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Giemsa C-Banding Pattern of the Chromosomes in the Macrogametophyte of Norway Spruce

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Abstract

Giemsa C-banding method was applied on chromosomes in the female gametophyte tissue of Norway spruce (*Picea abies* (L.) KARST.). A karyogram of 1 single tree growing in the Botanical Garden of the University of Graz, Austria, was made by using an image analysis system. All chromosomes (n = 12) showed pericentric C-bands. Chromosomes II, V and IX possessed intercalary C-bands located at secondary constrictions. Telomeric C-bands appeared in chromosomes II and III on both arms. Stickiness between non-homologous chromosomes was observed and discussed in the paper.

Key words: Picea abies (L.) KARST., chromosomes, Giemsa C-banding method, macrogametophyte, stickiness, karyotype, image analysis.

FDC: 165.3; 174.7 *Picea abies.*

Zusammenfassung

Die Giemsa-C-Bänderungsmethode wurde an Makrogametophyten der Fichte (*Picea abies* (L.) KARST.) durchgeführt. Das Karyogram eines Baumes aus dem Botanischen Garten der Universität Graz, Österreich, wurde unter Zuhilfenahme eines Bildanalysesystems erstellt. Alle Chromosomen (n = 12) zeigten perizentrische C-Banden. Die Chromosomen II, V und IX weisen interkalare C-Banden, die an den sekundären Einschnürungen liegen, auf. Telomerische C-Banden wurden an den Chromosomen II und III an beiden Chromosomenarmen gefunden. Sticky-Effekte zwischen nicht homologen Chromosomen wurden beobachtet und ihr Auftreten wird in dieser Arbeit diskutiert.

Introduction

Karyotyping of gymnosperm macrogametophytes (= female gametophyte tissue, endosperm tissue in development) has been rarely used, but it does offer advantages in the preparation and finding of well-spread metaphase chromosomes. This haploid endosperm tissue in development was investigated by SAX and SAX (1933), SANTAMOUR (1960), PEDERICK (1967, 1970), BORZAN (1977a and b, 1981, 1988), BORZAN and PAPEŠ (1978), MACPHERSON and FILION (1981) and by some other cytogeneticists. Norway spruce (*Picea abies* (L.) KARST.) was investigated by SAX and SAX (1933), SANTAMOUR (1960) and KÖHLER et al. (1995).

Giemsa C-banding method was applied to gymnosperm chromosomes by BORZAN and PAPEŠ (1978), MURATOVA (1978), BORZAN (1981, 1988), MACPHERSON and FILION (1981), TANAKA and HIZUME (1980), WOCHOK et al. (1980). TEOH and REES (1977) applied this method to root tips meristem chromosomes of white spruce (*Picea glauca*) illustrating one A chromosome with pericentric C-band and B chromosome with no Giemsa banding.

In order to improve the identification of Norway spruce chromosomes, we have opted for the application of Giemsa C-banding method to the endosperm tissue in development of 1 Norway spruce tree of unknown origin, growing at the Botanical Garden of the University in Graz. The processing of data was possible by digital image analysis, and the revealed karyotype can be used as a reference for comparison with Norway spruce karyotype analyses from studies to detect intraspecific variation and interspecific differences.

Materials and Methods

Plant material, ovules fixation and slide preparation

During May, cones from a single tree of Norway spruce were collected. Ovules were extracted, fixed in 3:1 ethanol : acetic acid for 24 hours at room temperature and stored in 70% ethanol at 4 °C.

Stored macrogametophytes were sequentially placed in 50% and 30% ethanol for 10 minutes. After rinsing in distilled water they were placed in 45% acetic acid for 15 minutes. A small incision was made through the integument, the endosperm teased and gently squashed in 45% acetic acid. The coverslip was removed using liquid nitrogen. The slides were dehydrated for a few minutes in 96% ethanol and dried at room temperature overnight.

