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Quantitative Karyotyping of Norway Spruce Root Meristems by Image Analysis Methods and Pattern Recognition

By

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With 3 Figures

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Summary

GUTTENBERGER H. & MÜLLER M. 1996. Quantitative karyotyping of Norway spruce root meristems by image analysis methods and pattern recognition. – *Phyton* (Horn, Austria) 36 (1): 127–133, with 3 figures. – English with German summary.

Metaphase chromosomes of Norway spruce (*Picea abies* (L.) KARST.) root tips were prepared for quantitative karyotyping. Digital image analysis of chromosomes permitted fast and accurate measurement for the karyogram. One hundred spreads of Feulgen stained metaphase chromosomes were measured for total length, arm ratio, and localization of secondary constrictions. The data were subjected to t-test because of unequal variances. An expert-system that based on pattern recognition in the n^{th} dimension was used to further analyze the data. The expert system routinely identified five of the twelve different metaphase chromosome pairs. The remaining seven chromosome pairs could be divided into two groups. Within the two groups it was not possible to distinguish individual chromosomes because of similarity in morphology.

Zusammenfassung

GUTTENBERGER H. & MÜLLER M. 1996. Erstellung eines quantitativen Karyotyps von Wurzelmeristemen der Fichte unter Zuhilfenahme von digitaler Bildanalyse und Mustererkennung. – *Phyton* (Horn, Austria) 36 (1): 127–133, mit 3 Abbildungen. – Englisch mit deutscher Zusammenfassung.

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Chromosomen von Meristemen der Wurzelspitzen der Fichte (*Picea abies* (L.) KARST.) wurden für ein quantitatives Karyogramm präpariert. Durch die Verwendung digitaler Bildanalyse war ein genaues und schnelles Vermessen der Chromosomen möglich. Von einhundert gut gespreiteten Metaphaseplatten wurde die Länge der einzelnen Chromosomen, das Verhältnis ihrer Arme und die Lage der sekundären Einschnürung bestimmt. Die erhaltenen Daten wurden anhand des t-Testes statistisch geprüft. Weiters wurde ein Expertensystem, welches auf Mustererkennung beruht, zur weiteren Datenanalyse herangezogen. Fünf der zwölf Chromosomen konnten durch das System automatisch erkannt werden. Die restlichen Chromosomen sind morphologisch zu ähnlich, um exakt erkannt zu werden, aber eine Einteilung in zwei Gruppen war möglich.

Introduction

The family *Pinaceae* is the largest in the *Gymnospermae*, represented by ten genera and over 200 species (SPORNE 1965). Cytology of the *Pinaceae* began in the early 20th century. The genera of the *Pinaceae* usually possess the basic chromosome number $n = 12$ (e.g., SAX & SAX 1933, MEHRA & KHOSHOO 1956, HIZUME 1988). In Austria Norway spruce (*Picea abies* (L.) KARST.) is the most important forest tree species. DUMITRESCU 1972 investigated different provenances of Norway spruce from Europe, mainly from Rumania, and he found differences in the chromosome length and arm ratio. Different karyograms of Norway spruce (e.g., SAX & SAX 1933, TERASMAA 1971, 1972, 1975, PRAVDIN et al. 1976, HIZUME 1988, DRUŠKOVIČ 1988, KÖHLER et al. 1995) have been reported. All showed the same number of chromosomes $2n = 24$. However, the karyograms show differences in detail. The question was to work out a method for the objective measurement of chromosomes and statistical evaluation of the morphometric data.

In the late 80s new methods for chromosome research – concerning their morphology – arised. For instance FUKUI 1986, 1988, FUKUI & KAKEDA 1990, used an image analysis system for karyotyping plant chromosomes. Image analysis units of Zeiss/Kontron Inc. were used, and CHIAS (chromosome image analyzing system) was developed. Recently a new version to analyse the data by using a personal computer was described (KAMISUGI & FUKUI 1990, KAMISUGI et al. 1993).

We used a different system for our image analysis. This equipment allows a very fast and exact analysis of a large number of chromosomes. Using image arithmetic, one can decalibrate the images for the effects of reflected light and/or uneven illumination for making accurate measurements.

The aim of this study was to use an image analysis method to develop a new quantitative karyogram of meristematic tissues of root tips of spruce. Furthermore we demonstrate and discuss the efficiency and applicability of an expert system for automatic identification of the chromosomes.

Materials and Methods

The actively growing root tips of ten potted four-year-old Norway sprucetrees, *Picea abies* L. (KARSTEN), (one clone of 1039/116 – Forstliche Versuchsanstalt Hannover-Münden) were collected for examination. Sample treatment was according to MÜLLER et al. 1991, 1992. The collected root tips were immersed in 0.05% 1-bromonaphthalene at room temperature for pretreatment. Duration of pretreatment was 24 hours for adequate condensation in order to observation chromosome morphology. Then, the material was fixed in ethanol : glacial acetic acid (3:1/v:v) at 4°C for overnight. The root tips were hydrolyzed in 3N HCl at 63°C for 3 min and then transferred into cold aqua destillata and stained with Schiffs reagent for Feulgen reaction for 30 min and washed for 10 min. Under a binocular the root tips were longitudinally dissected and then the meristematic tissues were picked up onto glass slide by a needle with flattened tip. The isolated meristematic tissues were parted and well squashed in a aceto-carmine solution.

