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Karyotype of Norway spruce by multicolor FISH

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Abstract The chromosomes (2n = 2*x* = 24) of Norway spruce are very large since their size reflects the huge amount of genomic DNA (2C = 30 × 10^9 bp). However, the identification of homologous pairs is hampered by their high degree of similarity at the morphological level. Data so far presented in the literature were not sufficient to solve all the ambiguities in chromosome identification. Several genomic Norway spruce DNA clones containing highly repetitive sequences have been identified and characterised in our laboratory. Three of them were selected for fluorescent in situ hybridization (FISH) experiments because of their strong signals and suitability for chromosome identification: PATR140 hybridized at the centromeric site of three chromosome pairs; PAF1 hybridized in six subtelomeric and two centromeric sites; 1PABCD6 co-localized with the subtelomeric sites identified by PAF1. The statistical analysis of microscopic measurements of chromosomes in combination with the FISH signals of these probes allowed the unambigous construction of Norway spruce karyotype. We also compared the karyotype of Norway spruce with that of other spruce species to infer the number and kind of rearrangements that have occurred during the evolution of these species.

Keywords Norway spruce · *Picea abies* · Repetitive DNA · FISH · Karyotype

Introduction

The chromosomes of Norway spruce are very large as their dimensions reflect the considerable haploid genome size of spruce (2C = 29 × 10^9 bp, Murray 1998). The diploid chromosome number (2n = 2*x* = 24) is very conserved in the family of Pinaceae, with little karyotype differentiation between species. The chromosomes are mainly metacentric and the identification of homologous pairs is hampered by similar length and slight differences at the morphological level. Chromosome size is a helpful hint for microscopic observations, but on the other hand demands a high level of technical skill to get well-spread chromosomes without regions of overlap (Murray and Davis 1996). To circumvent this problem the first karyotype proposed for Norway spruce was based on the statistical analysis of several microscopic measurements (length, arm ratio, secondary constrictions and short arm/total length ratio) of the haploid chromosome complement from female gametophyte tissue (Köhler et al. 1995). Such haploid material is available for a short period of the year and many variables may influence morphological karyotyping: methodology in tissue preparation, quality of slide preparation, intraspecific variation and chromosome condensation. An improvement was proposed by Lubaretz et al. (1998) joining molecular genetics with cytogenetics. A single fluorescent probe approach was used. In this case signals of fluorescent in situ hybridization of rDNA probes aided in the identification of chromosomes, but some ambiguities still remain due to the small number of metaphases examined and the limited number of signals detected. A further improvement for chromosome identification was recently reported in Siljak-Yakolev et al. (2002) by using heterochromatin CMA/DAPI banding and rDNA probes, but even this approach could not distinguish every chromosome pairs.

Several genomic DNA clones containing highly repetitive sequences have been identified and characterised in our laboratory (A. Zuccolo, F. Cattonaro, I. Jurman, M. Morgante, manuscript in preparation). In the present paper we report a multicolour fluorescence in situ hybridization (FISH) approach to the construction of the karyotype of Norway spruce using three different probes corresponding to tandemly repeated sequences. We also compare the karyotype of Norway spruce with that of other spruce species to try and infer the number and kind

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of rearrangements that have occurred during the evolution of these species.

Materials and methods

Plant material and chromosome preparation

Root tips of 1-week-old seedlings grown on wet filter paper were used for chromosome preparations. Seeds were exposed to thermal cycles in the dark at 22–30 °C for 6–18 h respectively to increase the mitotic index. Root tips were collected at the end of the 30 °C cycle after 2 h of 22 °C treatment. After overnight treatment in 0.05% (w/v) colchicine they were fixed for 24 h in 3:1 ethanol:acetic acid and stored in 70% ethanol at 4 °C until use. After these steps a squashing and a dropping method for chromosome preparation were compared. Squashes were performed according to Brown et al. (1993) with slight modifications. Prior to squashing, root tips were rinsed in distilled water and digested in a mixture of 2.2% cellulase (Calbiochem), 8.4% Macerozyme (Serva), 1% Pectinase (solution in 40% glycerol, Sigma) for 30–60 min. Meristematic regions were excised under the stereomicroscope and the external epithelial layer was carefully removed with microdissectors. Two root tips per slide were teased apart in a drop of 45% acetic acid and squashed under a coverslip. Slides were pretreated with Vectabond according to supplier instructions (Vector Laboratories, Inc., U.S.A.). Coverslips were removed after freezing on dry ice. Dropping preparation of chromosomes was carried out according to Morgante et al. (1997).

