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A Statistical Analysis of Karyotypes of European Black Pine (Pinus nigra Arnold) from Different Sources

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Summary

A comparative, intraspecific analysis of karyotypes was performed on 2-week-old seedlings of European black pine (*Pinus nigra* Arnold) by examining the meristemic cells of root-tips. The metaphase chromosomes were studied by a combination of Feulgen and Aceto-carmine staining methods. Seedlings were categorized according to the country of seed source and geographic locality of collection. There were three collection localities each in France, Turkey, Yugoslavia, and Greece, and two localities in Austria. Several karyotypic variables were determined for each cell: chromosome number, short arm length (SL), long arm length (LL), total chromosome length (TL), arm ratio (AR), relative chromosome length (RL), centromere index (CI), morphological index (MI), presence of secondary constrictions on short arms (S_{sec}) and long arms (L_{sec}), and presence of satellites (SAT). Aneuploidy (2n = 18) was observed in one seedling of each of the Yugoslavian and Greek seed sources.

Analyses of variances for the means of chromosomal variables indicated that there were significant ($p < .05$ or $p < .01$) differences among the seed sources; chromosomes XI and XII were especially variable with significant ($p < .01$) variation found in SL, LL, TL, AR, CI, and MI. The frequency of occurrence for secondary constriction was relatively higher on the long arms of chromosomes and on the longer chromosomes. Satellites were infrequent and only observed in the cells of Yugoslavian and Greek seedlings, especially on the longer chromosomes. French and Austrian seedlings had only one submetacentric chromosome (chromosome XII) whereas the others had two (chromosomes XI and XII). By integrating all the variables measured on all haploid chromsomes, a karyotypic cluster analysis among seed sources was performed. The Austrian and French seedlings clustered closely together, as did the Greek and Yugoslavian. The Turkish seedlings, however, were the least similar to those from any of the other countries. Seed collections within a country always clustered as a group.

Key words: Karyotype, aneuploidy, cluster analysis, *Pinus nigra* Arnold.

Zusammenfassung

Bei 2 Wochen alten Sämlingen von *Pinus nigra* Arnold wurde eine vergleichende intraspezifische Analyse von Karyotypen durchgeführt, indem die Meristemzellen von Wurzelspitzen untersucht wurden. Die Chromosomen wurden in der Metaphase mit Hilfe einer Kombination aus Feulgen- und Aceto-Karminfärbungsmethode untersucht. Die Sämlinge wurden nach den Herkunftsländern und dem geographischen Ort der Einsammlung geordnet. Es gab je drei Sammelorte in Frankreich, der Türkei, Jugoslawien und Griechenland und zwei in Österreich. Für jede Zelle wurden verschiedene karyotypische Variablen bestimmt: Chromosomenzahl, die Kurz- (SL) und Langschenkel-Länge (LL), die Chromosomengesamtlänge (TL), das Schenkelverhältnis (AR), die relative Chromosomenlänge (RL), der Centromer-Index (CI), der morphologische Index (MI), das Vorhandensein sekundärer Einschnürungen an den kurzen (S_{sec}) und langen Schenkeln (L_{sec}) und das Vorhandensein von Satelliten (SAT). In je einem Sämling der jugoslawischen und griechischen Herkünfte wurde Aneuploidie (2n = 18) beobachtet.

Varianzanalysen für die Mittelwerte der Chromosomenvariablen zeigten, daß es dort signifikante Unterschiede zwischen den Saatgut-Herkünften gibt ($p - 0{,}05$ oder $p - 0{,}01$); die Chromosomen XI und XII waren besonders variabel mit einer signifikanten Variation für SL, LL, TL, AR,

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CI und MI für p — 0,01. Die Häufigkeit des Auftretens von sekundären Einschnürungen war bei Chromosomen mit langen Schenkeln und bei längeren Chromosomen relativ höher. Satelliten waren selten und wurden nur in den Zellen der jugoslawischen und griechischen Sämlinge beobachtet, besonders bei den längeren Chromosomen. Sämlinge aus Frankreich und Österreich hatten nur ein submetazentrisches Chromosom (Chromosom XII), wogegen die anderen zwei hatten (Chromosomen XI und XII). Bei Einbeziehung aller Variablen, die bei allen haploiden Chromosomen gemessen worden waren, wurde eine karyotypische Cluster-Analyse zwischen den Saatgutherkünften durchgeführt. Die österreichischen und französischen Sämlinge lagen dicht beieinander, wie die griechischen und jugoslawischen. Die türkischen Sämlinge jedoch, waren denjenigen aus den anderen Ländern am wenigsten ähnlich. Die Saatgutproben eines Landes lagen immer als Gruppe dicht beieinander.

Introduction

European black pine (*Pinus nigra* Arnold) is a widespread, commercial tree species found principally in the mountains of southern Europe and Asia Minor, with outliers in many of the Mediterranean islands and in northern Africa (Critchfield and Little 1966, Vidaković 1974). The species shows high variability in phenology, growth form, adaptability, and monoterpene and isozyme composition. Even though a number of sub-specific categories have been described, there is no general consensus on its taxonomy (Röhrig 1966, Read 1976, Wilcox and Miller 1975, Wheeler *et al.* 1976, Vidaković 1974, Arbez *et al.* 1974, Bonnet-Masimbert and Bikay-Bikay 1978).

