differences among replications (chicken blood standard-per slide or total mean: 5150 pine standard-total mean), a one-way analysis of variance was performed grouping the readings from the three replications from each provenance. Significant differences were found among the provenance means in all three tests. When converted with chicken blood per slide a 1.18-fold increase in DNA value per cell was found, and a 1.12-fold increase was found when extinction values were converted with either total chicken nuclei or total 5150 pine nuclei means.

The increase reported here is considerably lower than the 1.5-fold increase in DNA reported by Miksche (1968). The difference may be due, in part, to differences in experimental techniques (lack of an internal standard) or cytophotometric methods, but it is also likely due to the fact that different provenances were analyzed in the two studies.

DNA variation in Pinus banksiana displayed no correlation with latitude in previous studies (Miksche, 1968; Hall, 1974). The results presented here agree with these previous findings. (see Figure 5).

This study in no way explains the question of intraspecific variation. However, it does indicate that differences among P. banksiana seed sources are evident

and casts doubt on DNA constancy concepts within a species.

Results of the analysis of 48 slides of one local source (5150) of P. banksiana indicate that extreme within stand variation may occur, which suggests that for an experiment of this nature, ie., requiring an internal standard, seed from a single tree should be used. Further research on DNA variation with individual trees within a stand deserves investigation.

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Table 1 Reported intraspecific variation in nuclear DNA within coniferous species.

Species	Factor of variation	Reference
Picea glauca	1.6	Miksche (1968)
Pinus banksiana	0	Teoh and Rees (1976)
	1.5	Miksche (1968)
Pseudotsuga menziesii (inland and coastal forms)	1.9	El-Lakany and Sziklai (1971)
Picea sitchensis	1.36,1.92,1.72	Miksche (1971)
Pinus resinosa	2.2	Dhir and Miksche (1974)
Pinus contorta	0	Teoh and Rees(1976)

Table 2 Seed source, latitude, longitude, altitude, and DNA amounts ($\pm S_{\bar{x}}$) as converted from Feulgen absorption units to: 1) absolute DNA value by within slide mean of chicken blood; 2) absolute DNA value by total mean (n=1200) of chicken blood; 3) relative DNA values by within slide mean of 5150 pine; and, 4) relative DNA values by total mean (n=1200) 5150 pine.

Table 2

Seed source	Lat. °N	Long. °W	Alt. (ft)	DNA(pg) chicken blood/slide conversion Slide 1	Slide 2	Slide 3	Mean
2215	47°58'	75°22'	1600	33.2± .71	33.1± .63	32.4± .67	32.9± .29
2197	48°56'	71°45'	600	31.4±1.06	33.9± .51	34.3± .64	33.2± .90
2223	46°50'	80°58'	1000	36.0± .76	32.8± .77	33.3±1.00	34.0±1.00
2246	49°30'	95°45'	1050	34.6± .69	37.1± .66	35.0± .83	35.6± .78
2207	45°30'	76°56'	500	34.8± .65	34.8± .62	37.5± .85	35.7± .92
2247	49°51'	95°21'	1300	32.4± .83	38.2± .90	36.6± .86	35.7±1.75
2237	44°31'	84°47'	1145	36.8±1.07	37.7± .72	33.6± .82	36.0±1.25
2194	47°37'	71°45'	600	36.5± .64	35.9± .73	35.7± .77	36.1± .25
2236	44°31'	84°44'	1193	35.9± .92	35.8± .97	36.8± .79	36.1± .33
2244	48°46'	93°30'	1100	37.6± .77	37.1± .89	33.9± .58	36.2±1.15
2195	48°15'	70°53'	825	34.9± .62	36.9±1.10	36.9± .81	36.2± .66
2189	44°20'	73°47'	950	37.7± .73	36.0± .81	35.2± .64	36.3± .75
2212	46°50'	76°05'	800	35.1± .73	35.9± .65	40.3± .55	37.1±1.61
2210	45°47'	77°23'	600	40.3± .71	35.3± .85	39.1± .67	38.2±1.49
2199	46°25'	72°35'	250	38.3± .64	36.9± .89	40.9±1.13	38.7±1.17
2192	47°40'	74°08'	1400	39.8± .67	37.3± .73	39.1± .63	38.7± .74
5150	46°17'	89°10'				Mean for 48 slides	35.8± .47

