Karyotype analysis in Pinus: A contribution to the standardization of the Karyotype analysis and review of some applied techniques*)

By Ž. Borzan and D. Papeš

Department of Forest Genetics and Dendrology, Faculty of Forestry, University Zagreb, 41001 Zagreb, P.O. Box 178; and Department of Botany, Biology Division, Faculty of Sciences, University Zagreb, 41001 Zagreb, P.O. Box 933, Yugoslavia

(Received December 1977 / July 1978)

Summary

Comparative intraspecific karyotype analysis was performed on two European black pine trees (*Pinus nigra* Arn.) by examining 18 endosperm cells for each tree ("ni 47" and "ni 221"). The standard Feulgen technique was used for staining chromosomes (*Fig. 1*).

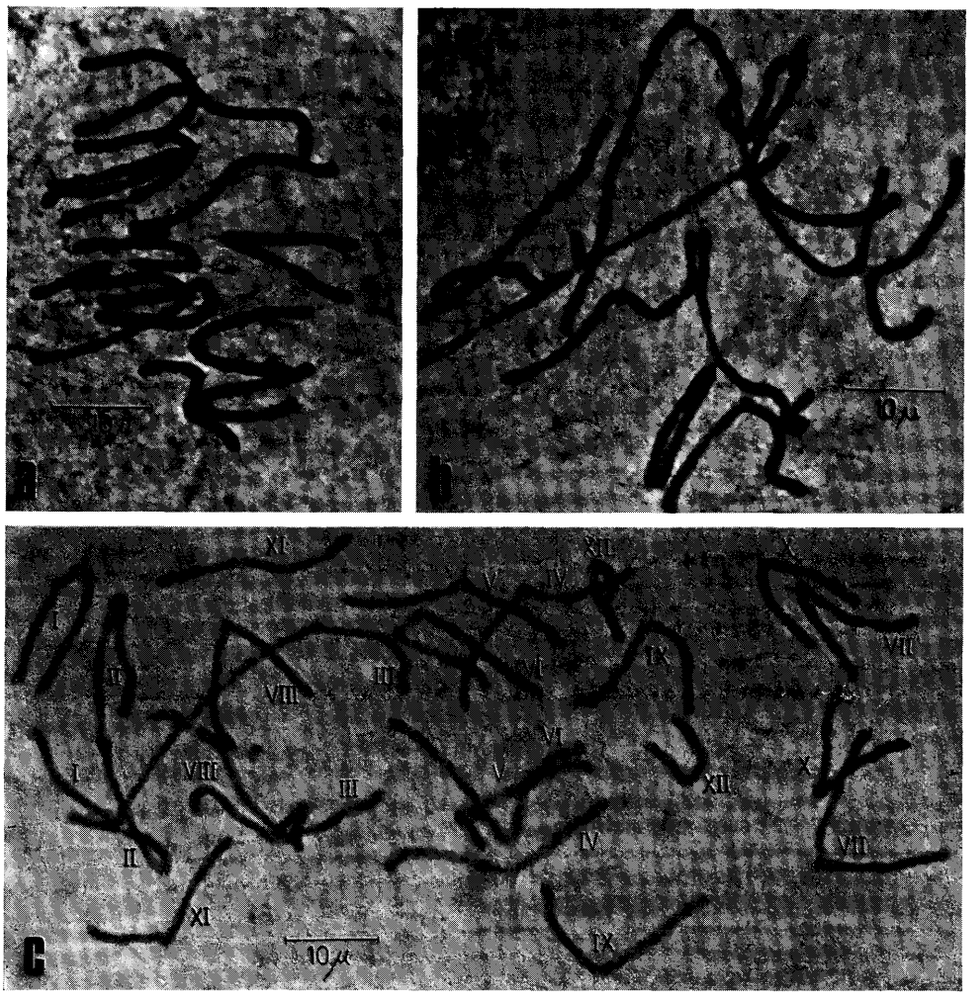

Figure 1. — Photomicrographs of cells from endospermal tissue of *Pinus nigra* Arn. The preparations are made with the Feulgen squash method for endosperm: a — One of eighteen metaphase cells ("ni 221"), which was used for comparative analysis of two European black pine trees; b — Sticky chromosomes in the endosperm of pines; this is one of the sources of chromosome length variation; c — Anaphase with identified chromatids; sticky connections are one of the noticeable causes of sister chromatid length variation (from Borzan, 1977 a).

*) The research reported here was supported by the Council for Science of the Socialist Republic of Croatia, Faculty of Forestry, University Zagreb and through Grant No: YO-FS-88-JB-6 by the U. S. Ministry of Agriculture, Forest Service, through funds made available by the U. S.-Yugoslav Joint Board on Scientific and Technological Cooperation. Results have been presented as a poster demonstration at the VI international Chromosome Conference held in Helsinki, August 29th—31st, 1977.

Chromosome XII of tree "ni 221" is significantly longer (at the five percent level) than chromosome XII of tree "ni 47". The short arm of chromosome VI of tree "ni 221" has noticeably more constrictions than short arm of chromosome VI of tree "ni 47" (*Fig. 2*).

Varisus methods for karyotype analysis of pines were discussed to point out the necessity for standardization of

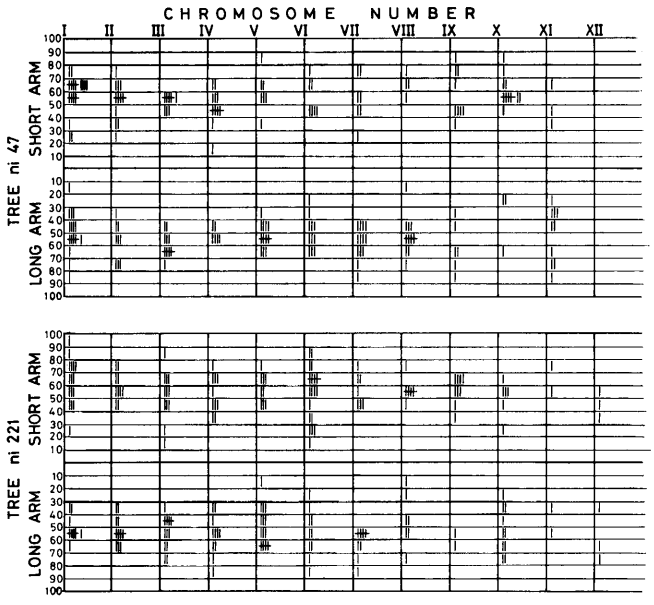

Figure 2. — Schematic overview of frequency distributions of secondary and tertiary constrictions in two trees of *Pinus nigra* Arn. All noted constrictions in 18 endospermal cells of tree "ni 47" and "in 221" are recorded as to their relative location (i.e. as a percentage of their distance from the centromere in relation to the entire length of the arm on which they are located).