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Giemsa C-banding

The procedure we used is a combination of a method used by 4 authors: SCHWEIZER (1973, 1974), MARKS and SCHWEIZER (1974), MARKS (1975) and BORZAN and PAPEŠ (1978):

1. The slides were incubated in 45% acetic acid at 60 °C for 20 minutes and rinsed for 15 minutes in running tap water.

2. After incubation in 5% $Ba(OH)_2 \bullet 8H_2O$ at 55 °C for 10 minutes, slides were rinsed in distilled water and washed in tap water for 1 hour.

3. The slides were immersed in 2 x SSC (0.3 $mol.l^{-1}$ sodium chloride and 0.03 $mol.l^{-1}$ sodium citrat, pH 7.0 with 0.1 $mol.l^{-1}$ HCl) at 60 °C for 1.5 hours.

4. After rinsing in distilled water, slides were placed in a 2% Giemsa (Giemsas-Lösung, Azur-Eosin-Methylenblaulösung, Merck) solution, diluted with 0.15 $mol.l^{-1}$ SÖRENSEN's phosphate buffer (pH 6.9). The staining time was monitored and varied from 30 minutes to 60 minutes.

Technical equipment and software

The image analysis system consists of a 3-chip colour video camera Sony DXC 930 P, with Sony-Control-System to check all camera functions with the computer, a frame grabber ITI MFG-3M-V (Image Technology Inc.), variable scan module AM-VS-VP and colour recording module AM-CLR-VP included. The frame grabber has a resolution of 1024 x 1024 pixels with 24 Bit true colour (24 Bit), 4 Bit overlay, 0.5 MB overlay memory, 3 MB image memory, 4 MB programme and data memory. The image-CPU is a 40 MHz TI 34020. The central computer is a 66 MHz i486 DX/2 AT (R+R Inc.) with a Cirrus VGA-board, two 17" monitors Flexscan F550i-w (Eizo Inc.) and a 1 GB hard disc. The computer works under the operating system DOS 6.2 and Windows 3.11 (Microsoft Inc.). The image analysis software used was Optimas 4.02 (BioScan Inc.). The image manipulations (autotracing, rotations, arrangements, inscriptions etc.) were done with the graphic package Corel Draw 4.0 (Corel Inc.), and Picture Publisher 3.1 (Mikrografix Inc.). The printing was done with a laser printer Laserjet 4/4M (HP Inc.). The data were transferred by DDE dynamic data exchange directly to the spreadsheet programme Excel 5.0 (Microsoft Inc.). The microscope used was the Axioplan with an oil-immersion objective Plan Neofluar 100 x 1.3, and the C-mount adapter for the video camera (Zeiss Inc.).

Statistical analysis

For the numerical karyotype analysis 16 metaphase plates were measured with the image analysis software. Total chromosome lengths (S + L), short arm lengths (S) and long arm lengths (L) were measured in µm, and converted in relative values as a percentage of the calculated cell average chromosome length. Standard deviations (s) and coefficients of variability ($s/\bar{x} \bullet 100$ = C. V.%) were calculated. Chromosomes were identified, according to their total length and position of secondary constrictions and Giemsa C-banding patterns, labelled with Roman numerals from I to XII in a manner that the chromosome I is the longest and chromosome XII the shortest. Arm ratios (S/L) and centromere indices ($[S/S+L)x100$ were added as helpful tool in karyotype analysis. The centromere position was defined according to SAYLOR (1961, 1964) by the arm ratio S/L. The centromere is in the metacentric zone if the arm ratio value is between 0.75 and 1.00, and in the submetacentric zone if that ratio is between 0.50 and 0.75. Location of the secondary constriction on chromosome arm was recorded after PEDERICK (1967, 1970): as a percentage distance from the centromere in terms of the total arm length on which they are located. Thus, as an example, II L 63 marks a constriction on chromosome II, which is located on the long chromosome arm at about 63% of the arm length from the centromere.

