STANDARDIZATION OF GYMNOSPERM KARYOTYPES USING *PICEA OMORIKA* AS AN EXAMPLE

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ABSTRACT

The karyotype analysis of a single *Picea omorika* (Panč.) Purk. tree was completed using female gametophyte tissue. Most chromosomes ($n = 12$) were metacentric, but chromosomes IX and XII were submetacentric. The positions of the centromeres of chromosomes X and XI were close to submetacentric, but were classified as metacentric. Some authors, however, could have considered the smallest four chromosomes as submetacentric. This variance among authors in describing karyotypes creates a problem when comparisons are made among studies. A standardization for gymnosperm karyotypes is presented, based on a review of literature on karyotype analyses of gymnosperms. Recommendations for standardization are given, including karyotype analyses using metaphase or anaphase cells, presentations of numerical karyotypes in absolute or relative length, definitions of centromere position, karyotype graphs, and reliability of position and frequency of secondary constrictions.

Key words: *Picea omorika* (Panč.) Purk., karyotypes, gymnosperms, centromere nomenclature

INTRODUCTION

Many years of cytological research on pines by the authors and extensive review of the cytological literature on gymnosperms reveal numerous problems that every cytogeneticist encounters, especially a beginner. Ideally, a scientist should be familiar with the available literature before initiating research on a topic. Unfortunately, this is usually not the case. Previously-published articles concerning the research in progress are, therefore, obtained successively as the research goes on. It may be evident as the research progresses that a different method or a different material should have been used, but usually a project termination deadline prevents the researcher from changing the current technique and starting with a new approach. Although the work may lead to an excellent original paper, it is very often full of unknown terminologies (newly introduced terms in place of already existing ones), or it shows results which are not comparable with previously published works.

This paper discusses examples of such work and proposes standardization of the materials and research methods used in gymnosperm karyotyping, particularly for species in the family *Pinaceae*. Based on our research on the *Picea omorika* (Panč.) Purk. karyotype, it presents different possible formats for the results, following the various formats found in the literature. The authors solicit any comments that might further aid in the standardization of gymnosperm karyotyping.

MATERIALS AND METHODS

Conelets for investigating the haploid female gametophyte tissue (endosperm tissue during development, megagametophyte) were collected on 7 June 1976 from a single *Picea omorika* tree growing at the Department of Forest Genetics and Dendrology in Zagreb. Permanent slides were prepared as described in earlier papers (BORZAN 1977, 1981, 1988) using a modified classical Feulgen squash method. In 1995, four slides were selected from these 18-year-old permanent slides. Images of 27 suitable 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. Absolute arm lengths of all chromosomes were measured directly from the computer image by the use of image-analysis software. Morphometric values were calculated and statistically analyzed after transferring the data to the Excel spreadsheet. An idiogram was developed from the database, the chromosomes were numbered according to relative length and morphology, and the basic karyotype of the tree was then constructed. A more detailed description of the equipment and software used was given by KÖHLER *et al.* 1995. Different possible formats that were developed from the results are shown

Table 1 Numerical karyotype of the *Picea omorika* (Panè.) Purk. m = metacentric, sm = submetacentric

Chromosome number	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Total length												
$\bar{x}$	123.55	115.86	110.37	107.99	105.99	103.63	101.41	99.03	95.78	91.62	77.03	67.71
Length differences	7.69	5.48	2.38	2.01	2.36	2.22	2.39	3.24	4.14	14.6	9.32	
s	4.47	3.78	2.95	2.21	2.01	2.59	2.49	2.14	2.34	3.59	5.35	3.74
C.V.%	3.62	3.26	2.67	2.04	1.89	2.49	2.45	2.16	2.44	3.92	6.95	5.53
Long arm												
$\bar{x}$	57.06	53.27	49.78	48.12	48.35	46.65	45.85	44.33	38.76	39.15	33.49	25.99
s	4.48	3.92	5.00	4.94	4.73	3.57	4.64	3.94	5.56	5.27	4.36	4.96
C.V.%	7.85	7.36	10.04	10.26	9.79	7.66	10.12	8.88	14.57	13.47	13.00	19.07
Short arm												
$\bar{x}$	66.48	62.70	60.59	59.88	57.64	56.99	55.57	54.70	57.02	52.50	43.53	41.72
s	2.96	4.86	5.05	5.20	5.16	3.54	4.50	4.04	6.10	5.10	3.56	5.09
C.V.%	4.45	7.75	8.33	8.69	8.95	6.22	8.09	7.38	10.70	9.72	8.18	12.19
Arm ratio (S/L)	0.86	0.86	0.82	0.83	0.85	0.82	0.83	0.82	0.70	0.76	0.77	0.64
Centromere index	46.15	45.96	45.10	44.56	45.64	45.01	45.20	44.77	40.49	42.69	43.41	38.35
Chromosome designation	m	m	m	m	m	m	m	m	sm	m	m	sm
Secondary constrictions position on arms			III L 47		V L 47				IX L 42	X S 61		