methods. On the basis of the results presented, it is possible to emphasize certain steps in the analysis of pine karyotypes for the standardization of work. These include such things as:

1. The use of the haploid tissue of the female gametophyte, and analysis with a sufficient number of suitable cells.

2. The use of one of the differential staining techniques (e.g. the C-banding method), which will make chromosome identification possible and allow the comparison of results of different investigations.

3. The presentation of Giemsa C-banding patterns in idiograms.

4. The use of statistical methods in karyotype analysis for the comparison of results (e.g. *Table 2)* such as the total chromosome lengths, short arms, long arms, the arm ratios and the centromere index values.

The appearance and behaviour of sticky chromosomes in pine endosperm was discussed *(Fig. 1 b and c).*

Chromosome C-banding patterns of the European black pine were demonstrated on chromosomes of female gametophyte tissue of tree "ni 221". *Pinus nigra* Arn. was shown to have a very small per cent of heterochromatin. In metaphase, heterochromatic regions appeared centromerically, as well as in secondary constriction regions. Seven to eight chromosomes with bands in secondary constriction regions were found in the haploid set of the endosperm of the European black pine tree "ni 221" *(Fig. 3).* It is easy to identify submetacentric chromosomes XI and XII which are char-

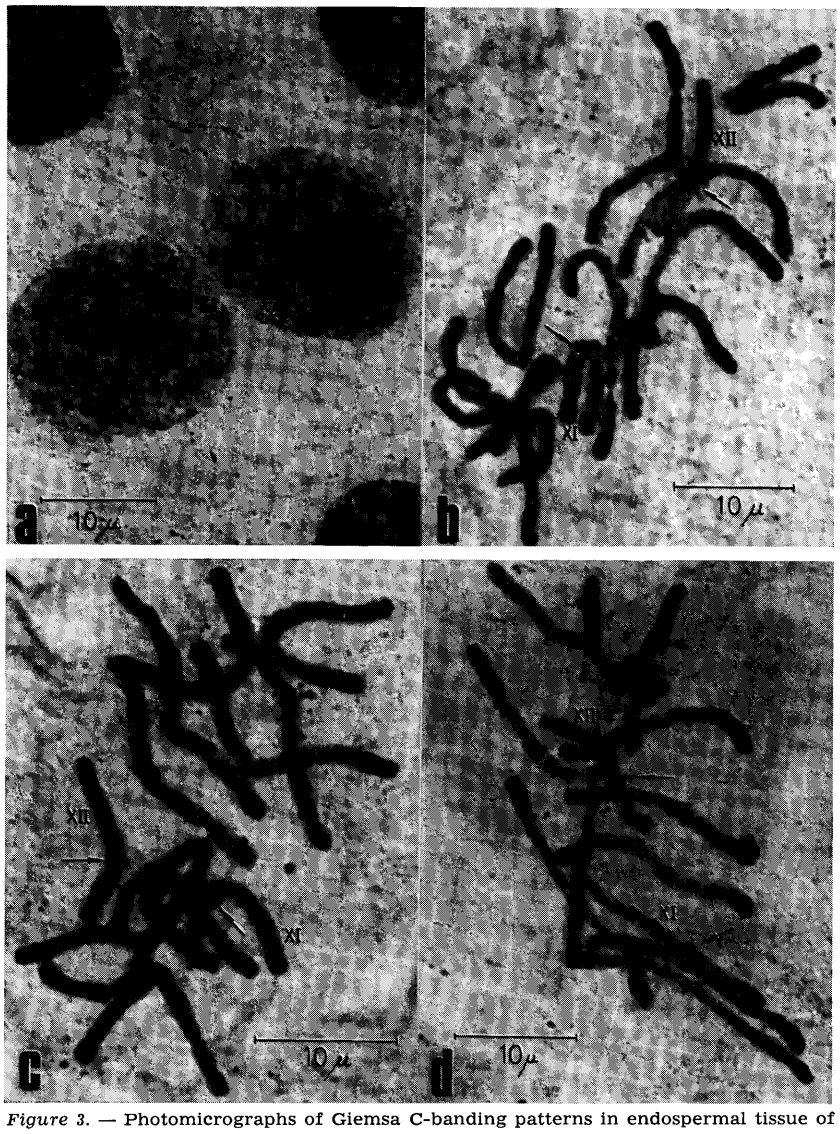

Figure 3. — Photomicrographs of Giemsa C-banding patterns in endospermal tissue of the *Pinus nigra* Arn. tree "ni 221": a — Interphase nuclei show an indistinct number of chromocentres; b, c and d — Metaphase C-bands showing chromosomes with centromeric and nucleolar bands. Chromosomes XI and XII have clear centromeric bands (indicated by arrows) and no secondary constriction.

acterised by clear centromeric C-bands and an absence of intercalary arm bands *(Fig. 3 b, c and d).*

Key words: *Pinus nigra* Arn., karyotype analysis, Giemsa C- banding, female gametophyte, endosperm, heterochromatin, sticky chromosomes.

Zusammenfassung

Auf Grund der Ergebnisse aus Karyotypenanalysen bei zwei Einzelbäumen von *Pinus nigra* Arn. wird die Notwendigkeit einer Standardisierung des Vorgehens begründet, wobei folgende Schritte empfohlen werden: 1. die Verwendung von haploidem Gewebe des weiblichen Gametophyten, 2. die Anwendung von Differenzialfärbemethoden zur Chromosomenlängsdifferenzierung, 3. die Darstellung von Idiogrammen — Chromosomenbänderung — und 4. die statistische Bearbeitung zur Sicherung der Ergebnisse, insbesondere im Vergleich zu den Ergebnissen anderer Autoren.