DNA probe preparation

Three Norway spruce tandem repeat DNA sequences were selected as probes in this study: PATR140, PAF1 and 1PABCD6. The characteristics of these probes are listed in Table 1, together with their GenBank accession numbers.

Digoxigenin-11-dUTP and Biotin-16-dUTP (Roche, Germany) were used to label the probes by nick-translation. The protocol was based on the work of Langer et al. (1981). DNase-I concentration and reaction time were controlled in order to obtain labelled fragments between 100 and 500 bp in length. Labelled probes were separated from un-incorporated nucleotides by Sephadex G-50 filtration using a spin column.

Fluorescent in situ hybridization (FISH)

FISH protocol was developed according to Pinkel et al. (1986), Doudrick et al. (1995) and Zhu et al. (1995). Briefly, chromosome spreads were incubated with 100 μl of 340 μg/ml of RNase A (in 2× SSC) under a coverslip for 1.5 h at 37 °C. Slides were washed for 3 × 5 min with 2× SSC, and incubated with 100 μl of 10 μg/ml pepsin in 100 mM of HCl for 15 min at 37 °C. After washing 3 × 5 min with 2× SSC, slides were post-fixed for 10 min in 4% formaldehyde, dehydrated in an ethanol series and rinsed again for 3 × 5 min with 2× SSC. The probe hybridization solution consisted of 62.5% formamide, 2.5× SSC, 12% dextran sulphate, 62.5 μg/μl of sonicated herring sperm DNA and 4 μg/ml of denatured DNA probe. The mix was denatured at 80 °C for 10 min and chilled on ice. Fifty microliters were applied on each spread of chromosomes. Slides were covered with slips sealed by rubber cement and denatured at 80 °C for 5 min. After overnight hybridization at 37 °C, washes were performed twice at 42 °C in 20% (v/v) formamide in 1× SSC (low stringency) or in 50% formamide in 2× SSC (high stringency), followed by 1× or 2× SSC, respectively.

The detection of labelled probes was performed according to Doudrick et al. (1995) using anti-dig conjugated with Rhodamine and Streptavidin conjugated with fluorescein isothiocyanate (FITC). Chromosomes were counterstained with DAPI (4′, 6 diamidino-2-phenylindole).

Observations were made with a Zeiss Axiovert epifluorescence microscope with different combinations of Zeiss excitation and emission filters: 487901 for DAPI, 487909 for FITC, 487915 for Rhodamine and 487925 for triple signal detection. Images were recorded with Kodak Ektachrome 400 colour slide film, digitalized with a Nikon scanner LS2000, superimposed and optimized using Adobe Photoshop 6.0.

Image processing and data analysis

The chromosomes were randomly numbered 1–24 and measured according to Brown and Carlson (1997), taking into consideration the presence of FISH signals and their distance from centromeres. These data were compiled and analysed in an Excel datasheet for each of the 15 metaphases considered. The pairs of homologous chromosomes were identified and ordered according to their decreasing length. Once paired, the relative lengths, centromere indices, presence/absence of signals and their localization on the chromosome arms were averaged from all the cells. The standard deviation and confidence intervals were then calculated.