numerically and graphically, leading to a proposal for standardization in karyotype research on the *Pinaceae*.

RESULTS

A metaphase plate with identified chromosomes is shown in Figure 1. The numerical karyotype is presented in Tables 1 and 4. Most chromosomes ($n = 12$) are metacentric. Strict adherence to SAYLOR's (1961, 1964) definition of chromosome submetacentricity leads to the conclusion that only chromosomes IX and XII fall into that category. However, the arm ratio of the chromosomes X and XI is so close to the border value S/L = 0.75, that the karyotype can be defined as having the four smallest chromosomes (IX, X, XI and XII) submetacentric. Similar results were obtained by HIZUME (1988) from karyotype analysis of *Picea omorika* root tip meristematic tissue.

Secondary constrictions were found on the long arms of chromosomes III, V and IX and on the short arm of the chromosome X. According to their distance from the centromere, the secondary constrictions on the long arms are medial, whereas the constriction on the short arm of the chromosome X is placed more terminally. Stickiness was observed between nonhomologous chromosomes.

The constructed idiogram is shown in Figure 2a.

DISCUSSION

Research material option: root tip meristem versus female gametophyte tissue

The usual approach to karyotype research is to analyze metaphase plates in diploid cells of the root tip meristem. This method was usual for gymnosperms until the research of SAX & SAX (1933) on 53 species in 16 families showed the advantages of using female gametophyte tissue. Subsequently, however, only a small number of scientists actually used female gametophyte tissue, including SANTAMOUR (1960), SARKAR (1963), MERGEN & BURLEY (1964), and ILLIES (1971). PEDERICK's work (1967, 1969, 1970) presented detailed chromosome morphology in pine tissue that stimulated other work on the conifer karyotypes. Efforts to solve some cytogenetic problems in the *Pinaceae* family were well documented by BORZAN 1977, 1981, 1988, BORZAN & PAPEŠ 1978, MACPHERSON & FILION 1981 and KÖHLER *et al.* 1995.

Table 2 Number and percent of submetacentric chromosomes in 18-cell samples of 7 different trees of four pine species and out of a total of 126 cells. ni = *Pinus nigra*, sy = *P. sylvestris*, de = *P. densiflora*, nisy = hybrid *P. nigra* × *P. sylvestris*

Chromosome No.	ni 47		ni 221		ni 336		sy 367		sy 77		de V 116		nisy 410		Total	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
I	3	16.7	1	5.6	1	5.6	0	0.0	2	11.1	1	5.6	0	0.0	8	6.4
II	1	5.6	0	0.0	0	0.0	0	0.0	1	5.6	0	0.0	1	5.6	3	2.4
III	3	16.7	0	0.0	1	5.6	1	5.6	0	0.0	0	0.0	2	11.1	7	5.6
IV	0	0.0	0	0.0	3	16.7	0	0.0	0	0.0	1	5.6	1	5.6	5	3.9
V	2	11.1	1	5.6	1	5.6	1	5.6	1	5.6	0	0.0	0	0.0	6	4.8
VI	3	16.7	0	0.0	1	5.6	2	11.1	0	0.0	0	0.0	0	0.0	6	4.8
VII	1	5.6	0	0.0	2	11.1	2	11.1	1	5.6	0	0.0	2	11.1	8	6.4
VIII	2	11.1	1	5.6	2	11.1	3	16.7	1	5.6	1	5.6	2	11.1	12	9.5
IX	0	0.0	3	16.7	1	5.6	1	5.6	2	11.1	1	5.6	1	5.6	9	7.1
X	1	11.1	5	27.8	6	33.3	1	5.6	2	11.1	4	22.2	1	5.6	20	15.9
XI	12	66.7	10	55.6	11	61.1	12	66.7	10	55.6	9	50.0	2	11.1	66	52.4
XII	17	94.4	17	94.4	16	88.9	12	66.7	16	88.9	10	55.6	15	83.3	103	81.8