Das Vorkommen und das Verhalten der „sticky" Chromosomen im Endosperm werden diskutiert. Außer der signifikant verschiedenen Länge des Chromosoms XII wurden zwischen den beiden Bäumen auch in der Zahl der sekundären Konstriktionen Unterschiede festgestelllt. Es zeigte sich, daß bei der Schwarzkiefer der Prozentsatz des Heterochromatins im Gewebe des sich entwickelnden weiblichen Gametophyten sehr gering ist. In der Metphase erscheinen die heterochromatischen Abschnitte im Bereich der Zentromere und der sekundären Konstriktionen. Die submetazentrischen Chromosomen XI und XII können durch klar sichtbare Bänder im Bereich der Zentromere und durch das Fehlen des interkalaren Bandes an den Armen leicht identifiziert werden.

Introduction

Pines are very similar in their basic karyotypes (Sax and Sax, 1933). Differences in the number of secondary constrictions between certain species of pine were noticed by Mehra and Khoshoo (1956). Analysing the relative length of chromosome arms in five species of pine Saylor (1961) confirmed interspecific variation between karyotypes, but did not find intraspecific variability. Pederick (1967) pointed to a more accurate way of identifying every individual chromosome, and of determining the number and location of secondary and tertiary chromosomal constrictions using analysis of the haploid female gametophyte tissue of the *Pinus radiata.*

In the course of our extensive research on the problem of incompatibility (Vidaković and Borzan, 1973; Vidaković, 1977 a, 1977 b) between the European black pine *(Pinus nigra* Arn.) and the Scots pine *(Pinus sylvestris* L.), we discovered the existence of special combining ability between a specific tree of the European black pine "ni 221" and a specific tree of the Scots pine "sy 77". With controlled hybridization on the European black pine tree (ni 221) using pollen of the Scots pine (sy 77) we regularly obtained F_1 progeny *(Pinus* × *nigrosylvis* Vid.) although in small amounts. Pederick's work (1967, 1970) led us to try to confirm intraspecific differences between the karyotypes of pines. Applying his methods (using haploid tissue), we hoped to identify the characteristics of the karyotype of tree "ni 221" and tree "sy 77" to find out if they differed in any morphological chromosome patterns from other European black pine and Scots pine trees. Since the other European black pine trees which we used for controlled hybridization with the pollen of various Scots pine trees did not cross interspecifically, we first established the basic karyotype of one of the "incompatible" European black pine trees, "ni 47" (Borzan, 1977 a). The results of the comparative karyotype analyses for the European black pine trees "ni 221" and "ni 47" are presented in this paper. We also discuss the difficulties of comparative analysis of pine karyotypes, with the discussion serving as a contribution to the standardization of methods for production and presentation of pine karyotypes. Also the results of methods used for differential staining chromosomes of the European black pine are presented.

Material and Methods

For karyotype analyses, slides were made from female gametophyte tissue ("endosperm") from European black pine trees "ni 47" and "ni 221". The technique of preparing the endosperm by the Feulgen method according to Darlington and La Cour (1962) was used. The gathering of conelets for fixing ovules and the details of making the slides are described by Pederick (1967, 1970) and Borzan (1977 a, 1977 b).

In our investigations we profited by the experience and methods of Sax and Sax (1933), Simak (1962), Saylor (1961, 1964), Pederick (1967, 1970) and concluded:

1. There is a lack of standard methods for pine karyotype analysis which allows comparative analysis of the results both of our research and the research of other authors,

2. It is difficult to identify metacentric chromosomes of pines because of a lack of clear morphological characteristics which would exclude chromosome "reversal of order" and "arm reversal" (a critically exhaustive study on this problem was published by Matern and Simak, 1968 and 1969).

Considering the second point, we decided to apply some of the already well-known "chromosome banding" techniques, in order to differentially stain chromosomes of the European black pine.

In recent years, numerous staining methods with fluorochromes and Giemsa have become well-known in cytological investigations, resulting in differential staining of chromosomes longitudinally (Hsu, 1973). With banding methods, it is possible to identify individual chromosomes accurately, and analyse chromosome polymorphism between and within species. Many chromosome banding techniques are now routinely used in animal and human cytogenetics, but not all of them provide good results in banding plant chromosomes. As far as we know, mainly fluorochromy and Giemsa techniques (for the preferential staining of constitutive heterochromatin, "C-banding") have been successfully applied to Angiosperm chromosomes. With the application of C-banding technique to pine chromosome, we could simplify karyotype analysis and avoid chromosomal reversal of order, and we would have methods on hand for intra- and interspecific comparative analysis of pine karyotypes, which is of importance not only in terms of incompatibility but also in terms of taxonomy.

The term "C-banding" is used as Schweitzer and Ehrendorfer (1976) proposed in their report, to describe darkly stained chromosome segments obtained by a particular Giemsa method.

Seeds from trees "ni 47" and "ni 221" served for testing the appropriate "banding method". Several C-banding and Hy-banding techniques for root-tip meristems (Linde-Laursen, 1975; Greilhuber, 1974) and flower buds (Klášterská and Natarajan, 1976) were applied without successful results. (The techniques of the above authors were not applied to endospermal tissue.

The first encouraging results were obtained with the C-banding technique when we used endospermal material, applying the combined and somewhat modified techniques of two authors: Schweizer (1973, 1974), Marks and Schweizer (1974), Marks (1975), Schweizer (personal comunication).

The Giemsa C-banding technique was successfully applied to endosperm as follows:

1. After 24 hours of fixing in 3 : 1 ethanol : acetic acid, ovules were stored for about 1 week in 90% ethanol at +4° C.

2. Ovules were squashed in 45% acetic acid and heated very slightly after gently pressing.

3. The coverslips were removed using dry ice, and slides were dried at room temperature overnight.

4. Preparations were incubated in 45% acetic acid at 60° C for 20 minutes.

5. After incubation, slides were washed for 15 minutes in tap-water and than rinsed in distilled water.

6. Incubation in hot barium hydroxide solution was than performed. The optimal condition was 5% Ba $(OH)_2 \cdot 8\ H_2O$ at 54—56° C for 15 minutes, followed by rinsing the slides in distilled water and washing them in tap-water for 1 hour.