Most karyotypic studies of *P. nigra* have been performed to determine interspecific karyotypic variation within the genus *Pinus;* therefore, sample sizes for those studies have been very small (Saylor 1964, Pederick 1970, MacPherson and Filion 1981). Intraspecific variation in karyotype has been strictly studied on an individual tree basis (Borzan and Papes 1978, Borzan 1981). Karyotypic relationships among and within pine species have been traditionally studied by comparing idiograms of haploid chromosome sets and by analyzing individual chromosomal variables (in most cases, only arm ratios and relative length of chromosomes) (Saylor 1961, 1964, 1972, Pederick 1967, 1970, Borzan and Papes 1978, Borzan 1981, MacPherson and Filion 1981). There has been no attempt to statistically analyze karyotypic information involving a number of traits and to explore relationships among populations of species. This study had two objectives: 1) assess intraspecific karyotypic variation in *P. nigra* Arnold qualitively and quantitatively; and 2) develop a statistical procedure for use in such karyotypic studies.

Materials and Methods

An attempt was made to obtain seeds from the entire range of *Pinus nigra* Arnold; however, communication difficulties limited the effort to five countries. The French, Greek, Yugoslavian, and Turkish seeds came from three collection localities in each country; seed could only be collected from two localities in Austria *(Table 1).* Despite the general lack of specific information regarding location, seed collections from each country are known to have come from well separated areas. The local seed collections were bulk samples and it is not known how many individual trees were involved in each. Possibly as many as 50 or more parent trees are represented in each collection, if the seeds were originally gathered for reforestation purposes (personal communication with seed suppliers).

Table 1. — Categorization of *Pinus nigra* Arnold seed sources and collections, and number of seedlings karyotyped in each collection.

Seed Source	Seed Collection	Number	Latitude	Elevation
Austria	Alland	16	——[1]	400–900 m
	Hernstein	16	——	500 m
Turkey	Adana	21	37°30'N	1250 m
	Balikesir	15	40°20'N	500–1000 m
	Kütahya	15	39°21'N	1000–1200 m
Yugoslavia	Zavidovici	10	44°20'N	600 m
	Kozina	10	45°31'N	500 m
	Visegrad	11	43°50'N	400–650 m
France	Noceta	10	——	(Coast)
	Tartagine	10	——	(Coast)
	St. Guilhem	20	——	(Inland)
Greece	Drama	15	——	——
	Sparta	15	——	——
	Kalambaka	17	——	——

[1] Data not provided by seed supplier.

Approximately 200 seeds from each collection were soaked in 1% H_2O_2 for two days to speed-up and assure more uniform germination. The seeds were then germinated in petri dishes inside a darkened incubator kept at a constant 25° C. After the radicles of the germinated seeds reached about 5 mm, the seedlings were transplanted into pots filled with soil and grown in a greenhouse for two weeks.

Karyotypes were determined by studying mitotic chromosomes in squash preparations of root-tip meristems from the 2-week-old seedlings. The root-tips were soaked in mono-bromo naphtalene for 24 hours at room temperature in order to obtain more cells in metaphase of mitotic cell division. Next, they were fixed in 3:1 ethylalcohol-acetic acid for 4—6 hours, hydrolyzed in 1N HCl for 12—15 minutes at 60° C, and stained with a combination of Feulgen and Aceto-carmine. This combined staining method gave better results than solely Aceto-carmine staining (Doerksen and Ching 1972). We also did some Giemsa staining with the materials available, but had limited success in resolution of chromosomal structures.

At least 50 seedlings were utilized for each seed collection. Over 100 slides (2 slides per seedling) were made from each seed collection within a seed source. However, only slides showing cells with well-spread metaphase chromosomes were chosen for analysis, and photographs of these cells (35-mm color transparency, ASA 200 daylight film) were taken through a phase contrast microscope. Additional slides were prepared from seed collections that showed greater variation in chromosome morphology. *Table 1* lists the number of seedlings from each seed collection that were photographed and subsequently analyzed.

Diagrams of the chromosomes in each analyzed cell were drawn from photographs magnified to 3756X with a standard slide projector (projection distance of 250 cm). The chromosomes of each cell were arbitrarily numbered from 1 to 24, and the following data were recorded from the diagrams of each chromosome: short arm length (SL), long

