KARYOTYPE ANALYSIS BASED ON THE FEMALE GAMETOPHYTE OF NORWAY SPRUCE

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ABSTRACT

The karyotype analysis was performed using the Feulgen squash method on slides made from the female gametophyte (= macrogametophyte, endosperm tissue in development) of a Norway spruce *Picea abies* (L.) KARST. tree growing at the Botanical Garden of the University of Graz. Twenty-two images of well spread metaphase-plates of the haploid tissue were saved using image analysis equipment consisting of a video camera mounted on a light microscope and connected to the central computer. The absolute arm lengths of all chromosomes (n = 12) were measured directly on the computer image by image-analysis software. The morphometric values were calculated after transferring the data to the Excel spreadsheet, and statistically analyzed. From the base, an idiogram was developed, and the chromosomes were numbered according to the relative length and morphology. Chromosomes I, IV and VIII are m-types, with median centromeres, while the chromosomes II, III, V, VI, VII, IX, X, XI and XII have the centromere in the median-submedian region. Two chromosomes (II and V) have secondary constrictions on the long arm and one chromosome (IX) on the short arm. The secondary constriction of the chromosome II is more terminal than the secondary constrictions of chromosomes V and IX. The use of digital image analysis technique allowed for fast selection of metaphase cells, storage of images on a hard disk and rapidly provided measurement for statistical analysis.

Key words: *Picea abies* (L.) KARST., chromosomes, Feulgen squash method, female gametophyte, digital image analysis

INTRODUCTION

Norway spruce (*Picea abies* /L./ KARST.) is one of the most important forest tree species in Europe. Decline of Norway spruce – dominated ecosystems has caused great concern, and teams of scientists in numerous institutions are working toward solutions for this complex problem. A considerable amount of research has been conducted on detection and assessment of damage in declining forests. DRUŠKOVIČ (1988) demonstrated that genetic disturbances found in cytogenetic studies can be used as bioindicators of early stages of decline in arborescent species affected by soil or air pollution. Subsequent research has shown that cytogenetic studies of forest trees are a reliable method for bioindication of forest decline or pollution damage, as some nuclear alterations can be visualized at the chromosome level (MÜLLER *et al.* 1991; MÜLLER *et al.* 1992; MÜLLER *et al.* 1994a; MÜLLER *et al.* 1994b).

The majority of cytogenetic studies for bioindication use root tip meristems for experimental materials, and the resulting karyotypes often include artifacts accrued in slide preparation and/or blurred features due to overlapping or different planes of focus. Moreover, the karyotypes developed from these studies can vary in the positions of primary and secondary constrictions. An artifact-free baseline karyotype is needed for a standard reference to detect variation that is due to seed source, genotype or pollution. In order to develop a baseline karyotype, however, the intraspecific variation in karyotypes should be well characterized from cells that have well separated chromosomes and are relatively free from artifacts. Despite the significant number of publications on spruce karyology, e.g., SAX & SAX (1933), BIAŁOBOK & BARTKOWIAK (1967), PRAVDIN *et al.* (1976), TERASMAA (1971, 1972, 1975), and DRUŠKOVIČ (1988), no karyotype of Norway spruce has been proposed for use as a standard for comparison.

SAX & SAX (1933), followed by PEDERICK (1967, 1970) and BORZAN (1977, 1981, 1988), previously have described the advantages of karyological research using the haploid female gametophyte in gymnosperms. Correspondingly, we have opted to combine classical slide preparation methodology using the female gametophyte (= macrogametophyte, endosperm tissue in development) excised from developing Norway spruce cones with modern computer imaging technology to develop a baseline karyotype. This karyotype can be

used as a reference for comparison with Norway spruce karyotype analyses from bioindication and cytogenetic studies to detect variation due to decline or evolutionary processes.