7. The slides were immersed in 2 × SSC (pH 7) at 60° C for 1—2 hours.

8. After rinsing in distilled water the slides were put into 2% Giemsa's (Merck) solution, diluted with M/15 Sörensen's phosphate buffer (pH 6.9). The optimum staining was found to occur after 1 hours.

9. When sufficiently stained, preparations were washed in distilled water, air-dried overnight, and mounted.

Results and Discussion

Pines are a suitable cytological object, which has already been established in papers by Dixon (1894), Lewis (1908) and especially in Ferguson's paper (1904). Sax and Sax (1933) make a more detailed karyological analysis, indicating at the same time the advantages of working with haploid endospermal material. Mehra and Khoshoo (1956) show the karyotypes of some pines along with those of other conifers. In addition there are the following papers: Aass (1957), Vidaković (1958), Santamour (1960), Natarajan *et al.* (1961), Saylor (1961, 1964, 1972), Nagl (1962, 1965, 1967), Kim (1962), Yim (1963), Sarkar (1963), Tarnavschi (1965), Fahmy (1966), Pederick (1967, 1970), Shidei and Moromizato (1971), Mihailescu and Dalu (1971, 1972). The difficulty of comparing the results of any two of these papers is a characteristic they all have in common. This is the case even when two papers deal with karyotypes of the same species. It is possible to compare chromosome number and perhaps even the number of secondary constrictions in inidividual plates, but it is not possible, even when working with the same species, to compare using statistical methods, relative lengths of the chromosomes, their arms, or the location of secondary constrictions on individual chromosomes. Althouhg some studies do solve the problem of identifying individual chromosomes and the location of their constrictions (Natarajan *et al.*, 1961; Saylor, 1964; Pederick, 1967, 1970), the results presented are difficult to compare to the results of other authors.

The basic reason for this is the lack of uniformity in the presentation of one's own research results in terms of the research of others. Sax and Sax (1933), for example, presented the karyotype by a decreasing series of shorter chromosome arms. Vidaković (1958) presented an increasing series of 2n chromosomes, and Pederick (1967, 1970) used a decreasing haploid series of the total relative chromosome length. The rest of the above-mentioned papers present series of individual numerically-expressed karyotype characteristics which are very useful for the author and conclusions in his material, but are of limited or no use for comparison to other author's results, especially when applying statistical methods. It is for this reason that systematization and standardization of methods is so important. In this paper we stress some details of karyotype research in our results and discussion with the hope that it will contribute to more uniform methods of presentation and explanation of results dealing with intra- and interspecific pine karyotype analysis.

Intraspecific comparisons of European black pine karyotypes were carried out on 18 cells of female gametophyte tissue in development from tree "ni 47", and on 18 cells from tree "ni 221". The gathering of conelets, working out of preparations and analytical method are described in detail by Pederick (1967, 1970) and Borzan (1977 a, 1977 b). Relative chromosome lengths are calculated as a percentage of the average chromosome length for each cell. For average chromosome arm lengths, there are no significant differences between trees "ni 47" and "ni 221". The values are:

Tree No:	ni 47		ni 221	
Average	Short arm	Long arm	Short arm	Long arm
chromosome	45.62%	54.38%	46.20%	53.80%

In *Table 1* chromosome arm length, arm ratio and centromere index are shown in the customary manner. However, for comparative analysis *Table 2* is more suitable. Results show that the first eight chromosomes of "ni 47" are somewhat longer than those for "ni 221". Chromosomes IX, X, XI and XII of "ni 47" are shorter than the same chromosomes of "ni 221". However we found a significant difference (at the 5% level) in length between the corresponding chromosomes of the two trees only for chromosome XII's.

Approximate differences in the lengths of adjacent chro-

Table 1. — Relative arm lengths, total chromosome lengths, arm ratios and centromere index values of *Pinus nigra* Arn. chromosomes. Standard deviations are given in the parenthesis.

CHROMOSOME NUMBERS	RELATIVE CHROMOSOME LENGTH						ARM RATIO $\frac{S}{L}$		CENTROMERE INDEX $\frac{100\ S}{S+L}$	
	SHORTER ARM S		LONGER ARM L		TOTAL S+L		MEAN VALUES		MEAN VALUES	
	TREE NUMBER									
	ni 47	ni 221	ni 47	ni 221	ni 47	ni 221	ni 47	ni 221	ni 47	ni 221
I	56.50 (5.88)	58.95 (4.27)	68.86 (6.63)	66.29 (5.79)	125.37 (8.58)	125.24 (8.70)	0.83 (0.11)	0.89 (0.07)	45.08 (3.58)	47.07 (2.06)
II	55.31 (3.10)	54.58 (2.40)	62.72 (3.51)	60.87 (3.28)	118.04 (4.30)	115.45 (3.57)	0.88 (0.07)	0.90 (0.07)	46.87 (2.13)	47.28 (1.91)
III	52.67 (2.71)	52.73 (2.06)	60.15 (4.49)	58.33 (2.55)	112.82 (4.54)	111.06 (2.94)	0.88 (0.09)	0.91 (0.06)	46.68 (2.54)	47.49 (1.58)
IV	51.13 (3.04)	51.03 (2.21)	57.91 (2.55)	56.76 (2.44)	109.05 (3.57)	107.80 (2.58)	0.88 (0.07)	0.90 (0.06)	46.88 (2.00)	47.34 (1.78)
V	50.44 (3.36)	49.44 (2.63)	56.00 (3.28)	55.95 (3.12)	106.44 (3.44)	105.39 (2.17)	0.90 (0.09)	0.89 (0.09)	47.38 (2.67)	46.92 (2.53)
VI	48.74 (2.72)	49.77 (1.57)	55.15 (3.29)	53.46 (1.88)	103.89 (2.41)	103.23 (2.26)	0.89 (0.09)	0.93 (0.05)	46.93 (2.64)	48.22 (1.27)
VII	47.98 (3.26)	47.26 (2.48)	53.73 (4.22)	52.68 (1.64)	101.72 (2.76)	99.94 (2.87)	0.90 (0.11)	0.90 (0.06)	47.18 (3.38)	47.29 (1.60)
VIII	45.37 (3.05)	46.05 (2.29)	52.87 (2.83)	51.32 (2.99)	98.24 (2.97)	97.38 (2.89)	0.86 (0.13)	0.90 (0.08)	46.17 (2.53)	47.29 (2.27)
IX	44.45 (2.25)	43.45 (3.00)	49.34 (2.57)	50.37 (3.07)	93.79 (3.75)	93.82 (3.39)	0.90 (0.05)	0.87 (0.09)	47.39 (1.64)	46.31 (2.69)
X	39.67 (3.23)	40.02 (4.23)	46.81 (3.56)	49.36 (3.95)	86.48 (6.30)	89.38 (4.88)	0.85 (0.08)	0.82 (0.12)	45.87 (2.32)	44.77 (3.68)
XI	31.39 (4.46)	34.77 (2.66)	47.64 (5.29)	47.14 (3.42)	79.02 (5.91)	81.91 (4.24)	0.67 (0.14)	0.74 (0.08)	39.73 (4.90)	42.46 (2.57)
XII	24.59 (2.42)	26.52 (3.66)	40.55 (5.97)	42.84 (4.42)	65.14 (5.88)	69.36 (6.17)	0.61 (0.07)	0.62 (0.14)	37.75 (2.87)	38.23 (3.63)