Seed source	DNA(pg) chicken blood tot. mean conversion Slide 1	Slide 2	Slide 3	Mean
2215	34.1± .73	37.0± .70	33.9± .67	35.0±1.01
2197	32.2±1.09	34.0± .51	34.2± .64	33.5± .64
2223	36.0±1.20	33.4± .78	33.3±1.00	34.2± .86
2246	36.3± .72	37.7± .67	38.0± .91	37.8± .52
2207	35.3± .65	33.4± .59	35.7± .80	34.8± .71
2247	34.9±1.07	36.6± .87	35.6± .84	35.7± .48
2244	35.2± .73	35.0± .82	34.4± .58	35.1± .40
2237	35.1±1.02	38.4± .71	33.9± .83	35.8±1.33
2194	36.8± .64	36.8± .76	35.7± .77	36.4± .34
2236	37.9± .97	35.9± .97	36.7± .78	36.8± .56
2195	35.2± .62	39.0± .87	37.2± .81	37.1±1.12
2189	37.2± .72	37.9± .85	35.0± .64	36.7± .87
2212	36.3± .75	36.4± .66	36.4± .49	36.3± .02
2210	39.0± .69	35.4± .81	37.7± .66	37.3±1.04
2199	38.0± .63	34.7± .84	35.2± .97	36.0± .99
2192	36.8± .62	37.9± .75	37.9± .61	37.5± .36
5150			Mean for 48 slides	35.7± .41

Table 2 cont.

Seed source	Extinction(test)/Extinction(5150/slide) Slide 1	Slide 2	Slide 3	Mean
2215	1.01 ± .02	1.05 ± .02	.97 ± .02	1.01 ± .02
2197	.98 ± .03	.97 ± .01	.97 ± .02	.97 ± .00
2223	1.10 ± .02	1.00 ± .02	.90 ± .03	.97 ± .05
2246	1.00 ± .02	1.07 ± .02	1.03 ± .02	1.03 ± .02
2207	1.02 ± .02	.94 ± .02	.96 ± .02	.97 ± .03
2247	.95 ± .02	1.03 ± .02	.99 ± .02	.99 ± .03
2244	1.01 ± .02	.94 ± .02	.98 ± .02	.98 ± .02
2237	1.02 ± .03	1.02 ± .02	.98 ± .02	1.01 ± .01
2194	1.10 ± .02	.99 ± .02	.97 ± .02	1.02 ± .04
2236	1.08 ± .03	1.01 ± .03	.98 ± .02	1.02 ± .01
2195	.90 ± .02	1.02 ± .02	1.10 ± .02	1.01 ± .06
2189	1.05 ± .02	1.10 ± .02	1.02 ± .02	1.05 ± .02
2212	1.10 ± .02	.94 ± .02	.98 ± .01	.99 ± .03
2210	1.20 ± .02	.96 ± .02	1.08 ± .02	1.08 ± .07
2199	1.08 ± .02	.92 ± .02	.96 ± .03	.99 ± .05
2192	1.01 ± .02	.99 ± .02	1.11 ± .02	1.04 ± .04

Seed source	Extinction(test)/Extinction(5150-tot. mean) Slide 1	Slide 2	Slide 3	Mean
2215	.96 ± .02	1.04 ± .02	.96 ± .02	.98 ± .03
2197	.90 ± .03	.95 ± .01	.97 ± .02	.94 ± .02
2223	1.01 ± .02	.94 ± .02	.94 ± .03	.96 ± .02
2246	1.02 ± .02	1.10 ± .02	1.07 ± .03	1.05 ± .01
2207	.99 ± .02	.93 ± .02	1.00 ± .02	.97 ± .02
2247	.97 ± .03	1.03 ± .02	1.00 ± .02	1.00 ± .02
2244	1.00 ± .02	.98 ± .02	.97 ± .02	.98 ± .01
2237	.99 ± .03	1.08 ± .02	.95 ± .02	1.00 ± .04
2194	1.03 ± .02	1.03 ± .02	1.00 ± .02	1.02 ± .01
2236	1.06 ± .03	1.01 ± .03	1.03 ± .02	1.03 ± .02
2195	1.04 ± .02	.99 ± .02	1.09 ± .02	1.04 ± .03
2189	1.04 ± .02	1.06 ± .02	.98 ± .02	1.03 ± .02
2212	1.03 ± .02	1.02 ± .02	1.02 ± .01	1.02 ± .00
2210	1.10 ± .02	1.00 ± .02	1.05 ± .02	1.05 ± .03
2199	1.07 ± .02	.97 ± .02	.99 ± .03	1.01 ± .03
2192	1.03 ± .02	1.06 ± .02	1.06 ± .02	1.05 ± .01

Table 3 Summary of two-way analysis of variance. (df= degrees of freedom: SS= sum of squares: MS= mean sum of squares: * indicates significant F-values).