Results and discussion

Specimen preparation

Chromosome preparation is a critical step in Norway spruce karyotyping. Well-spread metaphases are difficult to obtain due to the dimensions of Norway spruce chromosomes. The dropping method was abandoned after some preliminary tests because it was impossible to obtain chromosomes without extensive regions of overlap. On the other hand, the mechanical action of the pressure exerted in the squashing method, in combination with the chemical action of enzymes, were more suitable for chromosome spreading. In such a procedure it is very important to achieve a compromise between degradation and pressure so that spreads can be obtained without an excessive dispersion of the chromosomes. In our conditions 30 min were generally sufficient, as assessed by monitoring the degree of degradation with a stereomicroscope. Nevertheless, despite extensive manual intervention only 15 well-spread metaphases were obtained from more than 1,500 root-tip preparations.

Table 1 Norway spruce sequences used as probes in this study

Probe	Accession number	Category	Repetitive unit (monomer bp)
PATR140	AF180446	Satellite DNA, tandemly repeated	140
PAF1	AF105962	45S rDNA IGS (inter-genic spacer)	45
1PABCD6	AF305174 AF305175	Hypomethylated genomic sequence	133

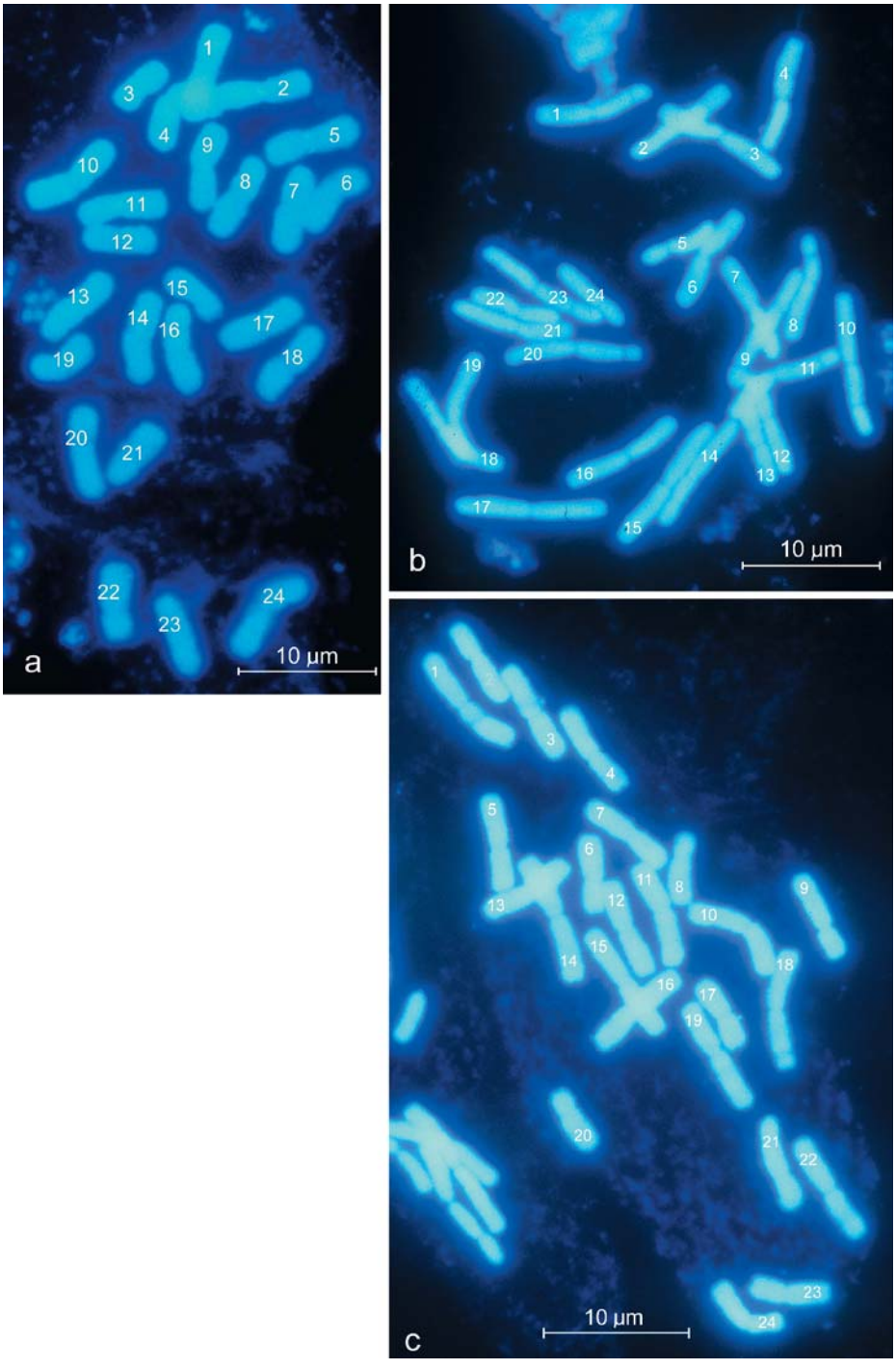

Fig. 1a–c Different level of chromosome condensation among Norway spruce metaphases. The chromosome length varied from a minimum of 4 μm (**a**) to a maximum of more than 10 μm (**b**). An intermediate length was detected in (**c**). The chromosomes were counterstained with DAPI and randomly numbered

Morphological observations