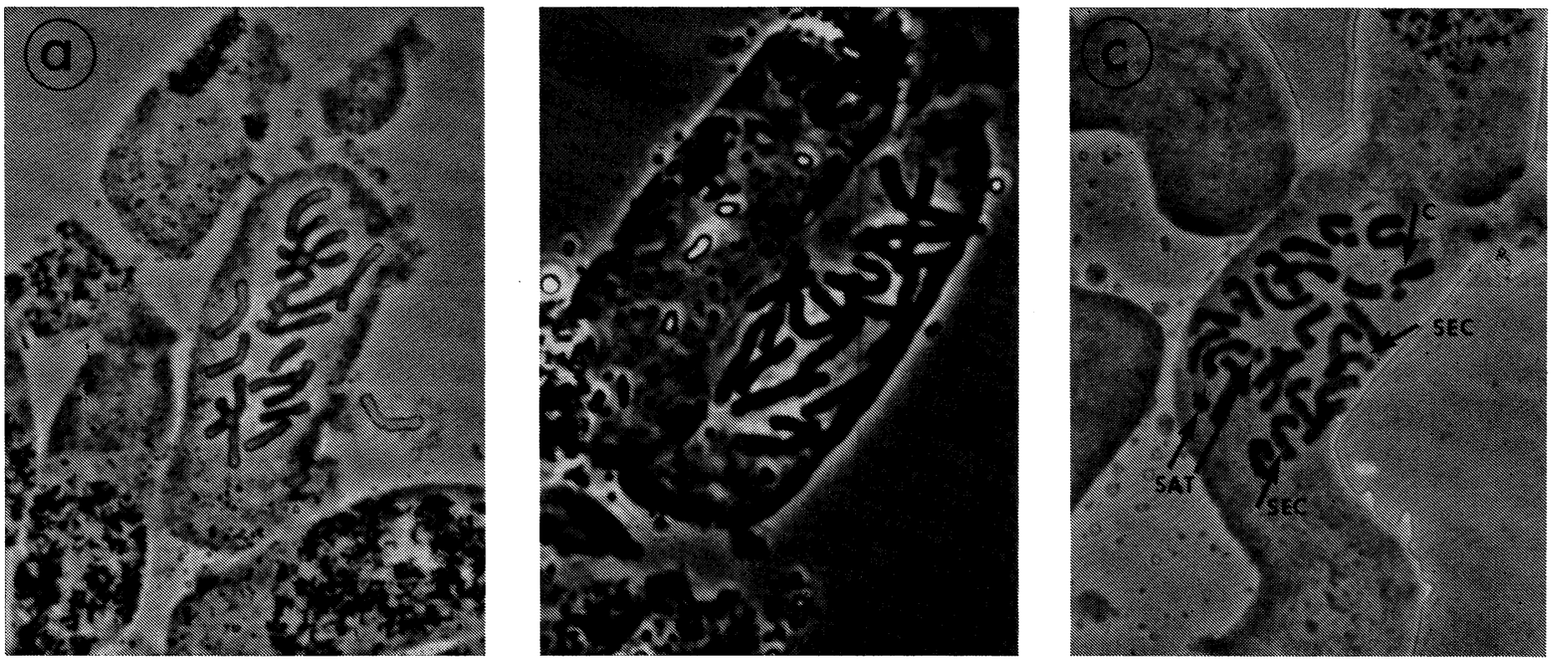


Figure 1. — Microphotographs of chromosomes of *Pinus nigra* Arnold Greek seed source (a) and Yugoslavian seed source (b) both show aneuploidy (2n = 18, not 24). c — Shows occurrence of satellites (SAT = satellites; SEC = secondary constriction; C = centromere). Magnification = 2692.4X.

arm length (LL), total chromosome length (TL = SL + LL), presence of a secondary constriction on the short arm (S_{sec}), presence of a secondary constriction on the long arm (L_{sec}), and the presence of a satellite (SAT). The locations of the above chromosomal structures were double-checked with the original microscope slides. For the evaluation of the homologous chromosomes, diagrams of chromosomes and microscope slides were again used to eliminate reduced resolution resulting from projector magnification.

In order to group the chromosomes into homologous pairs, they were evaluated according to SL, LL, TL, S_{sec}, L_{sec}, SAT, arm ratio (AR = SL/LL)(Saylor 1961), and relative chromosome length ($RL = [TL_i/(\Sigma TL_i/n)] \times 100$). Once the pairing was complete, the means of SL, LL, and TL, and the frequency of S_{sec}, L_{sec} and SAT, were computed for each pair. In addition to the above variables, the values of AR, RL, morphological index (MI = SL/LL × TL) (Giannelli and Howlett 1967), and centromere index (CI = (SL/TL) × 100) (Stephenson *et al.* 1972) were derived for each pair. The haploid set of chromosomes was numbered in descending order from I to XII with chromosome I the longest and chromosome XII the shortest. Thus, the karyotypic data in each cell were analyzed in the form of 12 haploid chromosomes, each with 10 variables.

Statistical Analysis

A statistical analysis was performed on each of the 12 haploid chromosomes from each seed collection. The frequencies of occurrence were computed for S_{sec}, L_{sec}, and SAT, and the mean values were calculated for the other variables. For example, the mean of SL for chromosome I in the French St. Guilhem seed collection was computed by averaging the SL values of 20 studied cells.

Analyses of variance were conducted for all variables except S_{sec}, L_{sec}, and SAT. These were excluded for two reasons: 1) the appearance of secondary constrictions was greatly affected by cytological techniques, such as squashing; 2) satellites appeared in only two seed sources, and the frequency was too low. Analyses of variance were done using a nested design with seed sources (countries) as treatments (degrees of freedom (df = 4) and seed collections (localities) within seed sources as subsamples (df = 9). Both seed sources and seed collections within seed sources were assumed to be random variables in the analysis.

In addition to analysis of variance, a "cluster analysis for cases" was conducted using the P2M:BMDP statistical package (Engelman 1979) on each of the 12 chromosomes; the mean values of the seed collection and seed source variables were used (in the case of S_{sec}, L_{sec} and SAT, the frequencies were used). Cluster analysis forms clusters of cases based on one of several possible amalgamation distances (similarity indices), such as Euclidean, Phi-square, or Chi-square statistics. The Euclidean distance was chosen as the basis for clustering in this study. It is the square root of the sum of squares of differences between values of variables for two cases. Two separate analyses were performed to determine the clustering pattern of chromosomes: in the first cluster analysis--between seed collections within seed sources--"cases" were chromosomes of seed collections; whereas in the second cluster analysis--between seed sources--"cases" were chromosomes of seed sources. For example, when a cluster analysis for chromosome I was performed between 5 seed sources, there were 5 cases and 10 determined variables for each case. All of the 10 variables were utilized together in the cluster analyses.

Initially, each case was considered to be in a cluster of its own. At each step, the two clusters with the shortest distance between them were combined (amalgamated) and treated as one cluster. This process of combining clusters continued until all the cases were combined into one cluster. This algorithm is called "average distance" or "average linkage." The theoretical basis of cluster analysis is thoroughly discussed in Johnson and Wichern (1982).