Data	Source	df	SS	MS	F
5150 DNA(pg) c.b./slide conv.	Total	1199	23873.6	19.911	
	Provenance	15	2994.1	199.603	13.654*
	Replication	2	533.1	266.569	18.235*
	Interaction	30	3506.0	116.867	7.995*
	Error	1152	16840.5	14.618	
5150 DNA(pg) c.b. total mean conv.	Total	1199	19402.4	16.182	
	Provenance	15	631.6	42.106	2.906*
	Replication	2	393.4	196.701	13.578*
	Interaction	30	1688.1	56.270	3.884*
	Error	1152	16689.3	14.487	
Test DNA(pg) c.b./slide conv.	Total	1199	24123.3	20.119	
	Provenance	15	3216.7	214.449	13.454*
	Replication	2	63.2	31.598	1.982
	Interaction	30	2480.8	82.692	5.188*
	Error	1152	18362.6	15.940	
Test DNA(pg) c.b. total mean conv.	Total	1199	20270.4	16.906	
	Provenance	15	1613.4	107.560	7.204*
	Replication	2	59.5	29.742	1.992
	Interaction	30	1398.6	46.619	3.123*
	Error	1152	17198.9	14.930	
Extinction (test) / Extinction (5150/slide)	Total	1199	17.749	.015	
	Provenance	15	.989	.066	5.598*
	Replication	2	.311	.156	13.215*
	Interaction	30	2.886	.096	8.170*
	Error	1152	13.563	.012	
Extinction (test) / Extinction (5150-total mean)	Total	1199	15.940	.013	
	Provenance	15	1.274	.085	7.265*
	Replication	2	.022	.011	.934
	Interaction	30	1.176	.039	3.353*
	Error	1152	13.468	.012	

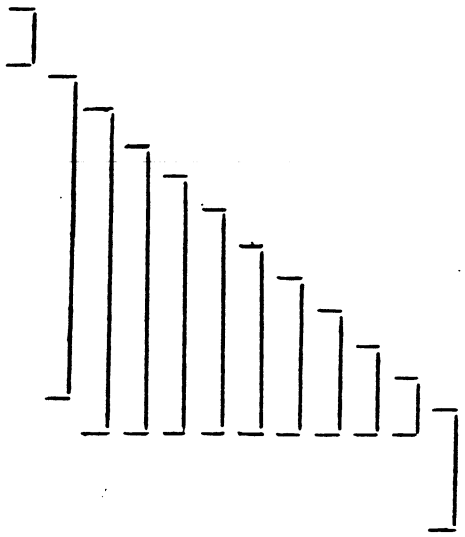
Table 4 Summary of one-way analysis of variance on 16 test provenances. (* indicates significant F-values).

Seed source	DNA(pg) (c.b./slide conv)	DNA(pg) (c.b.-tot. mean conv.)	Extinction ratio (test:5150)
2215	32.9 ± .66	34.5 ± .75	.98 ± .02
2197	33.2 ± .80	33.0 ± .80	.93 ± .02
2223	34.1 ± .87	33.7 ± .89	.96 ± .02
2246	35.6 ± .75	36.8 ± .77	1.04 ± .02
2207	35.7 ± .74	34.2 ± .71	.97 ± .02
2247	35.7 ± .98	35.2 ± .89	.99 ± .02
2244	36.1 ± .80	34.6 ± .72	.98 ± .02
2237	36.0 ± .93	35.3 ± .92	1.00 ± .03
2194	36.0 ± .71	36.0 ± .72	1.02 ± .02
2236	36.1 ± .88	36.3 ± .91	1.03 ± .03
2195	36.2 ± 1.10	36.9 ± .87	1.04 ± .02
2189	36.2 ± .75	36.2 ± .77	1.02 ± .02
2212	37.0 ± .78	35.9 ± .64	1.02 ± .02
2210	38.2 ± .85	36.8 ± .76	1.04 ± .02
2199	38.7 ± .95	35.4 ± .85	1.01 ± .02
2192	38.7 ± .70	37.0 ± .66	1.05 ± .02
F	12.78*	6.92 *	6.85*
df_1	15	15	15
df_2	1184	1184	1184
Error MS	16.56	15.97	.012

Table 5 Summary of Student-Newman-Keuls tests indicating provenances with significantly different mean DNA values. (A) readings converted by mean of chicken blood nuclei within slide: (B) readings converted by total chicken blood mean: (C) readings converted to ratio with total 5150 pine mean. Bars connect sources which are not significantly different from each other at the 5% level.