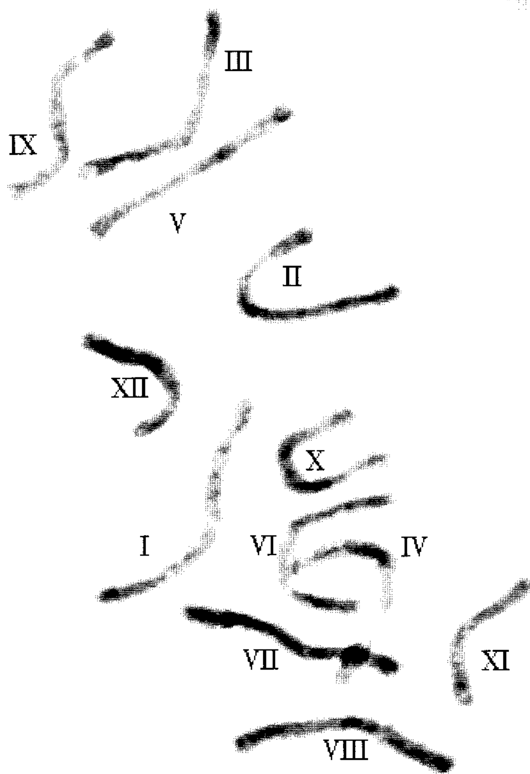

Figure 1 Metaphase plate from the female gametophyte tissue of *Picea omorika* (Panč.) Purk. tree. All chromosomes are identified.

Table 3 Differences in the centromeric positions of three presentations of *Picea omorika* (Panč.) Purk. chromosomes depending on the classification used after SAYLOR 1961, after LEVAN *et al.* 1964 and after SCHLARBAUM and TSUCHYA 1984. m = metacentric, sm = submetacentric, msm = metasubmetacentric

Chromosome numbers	According to SAYLOR'S deffinition (1961)	According to LEVAN'S *et al.* deffinition (1964)	According to SCHLARBAUM & TSUCHIYA'S deffinition (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	m	msm
X	m	m	msm
XI	m	m	m
XII	sm	m	msm

Among the advantages of using female gametophyte tissue in cytogenetic research, the haploid state of the cells is particularly important. There is also less dependency on treatment with chemicals such as enzymes, or pre-treatment by colchicine or l-bromonaphthalene, that lead to contraction of chromosomes. 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

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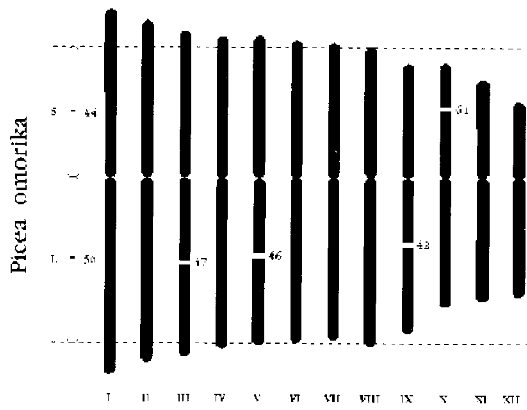
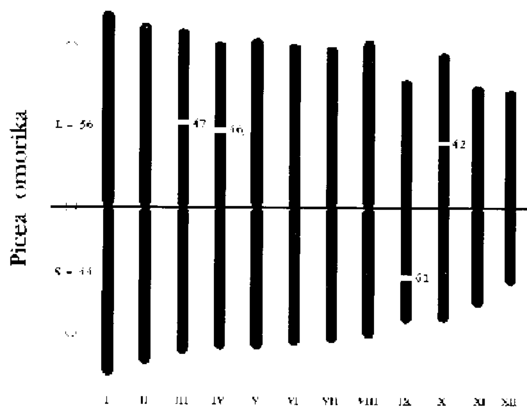
a) Karyogram presentation using Borzan's (1988) method