Results and Discussion

Karyotypes of Pinus nigra in general

The great majority (99%) of the seedlings sampled had the expected diploid chromosome number of 24. However, one seedling each from Yugoslavia and Greece had only 18 chromosomes in all the cells that we examined *(Figure 1 a, b)*. The remaining cells of these seedlings were in early or late stages of mitotic cell division; thus we were not able to count their chromosomes. We were unable to find previous reports of abnormalities in the chromosome number of *Pinus nigra*, and although rare, they have been observed consistently in numerous other pine species (Pederick 1967, Mergen 1958, Ching and Simak 1971). Possibly, the occurrence of aneuploidy may have arisen from somatic mutation, since aneuploid seedlings during sampling were phenotypically no different than normal seedlings (2n = 24). Presumably these aneuploid seedlings had plenty of normal diploids cells that retained full function. We rule out the possible origin of aneuploidy from unequal translocation (Burnham 1962, Stebbins 1971, Grant 1981, Schulz-Schaeffer 1980, Swanson *et al.* 1981) or selective chromosome elimination during interspecific hybridization (Ho and Kasha 1975, Gupta 1973, Pontecorvo 1971) because we did not observe increased length on the remaining 18 chromosomes nor occurrence of polyploidy, respectively.

The range in means of TL was almost two-fold, varying from 51.17 mm in chromosome I to 28.30 mm in chromosome XII, and relative chromosome length (RL) varied from 123.1 to 68.2 *(Table 2)* (values obtained from projection drawings magnified to 3756X). Chromosome lengths of members of homologous pairs often differed slightly. The longest chromosome (1) and the two shortest chromosomes (XI and XII) can be readily identified by visual means in *P. nigra;* the identification of the remaining 9 chromosomes is not possible because of their similar lengths and centromere locations. However, they can be identified consistently by evaluating them according to the values of chromosomal variables when multiple cells are observed for each seedling.

Median and submedian centromere positions were defined with AR values of 0.80—1.00 and 0.50—0.80, respectively. Centromeres of chromosomes XI and XII in *P. nigra* were found to be in the submedian position, whereas the remaining 10 chromosomes had their centromeres located in the median position *(Table 2)*.

In our material, the frequency of secondary constrictions was proportionate to the overall length of chromosomes, and was higher on long arms than short arms. Secondary constrictions were especially infrequent on the two shortest chromosomes (XI and XII) *(Table 2)*. In general, secondary constrictions occur only sporadically on pine chromosomes. This may be attributed in part to the cytological techniques used in karyotyping. Alternatively, it may be inherent in low activity of the Nucleolus Organizer Region (NOR)

Table 2. — Mean values and standard errors of chromosomal variables of *Pinus nigra* Arnold over all seed sources.

Variables[1]	Chromosome I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
SL	24.51 ± .49	23.19 ± .54	22.44 ± .52	21.91 ± .53	21.12 ± .46	20.67 ± .47	20.06 ± .34	19.36 ± .30	18.48 ± .29	17.34 ± .36	14.48 ± .41	11.44 ± .40
LL	26.66 ± .89	25.23 ± .85	24.42 ± .87	23.52 ± .73	23.18 ± .73	22.56 ± .62	21.92 ± .68	21.22 ± .58	20.43 ± .55	19.46 ± .50	18.40 ± .46	16.86 ± .40
TL	51.17 ±1.36	48.42 ±1.37	46.86 ±1.20	45.43 ±1.18	44.30 ±1.08	43.23 ±1.01	41.98 ± .87	40.58 ± .84	38.91 ± .84	36.80 ± .70	32.88 ± .75	28.30 ± .75
AR	0.92 ± .013	0.92 ± .013	0.92 ± .014	0.93 ± .006	0.91 ± .011	0.91 ± .006	0.92 ± .013	0.91 ± .011	0.90 ± .009	0.89 ± .009	0.78 ± .025	0.68 ± .016
RL	123.1 ± .66	116.4 ± .60	112.7 ± .60	109.2 ± .32	106.5 ± .33	104.0 ± .12	101.0 ± .25	97.6 ± .40	93.7 ± .47	88.5 ± .34	78.2 ± .95	68.2 ± .62
CI	47.9 + .37	47.9 + .33	47.9 + .36	48.2 + .12	47.7 + .28	47.9 + .23	47.8 + .37	47.7 + .32	47.4 + .26	47.1 + .26	44.0 + .79	40.4 + .57
MI	47.1 ± .76	44.6 ± .91	43.0 ± .80	42.4 ± .96	40.5 ± .64	39.6 ± .85	38.4 ± .50	37.0 ± .45	35.2 ± .33	32.8 ± .65	25.9 ± .99	19.2 ± .81
S_{sec}	.264	.228	.190	.224	.104	.140	.160	.124	.100	.070	.060	.005
L_{sec}	.428	.333	.260	.280	.209	.250	.280	.184	.124	.120	.080	.035
SAT	.030	.045	.025	.025	.030	.045	.010	.000	.005	.005	.005	.000

[1]SL = Short arm length (mm) (values obtained from drawings projected at magnification of 3756X).
LL = Long arm length (mm).
TL = Total length (mm).
AR = Arm ratio (short arm length/long arm length).
RL = Relative length [$(TL_i/\sum TL_j/n)X100$].
CI = Centromere index [(SL/TL)X100].
MI = Morphological index [(SL/LL)XTL].
S_{sec} = Frequency of secondary constrictions on short arm.
L_{sec} = Frequency of secondary constrictions on long arm.
SAT = Frequency of satellites.

failing to produce secondary constrictions (MacPherson and Filion 1981, Sato *et al.* 1980, Saylor 1964). When karyotyping pines, secondary constrictions should not be considered as a major criterion, but they can be used as additional chromosome markers. From this study, as well as earlier ones, it is apparent that at least two conventional staining techniques should be used when studying chromosomes of pine species: Giemsa staining for banding individual chromosomes and silver staining for identifying NORs.