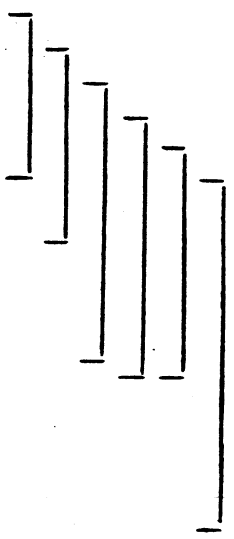
(A)

Seed source	Mean DNA values
2215	32.86
2197	33.17
2223	34.12
2246	35.56
2207	35.66
2247	35.67
2237	36.01
2194	36.01
2236	36.09
2244	36.14
2195	36.22
2189	36.25
2212	37.05
2210	38.20
2192	38.66
2199	38.67



(B)

2197	33.03
2223	33.73
2207	34.24
2215	34.49
2244	34.56
2247	35.16
2237	35.27
2199	35.41
2212	35.87
2194	35.96
2189	36.20
2236	36.35
2210	36.80
2246	36.84
2195	36.87
2192	37.01



(C)

2197	.936
2223	.956
2207	.969
2215	.977
2244	.980
2247	.993
2237	.999
2199	1.007
2212	1.015
2194	1.017
2189	1.023
2236	1.028
2195	1.036
2246	1.041
2210	1.042
2192	1.048

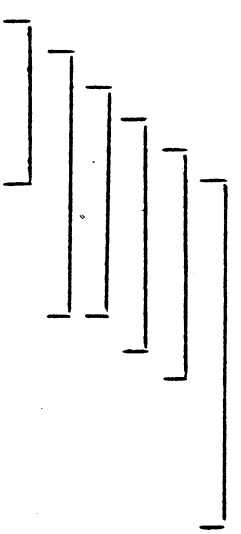


Figure 1 Hydrolysis curve. Left ordinate - Feulgen absorption units for chicken blood readings: right ordinate - Feulgen absorption units for 5150 pine readings: abscissa - hydrolysis time in minutes. o——o chicken blood; Δ——Δ 5150 pine.