DAPI staining enabled us to observe accurately the morphological characteristics of the spruce chromosome complement. In our experimental conditions we observed a different degree of chromosome condensation among metaphases (Fig. 1). Chromosome length varied between 4–6 and 6–10 μm at high and low levels of condensation respectively. Secondary constrictions were not visible in highly condensed chromosomes and therefore were not considered as morphological markers for chromosome identification (Mehra and Khoshoo 1956; Saylor 1961). The chromosomes of each metaphase were arranged according to decreasing length and classified according to arm ratio. It was not possible to match chromosomes by homologous pairs because of similar and/or overlapping measures from the 3rd to the 8th class when considering only these two criteria (Table 2).

Table 2 Numerical karyotype of Norway spruce based on the analyis of 15 metaphases. Chromosomes are arranged according to their relative length, arm length ratio (q/p) and position of probes. Abbreviations: SD = standard deviation; c = centromere q = long arm; p = short arm; a = distance of chromosome arm length from the centromere; m = metacentric (q/p = 1-1.5); sm = submetacentric (q/p>1.5)

N	Relative length SD	Confidence interval (95%)		q/p SD	PATR140	PAF1	PABD6	a (%) SD	Chromosome type
1	10.46 ± 0.19	10.83	10.09	1.08 ± –0.04	–	–	–		m
2	9.76 ± 0.41	10.56	8.96	1.13 ± –0.08	–	q	q	63.36 ± 6.66	m
3	9.02 ± 0.49	9.98	8.06	1.12 ± –0.06	–	p	p	68.50 ± 6.20	m
4	8.95 ± 0.49	9.91	7.99	1.17 ± –0.10	c	p	p	54.48 ± 3.16	m
5	8.78 ± 0.56	9.88	7.68	1.12 ± –0.07	–	q	q	65.45 ± 5.81	m
6	8.77 ± 0.41	9.57	7.97	1.14 ± –0.08	–	–	–	–	m
7	8.51 ± 0.47	9.43	7.59	1.09 ± –0.03	c	–	–	–	m
8	8.45 ± 0.65	9.72	7.18	1.31 ± –0.10	c	q	q	50.61 ± 5.40	m
9	7.68 ± 0.50	8.66	6.7	1.64 ± 0.15	–	c	–	–	sm
10	7.17 ± 0.43	8.01	6.33	1.32 ± –0.14	–	c/q	q	58.81 ± 4.41	m
11	6.60 ± 0.41	7.4	5.8	1.21 ± –0.15	–	–	–	–	m
12	5.86 ± 0.34	6.53	5.19	1.78 ± –0.21	–	–	–	–	sm