The image analysis system consisted of a 3-chip-color video camera (Sony DXC 930 P), controlled by a computer, and a frame grabber ITI MFG-3M-V (Imaging Technology Inc.). This frame grabber was mounted in a central computer and had a resolution of 1024×1024 pixels with 24 bit true color, and 4 bit overlay. The image-analysis software we used was Optimas 4.02 (BioScan Inc.). The bar was put in by the program Corel Photopaint 3.0 (Corel Inc.). The printing out was done with a laser printer (Laserjet IV, HP Inc.). The data were transferred by DDE (dynamic data exchange) directly to the spreadsheet program Excel 4.0.

The microscope used was a Zeiss Axioplan with an oil-immersion objective (Plan Neofluar $100 \times / 1.3$, Zeiss), and an adapter for the video camera.

For the quantitative karyogram we measured 100 stained metaphase spreads. Statistical analysis was carried out by means of the software package NCSS 5.03 (Unisoft Inc.). For normal statistics we used the t-test for unequal variances, because the F-test showed significant differences (ZÖFEL 1992). Degrees of freedom were based on the number of individual trees we used (10).

The pattern recognition program was Autoklas 5.05 (Schindler + Partner Inc.) version S, with the additional modules Z1 (compression of foreign data and their classification), Z2 (evaluation test of parameters), Z3 (sorting and printing of array-structures), Z4 (concentration of knowledge bases) and Z5 (analysis of the components). The program is able to manage 90 knowledge bases and to connect them. Each knowledge base can consist of 50 classes, 125 parameters, and 5000 data records.

Results and Discussion

Our image analysis system allows a very fast and exact measurement of a large number of chromosomes within a relatively short time. Furthermore: Using image arithmetic, one can decalibrate the images for the effects of reflected light and/or uneven illumination for making accurate measurements. We improved the original microscope image by using a median filter. Median filterings smooth the image, but they do not distort the image: edges and grey scale ramps are kept – only single disturbed pixels are removed (JÄHNE 1989). The result of a corrected image is presented in Fig. 1. The resolution and sharpness in comparison to the uncorrected image are much enhanced.

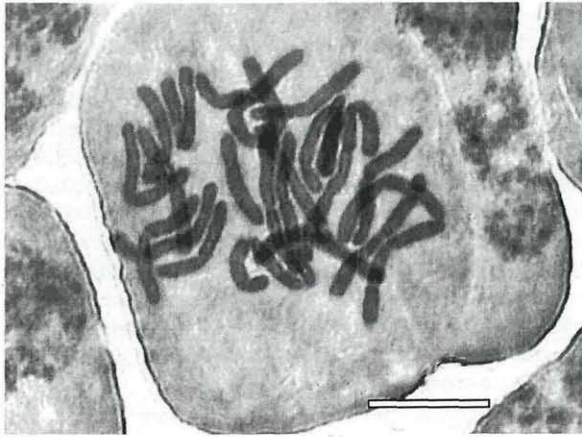
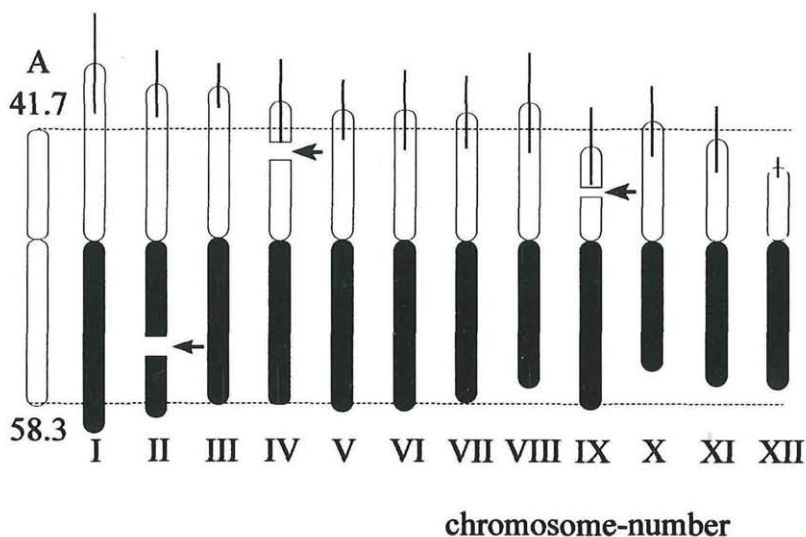


Fig. 1. Metaphase plate of Norway spruce. Bar = 10 μm . Image after background correction and median filtering. Printout was done with a laserprinter.