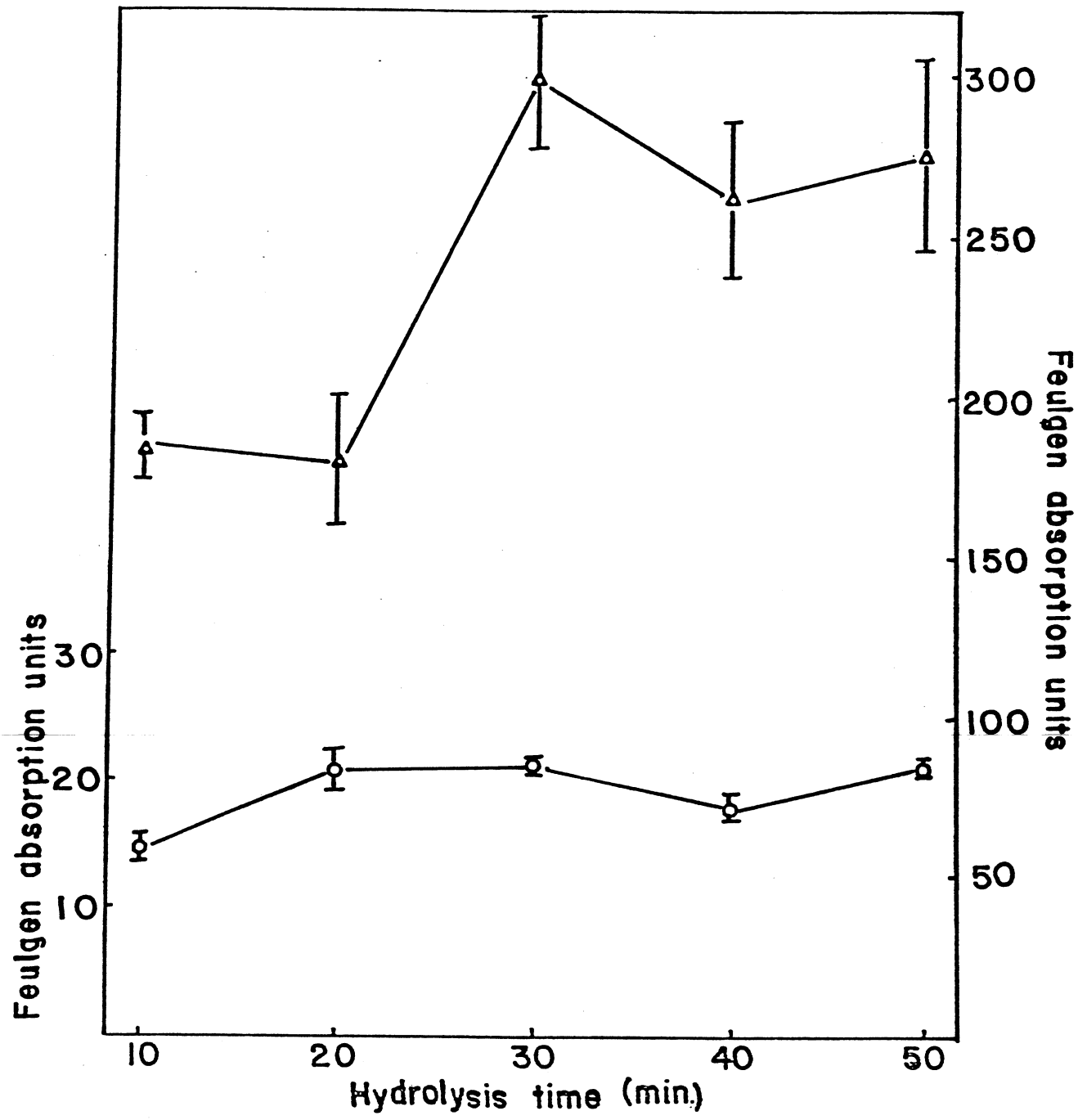

Feulgen absorption units
30
20
10
300
250
200
150
100
50
Feulgen absorption units
10
20
30
40
50
Hydrolysis time (min.)

Figure 2 Spectral curves. Left ordinate - chicken blood nuclear absorbance; right ordinate - 5150 test nuclear absorbance; abscissa - wavelength (nm). Chicken blood curve formed from equation: $y=-(.0199)\ x^2 + 22.818x - 6318.74$. 5150 test curve formed from equation: $y= -(.0728)x^2 + 82.874x - 228.62$. o——o chicken blood; Δ——Δ 5150 pine.