MATERIALS AND METHODS

Plant material, fixation and the slide preparation

In May, about 1.5 month after pollen was shed, conelets of Norway spruce were collected from a single tree growing at the Botanical Garden of the University of Graz. Ovules were extracted, placed in 3:1 ethanol : acetic acid fixative for 24 hours at room temperature and then stored in 70 % ethanol at 4 °C.

The slides were prepared by the Feulgen squash method described by DARLINGTON & LA COUR (1962) and BORZAN (1981) with the following modification:

1. The stored female gametophytes were sequentially placed in fresh 70 %, 50 % and 30 % ethanol and in distilled water, each for 10 minutes.
2. Hydrolysis followed for 3 minutes at 63 °C in 3 N HCl p.a. (Merck).
3. The hydrolysis was discontinued by placing the female gametophytes in distilled water.
4. The female gametophytes were stained in Feulgen solution for 30 minutes and rinsed in distilled water for 10 minutes.
5. The female gametophytes were moved into a 45 % acetic acid solution for 5 minutes.
6. Under a stereomicroscope a small incision was made through the integument and the cells were teased out and gently squashed in 45 % acetic acid.
7. Semipermanent slides were examined on the Axioplan (Zeiss Inc.) microscope with an oil-immersion objective Plan Neofluar 100x/1.3. A C-mount adapter was used for the video camera. The image was made of 22 selected cells with the chromosomes in the same plane of focus and minimal overlapping.

Technical equipment and software

The image analysis system consists of a 3-chip color 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 (Imaging Technology Inc.), variable scan module AM–VS–VP and color recording module AM–CLR–VP included. The frame grabber has a resolution of 1024 x 1024 pixels with 24 Bit true color (24 + 4 Bit), 4 Bit overlay, 0.5 MB overlay memory, 3 MB image memory, 4 MB program- 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 harddisk. The computer works under the operating system DOS 6.2 and Windows 3.11 (Microsoft Inc.). The image-analysis software was Optimas 4.02 (Bio-Scan 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 prints were made with a greyscale laser printer Laserjet 4/4M (HP Inc.). The data were transferred by DDE (dynamic data exchange) directly to the spreadsheet program Excel 5.0 (Microsoft Inc.).

Statistical analysis

Absolute measurements of the short (S) and long (L) chromosome arms were made. The chromosomes in each cell were arranged according to their lengths. Chromosome I was defined as the longest chromosome, and chromosome XII as the shortest chromosome. The position of secondary constrictions was designated as a percentage distance from the centromere in terms of the total arm length on which they are located. Using the data from the image-analysis software Optimas 4.02 in µm, relative chromosome lengths were calculated, based on average chromosome measurements in each cell (=100).

In presenting results of the karyotype analyses, chromosomal morphometric values were defined or modified after TEPPNER (1974), SAUER & LEEP (1979) and BORZAN (1988):

- $\bar{x}$ = total lengths of the chromosomes = short arm (S) + long arm (L);
- s = standard deviation;
- C.V. % = coefficient of variation = (standard deviation / average value) × 100;
- arm ratio = S / L = short arm / long arm;
- centromere index = (short arm / total length) × 100;
- chromosome index (r) = L / S = long arm / short arm;
- symmetry index (Si) = (total lengths of the short arms / total lengths of the long arms) × 100;
- size grading index (Gi) = (length of the smallest chromosome / length of the largest chromosome) × 100.

When calculating Si and Gi, the length of the gap formed by the secondary constrictions was included in the arm measurements.

The position of the centromere and the corresponding chromosome classification were made using SCHLARBAUM & TSUCHIYA'S (1984) modification of LEVAN *et al* 's (1964) nomenclature and classification system. The idiogram was constructed using the software package Origin 3.5 (Microcal Software Inc.).