Results

In all cells the haploid chromosome number (n=12) was present, except in some cells where due to the nature of the endospermal tissue in development and procedure of slide preparations the loss of one or more chromosomes occurs. Numerical karyotype is given in *table 1.* Three chromosomes (VI, XI and XII) are classified as submetacentric, and others as metacentric, though the arm ratio of chromosome X is very close to submetacentricity (*Table 2*).

Comparison of Norway spruce relative chromosome lengths presented by different authors is given in *table 3.*

Secondary constrictions were present on the second chromosome (II L 63), on fifth (V L 51) and ninth (IX S 52). Their location on arms could be classified as median.

Same Giemsa C-banding patterns were present in all 16 investigated metaphase plates. *Figure 1a* and *b* shows 2 meta-

Table 1. – Numerical karyotype of Norway spruce with relative chromosome lengths and corresponding statistical parameters. The position of secondary constriction is defined on the particular chromosome as the percentage of the arm length measured from the centromere.

Chromosome Numbers	Total Lengths S + L	Standard Deviations s	Coefficients of Variability C. V. %	Short Arms S	Long Arms L	Arm Ratios S/L	Centromere Indices (S/S+L)x100	Position of Secondary Constrictions
I	140.5	8.4	6.0	63.2	77.3	0.82	45.0	
II	120.3	5.7	4.7	57.4	62.9	0.91	47.7	II L 63
III	114.6	2.5	2.2	51.0	63.6	0.80	44.5	
IV	109.8	2.0	1.8	49.4	60.3	0.82	45.0	
V	106.7	1.8	1.7	47.6	59.1	0.81	44.6	V L 51
VI	102.8	2.2	2.1	38.9	63.9	0.61	37.8	
VII	98.9	2.9	2.9	44.8	54.1	0.83	45.3	
VIII	93.3	3.7	4.0	42.2	51.1	0.83	45.2	
IX	89.3	3.4	3.8	40.4	48.9	0.82	45.2	IX S 52
X	81.8	2.5	3.1	35.4	46.3	0.77	43.4	
XI	75.2	3.9	5.2	30.0	45.1	0.67	40.0	
XII	66.4	4.4	6.6	12.4	54.1	0.23	18.6	

phase plates with Giemsa C-banded chromosomes, and proves the reproducibility of the method we used. *Figure 1c* is the karyotype of the investigated Norway spruce tree, prepared from *figure 1a* and *1b* metaphase plates, to point out the reproducibility of heterochromatic banding patterns. The chromosomes were cut, rotated and ordered using a graphic-package tool Corel Draw 4.0.

All 12 chromosomes had pericentric bands (*Figure 1, Table 4*). Heterochromatic C-bands were present on secondary constrictions of chromosomes II, V and IX and as telomeric C-bands on both arms of chromosomes II and III. (The term C-banding is used as SCHWEIZER and EHRENDORFER (1976) defined it, as a description of the darkly stained chromosome segments obtained by a specific Giemsa method).

In almost all cells sticky connections between chromosomes were observed.

Discussion

The haploid chromosome number (n=12) is a confirmation of previous reports (SAX and SAX, 1933; SANTAMOUR, 1960 and KÖHLER et al., 1995). The corresponding number of chromosomes, but investigated in the diploid state (2n=24) was reported by BEVILACQUA and VIDAKOVIĆ (1963), BIAŁOBOK and BARTKOWIAK (1967), PRAVDIN et al. (1976), TERASMAA (1971), HIZUME (1988) and some others.