Table 2. — Comparative analysis of chromosome lengths, standard deviations, standard errors and coefficients of variabiilty for two European black pine trees. The arrows are oriented towards higher value of chromosome length. ** indicates significance at the 5 percent level.

	TREE NUMBER	CHROMOSOME NUMBERS I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
TOTAL (RELATIVE) CHROMOSOME LENGTH	ni 47	125.37 ↑	118.04 ↑	112.82 ↑	109.05 ↑	106.44 ↑	103.89 ↑	101.72 ↑	98.24 ↑	93.79 ↓	86.48 ↓	79.02 ↓	65.14 ↓ **
	ni 221	125.24	115.45	111.06	107.80	105.39	103.23	99.94	97.38	93.82	89.38	81.91	69.36
STANDARD DEVIATION S	ni 47	8.58	4.30	4.54	3.57	3.44	2.41	2.76	2.97	3.75	6.30	5.91	5.68
	ni 221	8.70	3.57	2.94	2.58	2.17	2.26	2.87	2.89	3.39	4.88	4.24	6.17
STANDARD ERROR $S_{\bar{x}}$	ni 47	2.02	1.01	1.07	0.84	0.81	0.57	0.65	0.70	0.88	1.48	1.39	1.39
	ni 221	2.05	0.84	0.69	0.61	0.51	0.53	0.68	0.68	0.80	1.15	1.00	1.45
COEFFICIENT OF VARIABILITY C.V. %	ni 47	6.84	3.64	4.02	3.27	3.23	2.32	2.71	3.02	4.00	7.29	7.48	9.03
	ni 221	6.95	3.10	2.65	2.39	2.06	2.19	2.87	2.97	3.61	5.46	5.18	8.89

mosomes are as follows:

"ni 47"
I-7-II-5-III-4-IV-3-V-2-VI-2 -VII-4-VIII-4-IX-8-X-7-XI-14-XII

"ni 221"
I-10-II-4-III-3-IV-3-V-2-VI-3 -VII-3-VIII-3-IX-5-X-7-XI-13-XII.

There are small differences in length between neighbouring chromosomes, particularly from chromosome II through chromosome IX. From *Table 2* it is obvious that these chromosomes have small coefficients of variability, i.e. small variation in length in relation to the size of chromosomes I, X, XI and XII. We are interested in the reason for this phenomenon which could be natural, or a result of research methods (e.g. artificial). We should point out that we tend towards the latter explanation for the following reasons.

The customary manner of researching karyotypes is to organize the chromosomes in a series from I to XII on the basis of the length they have on the photograph. This method does not take completely into consideration the division stages of the cell under investigation. Some of the cells could be, for example, at the prometaphase stage; most often cells are analysed at metaphase but could be analysed at anaphase also (Borzan, 1977 a). It has been thought that calculation of relative chromosome length avoids the variability of individual chromosome length caused by division stages of the cells, and that because of this it is possible to compare different cells. However, in the same cell there exists the possibility that two sister chromatids have different lengths, and solely on the basis of length one might be interpreted as a chromatid of chromosome I while the other was thought to belong to chromosome VIII (Borzan, 1977 a). Finally, the above-mentioned pair of sister chromatids may be identified in terms of the location of the constriction as corresponding to chromosome VII. If in the same cell one chromatid is assigned to chromosome I on the basis of length, and its sister chromatid to chromosome VIII when the chromatid pair actually belong to chromosome VII, it is to be expected that in karyotype analysis the variation in length of one particular chromosome is also noticable and high from cell to cell.

If, on the basis of measured chromosome length, any value in decreasing figure order in each cell is thought to be the real chromosome length, the variability in length of that chromosome would be quite limited. Variability is even less as the adjacent chromosome differs less in length *(Tab. 2)*. From this we can conclude why the variability of chromosomes I, X, XI and XII is greatest, because with the European black pine there is the least risk of these chromosomes being reversed in order. Chromosome I is the longest, often noticeably larger than the adjacent chromosome II; chromosomes XI and XII are submetacentric and the shortest, and noticeably differ in length from their adjacent chromosomes.

From the above, one can conclude that the variability of chromosomes I, X, XI and XII is close to the actual variability, and that the calculated coefficients of variability for the remaining chromosomes are too low, i.e. the actual variability of length is greater than the calculated values.

Matern and Simak (1968, 1969) have made detailed research into the risk of reversal of order for *Larix decidua* Mill. chromosomes; Chetty *et al.* (1970) have done the same for *Pinus roxburghii* Sarg. chromosomes. The conclusion of their investigation was that "the risk of reversal cannot be disregarded if the average difference is less than 11 per cent of the average length of the two *Larix decidua* chromosomes", and that "the risk of reversal of order cannot be disregarded if the average difference between the length of the two chromosomes is less than 34 per cent of the mean length of the two *Pinus roxburghii* chromosomes"

In our opinion one cause of length variation between corresponding chromosomes in various cells is the „sticky" phenomenon *(Fig. 1 b)*, which we originally came across as normal behaviour of chromosomes in endospermal pine cells (Borzan, 1977 b), but which also appeared in some of our slides of root-tip meristem made using the Giemsa technique. Stickiness is described often a meiosis (Andersson, 1947; Klášterská and Natarajan, 1975), resulting from gene mutation (Beadle, 1932), or induced by gamma-ray treatment (Rao and Rao, 1977). Mc Gill *et al.* (1974) show that ethidium bromide causes a high incidence of sticky chromosomes in mammalian cells in culture. Electron microscopic examinations show connections of submicroscopic chromatin strands of various widths between chromosomes. An interpretation of stickiness is therefore given as an entanglement of chromatin fibers between unrelated chromosomes. Similar results were obtained (Pathak *et al.*, 1975) when Indian muntjac and Chinese hamster cells in culture were treated with Actinomycin D.