RESULTS

Utilization of the Feulgen squash method for karyotyping Norway spruce chromosomes from female gametophyte proved to be very fast and efficient (Figure 2). With the digital image analysis equipment, it was relatively easy to measure chromosomes in the 22 selected metaphase plates within a short time. The total chromosome lengths, arm ratios, centromere and chromosome indices (r), the symmetry index (Si) and the size grading index (Gi) were calculated and shown in Table 1. Chromosomes II, III, V, VI, VII, IX, X, XI, and XII have the centromere in median-submedian region. Theother chromosomes, chromosome I, IV, and VIII are m-types, with the median centromere (Table 3). On the idiogram presented in Figure 1, three chromosomes (II, V, and IX) have secondary constrictions. Two chromosomes (II and V) have secondary constrictions, one on the long arm each, and one chromosome (IX) on the short arm (Figures 1 and 2). The secondary constriction of chromosome II is more terminal than the other secondary constriction.

DISCUSSION

The use of the digital image analysis equipment allowed for the analysis of a large number of metaphases within short time and rapidly provided measurement for

Table 1 Numerical karyotype of the investigated Norway spruce tree, based on the analysis of 22 haploid cells

Chromosome	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Total Length (S + L)												
x	140.7	117.1	112.3	109.9	105.7	103.3	100.2	96.8	90.6	81.9	74.7	66.6
Length Differences	23.6	4.	2.4	4.2	2.4	3.1	3.4	6.2	8.7	7.2	8.1	
s	17.3	4.6	3.4	3.4	3.9	3.7	4.3	4.0	5.7	5.2	5.8	5.9
C.V. %	12.3	3.9	3.0	3.1	3.7	3.6	4.3	4.2	6.3	6.3	7.8	8.9
Short Arm (S)												
x	62.2	50.5	47.9	48.0	45.7	42.5	42.4	42.1	39.3	34.2	30.4	25.4
s	8.0	6.0	8.6	4.2	6.6	7.6	4.6	5.3	6.0	5.9	5.5	5.6
C.V. %	12.9	11.9	18.0	8.6	14.5	17.9	10.9	12.7	15.2	17.3	18.1	22.0
Long Arm (L)												
x	78.5	66.6	64.4	61.9	60.1	60.9	57.8	54.7	51.3	47.7	44.3	41.2
s	14.5	7.8	8.4	4.5	8.0	8.5	4.8	4.6	5.5	7.8	6.1	7.0
C.V. %	18.4	11.7	13.0	7.2	13.2	13.9	8.4	8.3	10.8	16.3	13.8	17
Arm Ratio (S/L)	0.79	0.76	0.74	0.78	0.76	0.70	0.73	0.77	0.77	0.72	0.69	0.62
Centromere Index	44.2	43.1	42.7	43.7	43.2	41.1	42.3	43.5	43.3	41.8	40.7	38.1
Chromosome Index	1.26	1.32	1.34	1.29	1.32	1.43	1.36	1.30	1.31	1.40	1.46	1.62
Index Class	m	msm	msm	m	msm	msm	msm	m	msm	msm	msm	msm
Secondary Constrictions												
Position on Arms		II L 65.6			V L 50.3				IX S 50.6			
Symmetry Index (Si)	74.06											
Size Grading Index (Gi)	47.34											

Table 2 Relative chromosome lengths of the *Picea abies* (L.) Karst. Karyotypes obtained by different authors working with different examination material and different staining methods

Author Staining Method Investigated Tissue	Chromosomes											
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Guttenberger & Müller (unpublished) Feulgen Squash Method Root tip Meristem	109	98	93	89	88	87	85	84	77	74	72	56
Köhler, Guttenberger & Borzan (unpublished) Giemsa C-Banding Method Haploid Endosperm	141	120	115	110	107	103	99	93	89	82	75	66
Present Study Feulgen Squash Method Haploid Endosperm	141	117	112	110	106	103	100	97	91	82	75	67