A comparison of the data from reference literature shows that it is extremely difficult to determine similarities or differences in the results of investigation of karyotypes of identical or different species presented by various authors. We have nevertheless attempted to compare the lengths of haploid chromosomes of Norway spruce after recalculations of data published by different authors in the way used in the present study. The comparison is shown in *table 3*. It is evident that the chromosome I is the longest and significantly longer than the chromosome II. The chromosome XII is smaller than XI, and these characteristics are sufficient for the identification of chromosomes I and XII. Another common characteristic of the Norway spruce karyotypes is evident: the difference in lengths between neighbouring chromosomes from II to XI is smaller than differences between the first two and last two chromosomes. Therefore, the identification of neighbouring chromosomes from II to XI carries a relatively high risk of reversal of order unless there are additional identification characteristics that may aid identification.

Position of the centromere is the next feature which could help in the identification of particular chromosome, but this feature is defined by many authors in a different way. The idiogram of Norway spruce, shown by SAX and SAX (1933), has 3 last chromosomes as submetacentric. SANTAMOUR (1960), using classification after MEHRA and KHOSHOO (1956), for 15

Table 2. – Comparison of arm ratios in obtained karyotype by TERASMAA (1971) and in the present study.

Chromosome Numbers	Arm Ratios after Terasmaa S/L	Centromere Position	Arm Ratios in a Present Study S/L	Centromere Position
I	0.95	Metacentric	0.82	Metacentric
II	0.90	Metacentric	0.91	Metacentric
III	0.90	Metacentric	0.80	Metacentric
IV	0.96	Metacentric	0.82	Metacentric
V	0.76	Metacentric	0.81	Metacentric
VI	0.91	Metacentric	0.61	Submetacentric
VII	0.93	Metacentric	0.83	Metacentric
VIII	0.93	Metacentric	0.83	Metacentric
IX	0.57	Submetacentric	0.82	Metacentric
X	0.68	Submetacentric	0.77	Metacentric
XI	0.79	Metacentric	0.67	Submetacentric
XII	0.40	Submetacentric	0.23	Submetacentric

Table 3. – Relative chromosome lengths of Norway spruce karyotypes obtained by different authors. Values from SAX and SAX (1933) are presented by TERASMAA (1971). Data from the PRAVDIN et al. (1976) are recalculations of originally published data for the Lithuanian provenance of Norway spruce. HIZUME's (1988) data are recalculations of originally published numerical karyotype given in a diploid state. Data from BIALOBOK and BARTKOWIAK's (1967) paper are originally published relative mid values between the largest and smallest particular chromosome of 5 investigated provenances.

Author	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
SAX and SAX (1933)	146	114	108	107	105	101	99	93	86	85	85	73
BIALOBOK and BARTKOWIAK (1967)	134	114	111	111	107	103	100	95	94	87	77	70
TERASMAA (1971)	126	114	111	110	104	103	102	102	91	87	79	69
PRAVDIN et al. (1976)	125	113	113	105	104	104	104	104	91	89	80	71
HIZUME (1988)	120	112	111	110	108	106	101	100	96	88	80	69
KÖHLER and GUTTENBERGER (1995)	141	117	112	110	106	103	100	97	91	82	75	67
Present study	141	120	115	110	107	103	99	93	89	82	75	66