b) Karyogram presentation using Saylor's (1961) method



c) Karyogram presentation using Guttenberger's (1993) method

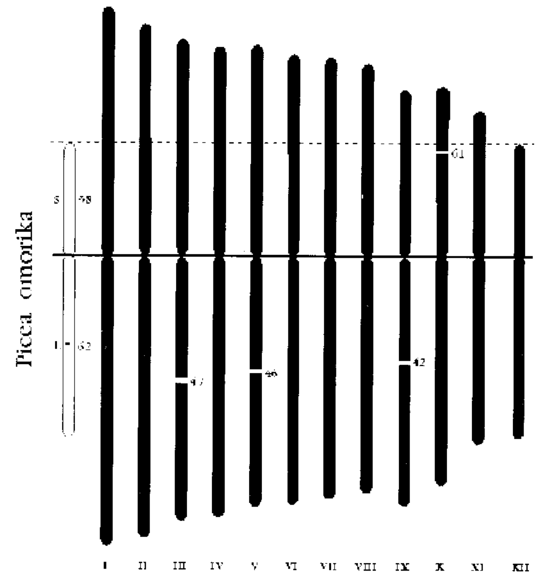


Figure 2 Idiograms of the same *Picea omorika* (Panč.) Purk. tree constructed after three different methods.

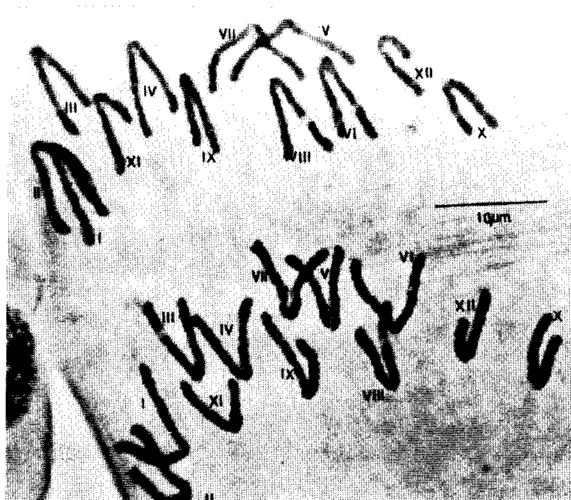


Figure 3 Scots pine (*Pinus sylvestris* L.) anaphase cell. No. IX chromatids have obviously different morphometric values. Reproduced from BORZAN 1988.

Figure 4 *Picea omorika* (Panč.) Purk. anaphase cell. It was not possible to identify chromatids with certainity

parental genomes. Each slide used in cytological analysis, if made from a single root tip of one seed, may represent a completely different genome, at best having in common only the female parent. Compared with root tip tissue, the variation in relative length among the chromosomes from female gametophytic tissue is

Table 4a Numerical karyotype of the analysed *Picea omorika* (Panč.) Purk. tree. Chromosome lengths

Chromosome number	Differences between neighbouring chromosomes	Relative mean length (S+L)	Standard deviation s	Coefficient of variation C.V. %
I		123.55	4.47	3.62
	7.69			
II		115.86	3.78	3.26
	5.48			
III		110.37	2.95	2.67
	2.38			
IV		107.99	2.21	2.04
	2.01			
V		105.99	2.01	1.89
	2.36			
VI		103.63	2.59	2.49
	2.22			
VII		101.41	2.49	2.45
	2.39			
VIII		99.03	2.14	2.16
	3.24			
IX		95.78	2.34	2.44
	4.14			
X		91.65	3.59	3.92
	14.62			
XI		77.03	5.35	6.95
	9.32			
XII		67.71	3.74	5.53

Table 4b Numerical karyotype of the analysed *Picea omorika* (PANČ.) PURK. tree. Mean, standard deviation and coefficient of variation of chromosome arm lengths

Chromosome number	Short arm (S)			Long arm (L)		
	$\bar{x}$	s	c. v. %	$\bar{x}$	s	c. v. %
I	57.06	4.48	7.85	66.48	2.96	4.45
II	53.27	3.92	7.36	62.70	4.86	7.75
III	49.78	4.99	10.04	60.59	5.05	8.33
IV	48.12	4.94	10.26	59.88	5.20	8.69
V	48.35	4.73	9.79	57.64	5.16	8.95
VI	46.65	3.57	7.66	56.99	3.54	6.22
VII	45.85	4.64	10.12	55.57	4.50	8.09
VIII	44.33	3.94	8.88	54.70	4.04	7.38
IX	38.76	5.65	14.57	57.02	6.10	10.70
X	39.15	5.27	13.47	52.50	5.10	9.72
XI	33.49	4.36	13.00	43.53	3.56	8.18
XII	25.99	4.96	19.07	41.72	5.09	12.19

greater, but there are fewer morphological details shown in the resulting idiogram. The only disadvantage of using the female gametophyte is the short period of availability.