Satellites were only rarely observed, but generally were most frequent on the longer chromosomes (I to VII) *(Fig-*

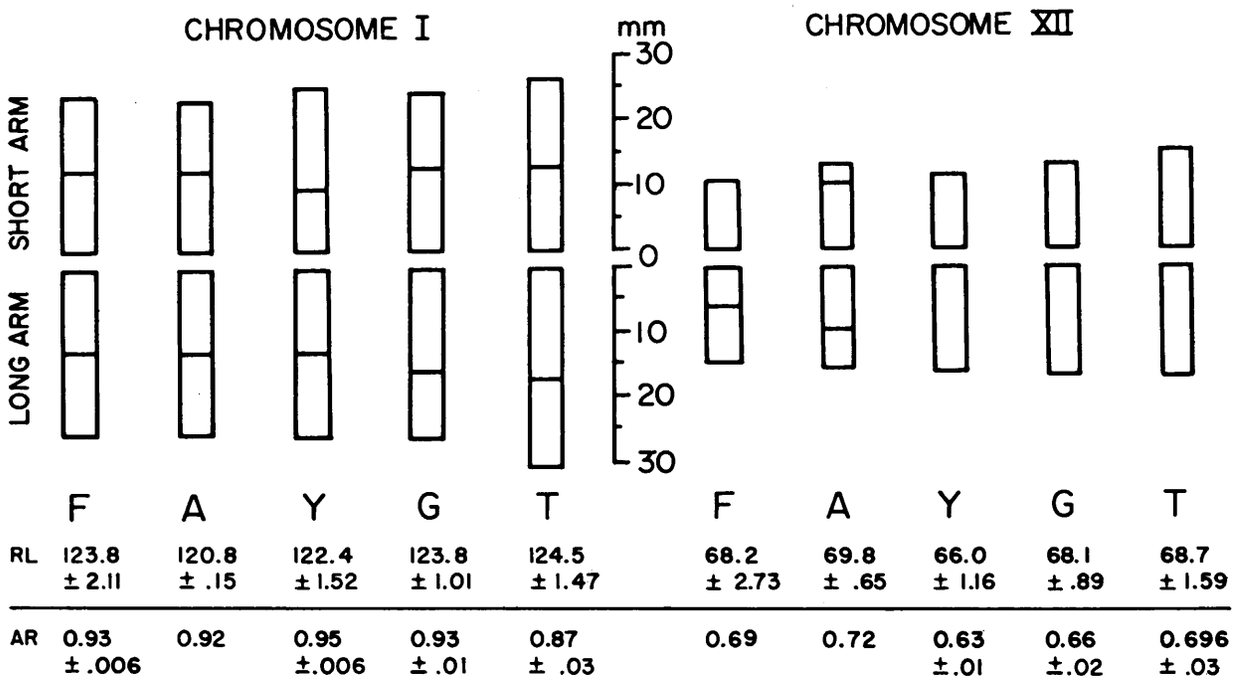


Figure 2. — Idiograms of chromosomes I and XII of *Pinus nigra* Arnold. Lines on chromosome arms indicate the average location of secondary constrictions (F = France, A = Austria, Y= Yugoslavia, G = Greece, and T = Turkey). Magnification = 2756X.