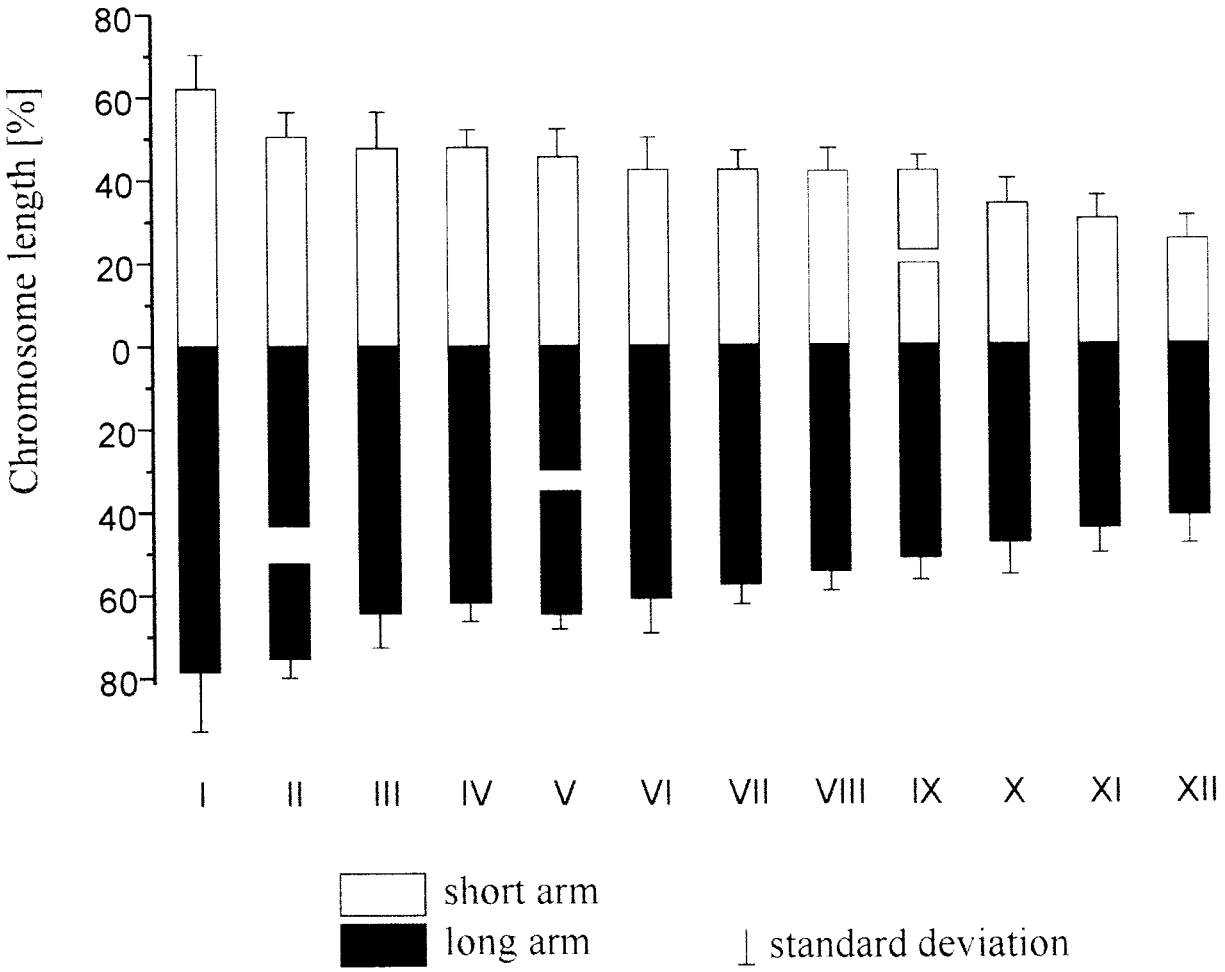

Figure 1 The idiogram of the investigated Norway spruce tree. The length of the gap at the secondary constriction stands for the standard deviations of the arm from centromere to secondary constrictions