On the contrary, Marks (1973) reported that in many species, when acetic ethanol fixative was omitted during the process of making chromosome preparations from root-tips, very few slides had "sticky" metaphases.

Regardless of the cause of the sticky phenomenon (i.e. whether it is natural or an artifact), it could be the cause of the "stretching" of certain chromosomes which leads to reversal of order in karyotype analysis.

The value of secondary and tertiary constrictions for pine karyotype analysis and chromosome identification was demonstrated by Pederick (1967, 1970). Using a slightly modified method from Pederick's investigation, we show in *Figure 2* schematically the order of secondary and tertiary constrictions on chromosomes of both trees. Taking into consideration the possibility of chromosome reversal of order, total comparison of constriction number and location on the chromosomes seems impossible. However, if one works with sufficiently large samples, taking into consideration that the risk of reversal of order is relatively high, it seems that in the manner it is also possible to identify individual chromosomes with reliability. This is particularly true in cases where the frequency distribution of constrictions on the arms of two or more trees of the same species coincide. From *Figure 2* we see that a relatively good agreement exists in the distribution of constrictions between the two trees, except for an obvious difference between the short arm of chromosome VI. We are inclined to interpret smaller inconsistencies as well as the low frequency of certain constrictions as the result of reversal of order of chromosomes, subjective interpretations in the observation of poorly visible constrictions, or the "stretching" and "contraction" of parts of chromosomes affecting some constrictions to change position in relation to the centromere, resulting in their registration in an adjacent group.

In this paper we succeeded to prove that morphological chromosome differences exist between two trees of the European black pine. One of these (ni 221), although it produces only a small number of hybrid seed, did cross with one particular Scots pine tree, while the other (ni 47) was incompatible in all interspecific crosses performed with the Scots pine. In spite of this, it is difficult to tie morphological

differences to the problem of incompatibility between the European black pine and the Scots pine. Intraspecific karyotype analysis on both species using a larger number of trees, is needed before any meaningful conclusions can be made.

It was hoped that the use of differential staining methods would make it possible to find some other intraspecific differences, or at least to improve the accuracy of chromosome identification. This led us to apply the already well-known methods of differential staining for plant chromosomes to root-tip meristem and endosperm tissue of the European black pine. However, in applying the method of GREILHUBER (1974), LINDE-LAURSEN (1975) and KLÁŠTERSKÁ and NATARAJAN (1976), we did not achieve hoped-for success.

The first satisfactory results of differential staining on pine chromosomes were achieved on endospermal material of the tree "ni 221", using the method described by SCHWEIZER (1973, 1974), MARKS and SCHWEIZER (1974), MARKS (1975). From 10 preparations made using the method described in the Material and Methods section of this paper, clearly marked C-bands on the chromosomes were found in only two preparations. In the rest of the preparations chromosomes were either unstained or over-stained. Cells with over-stained or colorless chromosomes without bands were also found in the two above-mentioned successful preparations. SCHWEIZER (1974) also reported such variation in differential staining of plant chromosomes.

When discussing the question of why the C-banding method can be successfully applied to chromosomes in pine endosperm and not yet to chromosomes in root meristem, the following must be stressed. The stage of free nuclear divisions in developing endosperm is such that chromosomes are exposed more easily to chemical treatment for revealing bands, while meristems have cell-walls that can provide barriers. However, the cell-walls could be weakened by an enzyme treatment, as has been shown on *Triticale* chromosomes using a pectinase solution (MERKER, 1973) or a mixed solution of cellulase and pectinase (SACHAN and TANAKA, 1976) for *Zea* chromosomes. Another possibility could be the application of suitable temperatures or treatment length which would be sufficient to reveal C-bands on root-tip meristem chromosomes.

At the moment we have too few successful preparations to state much about the characteristics of heterochromatin in the European black pine, except that there is relatively little, and that it appears centromerically as well as in the secondary constriction region on some chromosomes. Seven to eight chromosomes with bands in secondary constriction regions are visible in the haploid set of the endosperm of the *Pinus nigra* ARN. tree "ni 221" *(Fig. 3)*.

The submetacentric chromosomes of the European black pine (XI and XII) are easily identifiable. They are characterized by clearly defined centromere bands (arrows on *Fig. 3 b, c* and *d)* and by the absence of bands on the arms. These data agree with the earlier results of our investigations (BORZAN, 1977 a) showing that European black pine has no secondary constrictions on chromosomes XI and XII. (Constrictions on chromosomes XI and XII shown in *Figure 2* were found in only a small number of slides; their frequency was very low and they were barely visible so we are inclined to consider them as a subjective interpretation).

However, improvement of differential staining techniques will allow more accurate identification of chromosomes and will facilitate the use of these methods for comparative karyological inter- and intraspecific analysis of pines.