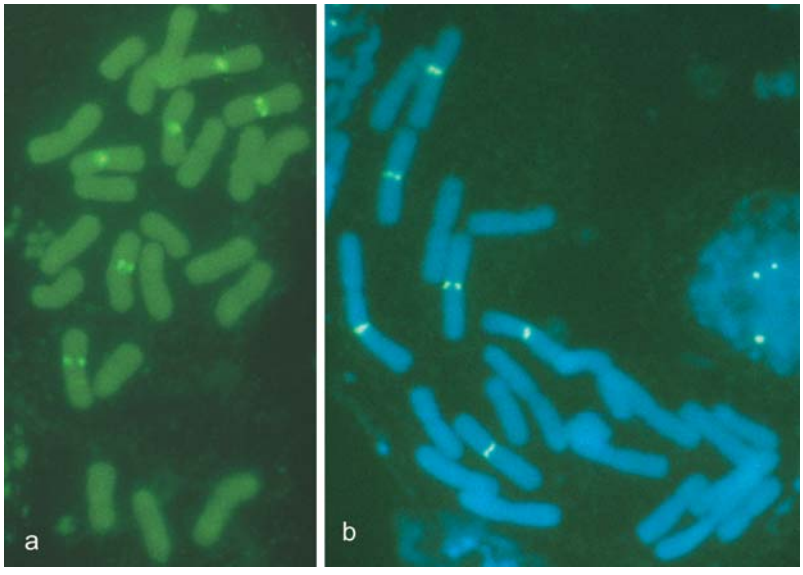

Fig. 2a, b Fluorescent in situ hybridization of PATR140. The hybridized probe was detected with avidin-FITC (fluorescein isothiocyanate) (*green*). Three pairs of signals were detected both at low (**a**) and high (**b**) stringency conditions. In (**a**) chromosomal DNA was not counterstained, in (**b**) it was counterstained with DAPI

Fluorescent in situ hybridisation (FISH)

The PATR140 probe showed three pairs of strong signals at the centromeric level at all stringency conditions tested. Signal intensity varied within and among homologous pairs, and in some cases distinct signals were visible for each chromatid (Fig. 2).

The PAF1 probe exhibited a different number of signals depending on the stringency conditions. Seven pairs of homologous chromosomes showed a total of 16 signals at low stringency: three pairs on the long arms, two pairs on the short arms, one pair on the centromere, one pair with double signals on the centromere and the short arm. Twelve signals were detectable at high stringency conditions because the two centromeric signals disappeared (Fig. 3). The 12 signals observed at higher stringency coincided in double-labelling experiments with those revealed by a 28S rDNA probe from *Picea abies* that we isolated in our laboratory (data not shown), thus lending support to the hypothesis that the PAF1 probe may be part of the rDNA tandemly repeated unit.

The 1PABCD6 probe, exhibited in both low and high stringency conditions, six pairs of signals on the chromosome arms. The signals co-localized with the PAF1 signals that were detected at high stringency. Although this probe did not introduce any additional hybridization site, it was used after detection with fluorescein-conjugated streptavidin to produce a third yellow/orange signal when simultaneously hybridized with PAF1 (detected with Rhodamine-conjugated antibodies) that made the identification of some pairs of chromosomes easier (Fig. 4).

According to these results the signal distribution was as follows:

– four pairs of homologous chromosomes with no signals;
– two pairs of homologous chromosomes with a double signal, the green centromeric signal being produced by