Advantages of different staining techniques

Different staining techniques can be used successfully on dividing cells of the developing megagametophyte. Application of the Giemsa C-banding method to this tissue has been found to be particularly appropriate for chromosome identification (BORZAN & PAPEŠ 1978, TANAKA & HIZUME 1980, MACPHERSON & FILION 1981, BORZAN 1981, BORZAN 1988, KÖHLER *et al.* 1995). Another method is the differential staining of chromosomes, which has been successfully used on root

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Table 4c Numerical karyotype of the analysed *Picea omorika* (PANČ.) PURK. tree. Mean, standard deviation and coefficient of variation of the chromosome arm ratio and centromere index. Chromosome designations: m = metacentric, sm = submetacentrics

Chromosome number	Arm ratios (S/L)			Centromere indices S*100/(S+L)			Chromosome designation
	$\bar{x}$	s	c.v. %	$\bar{x}$	s	c.v. %	
I	0.861	0.09	9.93	46.145	2.55	5.53	m
II	0.858	0.11	12.98	45.962	3.40	7.40	m
III	0.832	0.13	16.20	45.102	4.33	9.60	m
IV	0.815	0.14	17.32	44.563	4.58	10.28	m
V	0.851	0.14	16.04	45.637	4.51	9.89	m
VI	0.824	0.10	12.65	45.006	3.20	7.11	m
VII	0.834	0.12	14.95	45.197	4.31	9.54	m
VIII	0.819	0.12	14.76	44.766	3.87	8.64	m
IX	0.698	0.17	24.51	40.490	5.98	14.77	sm
X	0.759	0.15	20.15	42.692	5.26	12.32	m
XI	0.775	0.12	15.03	43.405	3.87	8.92	m
XII	0.643	0.19	29.38	38.353	6.83	17.81	sm

Table 5 Frequency distribution of secondary constrictions in the analyzed *Picea omorika* (Panč.) Purk. Tree. A secondary constriction was placed on the arm of a chromosome on which it most often appeared and defined as: III L 47, V L 47, IX L 42 and X S 61

	%	Chromosome numbers											
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
		Frequncy distribution of secondary constrictions											
SHORT ARM (S)	95												
	85			1		1							
	75	1		1	1		1	2	2	1			
	65			1	1	1			1	2	3	1	
	55	2		2	1	1		1	2	2	3		
	45						1	1	1				
	35		2	2	1		1	1					1
	25						1						
	15									1			
	5												
	CENTROMERE												
LONG ARM (L)	5												
	15	1								1			
	25		1						1				
	35	2	2	2	1	3		1		3	1		
	45		1	3	2	1	1		2	3			
	55	1	2	2		3	1	1	1	1	2		1
	65		1	1	1	1			1	1	1		
	75		3	1	2		2	1	2		1		
	85		1	1	1								1
	95												

tip meristem, *e.g.* by SCHLARBAUM & TSUCHIYA (1981). In their work, chromosomes were differentially stained by low temperature pretreatment. The use of fluorescent stains also seems to be a promising technique (KONDO & HIZUME 1982, HIZUME *et al.* 1983 and KONDO *et al.* 1985).

Division phases, image analyses and the number of cells investigated

Advantages of using female gametophyte tissue in karyological analysis can be seen clearly in Figure 3. This figure shows the anaphase of a Scots pine (BORZAN 1988) and indicates the separated chromatids that can be identified as pairs. However, length measurements of separated chromatids showed that individual chromatids that belong to the same pair may differ significantly in total length. Thus, in the anaphase of the *Picea omorika* tree that was analyzed (Figure 4), the separate chromatids cannot all be clearly matched in pairs as they were in the previous figure. Matching was attempted from (1) the appearance of secondary constrictions and (2) chromatid length measurements by arranging the pairs in a sequence from I to XII. Following this procedure, we encountered the same problem that we had with analyses of some diploid metaphases of root tip meristem. The only difference was that this anaphase figure clearly showed that separated chromatids cannot possibly be matched according to length; this length difference could not be seen in the diploid cells. In diploid cells, we always joined pairs of chromosomes according to their lengths, and, in the best cell preparations, also according to the centromeric position or the presence of observed secondary constrictions.