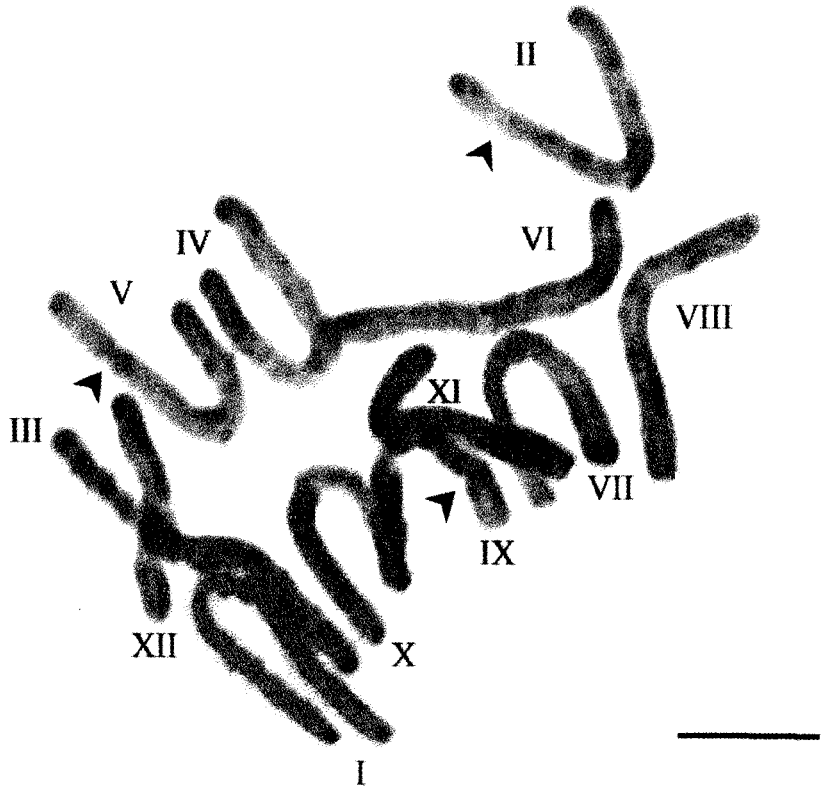

Figure 2 Computer image of a metaphase plate from the female gametophytic tissue of Norway spruce. Chromosomes are identified with Roman numerals I – XII. Arrowheads indicate the secondary constrictions (bar = 5 μm).

statistical analysis. Electronic data-processing-supported methods for karyotype representation have been available for nearly ten years. FUKUI (1986, 1988) presented a program for the automatic recording and processing of chromosome data specifically for application in botany. Other computer programs that analyse and present karyological data have been published by: WETSCHNIG (1992), VOSS *et al.* (1994), and BAUCHAN & CAMPBELL (1994).

Karyotype analysis using female gametophytic tissue has been seldom used, but it does offer advantages and facilitates the preparation and finding of well-spread metaphase chromosomes. This has been repeatedly confirmed and well documented in the works of SAX & SAX (1933), PEDERICK (1967, 1979), BORZAN (1977, 1981, 1988), BORZAN & PAPEŠ (1978) and MACPHERSON & FILION (1981). There is less dependency on chemical procedures, e.g. enzymes, or pretreatment by colchicine or 1-bromonaphtalene, that lead to contraction of chromosomes. The only disadvantage of using the female gametophyte is the short span of availability.

The chromosomes from female gametophytic tissue can show more morphological details, e.g., secondary constrictions, but less variation among cells. This could be due to the fact that the female gametophyte tissue represents the genome of the plant from which it has been taken. The root tip meristem tissue obtained from seeds is genotypically more complex, because each seed consists of parental genomes and therefore each slide (if made from a single root tip of one seed) used in cytological analysis may represent a completely different genome, at best having in common only the mother parent. The variation in relative length among the chromosomes from female gametophytic tissue is greater as the chromosomes have not been subjected to chemicals that cause contraction. In comparison, less variation in morphometric data analysis of chromosomes is obtained from root tip tissues, but there also are fewer morphological details shown in the resulting idiogram.