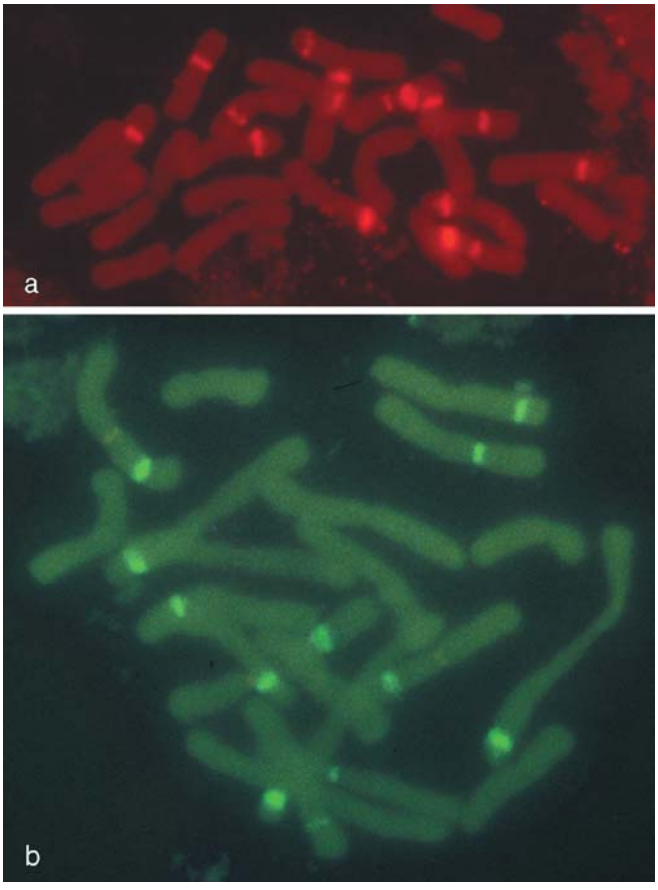

Fig. 3a, b Fluorescent in situ hybridization of PAF1. (**a**) At low stringency conditions 16 signals were detectable. The probe was detected with anti-dig rhodamine (*red*), chromosomes were not counterstained. (**b**) At high stringency the centromeric signals disappeared and 12 signals were detectable. Probe biotin-labelled was detected with avidin-FITC (fluorescein isothiocyanate) (*green*); chromosomes were not counterstained

PATR140 (fluorescein labelled) and the yellow/orange signal (PAF1 and 1PABCD6 combined labelling) being localized on the arms:
– one pair on the long arm,
– one pair on the short arm.

- One pair of homologous chromosomes with double signals: the red centromeric signal was produced by PAF1 (Rhodamine labelled) and the yellow/orange signal by the combined signal of PAF1 and 1PABCD6.
- Three pairs of homologous chromosomes with a single PAF1 and 1PABCD6 combined signal on the arms:
  - two pairs with a signal in the long arm (chromosome 2 and 5 in Fig. 3),
  - one pair with a signal in the short arm (chromosome 3 in Fig. 3), one pair of homologous chromosomes with a single red centromeric signal produced by PAF1.
- One pair of homologous chromosomes with a single green centromeric signal produced by PATR140.

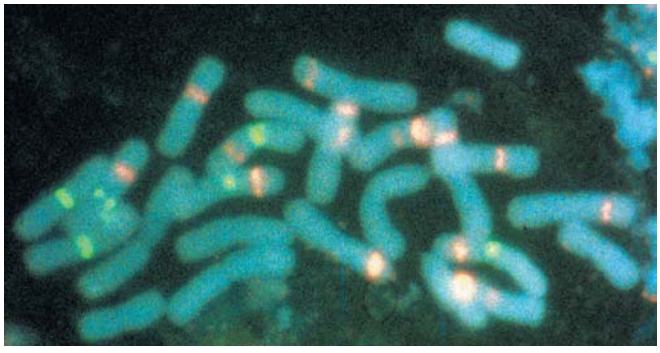

Fig. 4 Simultaneous hybridization of three probes PATR140 (*green signals*), PAF1 (*red signals*), PAF1 and 1PABCD6 (*orange signals*). PATR140 and 1PABCD6 were biotin-labelled and detected by avidin-FITC (fluorescein isothiocyanate) (*green*), PAF1 was digoxigenin-labelled and detected by anti-digoxigenin-rhodamine (*red*). Chromosomes were counterstained with DAPI. Triple signal detection was achieved with a Zeiss excitation and emission filter (no. 487925)