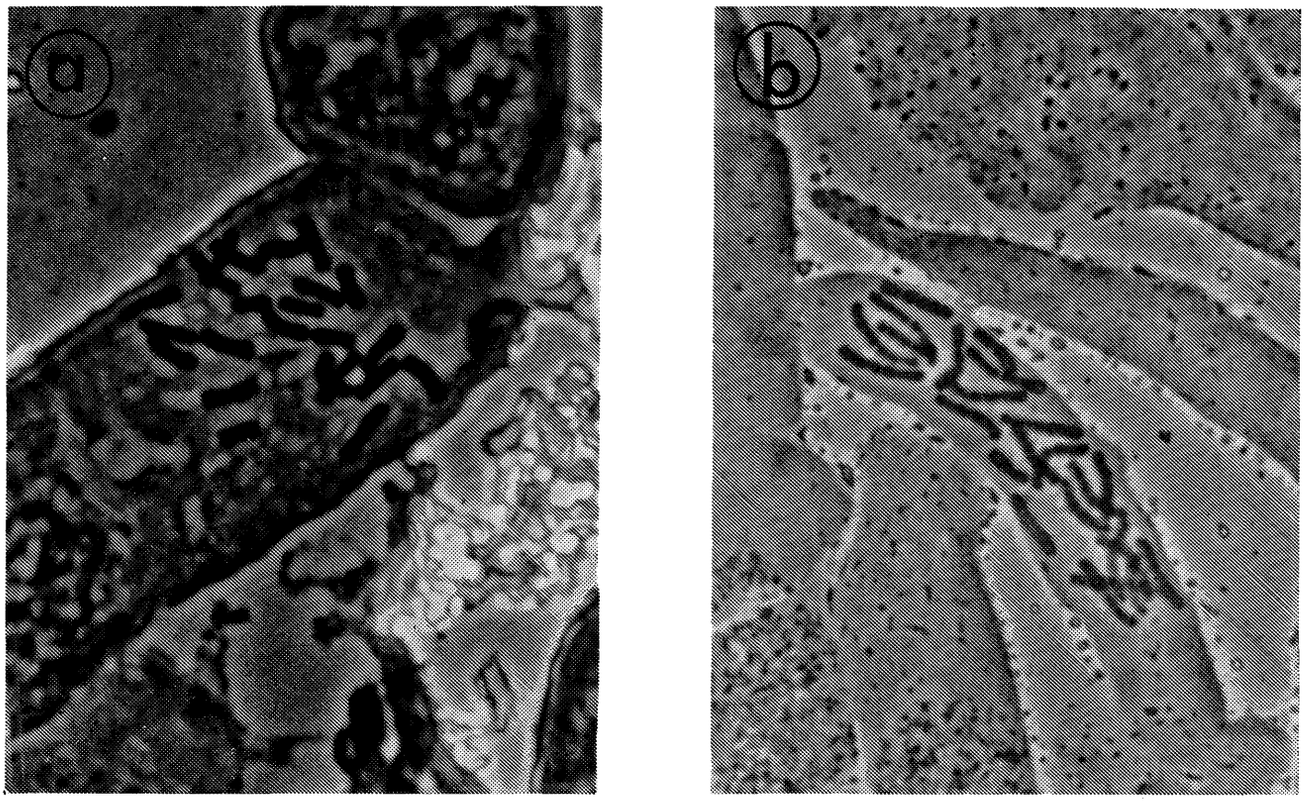


Figure 3. — Microphotographs of chromosomes of *Pinus nigra* Arnold from France (a) and Turkey (b). Magnification = 2692.4X.

ure 1 c and *Table 2)*. Chromosomes VIII and XII had no satellites and chromosomes IX, X, and XI had only a rare (.5%) occurrence of them *(Table 2)*. No previous study has reported satellites in *P. nigra*. Stebbins (1971) points out that satellites are heterochromatinized regions on chromosomes and are connected to chromosomes with NORs. Although the low frequency of satellites on any one chromosome limits their use for identifying chromosomes, they may be useful for separating *P. nigra* karyotypes from those of other closely related pines.

Karyotypic variation among seed sources

Differences in chromosome morphology among seed sources were quantified by the measured chromosomal variables for each haploid chromosome. The karyotype of each of the five seed sources was derived from the karyotypes of seed collections by averaging the values of their chromosomal variables. Idiograms of chromosomes for each chromosome set (chromosome set I to XII) were compared among the seed sources *(Figure 2)*.

Total chromosome length (TL) varied significantly ($p < .05$) among seed sources in all 12 chromosome sets (except for chromosomes VII, VIII, and IX) *(Table 3)*. On the average, chromosomes in the French seed sources were the shortest, whereas all chromosomes in the Turkish seed source were the longest of any source in the study *(Figures 3* and *4)*. Although variation in chromosome length between trees of single *P. nigra* population was reported by Borzan and Papes (1978), no compelling explanation on this matter has been offered to date. However, Miksche (1967, 1968) reported that duplication or deletion processes could cause a lengthening or shortening of chromosomes. Previous studies in plants have shown that the amount of DNA in cells can vary with geographic location and may have adaptive value (Price *et al.* 1981 a, 1981 b). Variation in chromosome length between sources could also be due to differences in chromosomal proteins present in chromosomes of sources. This could create an artifact as effected by differential chromosome contraction during the treatments (Miksche 1967, 1968, Sziklai and De-Vesconi 1978, Stebbins 1964, 1976, Dhir and Miksche 1974, Price *et al.* 1981 a, 1981 b).

Mean values of arm ratio (AR) and centromere index (CI) were significantly different ($p < .05$ or $p < .01$) in chromosome sets I, II, III, V, VII, VIII, X, XI and XII

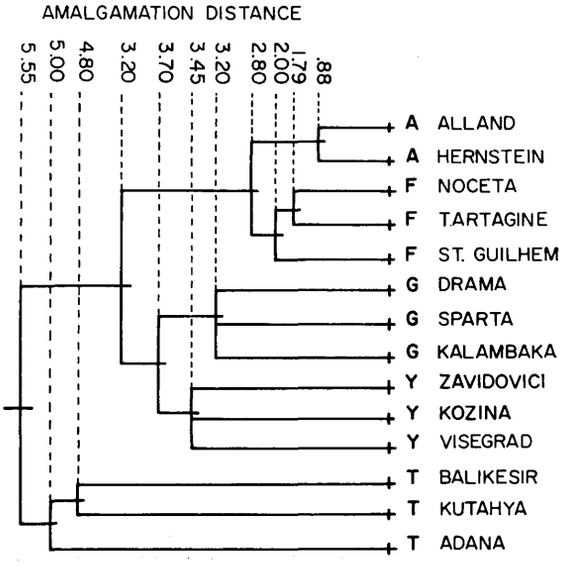


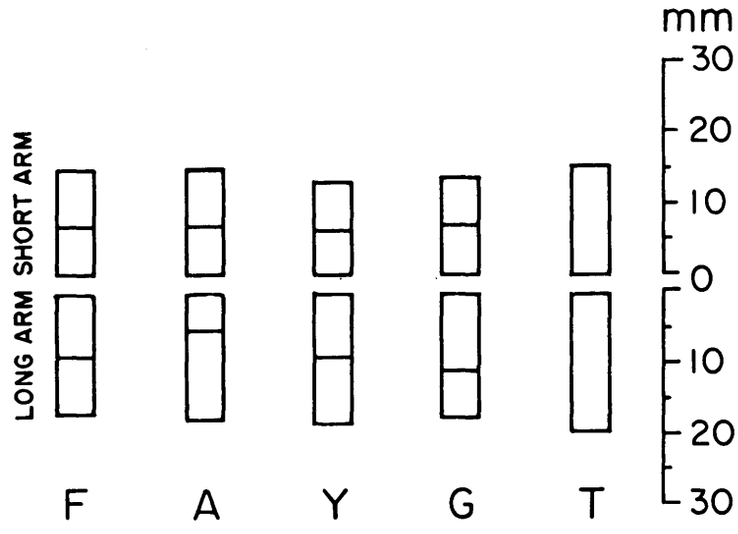
Figure 4. **— Dendrogram (not in scale) showing the general clustering patterns among the seed collections of five seed sources of *Pinus nigra* Arnold (A = Austria, F = France, G = Greece, Y = Yugoslavia, and T = Turkey).**

Table 3. — F-ratios[1] (mean square for seed sources/mean square for seed collections within sources) for seven variables measured on the 12 haploid chromosomes of *Pinus nigra*.