References

AASS, I.: A cytological analysis of Scots pine *(Pinus silvestris* L.) from Skjåk, Norway. Det norske Skogforsøksvesen **48**: 93—109 (1957). — ANDERSSON, E.: A case of asyndesis in *Picea Abies*. Hereditas **33**: 301—347 (1947). — BEADLE, G. W.: A gene for sticky chromosomes in *Zea mays*. Ztschr. f. ind. Abst. Vererbungslehre **63**: 195—217 (1932). — BORZAN, Ž.: Contribution to the karyotype analysis of the European black pine *(Pinus nigra* ARN.). Ann. Forest **8** (3): 29—50 (1977 a). — BORZAN, Ž.: Sticky chromosomes in the endosperm of European black pine and Scots pine. Genetika **9** (2): 139—147 (1977 b). CHETTY, C. K. R., UPADHAYA, L. P., and KEDHARNATH, S.: Estimation of risk of reversal of arms and order of chromosomes in karyotype analysis of *Pinus roxburghii* SARG. Indian Forester **96**: 811—816 (1970). — DARLINGTON, C. D., and LA COUR, L. F.: The handling of chromosomes. Georg Allen & Unwin Ltd., London, 263 pp. (1962). — DIXON, H. H.: Fertilization of *Pinus silvestris*. Annals of Botany **8**: 21—34 (1894). — FAHMY, T. Y.: Chromosome number and morphology in certain Conifers. Alexandria Journal of Agric. Res. **14**: 205—221 (1966). — FERGUSON, M.: Contribution to the knowledge of the life history of *Pinus* with special reference to sporogenesis, the development of the gametophytes and fertilization. Proc. Wash. Acad. Sci. **6**: 1—202 (1904). — GREILHUBER, J.: Hy-banding: A new quick technique for heterochromatin staining in plant chromosomes. Naturwissenschaften **61**: 170—171 (1974). — HSU, T. C.: Longitudinal differentiation of chromosomes. Ann. Rev. Genet. **7**: 153—176 (1973). — KIM, C. S.: The karyotype analysis in *Pinus rigida* MILL., *Pinus taeda* L. and their F_1 hybrid. Inst. For. Genet., Suwon (Korea) **6**: 21—28 (1962). — KLÁŠTERSKÁ, I., and NATARAJAN, A. T.: Stickiness in *Rosa* meiosis induced by hybridisation. Caryologia **28**: 81—88 (1975). — KLÁŠTERSKÁ, I., and NATARAJAN, A. T.: A modified C-band technique for staining of diffuse diplotene chromosomes. Stain Technology **51**: 209—211 (1976). — LEWIS, I. M.: The behaviour of the chromosomes in *Pinus* and *Thuja*. Annals of Botany **22**: 529—556 (1908). — LINDE-LAURSEN, I.: Giemsa C-banding of the chromosomes of "Emir" barley. Hereditas **81**: 285—289 (1975). — MARKS, G. E.: A rapid HCl/toluidine blue squash technic for plant chromosomes. Stain Technology **48**: 229—231 (1973). — MARKS, G. E.: The Giemsa-staining centromeres of *Nigella damascena*. J. Cell. Sci. **18**: 19—25 (1975). — MARKS, G. E., and SCHWEIZER, D.: Giemsa banding: Karyotype differences in some species of *Anemone* and in *Hepatica nobilis*. Chromosoma (Berl.) **44**: 405—416 (1974). — MATERN, B. and SIMAK, M.: Statistical problems in karyotype analysis. Hereditas **59**: 280—288 (1968). — MATERN, B., and SIMAK, M.: On some statistical problems connected with the identification of chromosomes. Research Note No. 11, from Dept. of Forest Biometry, Royal College of Forestry, Stockholm, 81 pp. (1969). — MC GILL, M., PATHAK, S., and HSU, T. C.: Effects of ethidium bromide on mitosis and chromosomes: A possible material basis for chromosome stickiness. Chromosoma (Berl.) **47**: 157—166 (1974). — MEHRA, P. N., and KHOSHOO, T. N.: Cytology of Conifers I. J. Genetics **54**: 165—180 (1956). — MERKER, A.: A Giemsa technique for rapid identification of chromosomes in *Triticale*. Hereditas **75**: 280—282 (1973). — MIHAILESCU, A., and DALU, M.: Comparative study on the karyotype of different provenances of *Pinus nigra* of the Şeţu Sinaia nursery. Rev. Roum. Biol.-Botanique **16**: 319—327 (1971). — MIHAILESCU, A., and DALU, M.: Comparative karyotype study in different provenances of *Pinus silvestris* and *Pinus nigra*. Rev. Roum. Biol.-Botanique **17**: 343—353 (1972). — NAGL, W.: Über Endopoliploidie, Restitutionskernbildung und Kernstrukturen im Suspensor von Angiospermen und einer Gymnosperme. Österr. Bot. Z. **109**: 431—494 (1962). — NAGL, W.: Karyologische Anatomie der Samenanlage von *Pinus silvestris*. Österr. Bot. Z. **112**: 359—370 (1965). — NAGL, W.: Mikrophotometrische DNS-Messungen an Interphase- und Ruhekernen sowie Mitosen in der Samenanlage von *Pinus silvestris*. Z. Pflanzenphysiol. **56**: 40—56 (1967). — NATARAJAN, A. T., OHBA, K., and SIMAK, M.: Karyotype analysis of *Pinus silvestris*. Hereditas **47**: 379—382 (1961) — PATHAK, S., MC GILL, M., and HSU, T. C.: Actinomycin D effects on mitosis and chromosomes: Sticky chromatids and localized lesions. Chromosoma (Berl.) **50**: 79—88 (1975). — PEDERICK, L. A.: The structure and identification of the chromosomes of *Pinus radiata* D. DON. Silvae Genetica **16**: 69—77 (1967). — PEDERICK, L. A.: Chromosome relationships between *Pinus* species. Silvae Genetica **19**: 171—180 (1970). — RAO, P. N., and RAO, R. N.: Gamma-ray induced meiotic chromosome stickiness in tomato. Theor. Appl. Genet. **50**: 247—252 (1977). — SACHAN, J. K. S., and TANAKA, R.: A banding method for *Zea* chromosomes. Japan. J. Genetics **51**: 139—141 (1976). — SANTAMOUR, F. S. Jr.: New chromosome counts in *Pinus* and *Picea*. Silvae Genetica **9**: 87—88 (1960). — SARKAR, P.: Chromosome studies on *Pinus* species (Abstr.). Canad. J. Genet. Cytol. **5**: 107 (1963). — SAX, K., and SAX, H. J.: Chromosome number and morphology in the Conifers. J. Arnold Arb. **14**: 356—375 (1933). — SAYLOR, L. C.: A karyotypic analysis of selected species of *Pinus*. Silvae Genetica **10**: 77—84