The cytology of Norway spruce, using female gametophyte tissue, has been investigated by SAX & SAX (1933) and SANTAMOUR (1960). Other karyograms shown by BIAŁOBOK & BARTKOWIAK (1967), PRAVDIN

Table 3 Differencews in centromere position of three presentations of Norway spruce karyotypes depending on the classification used after SAYLOR (1961), after LEVAN *et al.* (1964) and after SCHLARBAUM and TSUCHIYA (1984)

Chromosome Numbers	Centromere position of the chromosomes based on the Terasmaa's paper (1971)		
	According to Saylor's definiton (1961)	According to Levan's definiton (1964)	According to Schlarbaum & Tsuchiya's definition (1984)
I	m	m	m
II	m	m	m
III	m	m	m
IV	m	m	m
V	m	m	m
VI	m	m	m
VII	m	m	m
VIII	m	m	m
IX	sm	sm	sm
X	sm	m	msm
XI	m	m	m
XII	sm	sm	sm
	Centromere positions of the chromosomes based on Guttenberger & Muller's research (unpublished)		
I	m	m	m
II	sm	m	m
III	m	m	m
IV	m	m	m
V	m	m	msm
VI	m	m	msm
VII	m	m	m
VIII	m	m	m
IX	sm	sm	sm
X	m	m	m
XI	sm	m	msm
XII	sm	st	st
	Centromere positions of the chromosomes based on the present study results		
I	m	m	m
II	m	m	msm
III	sm	m	msm
IV	m	m	m
V	m	m	msm
VI	sm	m	msm
VII	sm	m	msm
VIII	m	m	m
IX	m	m	msm
X	sm	m	msm
XI	sm	m	msm
XII	sm	m	msm

et al. (1976), TERASMAA (1971, 1972, 1975), HIZUME (1988), DRUŠKOVIČ (1988) and recently by GUTTENBERGER & MÜLLER (unpublished) were made after preparation of root tips meristem cells. Each study reported slightly different results.

The differences in the karyograms shown by TERASMAA (1971, 1972, 1975), HIZUME (1988), DRUŠKOVIČ (1988), GUTTENBERGER & MÜLLER (unpublished), are in the length of individual chromosomes and in the number and location of secondary constrictions. TERASMAA (1971) observed five secondary constrictions on chromosomes II L, III S, V L, VI S and X S. HIZUME (1988) showed six secondary constrictions on chromosomes II S, V L, VI S, VII L, VIII L and IX S. As in the present study, DRUŠKOVIČ (1988) and GUTTENBERGER& MÜLLER (unpublished) noticed three secondary constrictions, but on different chromosomes: DRUŠKOVIČ (1988) on chromosome I S, V S and VII S, shown by BIAŁOBOK & BARTKOWIAK (1967), PRAVDIN mes is obtained from root tip meristem tissues, but there also are fewer morphological details shown in the resulting idiogram.

The cytology of Norway spruce, using female gametophyte tissue, has been investigated by SAX & SAX (1933) and SANTAMOUR (1960). Other karyograms shown by BIAŁOBOK & BARTKOWIAK (1967), shown by BIAŁOBOK & BARTKOWIAK (1967), PRAVDIN mes is obtained from root tip meristem tissues, but there also are fewer morphological details shown in the resul-ting idiogram.

The cytology of Norway spruce, using female gametophyte tissue, has been investigated by SAX & SAX (1933) and SANTAMOUR (1960). Other karyograms shown by BIAŁOBOK & BARTKOWIAK (1967), PRAVDIN-GUTTENBERGER & MÜLLER (unpublished) on chromosome II L, IV S and IX S. Such differences may be due to the fact that the observations were made on cells in different division phases ranging from prometaphase to metaphase. MACPHERSON & FILION (1981), found that the prophase-chromosomes of *Pinus resinosa* Ait. had 19 secondary constrictions, but only 12 constrictions were observed at full contraction in metaphase. Alternately, the differences among results obtained by previous studies may reflect intraspecific variation in the Norway spruce karyotype.