The orientation of chromatids toward the opposite poles can provide additional help in pairing the anaphase cells. The orientation to two opposite poles is not clearly visible in all chromatids at the anaphase (Figure 4). Therefore, this cell resembles diploid metaphases of root tip meristem and shows all the possible mistakes we can make in analysing diploid cells. Pairing chromatids solely by their length occasionally resulted in creating chromatid pairs which do not match by shape, orientation of the fiber attachment point, or secondary constriction. The chromatid pairs of this anaphase cell could not be identified even after other distinguishing criteria (position of secondary constrictions and centromeric position) gave acceptable results. Therefore, we conclude from this example of an anaphase cell that in analyses of diploid metaphase karyotypes of root tip meristem chromosomes are erronously matched in pairs. Because of this problem, SIMAK (1966) considered idiograms constructed from karyological analysis to be only a probable result of the analysis and referred to them as "apparent idiograms".

Computer technology provides a major advance in karyotype analysis by saving cell images on the hard disk without requiring permanent slide preparation. The selected cell image can be stored and saved on a variety of magnetic media for later computer analysis as needed. The faster analysis that computers provide allows evaluation of more data in a short period of time. Important advances have been made in the development of systems based on chromosome pattern recognition (GUTTENBERGER *et al.* 1995). This technology has already become an indispensable tool for cytogenetic research.

Karyotype variability

In karyotype research on some species, one of the main goals is to provide an answer to the question as to whether or not karyotype variability exists. Because we still do not have enough parameters for exact chromosome identification, we cannot answer the question. Since measurements are not sufficiently precise, intraspecific variability would not be noticeable in research unless a specific marker such as a B-chromosome, *i.e.* an identified banded chromosome could be identified that would separate one population or variety from any other one. For this reason, most research results on intraspecific karyotype variability in *Pinaceae* conclude that there is no such variability, and even that there is very little variation within a genus.

We support this conclusion primarily because of the reversal of chromosome order discussed by MATERN & SIMAK (1968), especially of chromosomes that are similar in total length and centromere position. This causes differences between chromosomes to become masked and regularly leads to the equating of data by averaging, which hides any possible differences in intraspecific chromosome morphometry.

In considering possible chromosome variability, it is important to emphasize a rarely-recognized characteristic of karyotypes. In every analysis of a metaphase figure it is assumed that a chromosome is fixed in length as well as in its relation to other chromosomes. It is assumed, for example, that chromosome III is always longer than chromosomes IV to XII, or that chromosome IV is always shorter than chromosomes I to III. This assumption ignores the fact that, while dividing, chromosomes act as individual bodies that stretch, contract, and bend. Therefore the moment of fixing only provides an image of their current physiological activity. In that state, for example, chromosome III may sometimes appear longer than chromosome II or shorter than chromosome IV. The same situation applies to the chromosome arms, which can also stretch or contract. For example, in analysis of karyotypes with metacentric chromosomes, the stretching of a shorter arm results in it being identified as a long arm, or the contracting of a long arm results in it being identified as a short arm. As a result, some metacentric chromosomes have often been classed as submetacentric, and *vice versa*. Such an example was well documented by BORZAN 1988, who determined the number and percentage of submetacentric chromosomes in 18-cell

samples of 7 different trees of 4 species of pines (Table 2).

This, of course, also causes inconsistencies in the secondary constriction data. SAYLOR (1961) and YIM (1963) concluded that specific chromosome identification was not possible considering the recorded secondary constriction positions. PEDERICK (1967, 1970) actually used the secondary and tertiary constrictions as indicators of karyotype differences among certain pine species. However, in comparing the black pine karyotypes published in PEDERICK's 1970 and SAYLOR's 1964 papers, BORZAN (1977) could verify neither the number nor the position of the constrictions reported by PEDERICK, nor the position of all secondary constrictions reported by SAYLOR.

It is impossible to explain what causes such differences in results of research on the same species until we agree on a standardization of research methods and their presentation format. Only then will it be possible to get a clearer picture of intraspecific diversity. Application of standard procedures to karyotype analysis of different trees of a certain species, which different authors can work on independently and at different times, will bring us closer to valid conclusions about the intraspecific karyotype features of that species. This makes all the research efforts valuable contributions to knowledge of possible karyotype diversity. We emphasize the importance of determining the extent of intraspecific differences among karyotypes, if they exist, because karyotype differences among species cannot be fully defined without such knowledge.