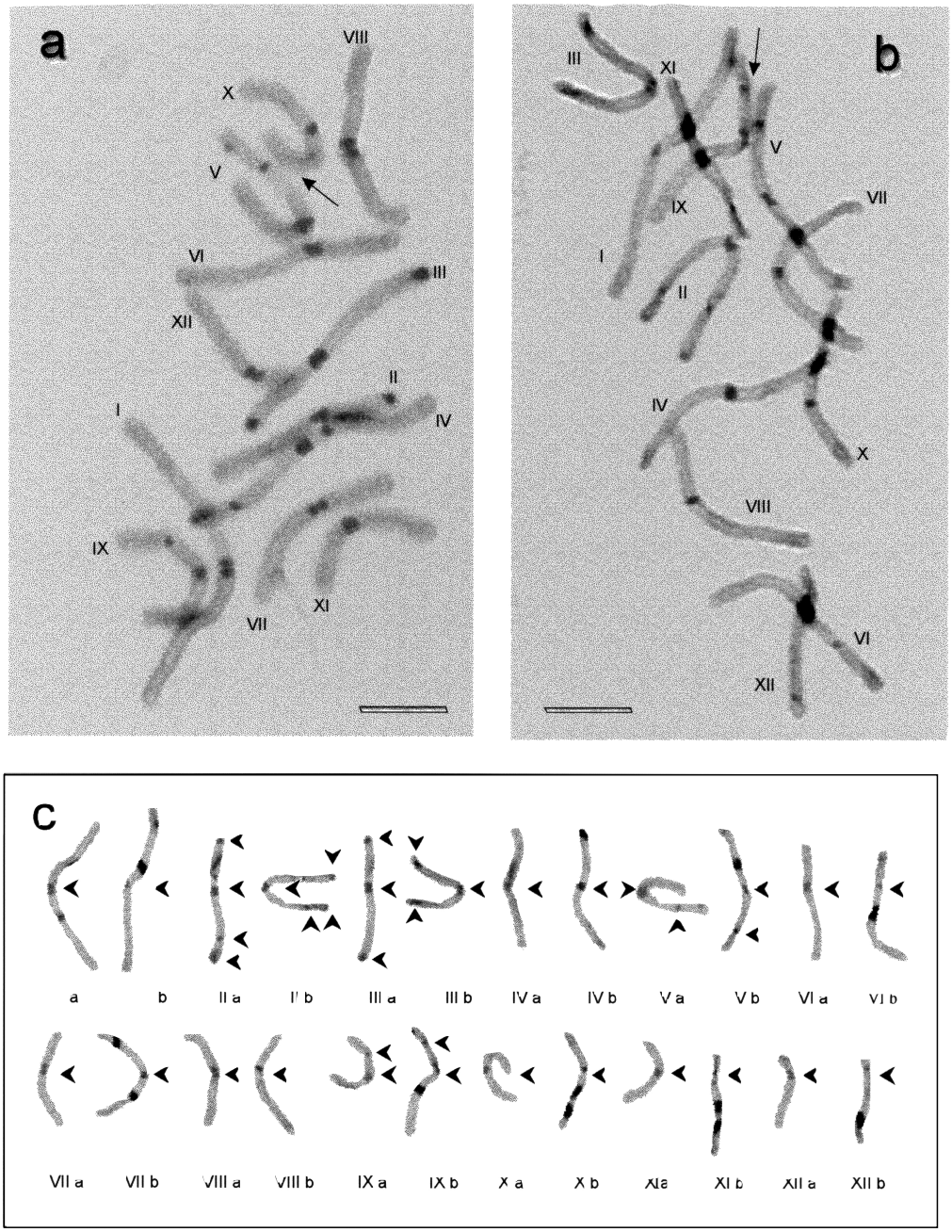

Figure 1. – Original computer images of 2 metaphase plates with Giemsa C-banded chromosomes (a, b). Chromosomes are identified. Arrow indicates sticky connection. Bar = 5 µm. Giemsa C-banded karyotype is shown on *figure c*. The chromosomes from a) and b) were cut, rotated and ordered according to length with the support of the graphic tools of Corel. Chromosomes are identified with Roman numerals I to XII and the letter indicates the a or b metaphase plate. Arrowheads indicate pericentric, intercalary and telomeric Giemsa C-bands.

Table 4. – Number of Giemsa C-bands per haploid genome of Norway spruce.

Chromosome Number	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Number of Pericentric Bands	1	1	1	1	1	1	1	1	1	1	1	1
Number of Intercalary Bands	-	2	-	-	1	-	-	-	1	-	-	-
Number of Telomeric Bands	-	2	2	-	-	-	-	-	-	-	-	-
Total Number of Bands	1	5	3	1	2	1	1	1	2	1	1	1

investigated *Picea* species claims 3 chromosomes as heterobrachial with subterminal centromere.