	Chromosome Sets											
Variables[2]	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
SL	2.65	3.63	2.72	3.66*	2.59	2.83	1.91	1.32	1.53	2.64	14.37**	8.61**
LL	6.22*	7.24**	10.49**	5.86*	7.29**	5.97*	5.25*	4.54*	5.71*	6.58**	7.27**	9.27**
TL	4.55*	5.53*	5.73*	4.74*	4.62*	4.36*	3.60	2.91	3.50	4.25*	7.06**	9.06**
AR	7.76**	6.42*	6.73**	2.52	12.00**	1.67	10.83**	3.73*	2.87	4.48*	31.09**	9.05**
RL	1.62	4.09*	9.32**	1.91	3.10	0.76	2.69	4.43*	6.02*	1.32	3.49	1.38
CI	7.54*	6.35*	6.54**	2.52	11.85**	1.67	10.84**	3.77*	2.93	4.44*	30.24**	9.19**
MI	1.54	2.43	1.53	2.88	1.48	1.97	0.92	0.56	0.58	1.92	23.87**	8.55**

[1] All F-ratios have (4/9) degrees of freedom.
[2] Descriptions of variables are the same as in *Table 2.*
* Significant at $\alpha = .05$ level of probability.
** Significant at $\alpha = .01$ level of probability.

(Table 3). When AR values showed significant differences between chromsomes of seed sources, CI values had similar results. This was expected because CI values were derived from AR values. Although we found centromeres of the two shortest chromosomes (XI und XII) generally in the submedian position, seedlings of France and Austria had the centromere of chromosome XI in the median position (AR of 0.86 ± 0.01 and 0.81 ± 0.77, respectively) *(Figure 5).* This result is contradictory to earlier findings in *P. nigra* and closely related species (Sax and Sax 1933, Mehra and Khoshoo 1956, Saylor 1961, 1964, 1972, Pederick 1967, 1970, Borzan and Papes 1978, Borzan 1981, MacPherson and Filion 1981). In those studies, samples were limited to either a single tree or a single population, regardless of the species' natural distribution. That limited sampling may explain why those authors were unable to detect intraspecific variation in centromere position of chromosome XI. Another possibility is that the evolution of centromere position of chromosme XI may be at a different stage in Austrian and French seed sources; prior research shows that variation in centromere position of a particular chromosome can originate from pericentric inversions or unequal translocations (Stebbins 1971, Saylor 1969, Pederick 1969). In our study, working with somatic tissues, we were unable to observe either pericentric inversions or unequal translocations. Either of the above would lead to irregularities in chromosome pairing during meiotic division and to decreased viability in gametes of F1 hybrids between seed sources with different centromere position (Schulz-Schaeffer 1980, Lewis 1962). Therefore, further



	F	A	Y	G	T
SL(MM)	14.79±.29	14.98±.11	13.30±.45	13.79±.22	15.52±.47
LL(MM)	17.14±.44	18.38±.06	18.76±.71	17.84±.55	19.88±.69
AR	0.86±.01	0.81±.007	0.71±.01	0.77±.02	0.78±.01
RL	80.7±2.55	81.8±.55	76.4±1.02	79.0±.29	78.1±1.89

Figure 5. — Idiograms of chromosome XI of *Pinus nigra* Arnold. French and Austrian sources show median centromere position, whereas other sources show submedian position. Lines on the chromosome arms indicate the average location of secondary constrictions. Note — Arm ratios are from different sources (F = France, A = Austria, Y = Yugoslavia, G = Greece, and T = Turkey). Magnification = 3756X.

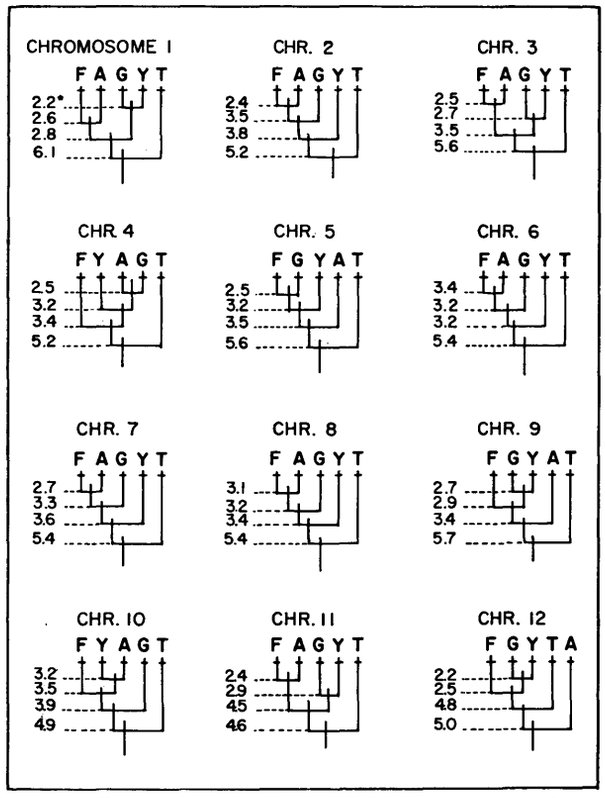


Figure 6. — Dendrograms (not in scale) showing cluster analysis of haploid chromosomes (n = 12) of *Pinus nigra* Arnold from five seed sources. *Amalgamation distance. (F = France, A =Austria, G = Greece, Y = Yugoslavia, and T = Turkey).

study of the behavior of chromosome XI of *P. nigra* in meiotic cell division will be valuable toward understanding the chromosomal evolution in pine species.