Difficulties and dilemmas in revealing karyotypes

In cytogenetics research, a major problem is the possibility of repeating the research results of previous authors. Understanding the author's methods used for the research, analysis and reporting of results can be a problem. Authors usually use methods discussed in previous publications, making modifications according to their needs. Many new and different papers have appeared, making it increasingly difficult to choose one's own method of work, data analyses and reporting. Certain authors defined the karyotype of a species solely from a metaphase plate figure. In this case, it is difficult to compare one's own results with a picture, which may not be a high quality reproduction of the original preparation. Others have described morphological characteristics of species karyotypes in great detail, but have failed to mention the number of analyzed cells used to define those karyotypes. One could conclude that only one cell has been analyzed. If this is the case, it is not possible to draw any conclusion about the intraspecific karyotype variability, nor about the variations within the sample, which was supposed to serve as a basis in revealing karyotype features.

In idiogram construction, chromosomes can be lined up in a sequence from the smallest to the largest or *vice versa*. Numerical values may or may not be published. Published values may be absolute values from chromosome measurements or, if recalculated, they may represent relative chromosome lengths. Published morphometric values that define the karyotype very often do not contain statistical parameters that would make it possible to define the variability of the results and statistically compare them with our own results. The system for designating the position of the centromere is usually given but varies with the authors (SAYLOR 1961, LEVAN *et al.* 1964, SCHLARBAUM & TSUCHIYA 1984). Table 3 shows the effect of interpreting our results using the definition of each of these authors. It is clear that different criteria for the classification of centromeric positions in the description of constructed karyotypes may lead to terminological confusion.

The position and occurrence of secondary constrictions is another problem we may encounter. Authors reporting their placement of secondary constrictions in the idiograms state the inability to use them for chromosome identification, but fail to explain how their position was finally determined.

Figure 2 shows the idiogram of *Picea omorika* derived from our karyotype research. It is based on analysis of 27 cells and displayed in 3 different ways, according to the methods of BORZAN (1988), SAYLOR (1961) and GUTTENBERGER *et al.* (1995) respectively. A whole new body of research would be needed to draw conclusions from any of the three idiograms, or to compare them with a newly-developed karyotype of *Picea omorika*. This becomes evident when we try to compare absolute values from the published numerical karyotype with our own results, calculated and presented as relative values. The comparison is especially difficult if, for example, the absolute values are measurements of the contracted chromosomes of diploid cells, while the relative values have measurements of the chromosomes from megagametophyte cells. In this case, we must first recalculate the absolute values from a published paper into relative values, in order to compare them to our own results.

By naming some of the problems encountered while choosing the methods for cytological research, our goal was to point out how much unnecessary work each scientist must carry out to compare results for a certain species with previously published results, simply because they differ in working or presentation methods. This is why we appeal for the standardization of research methods and presentation of karyological research of the species in the *Pinaceae*.