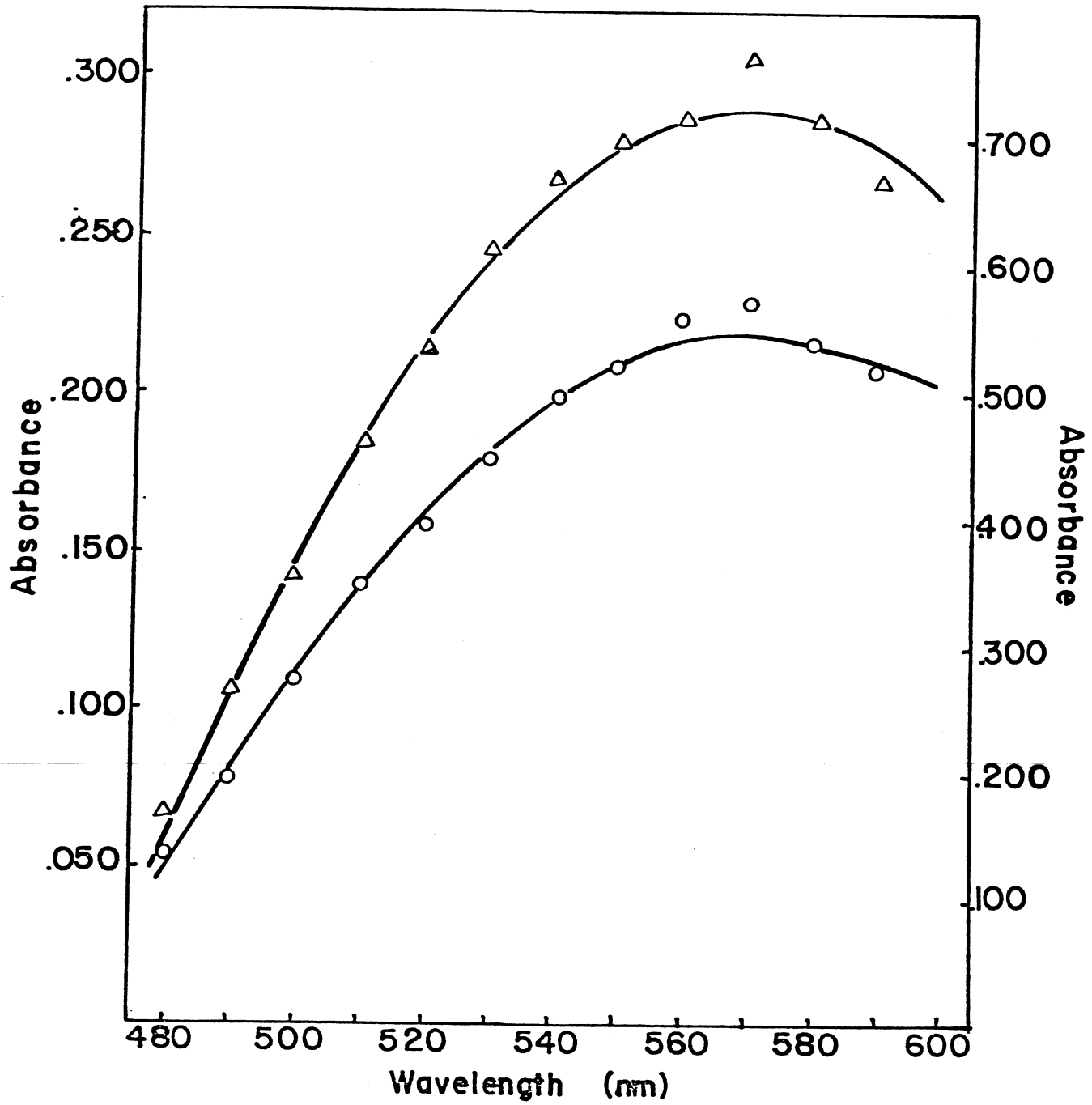

.300
.250
.200
.150
.100
.050
Absorbance
.700
.600
.500
.400
.300
.200
.100
Absorbance
480
500
520
540
560
580
600
Wavelength (nm)

Figure 3 Probability plots indicating normal distribution. Left ordinate - Feulgen absorption units for chicken blood; right ordinate - Feulgen absorption for 5150 pine; abscissa - cumulative percentage of Feulgen absorption unit frequencies. o——o chicken blood; △——△ 5150 pine.

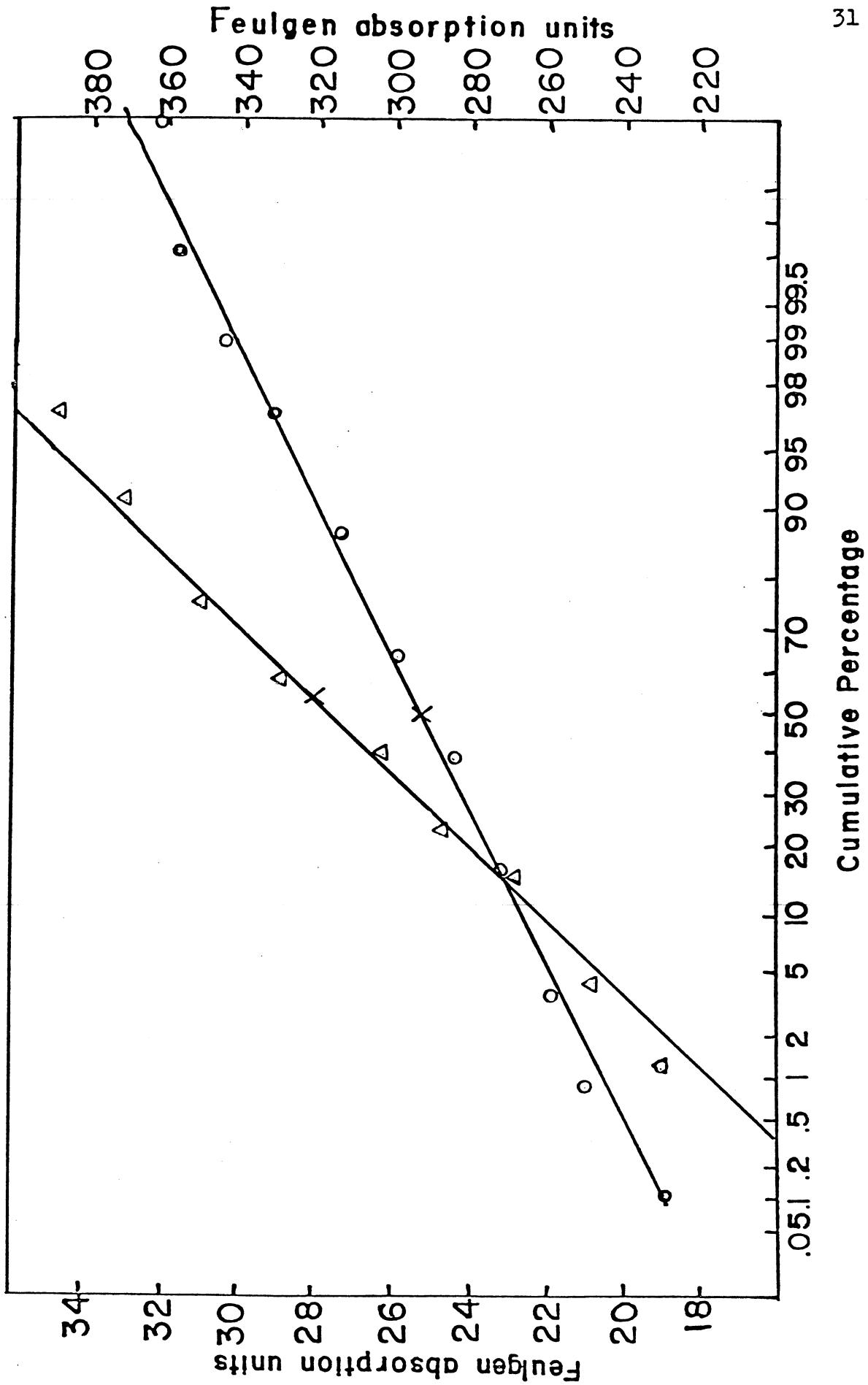

Feulgen absorption units
380
360
340
320
300
280
260
240
220
Cumulative Percentage
.05 .1 .2 .5 1 2 5 10 20 30 50 70 90 95 98 99 99.5
Feulgen absorption units
34
32
30
28
26
24
22
20
18