Frequency of secondary constrictions on short and long arms of the 12 chromosomes for the different seed sources is given in *Table 4.* Centromeres, secondary constrictions, and satellites are also shown in *Figure 1 c.* Secondary constrictions on chromosome XII were not observed in the Greek, Yugoslavian, and Turkish seedlings. Moreover, secondary constrictions were not found on chromosome V in the Austrian nor on chromosomes V and XI in the Turkish seedlings. The occurrence of secondary constrictions has often been used in pine karyotyping (Saylor 1964); therefore, the lack of secondary constrictions on chromosome V may be used to diagnose Austrian and Turkish seedlings of *Pinus nigra.*

Cluster Analyses

Cluster analyses were performed for each of 12 chromosome sets among seed collections by utilizing all variables (SL, LL, TL, AR, RL, MI, CI, S_{sec}, L_{sec}, SAT) together. A general clustering pattern among seed collections was developed by taking into consideration all the cluster analyses together. The similarity indices in *Figure 4* were computed by averaging the values for amalgamated distances of the 12 cluster analyses conducted between seed collections. Cluster analyses indicated that the seed collections within the seed sources were uniform in terms of their karyotypes, since the seed collections within seed sources tended to cluster together. It is evident from *Table 3* that the variables SL, LL, TL, AR, RL, CI and MI on some chromosomes among the seed sources showed significant variation. In order to determine a pattern of karyotypic variation among seed sources, cluster analyses were conducted for each chromosome set between seed sources. In more detail, the results of separate cluster analysis between seed sources for each chromosome are presented in *Figure 6.* In regard to karyotypes of seed sources, the French and Austrian seedlings appeared to have the most similar chromosomes, but also quite similar to this pair were the Greek and Yugoslavian seedlings. The Turkish seed source almost always clustered outside the other four sources. It displayed the least chromosome similarity with the others *(Figures 3 b, 4,* and *6)* because the Turkish seedlings had longer SL and LL, and most of the chromosomal variables included in the cluster analysis were a function of these two variables. Moreover, the Turkish seedlings had no satellites on their chromosomes and no secondary constrictions on chromosomes V, XI and XII *(Table 4).* Based on these results of cluster analysis, it seems that the Turkish seed source has karyotypically evolved somewhat independently from the rest of the seed sources. Further studies with material from a more complete sampling of the species range would be valuable to better understand the chromosomal evolution of *P. nigra.*

Conclusions

In contrast to many earlier studies in pine species, we found substantial intraspecific variation in chromosome morphology, probably because our study sampled more

Table 4. — Frequencies of secondary constrictions (on short arm, S_{sec}; on long arm, L_{sec}) and satelites (SAT) on *Pinus nigra* chromosomes from five seed sources.

Seed source	Variables	Chromosome I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
France	S_{sec}	.20	.27	.17	.30	.15	.15	.12	.10	.10	.07	.05	.02
	L_{sec}	.40	.27	.22	.12	.35	.17	.10	.18	.12	.15	.05	0
	SAT	0	0	0	0	0	0	0	0	0	0	0	0
Austria	S_{sec}	.31	.19	.03	.25	0	.12	.12	.12	.09	.09	.06	.06
	L_{sec}	.44	.34	.12	.41	0	.25	.28	.22	.09	.09	.03	.19
	SAT	0	0	0	0	0	0	0	0	0	0	0	0
Yugoslavia	S_{sec}	.22	.13	.32	.16	.22	.16	.13	.09	.13	.06	.09	0
	L_{sec}	.48	.23	.29	.23	.29	.26	.42	.13	.09	.19	.19	0
	SAT	.16	.06	.09	.09	.06	.13	.09	.06	0	.03	0	0
Greece	S_{sec}	.32	.32	.45	.23	.17	.15	.13	.19	.06	.02	.08	0
	L_{sec}	.53	.40	.38	.28	.38	.30	.28	.13	.15	.06	.11	0
	SAT	.15	.08	.13	.04	.06	.04	.13	0	0	0	.02	0
Turkey	S_{sec}	.22	.18	.18	.10	0	.08	.23	.08	.08	.12	0	0
	L_{sec}	.29	.37	.25	.25	0	.33	.27	.20	.19	.08	0	0
	SAT	0	0	0	0	0	0	0	0	0	0	0	0

intensively at the intraspecific level. Future studies comparing chromosome banding patterns and staining of NORs between populations of *P. nigra,* as well as other pine species, would be valuable in providing information about the evolution of chromosomes at the intraspecific level in *Pinus.* Moreover, a study on the behavior of chromosome XI at meiosis of F_1 hybrids between the French or Austrian seed sources and the remaining sources would help to determine if their seed production is reduced as a result of abnormalities in chromosome pairing.

Traditionally, karyotypic information has been presented merely by idiograms of chromosomes along with AR and RL values, thus lacking the quantification that a cluster analysis provides. An idiogrammatic interpretation of karyotypic data is especially difficult when large numbers of populations (species) and variables are involved. Since secondary constrictions in this study appeared quite inconsistent, the use of different staining techniques (i.e., C-banding with Giemsa staining) may be advised for the karyotyping of *P. nigra* and other pine species.

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