PRAVDIN et al. (1976) for the Lithuanian provenance of Norway spruce reports 4 smallest chromosome pairs as submetacentric. In TERASMAA's work (1971), using classification after LEVAN et al. (1964) 2 chromosomes of the *Picea abies* var. *acuminata* BECK. were heterobrachial. However, according to SAYLOR's classification (1961, 1964) submetacentric chromosomes have arm ratio S/L smaller than 0.75. Considering this classification, measurements made on the published idiogram in TERASMAA's work (1971) and compared with our own investigation (*Table 2*) have shown that in the karyotype published by TERASMAA 3 chromosomes are submetacentric. However, only the chromosome XII appears to be submetacentric in both investigations, while the results differ for other chromosomes.

Three chromosomes with secondary constrictions in our slides were clearly marked with C-bands. Karyotypes, published by most other authors have more than 3 chromosomes with secondary constrictions. Only BIAŁOBOK and BARTKOWIAK (1967) have shown on published idiogram 1 secondary constriction on the chromosome III. TERASMAA (1971) noticed 5 secondary constrictions (II and V on long arms, III, VI and X on short arms). In his paper he referred to the karyotype published by TOYAMA and KUROKI (1967) where 4 pairs of chromosomes had secondary constrictions. PRAVDIN et al. (1976) found secondary constrictions on chromosomes II, III, on 1 from the group IV to VIII and on the chromosome X of the Lithuanian population of Norway spruce. HIZUME (1988) reported 4 in the text (on "the 2nd, 4th, 7th, 9th"), but on the karyotype indicated with arrows 5 pairs of chromosomes with secondary constriction (on the II, V, VI, VII and X), and on one chromosome IX from the pair IX. However, most papers discussed here, mention chromosomes II, V and X to posses secondary constrictions.

Inconsistency in the number and position of secondary constrictions has been pointed out many times. Here, we can only stress once more this fact, and encourage researchers towards solutions of this problem, i. e. to study the problem of karyomorphological variations within and between species.

Because of the difficulties in the identification of certain Norway spruce chromosomes (particularly in distinguishing chromosomes III and IV, VI to VIII and X from XI, because of their similar length and absence of striking characteristics such as secondary constriction), it seemed appropriate to apply a method for differential staining of chromosomes. Successfully applied, the Giemsa C-banding method enabled us to distinguish the chromosome III, because of the additional heterochromatic bands telomerically located on both arms and no secondary constrictions. Chromosome V has a secondary constriction V L 51, and the chromosome VI is a submetacentric one. However, we were still unable to distinguish with 100% accuracy chromosomes VII from VIII and X from XI. For those chromosomes the risk of reversal in order is still high, as it was shown for larch chromosomes by MATERN and SIMAK (1968).

In respect to their size, chromosomes of Norway spruce contain relatively small amount of C heterochromatin, as shown for some other gymnosperms (BORZAN and PAPEŠ, 1978; TANAKA and HIZUME, 1980; WOCHOK et al., 1980; MACPHERSON and FILION, 1981).