Construction of the karyotype

The measurements of chromosome length and arm ratio were sufficient for the unambiguous identification of the four pairs that did not show FISH signals. In all the studied metaphases the longest chromosome pair did not show any signal and was therefore easily identified. Two of the other pairs without signals were the smallest ones. They were easily distinguished from each other because one pair is submetacentric (arm ratio >1.7) whereas the other is metacentric (arm ratio <1.3). The last pair of homologous chromosomes with no signal was identified by subtraction. The number, combination and distribution of the three probes simultaneously hybridized allowed the unambiguous identification of the remaining eight pairs of homologous chromosomes as described above. These data are summarized in Table 2 and graphically reported in Fig. 5. Chromosomes were conventionally ordered in the ideogram according to their decreasing length. The length estimate was averaged over all the metaphases observed. Four chromosomes, namely 1, 2, 11 and 12, were always classified in the same position in each metaphase, whereas the remaining chromosomes occupied variable positions in the different metaphases, with only one metaphase corresponding perfectly to the ideogram (Fig. 6). Chromosomes 9 and 12 were classified as sub-metacentric according to arm ratio, the remaining being metacentric (Essad et al. 1966). The variability between metaphases in chromosome length and arm ratio confirms the usefulness of FISH probes to identify single chromosomes.

The combination of morphological observations and of the FISH probes allowed the unambigous identification of each homologous pair of chromosomes. In comparison to the karyotypes previously reported for Norway spruce (Köhler et al. 1995; Lubaretz et al. 1996; Siljak-Yakolev et al. 2002) our karyotype shows several improvements: morphological observations on a higher number of

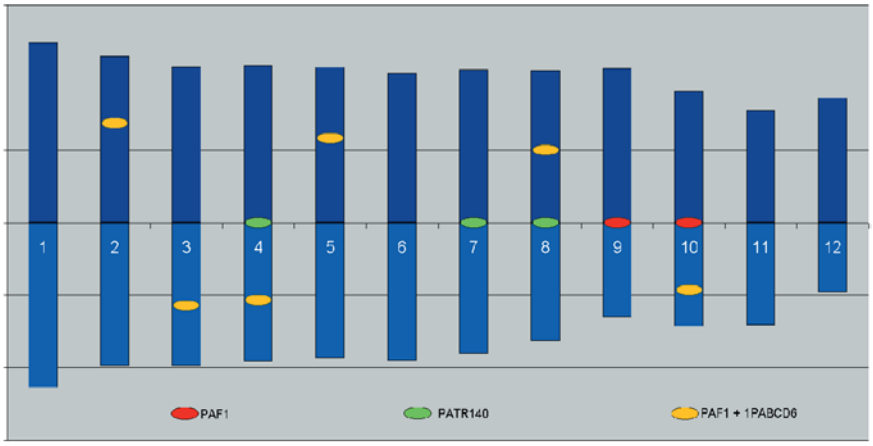

Fig. 5 Ideogram of *P. abies* chromosomes showing the chromosomal locations of PATR140 (*green circles*), PAF1 (*red circles*) and co-locations of PAF1 and 1PABCD6 (*orange circles*)

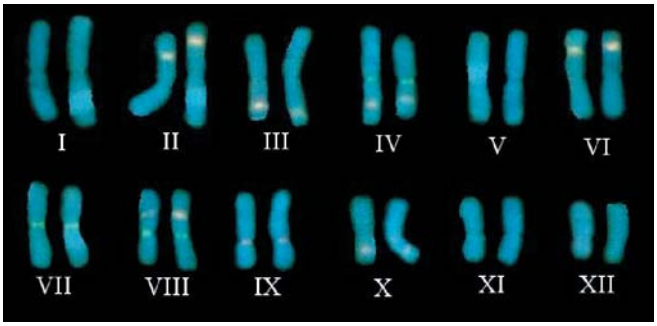

Fig. 6 Karyotype of *P. abies*. Each pair of chromosomes was identified by the combined use of morphometric data and distribution of the probes. Chromosomes were ordered according to decreasing length

metaphases, species-specific probes, a combination of centromeric and arm-specific signals and improved root-tip preparation. Chromosome preparation has proven to be the critical and time-consuming step for the success of the FISH experiments and thus the construction of the molecular karyotype. An unexpected problem was represented by the chromosome dimensions that make the application of the conventional squash techniques difficult for obtaining well-spread chromosomes.