In this way ten metaphase plates from the root meristems of each spruce tree (therefore altogether 100) were measured. For exact measurement the four fold hardware zoom of the framegrabber was used. First, all the data were extracted in absolute units of the measurements (μm). The data were put in order of the length of the chromosomes within the single metaphase plates. Relative lengths of the chromosomes were calculated, based on the average chromosome measurements in each cell (= 100%). The results are represented in Fig. 2: the mean total lengths of the chromosomes in percentage, the standard deviation of the length is indicated by a bar on top of the graphs. Because the existence of a secondary constriction is not always clear, a secondary constriction was set, when more than 30% of the investigated chromosomes showed a secondary constriction. Three chromosomes had a secondary constriction (II, IV, IX), only one on the long arm (chromosome II). Two of the chromosomes had an arm ratio > 1.55 (IX and XII), one of these (IX) having a second constriction (on the short arm). An arrow points to the position of the mean, the height of the gap indicates the standard deviation of it. Chromosome II was longer than the chromosome XII with high significance ($P < 0.001$). Significant differences ($0.01 < P < 0.001$) in the length of the chromosomes were observed between the Ist and the XIIth, and between the IInd and the XIth. With low significance ($0.05 < P < 0.01$) the results were different between chromosomes II and X, between I and VIII to XI, and between XII and III to VII. All the other differences were not significant. This study shows differences in relative chromosome lengths and in the location of the secondary constriction in karyotype analysis of Norway spruce shown by KÖHLER et al. 1995. They could be explained by the use of different



- short arm
- long arm
- | standard deviation for the length of the chromosome

Average length of the chromosomes = 100%

A = average Chromosome [%]

← position of the secondary constriction;
the gap represents the value of the
standard deviation of the position
of the secondary constriction

Fig. 2. Quantitative karyogram of the root meristem of Norway spruce.

examination tissues (gametophytic versus root-tip meristem), by different methodology in tissue preparation, or by intraspecific karyotypic variation.

The data were also transferred to an expert system. This expert system was used with success for solving other multifactorial problems (e.g., MEHLHORN 1990, FUCHS 1991). Fig. 3 shows the flow chart of the knowledge bases for the expert system "morphology of the spruce chromosomes". One can distinguish some of the chromosomes in several steps: two chromosomes have a ratio between long and short arm > 1.55 (chromosome IX and XII). The decision between these chromosomes is the secondary constriction on chromosome IX supported by the relative length of the whole chromosome. The next knowledge base (KB 3 in Fig. 3) separates the

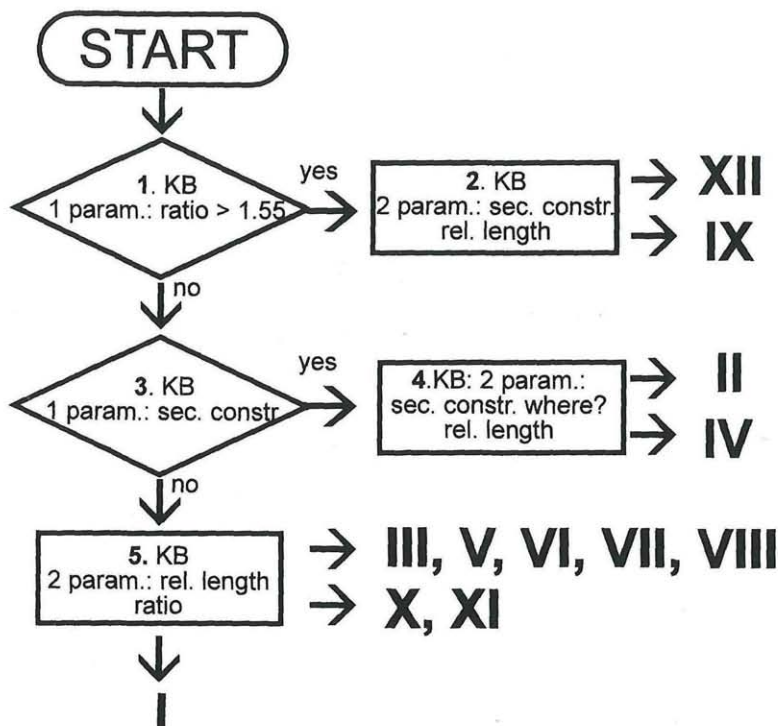


Fig. 3. Flow chart of the expert system "morphology of chromosomes of spruce". KB; knowledge base, param; parameter(s), chr; chromosome, ratio; ratio between long and short arm of a chromosome, sec. constr.; secondary constriction, rel. length; relative length.

chromosomes II and IV (secondary constriction) from all other chromosomes. It is possible to separate between II and IV: location of the secondary constriction and the length are helpful for decision. From all the other chromosomes only chromosome I can be separated by its length.

It was possible to recognize with the expert system automatically five chromosomes using their morphology: chromosomes I, II, IV, IX, and XII (fig. 3). The others could be separated into the two groups: X and XI; III, V, VI, VII, and VIII. The advantage of this program is that you are able to connect the data, and to recognize single chromosomes automatically, even if the data of a morphometric parameter are missing.

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