Figure 4 Probility plot for lightly stained pine nuclei. Ordinate - Feulgen absorption units; abscissa - cumulative percentage of Feulgen absorption unit frequencies. ——— estimated normal distribution; o——o pine nuclei distribution.

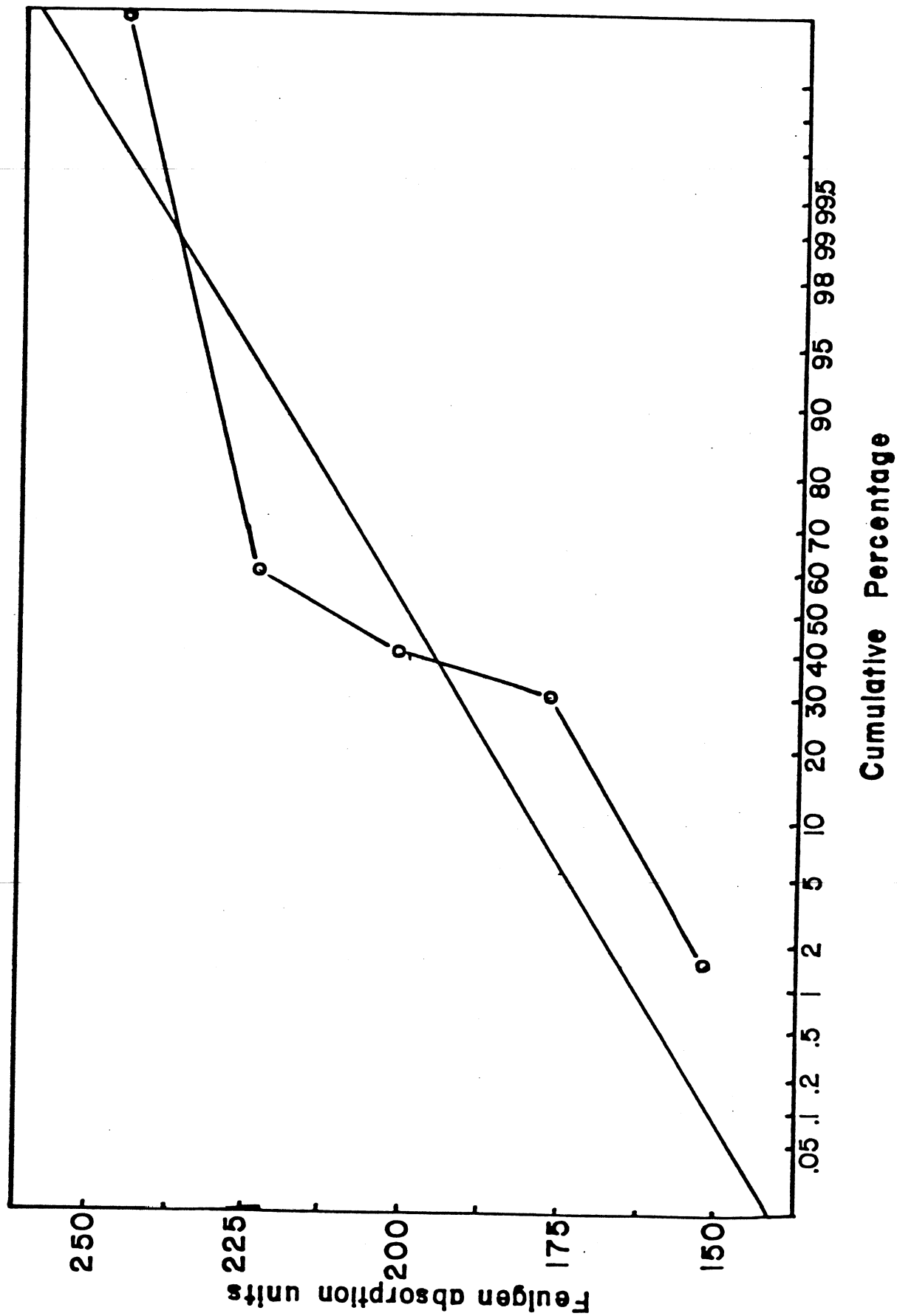
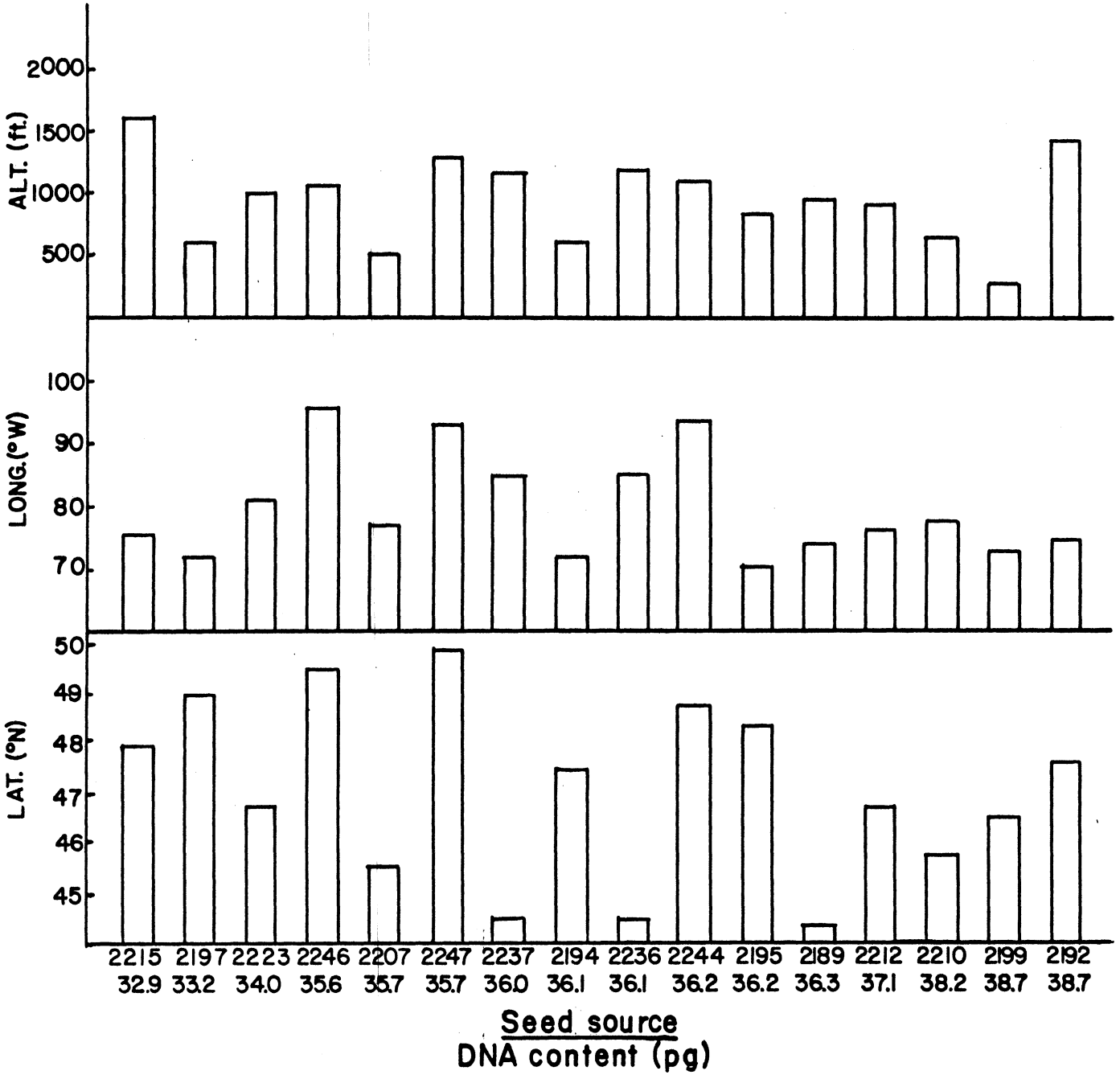

Feulgen absorption units
250
225
200
175
150
Cumulative Percentage
.05 .1 .2 .5 1 2 5 10 20 30 40 50 60 70 80 90 95 98 99 99.5

Figure 5 DNA content in relation to latitude, longitude, and altitude. Ordinate - latitude (°N); longitude (°W); altitude (ft.). Abscissa - seed source number and mean DNA content (pg).


ALT. (ft.)
2000
1500
1000
500
LONG.(°W)
100
90
80
70
LAT. (°N)
50
49
48
47
46
45
2215 2197 2223 2246 2207 2247 2237 2194 2236 2244 2195 2189 2212 2210 2199 2192
32.9 33.2 34.0 35.6 35.7 35.7 36.0 36.1 36.1 36.2 36.2 36.3 37.1 38.2 38.7 38.7
Seed source
DNA content (pg)