A substantial equivalence with the karyotypes of Lubaretz et al. (1996) and Siljak-Yakolev et al. (2002) is observed with respect to the morphological features of the chromosomes and the number of rDNA loci. When we used only morphological features, mainly the chromosome length and arm ratio, to align the three karyotypes, we observed discrepancies with respect to the location of the rDNA sites in chromosomes 3 to 9. Due to the very similar size of these seven chromosomes and the relatively large error in estimating their length (see Table 2, where all confidence intervals for these chromosomes are overlapping) we believe that the rDNA loci locations are a better predictor for identifying homologous pairs. Differences in chromosome length observed among the three karyotypes for the chromosomes aligned on the basis of molecular probes are in fact of similar magnitude to those observed for chromosomes that could be classified on the basis of morphology only (1, 2, 10–12). We observed, however, little correspondence between our karyotype and that of Köhler et al. (1995), where the karyotype was constructed only by morphological observations on haploid cells. A more comprehensive comparison can be done with other *Picea* karyotypes, namely those of *Picea glauca* and *Picea sitchensis* (Brown et al. 1998). The sequence of the *Sau*3A tandem repeated probe they used shows a high sequence identity (92%) with our PATR140 sequence, although the copy number of 2.0×10^4 they reported was remarkably lower than the one we estimated for *P. abies* (6.2×10^4). The chromosomal location is always around the centromeres, but the number of signals is different for each species: 3, 4 and 5 for *P. abies, P. glauca* and *P. sitchensis*, respectively. The number of rDNA loci differed also among the three species with 6, 7 and 5 loci for *P.abies, P. glauca* and *P. sitchensis*, respectively. Despite these differences we could align without difficulties the *P. abies* and *P. glauca* karyotypes. We assumed that the highly similar size of chromosomes 6 and 7 are in inverted order and that there has been a loss of an rDNA site (chromosome 7 in our karyotype) and of a PATR140 centromeric site (chromosome 12) in *P. abies*. Conversely the alignment of *P. sitchensis* with either of the two other species involves five losses or additions of loci (three events involving the PATR140 sites and two involving the rDNA sites for the alignment with *P. glauca*; two events involving PATR140 sites and three rDNA sites for that with *P. abies*). A closer relationship of *P. glauca* to *P. abies* than to *P. sitchensis* has been suggested on the basis of morphological traits (Schmidt 1989).

The construction of a karyotype and unambigous identification of chromosomes is a requirement for the integration of genetic and physical maps in Norway spruce. Genetic maps were developed in this species by using RAPD markers (Binelli and Bucci 1994; Bucci et al. 1997) and a combination of AFLP, SAMPL and SSR markers (Paglia et al. 1998). Improved sensitivity, using novel fluorochromes and image analysis, has allowed the

detection of single- and low-copy sequences especially through the use of BAC clones as probes (Ohmido et al. 1998; Peterson et al. 1999; Cheng et al. 2001; Khrustaleva and Kik 2001; Kubalakova et al. 2001; Wambugu et al. 2001). The simultaneous hybridization of genetically mapped BACs or other sufficiently long single-copy sequences with our three probes could allow the assignment of the linkage groups to chromosomes. An attractive strategy for Norway spruce, which has a very large genome (2C of about 30,000 Mbp) is the use of flow-sorted chromosome fractions. Flow cytogenetics has been developed for a number of species (Arumuganathan et al. 1994; Lee et al. 1996; Lysak et al. 1999; Dolezel et al. 2001). Flow-sorted plant chromosomes were found suitable for physical gene mapping using PCR, PCR in situ and FISH, construction of chromosome-specific DNA libraries, and the targeted isolation of molecular markers. Recently a protocol for the preparation of chromosome suspension in Norway spruce was developed (Jarolav Dolezel, personal communication). Our flourescent probes could be useful to identify and determine the purity of the chromosome fractions and provide a sorting parameter for enhancing resolution in multivariate flow karyotyping.

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