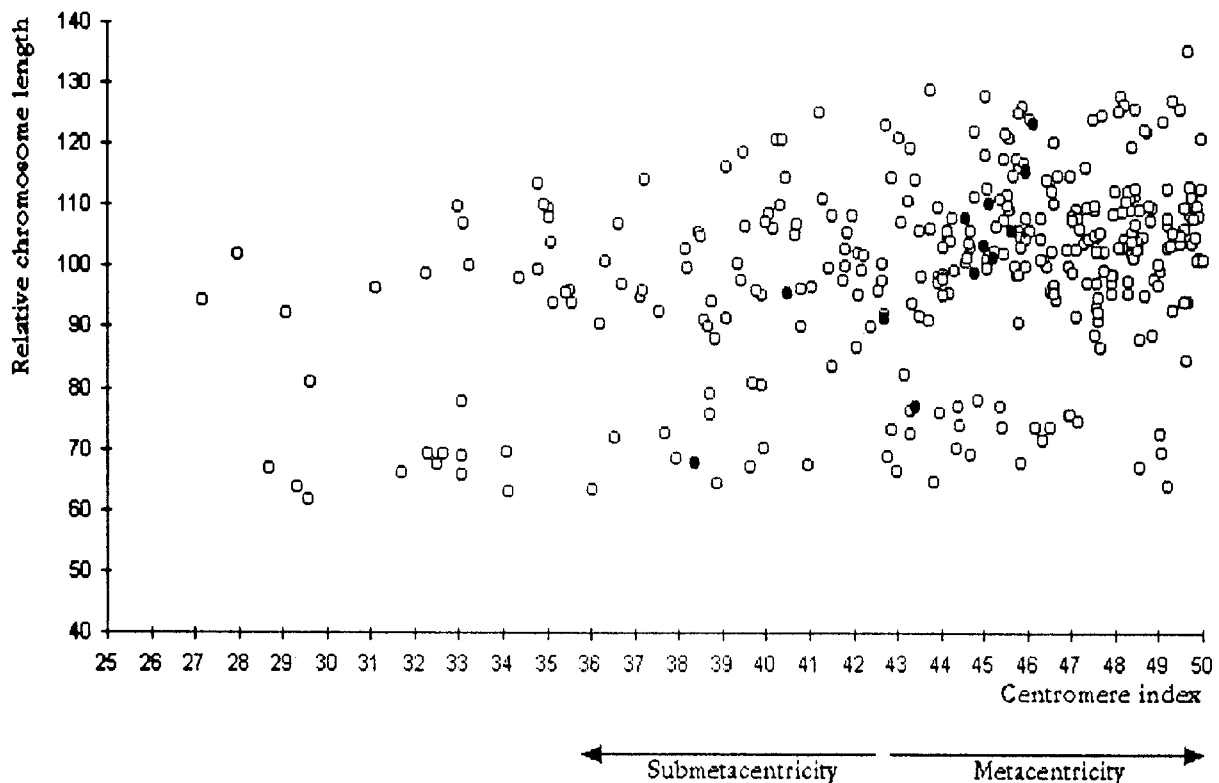

Figure 5 Polykaryogram of the *Picea omorika* (Panč.) Purk. tree analyzed. Solid dots are average values.

Proposed standard method

Unless there is a special reason for analysis of diploid cells (for example in bioindicator research), the haploid state of the female gametophyte tissue is recommended as the tissue to be used to reveal the karyotype of a species or to analyze intra- or interspecific karyotype diversities in the members of gymnosperm species.

Slides can be made either semipermanent or permanent. If semipermanent slides are made, it is necessary to store the cell images by photographing them, or by using a video camera mounted on a microscope and subsequently storing the images on a hard disk. The only disadvantage of working with semipermanent slides is the loss of the possibility of cytologically analyzing the same slides later.

Standardization in the staining of plant chromosomes seems to be the most difficult part of the microtechnique. However, any method which will facilitate the observation of well-contrasted chromosomes will be acceptable. The Feulgen squash method, the acetocarmine staining method and banding methods (Giemsa C and fluorescent methods) provide guidelines in the development of an individual worker's staining methods.

The number of cells analyzed is a very important consideration in obtaining information on sample variability. No one sample should be evaluated with less than 18 analyzed cells. Samples with 30 analyzed cells may be large enough for statistically well-documented results. F- and t-tests can be effectively used for statistical analysis and they may also be used for comparisons with results of other published data analysed in the same way.

For publication, only cells with well-spread chromosomes or with important and interesting details should be photographed. We recommend that the numbering of chromosomes in each cell be according to length, with chromosome I the longest chromosome and chromosome XII the shortest. Relative chromosome lengths should be calculated, based on the average chromosome measurements in each cell (=100).

The position of a secondary constriction should be designated as the ratio of its distance from the centromere to the total length of the arm on the which it is located, expressed as a percent. A secondary constriction should be placed on the arm of the chromosome on which it most often appears, and shown as the mean of its measured location. Its length should not be included in the total arm or chromosome length. The number and frequency of the most prominent secondary constrictions should be clearly designated (Borzan 1988). An example of the recommended method of graphical presentation is shown in Table 5.

In the *Pinaceae*, the position of the centromere and the corresponding chromosome classification should

follow SAYLOR's (1961) definition, in which submetacentricity is the short/long arms ratio (S/L); submetacentric chromosomes have values less than 0.75 and metacentrics have higher ratios.

High-precision optical equipment is needed for this type of work, combined with a computer to store image data, in order to measure chromosomes, analyze the measurements and construct them numerically and graphically. the "Expert System" software package is a useful method for identifying chromosomes (GUTTENBERGER *et al.* 1995). A similar approach may be used when the computer is not available as a tool in the cytological research.

Numerical karyotypes should be presented as shown in Tables 4a–4c. Graphical presentation of an average image of the karyotype is necessary and informative. We recommend presentation in the form of the Figure 2a idiogram. Additional information can be provided by the use of a "polykaryogram" (ILCHENKO 1975, MURATOVA 1978, BORZAN 1988) as shown in Figure 5.

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