(1961). — Saylor, L. C.: Karyotype analysis of *Pinus* group *Lariciones*. Silvae Genetica **13**: 165—170 (1964). — Saylor, L. C.: Karyotype analysis of the genus *Pinus* — subgenus *Pinus*. Silvae Genetica **21**: 155—163 (1972). — Schweizer, D.: Differential staining of plant chromosomes with Giemsa. Chromosoma (Berl.) **40**: 307—320 (1973). — Schweizer, D.: An improved Giemsa C-banding procedure for plant chromosomes. Experientia **30**: 570—571 (1974). — Schweizer, D., and Ehrendorfer, F.: Giemsa banded karyotypes, systematics and evolution in *Anacyclus (Asteraceae-Anthemideae)*. Plant Syst. Evol. **126**: 107—148 (1976). — Shidei, T., and Moromizato, S.: Karyotype analysis of Luchu pine *(Pinus luchuensis* Mayr.). J. Jap. For. Soc. **53**: 13—18 (1971). — Simak, M.: Karyotype analysis of *Larix decidua* Mill. from different provenances. Medd. Stat. Skogsforskningsinst. **51**: 1—22 (1962). — Tarnavschi, I. T., and Ciobanu, I.: Karyologische Untersuchungen an *Pinus nigra* Arn. ssp. *nigricans* Host. var. *banatica* Georg. et Ionescu in Vergleiche mit *Pinus nigra* Arn. var *austriaca* Moess. Rev. Roum. Biol., Ser. Bot. **10**: 371—375 (1965). — Vidaković, M.: Investigations on the intermediate type between the Austrian and the Scots pine. Silvae Genetica **7**: 12—18 (1958). — Vidaković, M.: Savladjivanje inkompatibilnosti pri križanju nekih borova (Overcoming the incompatibility in crossinig some pine species — Engl. Sum.). Genetika **9**: 51—63 (1977 a). — Vidaković, M.: Some morphological characteristics of *Pinus* × *nigrosylvis (Pinus nigra* × *Pinus sylvestris)*. Ann. Forest. **8** (2): 15—27 (1977 b). — Vidaković, M., and Borzan, Ž.: Contribution to the investigations of incompatibility by crossing Scots pine and European black pine. Int. Symp. on Gen. of Scots pine, Kornik: 1—19 (1973). Yim, K. B.: Karyotype analysis of *Pinus rigida*. Hereditas **49**: 274—276 (1963).

Die Frostresistenz der Douglasie (Pseudotsuga menziesii (Mirb.) Franco) verschiedener Herkünfte mit unterschiedlichen Höhenlagen

Von J. Bo Larsen

Institut für Waldbau der Universität Göttingen

(Eingegangen November 1977 / Mai 1978)

Zusammenfassung

Um die Bedeutung der Höhenlage für die Frostresistenz der Douglasie zu untersuchen, wurden Pflanzen der Herkunft Darrington/Wash. von 5 verschiedenen Höhengürteln und der Herkunft Randle/Wash. von 4 verschiedenen Höhengürteln auf ihre Frostresistenz hin getestet. Die Resistenz der einzelnen Höhenlagen wurde durch künstliche Frosttests zu jeweils einem Termin im Herbst und im Winter, sowie zweimal im Frühjahr ermittelt.

Es wurden signifikante Unterschiede in der Frostresistenz zwischen verschiedenen Höhenlagen des gleichen Herkunftsortes sowohl bei Darrington als auch bei Randle nachgewiesen.

Die niedrigsten Lagen (350—500 m) erwiesen sich als besonders früh- und spätfrostresistent. Die Hochlagen (oberhalb etwa 800 m) zeigten sich als besonders früh- und winterfrostresistent. Die mittleren bis unteren Lagen (500—650 m) zeigten sich bei allen 4 Tests am empfindlichsten. Die Herkunft Salmon Arm aus dem Inneren von Br. Columbia erwies sich als besonders früh- und winterfrostresistent, aber als relativ spätfrostempfindlich. Die Herkunft Matlock (südlich der Olympic Mountains) war besonders spätfrostresistent.

Die Unterschiede in den Frostresistenzeigenschaften der verschiedenen Höhenlagen werden als eine Anpassung an Kaltluftstauungen erklärt.

Schlagworte: *Pseudotsuga menziesii*, Frostresistenz, Herkünfte, Höhenlagevariation.

Summary

Frost resistance was tested on four-year old plants from 5 and 4 different altitudes respectively of two seed sources in the state of Washington, i.e. Darrington and Randle. Two additional seed sources from Salmon Arm from the interior part of British Columbia, and Matlock from the low land south of the Olympic Mountains, were also included.

The frost hardiness of the seedlings was tested once in autumn, once in winter, and twice in spring, each time at 5 different temperature levels with the following standard conditions:

1) Pre freezing conditions : 3 hours by $+2^0$ C
2) Cooling rate : -6^0 C per hour
3) Duration of test temperature : 4 hours
4) Thawing rate : $+6^0$ C per hour
5) Post thawing conditions : 3 hours by $+2^0$ C

The results showed significant differences in frost hardiness between altitudes both from Darrington and from Randle. The low elevation seed sources (350—500 m) showed high resistance to early and late frosts. The high elevation seed sources (above 800 m) were especially resistant to winter frosts. The seed sources from intermediate altitudes (500—700 m) showed a low resistance to both early, winter, and late frosts. The provenance Salmon Arm was highly resistant to early and winter frosts but very susceptible to late frosts. Matlock showed a very high late frost resistance.

The differences in frost hardiness related to the elevation of the seed source are explained as an adaptation to cold air drainage in autumn and especially in spring.

Key words: *Pseudotsuga menziesii*, frost hardiness, provenances, altitudinal variation.

1. Einleitung

Kaum eine für NW-Europa wichtige Wirtschaftsbaumart zeigt eine so große phänotypische Variation wie die Douglasie. Die Herkunftsfrage bei der Douglasie ist im Vergleich mit anderen Baumarten deshalb von besonders großer Wichtigkeit, und dementsprechend sind in den letzten Jahren eine Reihe von Untersuchungen gemacht worden, um die geographische Variation in Wachstum und Resistenz gegenüber biotischen und abiotischen Schadfaktoren näher zu analysieren.

Einen der bedeutendsten Faktoren für einen erfolgreichen Anbau der Douglasie in Nordwesteuropa bildet die Frostresistenz des verwendeten Pflanzenmaterials. Eine Reihe von Untersuchungen ist darauf gerichtet, das geo-