In our slides in most cells we have observed sticky connections (*Figure 1a* and *1b*) between non-homologous chromosomes, similar to those described and discussed in detail in pine female gametophyte tissue by BORZAN (1977b, 1988). The occurrence was alternatively called "stickiness", according to RIEGER et al. (1976), who defined it as "a sort of chromosomal 'agglutination' of unknown nature which results in a picnotic or sticky appearance of chromosomes. Stickiness may give rise to sticky adhesions between 2 or more chromosomes and to the formation of 'sticky bridges' at anaphase". The term sticky chromosomes was first used by BEADLE (1932) who reported on "the apparent increase of frequency of non-disjunction, frequency of translocation and rate on gene mutation in some of maize plants, caused by the sticky chromosome gene". For the similar occurrence in mitosis MELANDER (1963a, b and c) used the term "pseudo- chiasmata" and in the paper from 1965 the term "adhesions". He deems them to be normal occurrence among chromosomes at anaphase, and suggests the explanation that pseudo-chiasmata are one of the first steps in the cell differentiation, because he found anaphase with connected chromosomes in early stages of the embryo development of some planarians (*Paludicola, Tricladida*), some flies and some worms. SAX and SAX (1933) seem to describe the stickiness in the endosperm of *Pseudolarix*: "In one metaphase figure, two chromosomes were in contact at all loci and several other chromosomes were found closely associated in pairs". BORZAN (1977a) found stickiness in prometaphase, metaphase and anaphase cells of 3 investigated pines, their numbers varying from cell to cell. There was no regularity or repetition in appearance of sticky connections, i. e. in each nucleus a different pair of non-homologous chromosomes was connected. He has noticed that at the point where the sticky connection appears, a flexion occurs on the chromosome, and suggests that this fact supports the natural character of this phenomenon.

However, so far the literature has provided no explanation for stickiness, which was often described in meiosis (ANDERSSON, 1947; KLÁŠTERSKÁ and NATARAJAN, 1975) resulting from gene mutation (BEADLE, 1932), or induced by gamma-ray treatment (RAO and RAO, 1977). ANDERSSON (1965) explained pollen mitoses with "sticky" chromosomes, during the meiotic divisions in pollen mother cells of Norway spruce, as the effect of minus temperatures below −4 °C. Stickiness and other meiotic anomalies in three asyndetic individuals of Scots pine have been discussed in details by RUNQUIST (1968). MCGILL et al. (1974) gave an interpretation of stickiness as an entanglement of chromatin fibres between unrelated chromosomes, showing that ethidium bromide causes a high incidence of sticky chromosomes in mammalian cells in culture. Similar results were obtained by PATHAK et al. (1975), when Indian muntjac and Chinese hamster cells in culture were treated with actinomycin D. MARX (1973) reported that in many species, when acetic ethanol fixative was omitted during slide preparations of root tips, very few slides had sticky metaphases.

BORZAN (1988) holds that the fact that stickiness is visible after Feulgen reaction, which is DNA specific, and sometimes similar in appearance to a fibre of a spindle, supports its natural origin in the endosperm in development of female gametophyte tissue of conifers. Keeping in mind that this tissue is highly specialised, it is useful to quote NAGL (1967) who cytophotometricaly investigated the female gametophyte tissue of Scots pine. He found that certain nuclei have twice as much DNA as can be expected in haploid nuclei, and that there is a positive correlation between the DNA content in the nuclei and chromosome volume. NAGL attributes the superfluous DNA to endospermal shortlasting tissue, characterising it in ontological development as physiologically highly active but lacking in "genetic future". BORZAN (1988) concluded that in endospermal tissue there are polytene (ditene) chromosomes and considering this, further investigation should be carried out by using other methods of fixing, staining and preparing this

material, including cytophotometry, physiological and molecular investigation, before we can consider sticky connections as a reflection of the high physiological activity of this tissue in development. At this point current knowledge allows hypotheses interpreting the sticky connections as "recognition" of identical, homologous parts of repetitive sequences between non-homologous chromosomes.

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Megagametophyte Salt-soluble Proteins as Genetic Markers in *Pinus pinaster* AIT.

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Summary

Salt-soluble proteins from haploid megagametophytes of *Pinus pinaster* seeds were analyzed by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). Seven polymorphic proteins were observed and their inheritances are reported. The genes encoding 6 of these proteins were found to be clustered into 2 linkage groups, each consisting of 3 loci. Variation at these proteins was estimated in seeds from nine locations within the natural area of distribution of *P. pinaster*. Our results demonstrate a close relationship between Tamjout (Morocco) and Ronda (Southern Spain) populations, as well as the uniqueness of the Corsican population within the Mediterranean group.

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