Discrepancies can occur between studies at the same institution. Two Norway spruce karyotype analyses were made at the same institute (GUTTENBERGER & MÜLLER in 1995, unpublished, and the present study), but assigned a different position of a secondary constrictions. GUTTENBERGER & MÜLLER (unpublished) observed the secondary constriction on the short arm of the chromosome IV, while the present study assigned the constriction to the long arm of the chromosome V. This difference may be due to the different tissues, root tips meristem versus female gametophyte, used in the respective studies, the possible different origin of the investigated plants or the differences in the method of investigation as GUTTENBERGER & MÜLLER (unpublished) used a pretreatment of l-bromonaphtalene. When

digital image analysis software is used for the evaluation of the length of each chromosome in cells of the endospermal tissue, the secondary constriction appears always on chromosomes II, V and IX. This approach greatly facilitated chromosome identification, while GUTTENBERGER & MÜLLER (unpublished) recognizes the difficulty in distinguishing between chromosomes IV and V by total length and centromeric position. Therefore it was uncertain on which chromosome (IV or V) the secondary constriction exists. Additionally, GUTTENBERGER & MÜLLER (unpublished) were able to identify five pairs of Norway spruce chromosomes (I, II, IV, IX and XII), while the others were divided into two groups based on similarity of centromere position and total length. The first group contains five chromosome pairs: III, V, VI, VII, VIII, and the second group contains chromosome pairs X and XI. In the present study we can distinguish chromosomes I, II, V, IX and XII, but specific identification of other chromosomes could result in the reversal of order or arm reversal (cf. MATÉRN & SIMAK 1968) due to morphological similarities.

The differences in relative chromosome lengths obtained in karyotype analyses of the Norway spruce shown by GUTTENBERGER & MÜLLER (unpublished) and in the present study (Tab.2) could be explained by the use of different examination tissues. Different criteria in classification of centromeric positions (cf. SAYLOR 1961; LEVAN *et al.* 1964; SCHLARBAUM & TSUCHIYA 1984) in description of constructed karyotypes may lead to terminological confusion (Table 3).

In general, the difference among studies may be due to: (1) methodology in tissue preparation, (2) intraspecific karyotypic variation, (3) quality of slide preparation, and/or (4) gametophytic versus root-tip meristematic tissues. Natural variation due to evolutionary processes or induced variation from forest decline /pollution are difficult to distinguish when optimum tissue and slide preparation and cell analysis methodologies are not used. We believe that the use of female gametophytic tissue coupled with digital image analysis has the greatest probability of producing karyotype analyses of consistent quality for comparisons.

Further refinements in chromosome identification can be made using banding techniques. Recently, at the Department of Plant Physiology of the University in Graz successful Giemsa C-banding preparations of the Norway spruce female gametophyte were made (KÖHLER, GUTTENBERGER & BORZAN, unpublished). These investigations revealed intercalary C-bands on secondary constrictions and telomeric C-bands on both arms of the chromosomes II and III. Using this method, it was possible to identify one chromosome more (chromosome No. III) than in the present study.

We believe that the present study contributes to the knowledge of spruce karyotype variation, and that further karyological investigation of various spruce provenance and individual genotypes with conspicuously different morphological characteristics will reveal intraspecific karyotypic variation in Norway spruce. In our opinion, this Norway spruce karyotype analysis can be regarded as the baseline karyotype for comparison with further karyotype analyses of other Norway spruce trees using these methods. Comparisons of karyotypes will then be possible among trees, among provenances and among individual genotypes with defined morphological characteristics i.e. among known cultivars, clones, varieties and forms. Correspondingly, comparison of normal populations with declining or polluted populations by cytological bioindication will become increasingly reliable.

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